Comparative Diagnosis of Viral Diseases

Volume II

HUMAN AND RELATED VIRUSES, Part B

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Preface

In view of the possibility of an epidemic, the swine influenza virus recently preoccupied public health departments to a great extent and mobilized uncommon efforts for its diagnosis and prevention. This single example of a virus that can infect animals and man at the same time confirms the importance of comparative virology, not only from the point of view of basic research but from that of the diagnosis, control, and prevention of viral diseases.

Although treatises on fundamental comparative virology are available, one will note that among several books devoted to the diagnosis and control of viral infections none is based on the comparative approach essential for several groups of viruses infecting animals and man. This four-volume treatise was conceived to fill this gap. It is even more necessary because it is difficult to diagnose specifically and rapidly numerous viral diseases without considering the comparative biological, serologic, and physicochemical properties of the viruses involved. Thus, it is devoted to the diagnosis of viral diseases, and is based on the new comparative unifying concept of the viral world. The work demonstrates that the comparison of and the discrimination among viruses, according to the criteria of classification of the International Committee on Taxonomy of Viruses and to the diseases caused by these viruses irrespective of the species involved, are essential for their diagnosis and prevention.

For reasons of classification and cataloguing, the first two volumes of this treatise deal with classified human and related viruses (Volume I) and unclassified human and related viruses (Volume II). In Volume II, one can also find the latest data on the possible implications of viruses in certain cancers and slowly evolving diseases. An important portion of this volume is devoted to the modern approach to the diagnosis of viral diseases and to their control by vaccination and chemotherapy, as well as to the system of Virus Information of the World Health Organization. Volumes III and IV (in preparation) will complete the treatise and will

be devoted to animal and related viruses (Volume III) and to invertebrate and plant viruses (Volume IV).

The treatise was conceived to cover in separate chapters the diseases caused by each virus family. However, in some cases in which a particular interest is manifested for a virus or for the disease(s) it induces, a whole chapter is devoted to the subject. This is the case for the Epstein-Barr virus, cytomegalovirus, rotavirus, rubella virus, and the Marburg virus which has elicited great interest in the last few years.

This treatise, which is based on a new concept, gives a comparative description of the principal physicochemical, molecular, structural, genetic, immunological, and biological characteristics of viruses implicated in various diseases. With a view to a comparative diagnosis, the symptoms and the evolution of the diseases are described in detail, as well as the modern methodology for their rapid and specific diagnosis and for their control and prevention. In this respect, Volumes I and II will interest all clinical virologists and immunologists and veterinarians and research workers. The first two volumes are addressed particularly to the professionals of public health and veterinary sciences who work in the area of diagnosis and control of viral diseases and to those who are interested in the problem of the relation between viruses and cancer.

Each of the contributors to this treatise is well known for his expertise in his field; each has prepared a thoughtful and well-documented treatment of his subject. Personal interpretations and conclusions of the authors, as well as the numerous illustrations and unpublished material, provide a large body of information which brings into sharp focus current findings and new directions in the comparative diagnosis and prevention of viral diseases.

It is our hope that the first two volumes of this treatise will provide a useful tool for all concerned with viral diseases, particularly in hospitals, schools of medical and veterinary sciences, and in diagnostic and control centers of infectious diseases.

We wish to express our sincere gratitude to the contributors for the effort and care with which they have prepared their chapters; to the members of the International Advisory Board of the treatise for their advice; to Professor Maurice L'Abbé, Vice-Rector for Research of the Université de Montréal, and to Doctor Pierre Bois, Dean of the Faculty of Medicine of the Université de Montréal, for their support, permitting the completion of the book; and last, but not least, to the staff of Academic Press for their part in the editing, indexing, proofreading, and other aspects of production of this treatise.

EDOUARD KURSTAK
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Contents of Volume I

HUMAN AND RELATED VIRUSES, Part A

Chapter 1

Marburg Virus Disease

HERTA WULFF AND J. LYLE CONRAD

I. INTRODUCTION

In an early publication concerning an unusual outbreak of a hitherto unknown hemorrhagic disease in an industrial laboratory setting, the name "Marburg virus" was chosen for the etiological viral agent by Siegert and his colleagues (1967, 1968a) because it was first isolated from patients in Marburg, Germany. This name has prevailed.

So far, only two outbreaks and 34 cases of Marburg virus disease have

come to the attention of the medical profession. The outbreaks occurred in entirely different environments. In the first, which occurred in August 1967, all primary cases were in laboratory workers who had close contact with blood and organs of African green or vervet monkeys (*Cercopithecus aethiops*). The monkeys had been imported by air from Uganda with a short intermediate stop in London. Hemorrhagic illness later occurred in Marburg and Frankfurt, Germany, and in Belgrade, Yugoslavia. A total of 31 persons became ill; seven patients died.

The second outbreak occurred in February 1975 in Johannesburg, South Africa. Of the three persons affected, there was one fatality (Center for Disease Control, 1975). The first patient had traveled in Rhodesia for 2 weeks before the onset of his illness. The patient had no known direct contact with monkeys. The natural reservoir for Marburg virus is still unknown.

In this chapter, current knowledge of Marburg virus disease is summarized, and the causative agent and its properties are described.

II. DESCRIPTION OF VIRUS

A. Morphology

The structure of the Marburg virus particle has been investigated in detail by the negative contrast technique; it was identical to that also observed in thin section preparations.

Electron microscopy of infected tissue cultures, guinea pig or monkey plasma, and human or animal organs shows cylindrical particles with a diameter of 65 to 90 nm. The length of each particle varies between 130 and more than 2600 nm, with a modal of 665 nm (Siegert *et al.*, 1967, 1968a; Kissling *et al.*, 1968; Peters and Mueller, 1968; Zlotnik *et al.*, 1968; May *et al.*, 1968). The particles consist of a helical wound core and an envelope with surface projections; they have one rounded end and are often coiled at the other. Terminal blebs are formed from a distention of the membrane envelope around the convoluted internal structure of one end of the particle. The membraneous material or "envelope" is about 20 nm thick. The inner cylindrical structure, which has a diameter of 45 nm, is considered the "nucleocapsid." It has transverse striations occurring at 5-nm intervals. Surface projections have a diameter of 10 nm (Figs. 1–3).

Further details of the structure of the virion are discussed by Peters and colleagues (1971), who describe different layers of the "envelope" with striation patterns of different periodicity, and by Almeida and colleagues

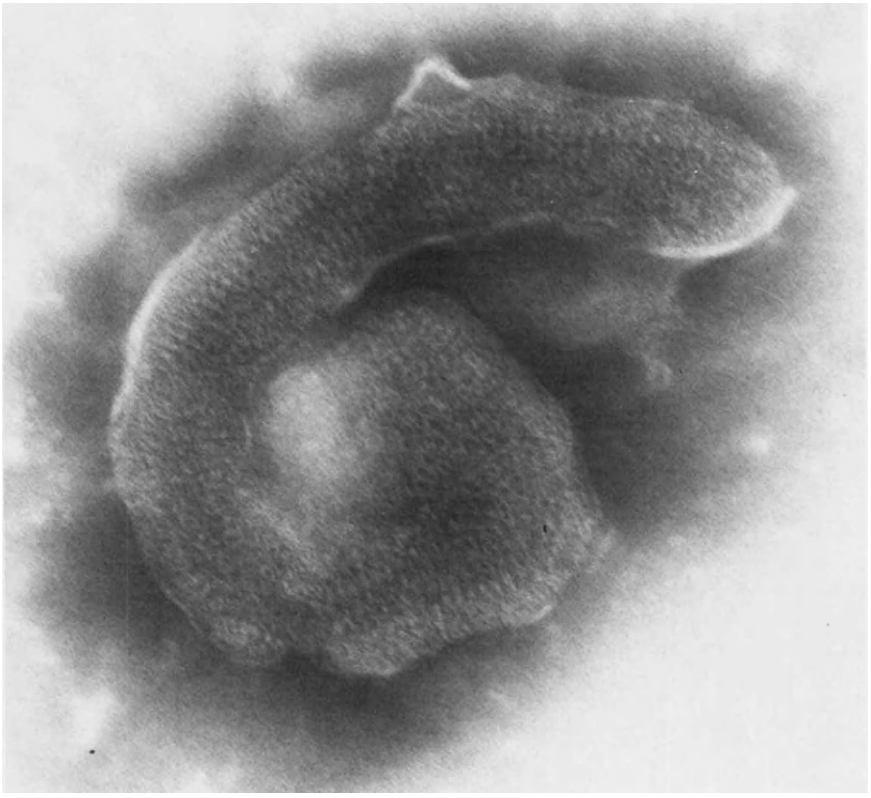

Fig. 1. Electron micrograph of Marburg virus particle in Vero cells inoculated with human blood specimen 4 days previously. Negative contrast preparation. Note cross-striation and surface projection. ×238,000. Courtesy of Dr. F. A. Murphy.

(1971), who speculate on the nature of the structure seen by negative contrast preparation. The latter authors distinguish between a "naked helix" with a linear structure of varying length but with a sharply defined central core and a sinuous membrane-covered form which is coiled at one end and is described as "covered," "full," or "empty." Only the "full" forms are considered to be able to replicate.

Since there are some similarities in the structure of Marburg virus and the vesicular stomatitis and rabies viruses, the name "Rhabdovirus simiae" was proposed (Kunz *et al.*, 1968a). However, Murphy and colleagues (1971), who studied the maturation of Marburg viral particles in liver cells of experimentally infected monkeys, found significant differences between rhabdoviruses and Marburg virus, especially in central

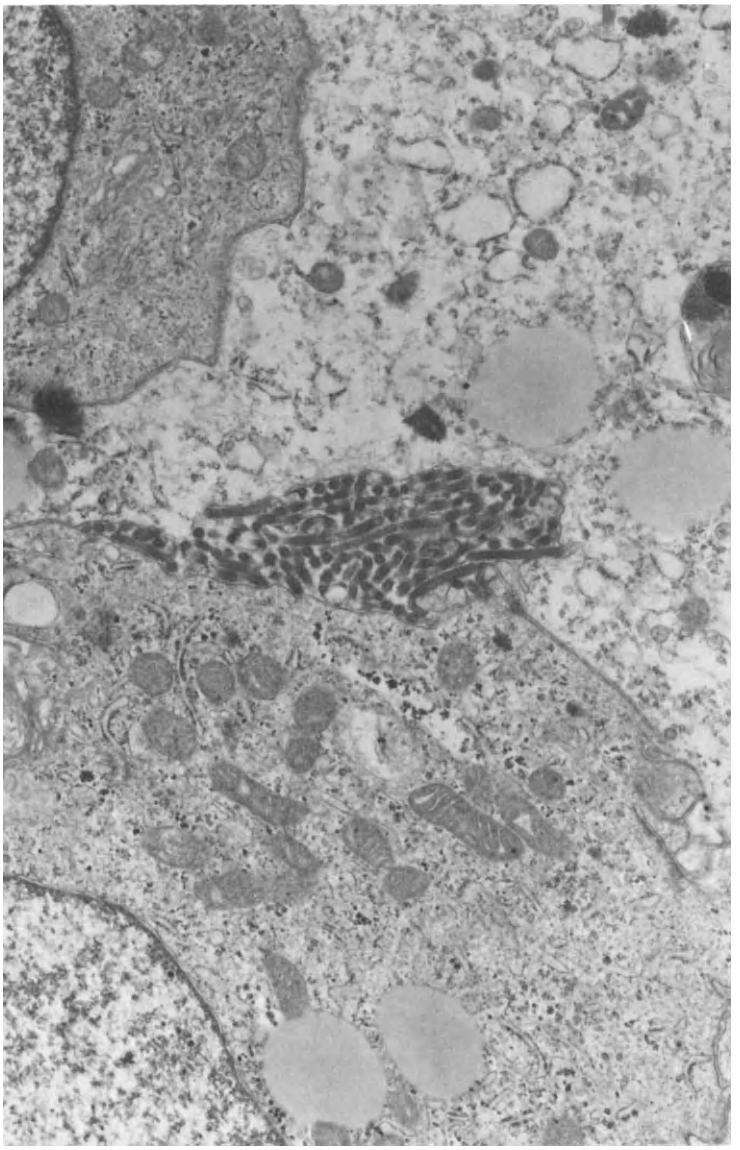

Fig. 2. Electron micrograph of aggregate of Marburg virus particles in space between two Vero cells, 4 days postinoculation with a human liver specimen from a fatal case. Two nuclei are seen at upper left and lower left corners. Ultrathin section. ×17,400. Courtesy of Dr. F. A. Murphy.

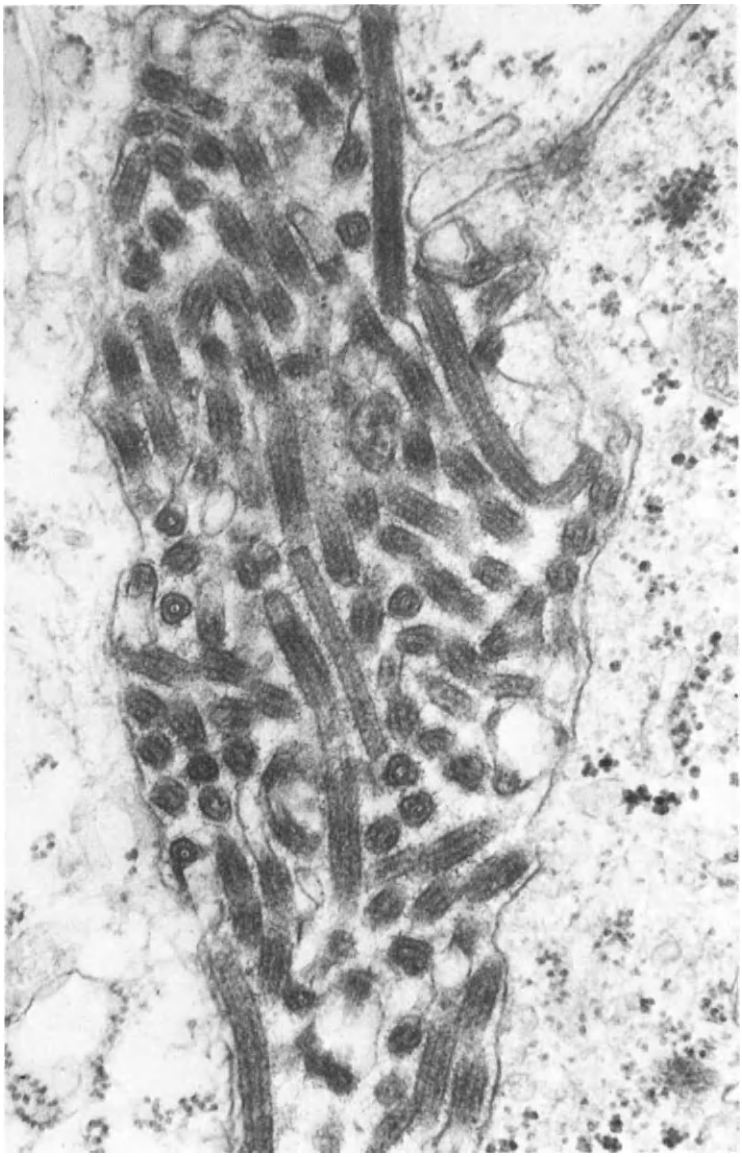

Fig. 3. Electron micrograph of higher magnification of aggregates of virus particles in Fig. 2. Longitudinal and transversely cut virus particles are seen. ×63,750. Courtesy of Dr. F. A. Murphy.

core size and structure of inclusion bodies. The virus has therefore remained unclassified.

B. Physiochemical Properties and Resistance to Treatment

The physiochemical composition of Marburg virus is still largely unknown; so far only a few indirect tests have been performed.

Replication of the virus in cell cultures in the presence of the metabolic inhibitor 5-bromodeoxyuridine (40 μg/ml) is presumptive evidence of an RNA genome (Kissling *et al.*, 1968). Other investigators (Shu *et al.*, 1968, 1969) used 5-iododeoxyuridine for their inhibition studies with the same results. Slenczka (1969) and Malherbe and Strickland-Cholmley (1971) failed to inhibit the multiplication of Marburg virus by actinomycin D (1 μg/ml). Ultracytochemical studies with DNA-specific HCl-silver-methenamine did not demonstrate any reaction with intracytoplasmic inclusions. It was also concluded from these studies that the genetic material of Marburg virus is RNA (Peters *et al.*, 1971).

Purified virus suspensions have not been prepared for chemical analysis. Experiments with lipid solvents and enzymes have provided evidence that the infectious particle contains lipoprotein (Murphy *et al.*, 1971; Siegert, 1972). Preliminary experiments did not reveal hemagglutinins and hemolysins in virus-containing serum or in the supernate of infected cell cultures when erythrocytes from man, chicken, goose, or guinea pig were used (Siegert, 1972; Malherbe and Strickland-Cholmley, 1971).

Inactivation experiments with blood plasma containing Marburg virus have shown that the infectivity is destroyed within 30 minutes at 60°C, (Siegert, 1972). At 56°C, however, Bowen and colleagues (1969) found that 60 minutes were required to completely inactivate an infected monkey liver suspension with a titer of 10^6 ID_{50}/0.1 ml for guinea pigs. The latter authors also noted that storage of an infectious liver suspension at room temperature or at 4°C for 5 weeks resulted in only a slight decrease in virus titer; after 8 weeks the titer was markedly reduced. Infectivity is not reduced after storage for several years at −70°C.

Marburg virus is sensitive to ether, chloroform, and deoxycholate (Siegert *et al.*, 1968b,c; Kissling *et al.*, 1968; Kunz *et al.*, 1968a; Malherbe and Strickland-Cholmley, 1971). Magnesium chloride in a concentration of 0.05 *M* did not protect the virus from heat inactivation (1 hour at 50°C) (Malherbe and Strickland-Cholmley, 1971). One percent formalin destroyed the infectivity of a monkey liver suspension within 1 hour at room temperature. However, the infectivity was not completely destroyed after treatment with 0.5% phenol under the same conditions.

The virus is inactivated by β-propiolactone at a final concentration of 1:2000 during a 24-hour period at 4°C (Kissling *et al.*, 1970).

C. Antigenic Properties

Details of the antigenic composition of Marburg virus have not been investigated.

Marburg virus can be neutralized by specific antiserum (Siegert *et al.*, 1968b,c); an antigen for the complement fixation test has been prepared from infected animal organs and tissue cultures (Smith *et al.*, 1967; Kissling *et al.*, 1968; Slenczka *et al.*, 1970), and virus-specific antigen has been demonstrated by the direct and indirect immunofluorescence technique (Siegert *et al.*, 1968d,e; Slenczka *et al.*, 1968a,b, 1970).

III. COMPARATIVE BIOLOGY

A. Clinical and Laboratory Features of Human Disease

In humans, onset of the disease is sudden. Early symptoms usually include high fever, headache, conjunctivitis, severe myalgia, and malaise. Shortly thereafter, nausea and vomiting occur, followed by profuse watery diarrhea necessitating intravenous fluid therapy to maintain adequate hydration. A characteristic maculopapular rash (Fig. 4) appears on the fourth or fifth day and rapidly coalesces to a diffuse erythema which blanches on pressure, and sometimes is associated with a deep red enanthema of the soft and hard palate (Martini *et al.*, 1968a,b; Martini, 1971; Stille *et al.*, 1968a,b; Stille and Böhle, 1971; Todorovitch *et al.*, 1971). The rash disappears by day 10. The fever usually rises rapidly to 38°–40°C and remains at this level for 5 to 7 days despite administration of antibiotics and antimalarials. Patients who survive may develop a scrotal or labial dermatitis, and the skin of the palms, soles, and extremities may desquamate after 2 weeks, during the recovery period.

On about the fourth day of illness hemorrhagic complications will commence in some patients and will present the greatest therapeutic challenge. Blood may be seen in the urine, stool, and vomitus, and, in severe cases, bleeding begins at needle puncture sites. Terminal anuria may develop, associated with shock, a rising white blood count, and a falling temperature; death is usually due to severe hemorrhagic diathesis.

The important laboratory findings in this disease include leukopenia, sometimes as low as 1000/mm^3, from onset to the sixth or eighth day; a

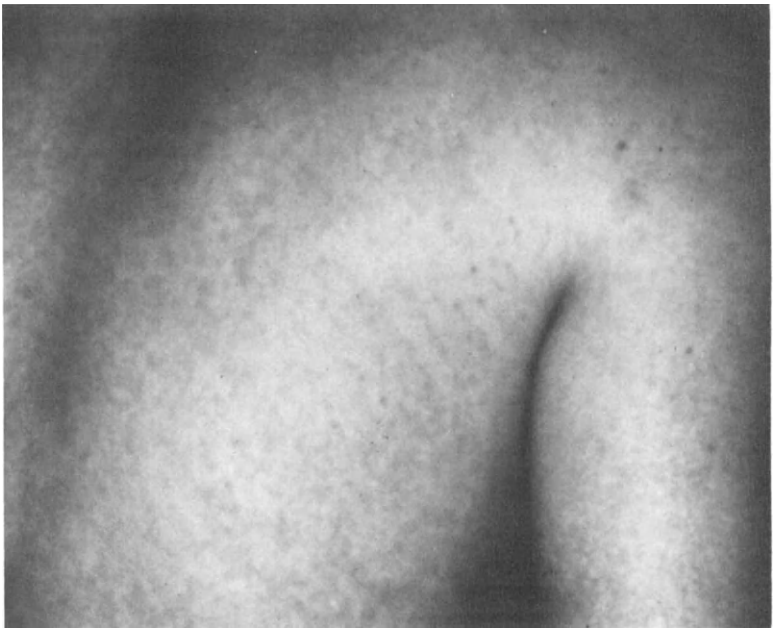

Fig. 4. Typical maculopapular rash, 6 days after onset. Courtesy of Dr. Lavinia Clausen, Department of Medicine, University of Witwatersrand, Johannesburg.

marked fall in thrombocytes (platelets), sometimes as low as 10,000–50,000/mm^3; and elevated serum liver enzymes. Some investigators regard the hematological picture seen in Marburg patients as characteristic of disseminated intravascular coagulopathy (Gear *et al.*, 1975), which is seen in a variety of exanthematous viral and arboviral diseases, with prolonged prothrombin times and increased fibrinogen degradation products. A marked increase in the serum glutamic-oxaloacetic transaminase (SGOT) and serum glutamic-pyruvic transaminase (SGPT) has been noted in virtually all the patients; SGOT levels are usually higher than SGPT levels. When SGOT levels are higher than 2500 units/ml the prognosis is grave because this signifies almost irreparable liver damage. Increases in serum bilirubin levels have been noted only in rare cases, and those only in the terminal stages of the disease.

Several investigators have noted that in severely ill patients, leucocytosis will occur after day 8; the temperature may defervesce preagonally, and shock and anuria may complicate the terminal picture. In a few patients, elevations of serum amylase levels have been noted; these elevated levels tended to persist longer after clinical recovery than those of liver enzymes and may signify pancreatic involvement.

Latent persistent infection has been observed in three persons. Marburg virus was isolated from liver biopsy specimens 31 and 73 days after onset of illness in a patient whose SGOT and SGPT has risen again after apparent clinical recovery (Siegert *et al.*, 1968d,e). Marburg virus was also isolated from the semen of a patient who had infected his wife 12 weeks after onset of illness (Siegert *et al.*, 1968d,e; Shu *et al.*, 1968, 1969). One patient developed unilateral uveitis 3 months after onset of illness, and Marburg virus was cultured from the anterior chamber of the eye; a second culture for virus was negative $2\frac{1}{2}$ months later (Gear *et al.*, 1975).

B. Experimental Hosts

1. Animals

Marburg virus was first isolated in guinea pigs by intraperitoneal inoculation with blood from acutely ill patients and with suspensions of autopsy specimens from fatal cases. The guinea pigs developed fever (40.5°C) after an incubation period of 4 to 10 days but recovered from the illness. The febrile stage of the disease usually lasted for 6 days. Serial passaging of heparinized blood of febrile guinea pigs in additional guinea pigs led to a more severe clinical disease with a high fatality rate and a shorter incubation period. The concentration of virus in the guinea pig blood was 10^6 ID_{50} in the third passage. Occasionally, blood that was collected immediately before death failed to clot. No contact infections were observed in guinea pigs (Smith *et al.*, 1967; Siegert *et al.*, 1967, 1968a,b,c; Kissling *et al.*, 1968; Simpson *et al.*, 1968b).

Marburg virus caused severe illness in experimentally infected vervet and rhesus monkeys. Viremia could be demonstrated before a rise in body temperature occurred. Virus concentrations of up to 10^6 ID_{50} have been found in urine of infected animals. All monkeys developed a fatal illness irrespective of route or dose of infection. Even a 10^{-10} dilution of infected monkey blood inoculated subcutaneously into normal monkeys resulted in illness and death. All inoculated monkeys died 5–25 days after infection. A petechial skin rash in late stages of illness was occasionally seen. Normal monkeys in cages adjacent to infected monkeys also succumbed to the disease when contact between monkeys through the lattice work was possible. They died 15–36 days after first possible contact exposure. Monkeys have also been infected by aerosols (Haas *et al.*, 1968; Simpson *et al.*, 1968b; Simpson, 1969a; Haas and Maass, 1971).

Marburg virus could be adapted to the hamster after nine passages in guinea pigs and three passages in monkeys. A uniformly fatal illness was produced in suckling hamsters after nine passages in hamsters, with encephalitis and high levels of virus in the brain (Simpson, 1969b).

Slenczka and Wolff (1971) describe infection in newborn mice without gross pathological alterations in liver, spleen, and brain. The brains of animals that died in the first 2 weeks after infection contained foci with intracytoplasmic inclusions. Inclusion bodies were also demonstrated in visceral organs by the immunofluorescence technique. All mice died within 3 to 4 weeks. Gross and histological mouse pathogenicity could be induced after three passages in hamsters. The baby mice died of encephalitis. Adult mice developed antibodies but did not show overt illness (Slenczka and Wolff, 1971; Hofmann and Kunz, 1970).

Marburg virus could also be propagated in intrathoracically inoculated *Aedes aegypti* mosquitoes, but not in *Anopheles maculipennis*. Infection of *Ixodes ricinus* was also not possible (Kunz *et al.*, 1968b). Transmission of the virus from infected mosquitoes has not been attempted.

Virus propagation in infected animals was demonstrated by isolation and neutralization, the immunofluorescence method, electron microscopy, and antibody determination.

2. Tissue Cultures

Since tissue cultures generally offer distinct advantages over animals for the isolation of viruses, many tissue culture systems have been evaluated for their ability to support the multiplication of Marburg virus. Siegert (1972) has reviewed in detail the work that has been done thus far. Marburg virus replicates in primary cell cultures of rhesus and vervet monkey kidney, human amnion, chick embryo, and guinea pig fibroblasts. A cytopathic effect has been observed only in vervet monkey kidney cells and then only after serial passage (Maass *et al.*, 1969).

A large number of established cell lines have also been investigated. Marburg virus replicates in AH-1 and Vero cells derived from vervet monkey kidney, in LLC-MK_2 cells originated from rhesus monkey kidney, in the CMH cell line of cynomolgous monkey heart, and in L cells (mouse embryo), guinea pig heart, BHK-21, and HeLa cells. Virus multiplication has also been demonstrated in diploid human lung and foreskin fibroblasts. A cytopathic effect was observed in AH-1, Vero, BHK-21, and human lung fibroblasts, but not by all investigators and, in some cases, only after several passages. In most instances, a complete destruction of the cell sheet could not be achieved. We can confirm the observation by Slenczka (1969) that a cytopathic effect in Vero cells does not develop before the third tissue culture passage. We might further add that the end point of a virus titration is usually reached in cultures which do not show a cytopathic effect; we evaluated the end point of virus titrations by the indirect immunofluorescence technique. The titer of Marburg virus in the third Vero cell passage varies between $10^{5.5}$ and $10^{6.5}$ ID_{50}/ml.

C. Pathology

1. Human Pathology

At autopsy, the gross morphological changes noted are generally limited to the gastrointestinal tract and lungs, where frank blood due to diffuse bleeding has been found.

On microscopic examination of various tissues, the important findings have been limited to the liver, kidney, and lungs. In the liver, severe hepatic necrosis occurs, sometimes with the destruction of as many as two-thirds of the hepatocytes (Fig. 5). Eosinophilic cytoplasmic degeneration with Councilman bodylike formation and nucleolysis is noted (Fig. 6), although the basic architecture of the liver is intact. The necrosis is not midzonal as in yellow fever, but may have a similar appearance because of the confluence of multiple randomly located foci of necrosis, with minimal inflammatory response (Gedigk *et al.*, 1968, 1969, 1971).

In the kidneys, marked tubular necrosis is noted with multifocal fibrin thrombi occluding the glomerular capillary tufts (Fig. 7, evidence of disseminated intravascular coagulopathy). In the lungs, pulmonary edema has been seen with accumulation of alveolar macrophages in fluid-filled alveoli (Fig. 8). No particular remarkable findings are noted in spleen, lymph nodes, adrenals, heart, pancreas, or stomach. In the first Marburg outbreak, some diffuse encephalitis was described in brain specimens examined microscopically, with perhaps some increased perivascular lymphocytic infiltrate and clear evidence of cerebral hemorrhages (Jacob, 1971).

2. Animal Pathology

The histopathological changes observed by various workers in tissues of guinea pigs, monkeys, and hamsters are similar, provided that the virus is well adapted to the animal species (Haas *et al.*, 1968; Zlotnik and Simpson, 1969; Zlotnik, 1969, 1971; Oehlert, 1971). Many findings in animal tissues are comparable to the histopathological changes seen in human tissues. In most animals studied, a generalized virus infection was noted, with involvement of all organs. Intracytoplasmic inclusion bodies were found in liver, kidney, spleen, and lung.

The liver is the primary target organ, with pronounced cytonecrotic changes of the hepatic parenchyma. Some parenchymal cells in the guinea pig liver were seen to contain basophilic inclusions, which occasionally were distended with masses of strongly basophilic bodies. These basophilic bodies gave a histochemical reaction for calcium.

The infection has a destructive effect on the reticuloendothelial system and causes depletion of lymphocytic elements in tissues and peripheral

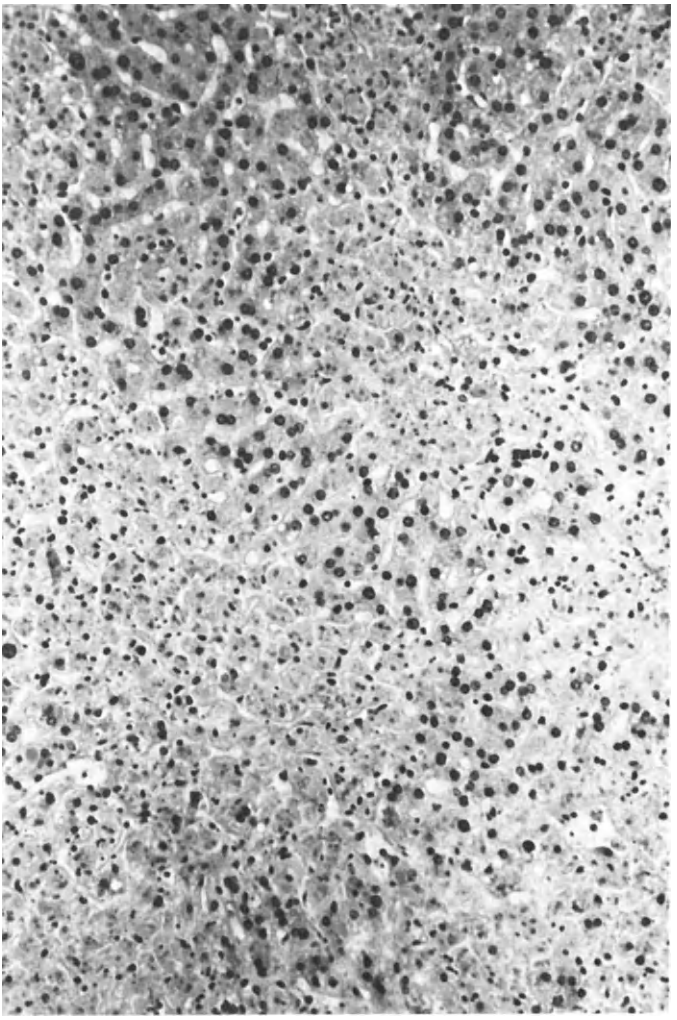

Fig. 5. Light micrograph of section of human liver from patient whose Marburg virus infection was fatal. Patient died 6 days after onset of illness. Severe focal necrosis of hepatocytes. ×238. Courtesy of Professor J. H. S. Gear and Dr. F. A. Murphy.

blood. Spleens and lymph nodes were congested, and necrosis of the germinal centers was very pronounced.

The lungs of guinea pigs and monkeys showed edema and interstitial pneumonia; interstitial pneumonia was also observed in hamsters.

Kidney changes consisted of tubular degeneration. The glomerular tufts were occasionally adherent to the capsular epithelium.

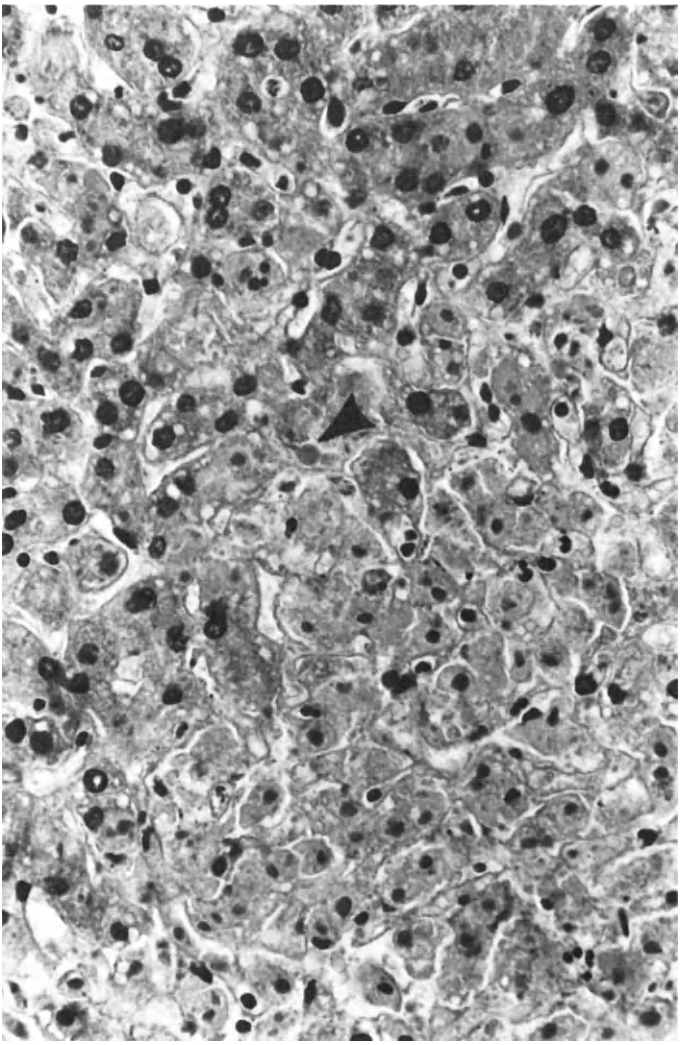

Fig. 6. Light micrograph of higher magnification of section of human liver of fatal case. Severe necrosis and Councilman bodylike formation (arrowhead). ×374. Courtesy of Professor J. H. S. Gear and Dr. F. A. Murphy.

Zlotnik and Simpson (1969) have described inflammatory changes in the brains of suckling hamsters. The infiltrating cells were either mesodermal mononuclear cells or astrocytes. Many neurons became necrotic in the vicinity of hemorrhages.

The kinetics of viral maturation were investigated in serial vervet monkey liver specimens by Murphy and colleagues (1971). Viral cyto-

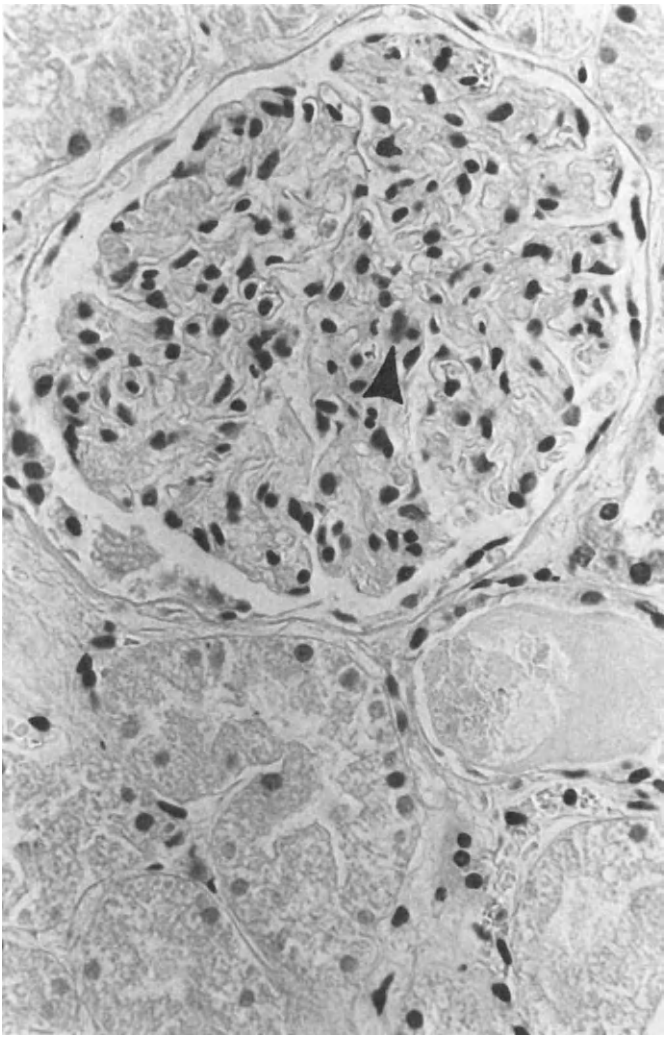

Fig. 7. Light micrograph of section of human kidney from patient who died of Marburg virus infection. Marked tubular necrosis with multifocal fibrin thrombi (arrowhead) occluding the glomerular capillary tufts. ×374. Courtesy of Professor J. H. S. Gear and Dr. F. A. Murphy.

plasmic matrix, which appeared rather dispersed early in infection, increased greatly in amount and complexity by the seventh day after inoculation. Many cells contained discrete matrixes at multiple sites in their cy-

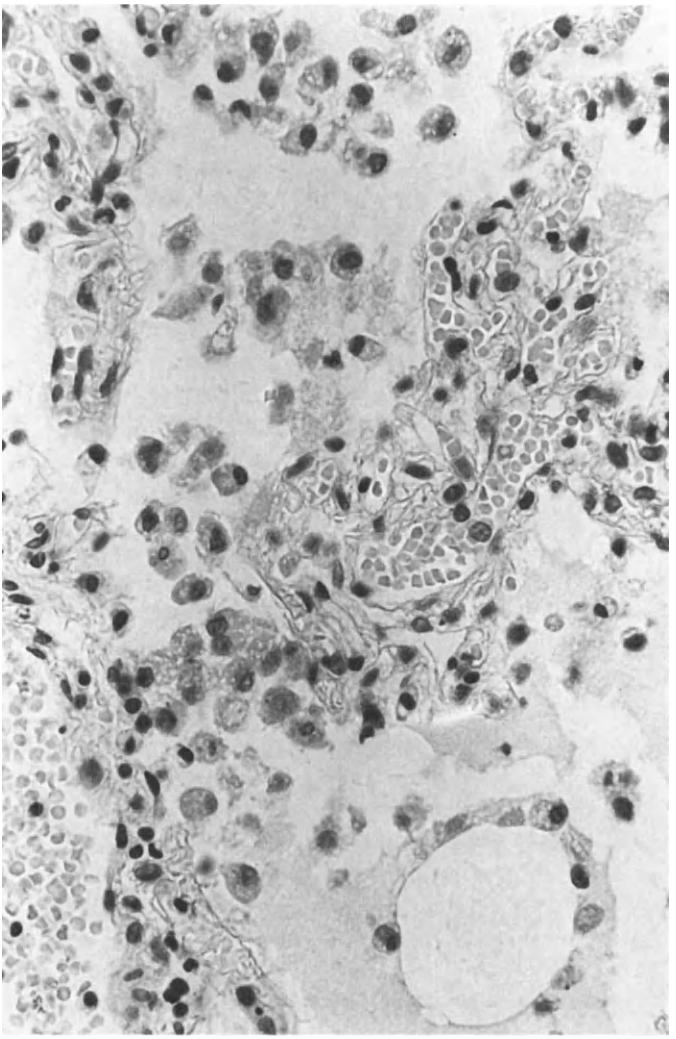

Fig. 8. Light micrograph of section of human lung from patient who died of Marburg virus infection. Pulmonary edema with accumulation of alveolar macrophages in alveoli. × 374. Courtesy of Professor J. H. S. Gear and Dr. F. A. Murphy.

toplasm. Structuring of inclusions could be recognized. These inclusions became massed filaments in parallel array. When sectioned transversely, the inclusions were seen to consist of uniformly packed cylinders (Figs. 9

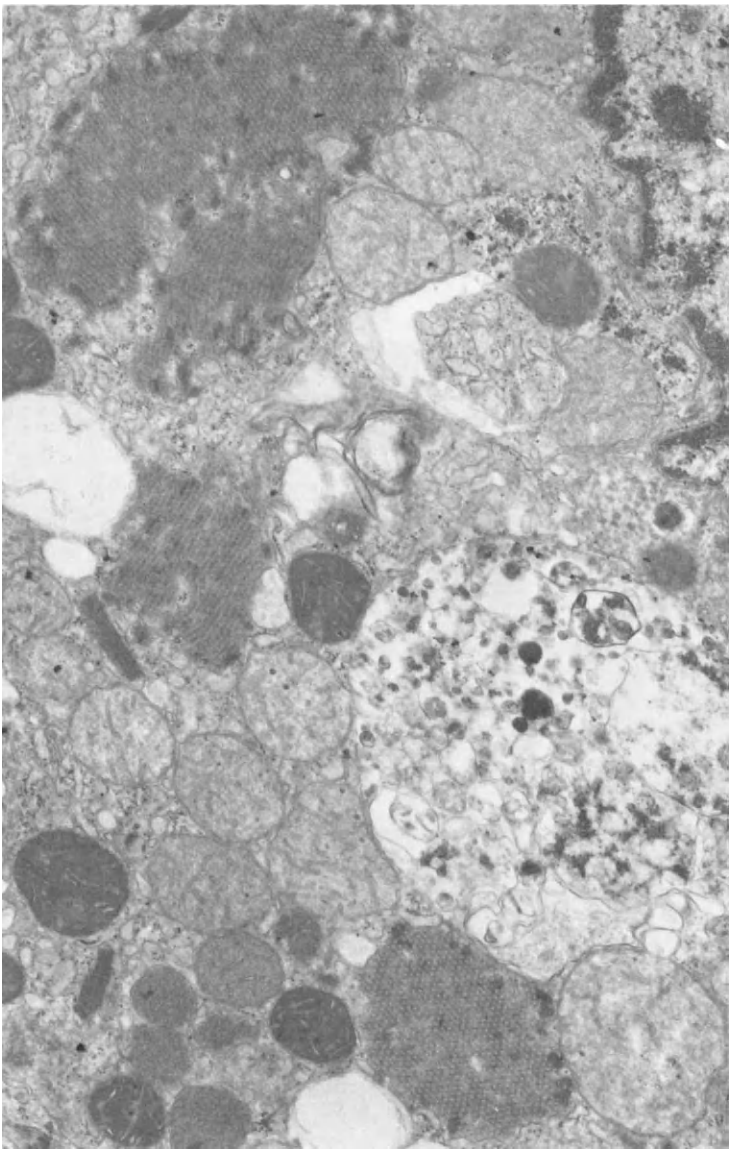

Fig. 9. Electron micrograph of portion of the cytoplasm of an infected hepatocyte in monkey liver. The monkey had been infected with Marburg virus 8 days previously. Viral inclusion bodies appear as arrays of filaments which are sectioned longitudinally or at an angle, but one is cut transversely (at bottom to the right). Part of the nucleus is at the top to the right and a necrotic area is at right from the center. × 15,300. Courtesy of Dr. F. A. Murphy.

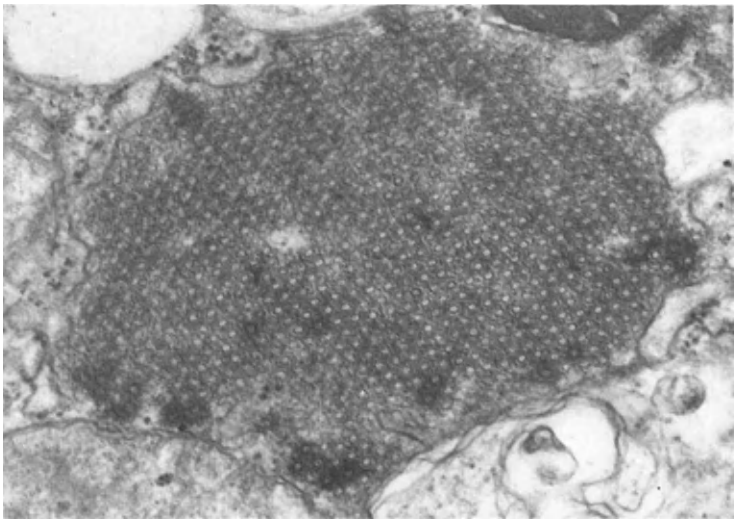

Fig. 10. Electron micrograph of higher magnification of the transversely cut inclusion of Fig. 9. Note close packing of tubular viral structures. × 43,350. Courtesy of Dr. F. A. Murphy.

and 10). These tubules developed into the core structure of virus particles as maturation occurred by budding through plasma or intracytoplasmic membranes. The cross-sectional conformation of particles is highly characteristic of Marburg virus.

Late events in the destruction of hepatic parenchyma cells involved mainly plasma and intracytoplasmic membranes and mitochondria. The nuclear morphology remained normal until the very late stages of cytonecrosis.

IV. IMMUNITY

All known cases of Marburg virus disease have occurred in adults 18–64 years of age. Reinfection in man has not been described, but it has been investigated in laboratory animals. Guinea pigs which have survived an initial infection with Marburg virus have remained afebrile after challenge. It was concluded from these experiments that the animals had acquired immunity (Siegert *et al.*, 1967, 1968a,b,c).

Marburg virus patients and experimentally infected animals develop neutralizing, complement-fixing, and immunofluorescent antibodies (Smith *et al.*, 1967; Siegert *et al.*, 1967, 1968a; Kissling *et al.*, 1968; Hofmann and Kunz, 1969). It is not known how long immunity lasts.

TABLE I
Development of Indirect Immunofluorescent Antibody in Marburg Virus Patients

Time after onset of illness	Patient[a]	Antibody titer with antigen of	
		Popp strain (1967)	Hogan strain (1975)
Days			
2	Cr.	<2[b]	<2
5	Oz.	<2	<2
7	Oz.	<2	<2
7	Cr.	2	<2
12	Cr.	8	16
14	Oz.	≥16	≥16
19	Oz.	32	32
26	Cr.	64	64
27	Oz.	128	64
119	Ul.	128	64
122	Kl.	64	ND[c]
128	Cr.	64	128
Years			
8	Kl.	8	ND
8	Ul.	16	16
8	Ha.	16	32
8	Pa.	2	<2
8	Fl.	16	ND
8	Hz.	8	ND

[a] Patients Cr. and Oz. contracted Marburg virus disease in 1975; sera were submitted by Prof. J. H. S. Gear, Poliomyelitis Institute, Johannesburg. The other sera are from the 1967 outbreak. They were supplied by Dr. W. Slenczka, Institute of Hygiene, Marburg; Prof. O. Bonin, Paul Ehrlich Institute, Frankfurt; and Prof. W. Hennessen, Behringwerke, Marburg.

[b] Titers are given as reciprocal of serum dilution.

[c] Not done.

Immunofluorescent antibodies develop within the second week after onset of illness. They reach a peak 1–2 weeks later and decline very slowly. Patients who acquired the disease in 1967 still had immunofluorescent antibodies 8 years later (Table I). Immunofluorescent antibodies reach a titer of up to 1:512. The titer is at least fourfold higher than the complement fixation titer (Slenczka *et al.*, 1971).

Complement-fixing antibodies are not as long lasting. They usually develop in the third week after infection, and 1–2 weeks later reach a maximum titer of 1:16 to 1:128. They markedly decline, starting in the eighth week after infection. They could still be demonstrated 2 years after

infection, but only at a very low titer (Slenczka *et al.*, 1970). A satisfactory test method for demonstrating neutralizing antibodies has not yet been developed; therefore, it is not possible to make a statement concerning their development, peak of titer, and duration.

Antibodies are transplacentally transmitted. Infants born to mothers who had the disease 1½ years earlier had complement-fixing antibodies. This evidence of passive immunity disappeared within 3 months (Siegert, 1972).

V. EPIDEMIOLOGY

Only two outbreaks of Marburg virus disease have been reported in the medical literature, with a total of 34 known cases. There was considerable difference on their basic epidemiology; therefore, they will be discussed separately.

Between July 20 and August 10, 1967, four shipments of vervet monkeys, *Cercopithecus aethiops,* numbering between 500 and 600 animals, were sent from Uganda to Europe via London's Heathrow Airport. They were sent to three institutions located in Marburg and Frankfurt, Germany, and in Belgrade, Yugoslavia, for laboratory and vaccine production usage. Human cases were reported in each city, and were directly associated with handling monkey blood and organs, especially kidneys; hence the earlier name for the disease "Green Monkey Kidney Disease." This outbreak (Fig. 11) which lasted 34 days, demonstrated a common source pattern (Hennessen, 1971) with a prolonged exposure; seven of the 31 patients died. Those at greatest risk were individuals involved in the

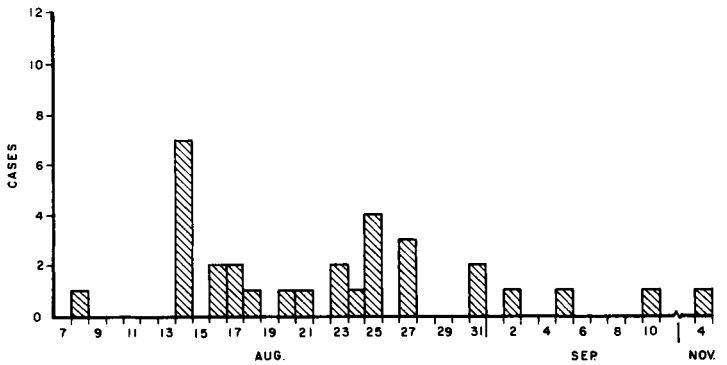

Fig. 11. Epidemic curve for Marburg virus outbreak in 1967. Cases of Marburg disease, by date of onset, are indicated for Belgrade, Yugoslavia and Frankfurt and Marburg, Germany. Adapted from Hennessen (1971, p. 162).

actual surgical removal of monkey organs from the animals; in this group, 20 out of 29 persons contracted the disease, whereas only five out of 15 exposed to tissue cultures were infected. The remaining six cases were contacts of the above patients.

During the 1967 outbreak the clinical course was somewhat milder in patients with secondary infections than in those with primary cases. Virus was probably transmitted through lesions of the skin and mucous membranes and perhaps the conjunctivas. The infectious medium was blood from the febrile phase and, in one case, from semen 12 weeks after onset of disease. Human throat secretions and urine specimens contained only low virus titers; therefore, it was felt they did not play a significant role in virus transmission. All secondary infections resulted in severe illness, but all patients with this type of infection made full recoveries.

The incubation period for primary cases was 3–7 days, while secondary cases occurred 5–8 days after exposure (Hennessen, 1971). In experiments with monkeys, incubation periods were 3–4 days after direct parenteral inoculation with infectious material and 6–9 days after exposure to infected animals in nearby cages (Haas *et al.*, 1968; Haas and Maass, 1971).

Control of the European outbreak was achieved by avoiding direct contact with infectious material and by cleaning and washing contaminated equipment. Protective clothing, including gloves and masks, was used while the contaminated areas were cleaned, all work with monkey tissues ceased, and all remaining monkeys were killed and incinerated.

Epidemiological studies were carried out in Uganda at the monkey source, but no Marburg-like agents were isolated from the blood of wild-trapped monkeys. No episodes of human disease were uncovered among the monkey trappers. Serological tests with a crude complement-fixing antigen prepared from guinea pig liver indicated presence of antibody in a number of vervet monkeys and also in three monkey trappers (Henderson *et al.*, 1971). However, this work was never confirmed; it is possible that the antigen reacted nonspecifically. Other investigators did not find antibodies in sera of wild monkeys that were trapped (Simpson *et al.*, 1968a; Strickland-Cholmley and Malherbe, 1971).

The most recent outbreak, in Johannesburg, South Africa, was a classic person-to-person transmission episode (Fig. 12) which occurred in February, 1975. Three cases were involved, and the index case died. This outbreak started when the first patient became ill 3 days after returning from a hitchhiking tour of Rhodesia. During this tour, the patient was not directly exposed to any animals, he slept out of doors unprotected on two occasions and received several bites by insects or other biting arthropods. Six days before onset of illness one of the bites was noted to be especially

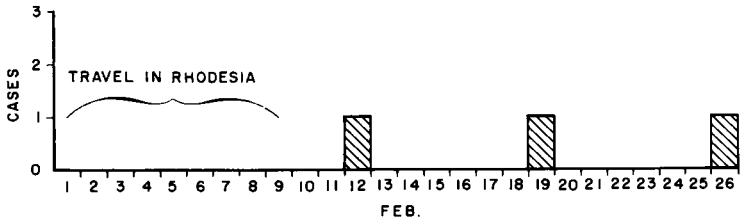

Fig. 12. Epidemic curve for Marburg virus outbreak in 1975. Cases of Marburg disease, by date of onset, is given for Johannesburg.

severe. His traveling companion, the second patient, became ill 7 days later; he presumably was her source of infection. She, in turn, apparently passed the infection to a nurse. The incubation periods appeared to be about 7 days. This outbreak was brought under control by strict hospital isolation of all close contacts of the three cases, plus double gown, glove, mask, and goggle protection of all patient attendants. Further studies are currently underway to attempt to uncover the natural source of the virus in Rhodesia.

VI. COMPARATIVE DIAGNOSIS

A. Differential Diagnosis of Human Disease

Both of the recorded outbreaks of Marburg virus disease have had an association with Africa, although the first was an indirect association through nonhuman primates. From what we now know, the disease is probably of African origin, and we can, therefore, limit our differential diagnostic considerations to African febrile hemorrhagic diseases.

The protozoal diseases, malaria and trypanosomiasis, can be ruled out by negative peripheral blood smears. Other important possibilities include certain virus diseases, such as yellow fever, chikungunya and Rift Valley fevers, Lassa fever, dengue, smallpox, and hepatitis. Bacterial diseases to be considered should include at least typhoid fever and meningococcal and plague septicemias; however, these can be ruled out by negative blood cultures and the lack of clinical response of suspect cases to high doses of broad-spectrum antibiotics. We agree with Monath (1974), that the most difficult differential diagnosis is among yellow fever, Marburg virus disease, and Lassa fever; this will be discussed here in further detail.

Lassa fever has an insidious onset of fever, chills, pharyngitis, malaise, headache, and to a lesser extent myalgia; Marburg virus disease has an

abrupt onset of essentially the same symptoms, and yellow fever usually does too. A severe painful sore throat, with exudative or ulcerative pharyngitis, is common in Lassa fever, but is rare or absent in Marburg virus disease and yellow fever; in Lassa it may be the earliest symptom and may be so severe that it is difficult or impossible for the patient to swallow. The earliest symptom in Marburg virus disease may be just myalgia. The course of yellow fever in severe cases is often biphasic with a short remission; Marburg and Lassa both appear unremitting in severe cases, although medical experience with these last two diseases is not extensive.

As mentioned in Section III,A, the maculopapular rash of Marburg virus disease is a prominent feature of this disease from days 4 to 10, and such a rash does not occur with yellow fever and only rarely with Lassa fever.

As yellow fever progresses and the temperature rises again, the pulse falls and a relative bradycardia develops; jaundice and hemorrhagic manifestations soon follow. For investigators having laboratory services available, it might be helpful to note that yellow fever patients frequently show hemagglutination inhibition (HI), fluorescent (FA), or neutralization antibodies to yellow fever while still clinically ill. In both Marburg and Lassa virus diseases, the fever tends to persist, and no jaundice develops except perhaps preterminally with Marburg virus disease. With Marburg virus disease, signs of severe liver impairment develop early and rapidly with abdominal pain, nausea, and vomiting; and, as with yellow fever, the patients may vomit blood and have melena in the stools. The management of Marburg virus disease involves monitoring the hepatitis and controlling the hemorrhagic tendencies. In severe cases the patients will bleed from needle sites as well as mucous membranes and gastrointestinal tract; this has been described as the picture of disseminated intravascular coagulopathy (Gear *et al.*, 1975). The management of hypotension and shock becomes the immediate medical problem with Lassa fever; shock in Marburg virus disease appears to be a terminal event due to blood loss and/or overwhelming bacterial superinfection.

In Johannesburg, the Marburg virus disease patients recovered relatively quickly after 10 to 14 days of severe illness; however, in the original outbreak in Marburg, Germany, a prolonged convalescent period was observed. Lassa fever has a prolonged recovery period of weeks before the patients are well enough to mobilize themselves unaided; their weakness is remarkably severe. Postural hypotension in Lassa in the third and fourth week of illness has been observed, with a prolonged severe sore throat as described.

B. Virus Isolation Procedures

Since Marburg virus causes a severe disease in man and is highly infectious, it is of utmost importance to make a laboratory diagnosis as early as possible. Since the virus titer of blood from the febrile phase ($10^{4.5}$ ID_{50}/ml evaluated in Vero cells) and of liver specimens (10^5 to 10^6 ID_{50}/g of tissue, also titrated in Vero cells) is high, it is feasible to make an early diagnosis. As Siegert and colleagues (1967, 1968a,d,e) have pointed out, virus-specific antigen can be detected by the immunofluorescence method in human specimens. An impression smear of a human liver suspension (Fig. 13) proved to be an excellent method for the demonstration of viral inclusion bodies (Wulff, 1977). The evaluation of a blood sample by the immunofluorescence method is more difficult. Siegert and Slenczka (1971) were able to find virus-specific antigen extracellularly in clumps, 1–10 μm in diameter in drops of citrated blood. However, they could not demonstrate antigen in blood smears and in drops of hemolyzed blood. The latter observation corresponds with our experience.

Electron microscopy seems to be the method of choice for direct demonstration of Marburg virus particles in serum and plasma (Peters and Müller, 1968).

Attempts to isolate the virus in tissue cultures should also be implemented immediately, because low virus concentrations might not be detected when a rapid method is used. The Vero cell line is an excellent choice. Marburg virus does not cause a cytopathic effect in the first passage, but we were able to demonstrate virus-specific antigen by the immunofluorescence method on day 4 after inoculation of human blood and liver suspensions (Fig. 14). Even earlier detection of antigen is probably possible.

Accumulation of antigen in the cytoplasm of the infected cells often have a sandlike appearance, but larger aggregates (up to 10 μm) are also seen. They are round, oval, elongated, and spindlelike, and some have very bizarre forms (Fig. 15). The fluorescent antigen can also be found extracellularly in cells with high antigen concentrations. Autopsy specimens that were titrated in Vero cells gave high infectivity titers (Wulff, 1977) which compared excellently with results of virus titrations in guinea pigs (Siegert *et al.*, 1968d,e).

Virus isolation from the blood is possible only during the febrile phase, which can last up to 14 days. Virus isolation from throat specimens and urine samples has also been attempted, but only one of four urine specimens tested and two of six throat washings were positive (Siegert *et al.*, 1968d,e).

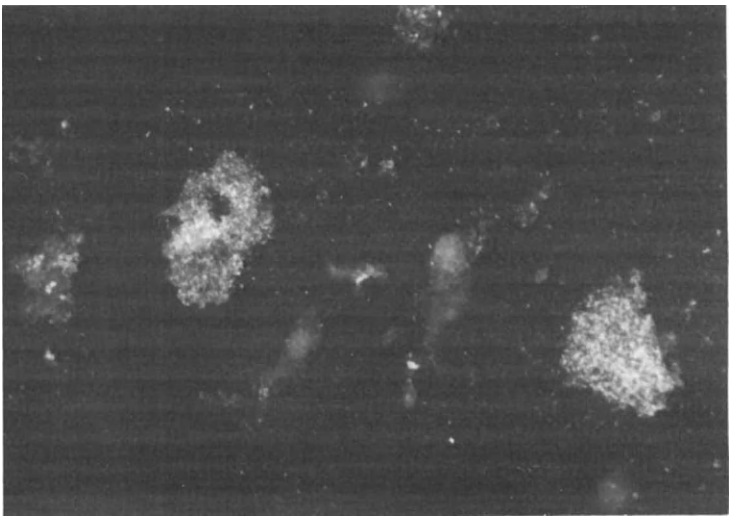

Fig. 13. Immunofluorescence of inclusion bodies in human liver cells from patient who died of Marburg virus disease. Impression smear. Some antigen is located extracellularly. ×441.

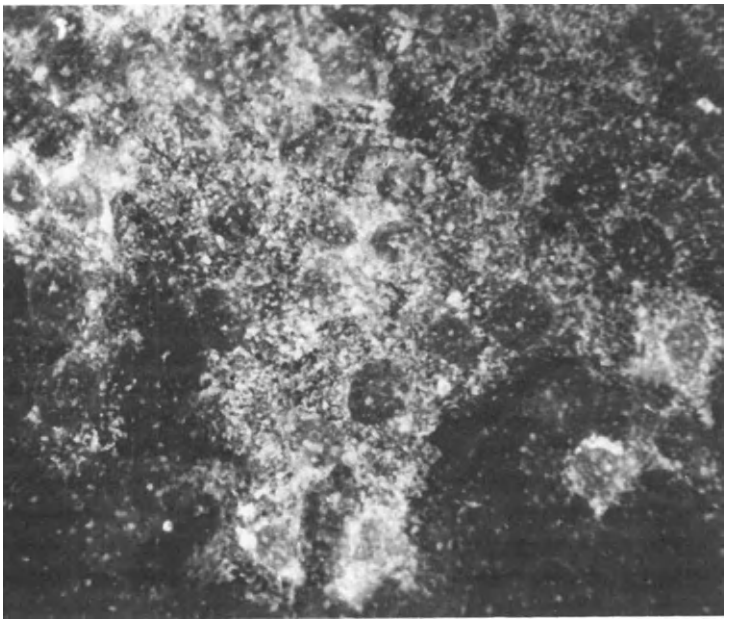

Fig. 14. Immunofluorescence in Vero cells which had been inoculated 4 days previously with human blood specimen from a Marburg virus disease patient. Many viral aggregates are located extracellularly. ×441.

In addition to being found in liver, virus-specific antigen can be demonstrated in spleen, kidney, lymph nodes, heart, lung, and sometimes brain material. In most of these organs, however, the virus titer is considerably lower than in liver, and direct demonstration of antigen by the immunofluorescence method might not be feasible.

As was pointed out in Section VI,A, it is difficult to distinguish between Marburg, Lassa, and yellow fever infections by clinical symptoms noted in the early stages of these diseases. It is fortunate that Vero cells support the propagation of Lassa, Marburg, and yellow fever viruses. Lassa virus-specific antigen can be detected in Vero cells on day 2 or 3 after inoculation (Wulff and Lange, 1975) by the indirect immunofluorescence technique. Yellow fever virus-specific antigen can also be demonstrated by the immunofluorescence technique in Vero cells, but it is advisable to inoculate baby mice as well. Yellow fever virus grows slowly in Vero cells; therefore, the cultures should be evaluated for the presence of virus-specific antigen over a period of 14 days before they can be considered negative.

When Marburg virus-infected Vero cell cultures are stained by hematoxylin and eosin, cytoplasmic inclusion bodies (Fig. 16) can be seen as early as day 3 after infection (Siegert and Slenczka, 1971). The inclusion bodies can also be recognized by phase contrast microscopy (Siegert *et al.*, 1967, 1968a).

C. Serological Procedures

Casals (1971) did not observe serological cross-reactions with other viruses. Sera from six patients were tested, with negative results, by the complement fixation and hemagglutination inhibition methods for antibodies against a large number of arbovirus and rhabdovirus antigens and antigens prepared from other viruses known to cause hemorrhagic fever in man and animals.

1. Neutralization

An easy and reliable neutralization test which can be used to evaluate large numbers of sera has not been developed. Neutralization of the virus in guinea pigs seems to be a reliable method (Siegert *et al.*, 1968b,c), but is very cumbersome and time-consuming.

Attempts to use tissue cultures in neutralization tests have not led to satisfactory results. The virus does not cause a consistent cytopathic effect, although the vervet monkey kidney cell line AH-1 has been used in a

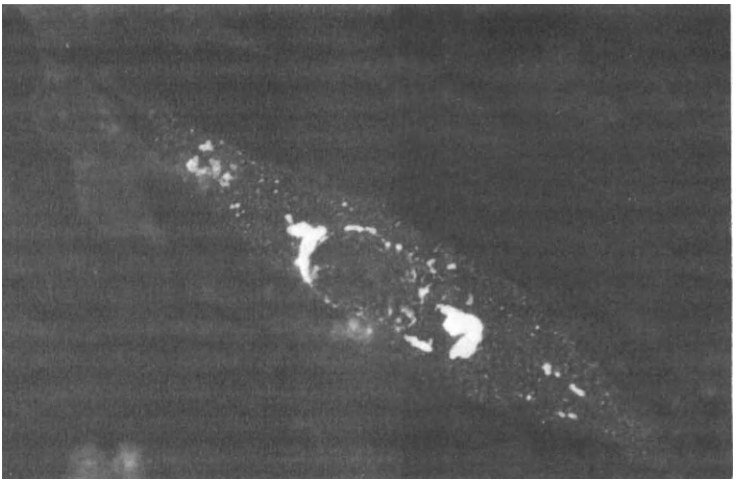

Fig. 15. Demonstration of viral antigen by immunofluorescence in a Vero cell. The culture was inoculated with third-passage Marburg virus material 6 days previously. ×441.

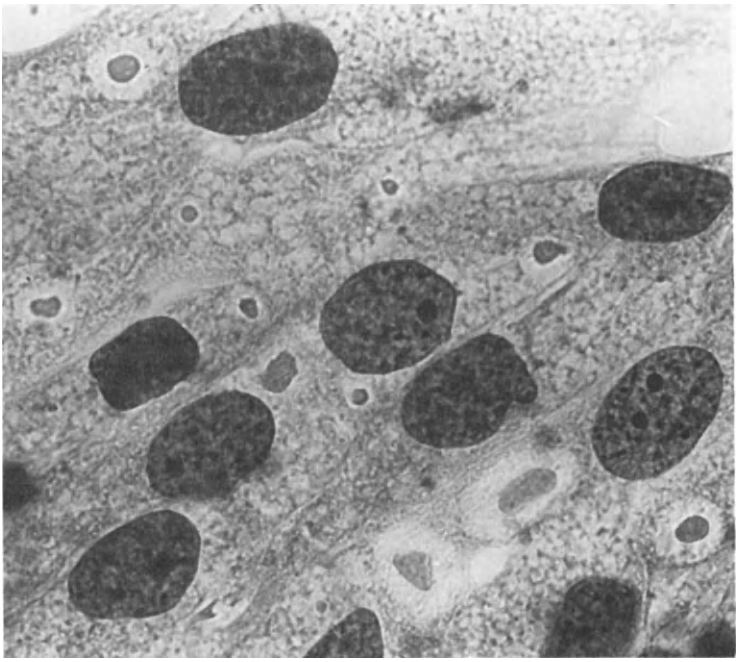

Fig. 16. Inclusion bodies in Vero cells inoculated with third-passage Marburg virus 6 days previously. Hematoxylin and eosin staining. ×935.

small number of experiments (Henderson *et al.*, 1971). Slenczka and Wolff (1971) describe a method in which they count the cells with cytoplasmic inclusions and evaluate their reduction as a measure for neutralization; however, this test is not useful for routine investigation.

2. Complement Fixation

Antigens for the complement fixation test have been produced in animals and tissue cultures. Most investigators (Smith *et al.*, 1967; Simpson *et al.*, 1968a; Kissling *et al.*, 1968; Hofmann and Kunz, 1969; Strickland-Cholmley and Malherbe, 1971) used infected guinea pig spleen and liver and vervet monkey liver. Several of these antigens gave nonspecific reactions when evaluated with human and monkey sera. Slenczka and his colleagues (1970) developed an antigen prepared from chronically infected Vero cells, which is far superior in quality to crude antigens derived from animal organs. Since complement-fixing antibodies develop rather late in infection and are not stable, the complement fixation test can only be regarded as an additional tool for antibody evaluation.

3. Immunofluorescence

As substrate for antigen preparation, Slenczka and his colleagues (1971) used Marburg virus-infected BHK-21 cells, and we used Vero cells. Our technical procedures are identical to those described for antibody determination in Lassa fever patients (Wulff and Lange, 1975). As outlined in Section IV, fluorescent antibodies develop within the second week after infection, and are long lasting. With the immunofluorescence method, we detected Marburg virus antibodies in specimens from persons who had had a Marburg virus-like illness 5–8 years previously. Thus the method appears to be well suited for surveys. It is now the method of choice, because we do not have any information about the persistence of neutralizing antibodies.

VII. PREVENTION AND CONTROL

Marburg virus disease appears to be a very rare African hemorrhagic disease, and since we understand so little of its epidemiology, natural reservoir, and modes of possible transmission, we cannot consider means of primary prevention. No vaccine is now (December 1975) available, and no antibiotic is known to be effective against the disease. We have to expect small episodes of disease from time to time. It is important that clini-

cians diagnose the disease as early as possible, in order to institute strict patient isolation. If possible, the diagnosis should be confirmed in the laboratory by virus isolation. Since work with Marburg virus is hazardous, specimens should be handled only in a Class III facility.

In Europe and South Africa, physicians familiar with the disease process examined all patients with suspected cases. Such patients were hospitalized in a single designated hospital with a competent staff capable of handling complications of the disease. All primary contacts of the patients were isolated and kept under close observation; daily surveillance was extended to all possible contacts. In Marburg, all work on the suspect monkeys and tissues ceased, and all the monkeys and associated animals were sacrificed and incinerated. Once the problem was recognized, patients in both outbreaks were treated with the utmost isolation precaution; in Johannesburg, use of double gown, gloves, masks, goggles, and boots by personnel attending the patients appeared to have been effective in terminating transmission in the hospital without compromising the meticulous medical care the patients required to survive.

Treatment must be supportive since there is no specifically effective antiviral drug for patients with Marburg virus disease. Convalescent plasma has been used in Frankfurt and may be effective in modifying the disease process (Siegert, 1972). The patient's hematological, liver, kidney, and spleen functions, as well as his (or her) clinical status, should be closely monitored. The patient must be promptly treated for any complications that arise. For example, if the patient's platelet level falls, transfusions of platelets and fresh blood must be given, as needed, to correct the condition. If disseminated intravascular coagulation begins, intravenous heparin may be effective in controlling bleeding, as in South Africa (Gear *et al.*, 1975). The close monitoring should be continued until biochemical functions and clinical status have returned to normal, a process that may require 14 or more days from the onset of disease. Specimens, especially urine samples, should be evaluated for virus isolation for several weeks. Marburg virus has a propensity for sequestering in the body in remote areas, such as the liver, semen, and eye, sometimes for as long as 3 months (Gear *et al.*, 1975; Siegert *et al.*, 1968d,e; Shu *et al.*, 1968, 1969), and late clinical complications must be considered part of the disease process (see Section III,A).

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REFERENCES

Almeida, J. D., Waterson, A. P., and Simpson, D. I. H. (1971). *In* "Marburg Virus Disease" (G. A. Martini and R. Siegert, eds.), pp. 84–97. Springer-Verlag, Berlin and New York.

Bowen, E. T. W., Simpson, D. I. H., Bright, W. F., Zlotnik, I., and Howard, D. M. (1969). *Br. J. Exp. Pathol.* **50,** 400–407.

Casals, J. (1971). *In* "Marburg Virus Disease" (G. A. Martini and R. Siegert, eds.), pp. 98–104. Springer-Verlag, Berlin and New York.

Center for Disease Control. (1975). *Morbidity Mortality Week. Rep.* **24,** 89–90.

Gear, J. S. S., Cassel, G. A., Gear, A. J., Trappler, B., Clausen, L., Meyers, A. M., Bothwell, T. H., Kew, M. C., Sher, R., Miller, G. B., Schneider, J., Koornhof, H. J., Gomperts, E. D., Isaäcson, M., and Gear J. H. S. (1975). *Br. Med. J.* **4,** 489–493.

Gedigk, P., Bechtelsheimer, H., and Korb, G. (1968). *Dtsch. Med. Wochenschr.* **93,** 590–601.

Gedigk, P., Bechtelsheimer, H., and Korb, G. (1969). *Ger. Med. Mon.* **14,** 58–67.

Gedigk, P., Bechtelsheimer, H., and Korb, G. (1971). *In* "Marburg Virus Disease" (G. A. Martini and R. Siegert, eds.), pp. 50–53. Springer-Verlag, Berlin and New York.

Haas, R., and Maass, G. (1971). *In* "Marburg Virus Disease" (G. A. Martini and R. Siegert, eds.), pp. 136–143. Springer-Verlag, Berlin and New York.

Haas, R., Maass, G., Müller, J., and Oehlert, W. (1968). *Z. Med. Mikrobiol. Immunol.* **154,** 210–220.

Henderson, B. E., Kissling, R. E., Williams, M. C., Kafuko, G. W., and Martin, M. (1971). *In* "Marburg Virus Disease" (G. A. Martini and R. Siegert, eds.), pp. 166–176. Springer-Verlag, Berlin and New York.

Hennessen, W. (1971). *In* "Marburg Virus Disease" (G. A. Martini and R. Siegert, eds.), pp. 161–165. Springer-Verlag, Berlin and New York.

Hofmann, H., and Kunz, C. (1969). *Zentralbl. Bakteriol., Parasitenkd., Infektionskr. Hyg., Abt. I: Orig.* **209,** 288–293.

Hofmann, H., and Kunz, C. (1970). *Arch. Gesamte Virusforsch.* **32,** 244–248.

Jacob, H. (1971). *In* "Marburg Virus Disease" (G. A. Martini and R. Siegert, eds.), pp. 54–61. Springer-Verlag, Berlin and New York.

Kissling, R. E., Robinson, R. Q., Murphy, F. A., and Whitfield, S. G. (1968). *Science* **160,** 888–890.

Kissling, R. E., Murphy, F. A., Henderson, B. E. (1970). *Ann. N.Y. Acad. Sci.* **174,** 932–945.

Kunz, C., Hofmann, H., Kovac, W., and Stockinger, L. (1968a). *Wien. Klin. Wochenschr.* **80,** 161–162.

Kunz, C., Hofmann, H., and Aspöck, H. (1968b). *Zentralbl. Bakteriol., Parasitenkd., Infektionskr. Hyg., Abt. I: Orig.* **208,** 347–349.

Maass, G., Müller, J., Seemayer, N., and Haas, R. (1969). *Am. J. Epidemiol.* **89,** 681–690.

Malherbe, H., and Strickland-Cholmley, M. (1971). *In* "Marburg Virus Disease" (G. A. Martini and R. Siegert, eds.), pp. 188–194. Springer-Verlag, Berlin and New York.

Martini, G. A. (1971). *In* "Marburg Virus Disease" (G. A. Martini and R. Siegert, eds.), pp. 1–9. Springer-Verlag, Berlin and New York.

Martini, G. A., Knauff, H. G., Schmidt, H. A., Mayer, G., and Baltzer, G. (1968a). *Dtsch. Med. Wochenschr.* **93,** 559–571.

Martini, G. A., Knauff, H. G., Schmidt, H. A., Mayer, G., and Baltzer, G. (1968b). *Ger. Med. Mon.* **13,** 457–470.

May, G., Knothe, H., Hülser, D., and Herzberg, K., (1968). *Zentralbl. Bakteriol., Parasitenkd., Infektionskr. Hyg., Abt. I: Orig.* **207,** 145–148.

Monath, T. P. (1974). *WHO Chron.* **28,** 212–219.

Murphy, F. A., Simpson, D. I. H., Whitfield, S. G., Zlotnik, I., and Carter, G. B. (1971). *Lab. Invest.* **24,** 279–291.

Oehlert, W. (1971). *In* "Marburg Virus Disease" (G. A. Martini and R. Siegert, eds.), pp. 144–156. Springer-Verlag, Berlin and New York.

Peters, D., and Müller, G. (1968). *Dtsch. Aerztebl.* **65,** 1831–1834.

Peters, D., Müller, G., and Slenczka, W. (1971). *In* "Marburg Virus Disease" (G. A. Martini and R. Siegert, eds.), pp. 68–83. Springer-Verlag, Berlin and New York.

Shu, H. L., Siegert, R., and Slenczka, W. (1968). *Dtsch. Med. Wochenschr.* **93,** 2163–2165.

Shu, H. L., Siegert, R., and Slenczka, W. (1969). *Ger. Med. Mon.* **14,** 7–10.

Siegert, R. (1972). *Virol. Monogr.* **11,** 98–157.

Siegert, R., and Slenczka, W. (1971). *In* "Marburg Virus Disease" (G. A. Martini and R. Siegert, eds.), pp. 157–160. Springer-Verlag, Berlin and New York.

Siegert, R., Shu, H. L., Slenczka, W., Peters, D., and Müller, G. (1967). *Dtsch. Med. Wochenschr.* **92,** 2341–2343.

Siegert, R., Shu, H. L., Slenczka, W., Peters, D., and Muller, G. (1968a) *Ger. Med. Mon.* **13,** 1–3.

Siegert, R., Shu, H. L., and Slenczka, W. (1968b). *Dtsch. Med. Wochenschr.* **93,** 604–612.

Siegert, R., Shu, H. L., and Slenczka, W. (1968c). *Ger. Med. Mon.* **13,** 514–518.

Siegert, R., Shu, H. L., and Slenczka, W. (1968d). *Dtsch. Med. Wochenschr.* **93,** 616–619.

Siegert, R., Shu, H. L., and Slenczka, W. (1968e). *Ger. Med. Mon.* **13,** 521–524.

Simpson, D. I. H. (1969a). *Trans. R. Soc. Trop. Med. Hyg.* **63,** 303–309.

Simpson, D. I. H. (1969b). *Br. J. Exp. Pathol.* **50,** 389–392.

Simpson, D. I. H., Bowen, E. T. W., and Bright, W. F. (1968a). *Lab. Anim.* **2,** 75–81.

Simpson, D. I. H., Zlotnik, I., and Rutter, D. A. (1968b). *Br. J. Exp. Pathol.* **49,** 458–464.

Slenczka, W. (1969). *Zentralbl. Bakteriol., Parasitenkd., Infektionskr. Hyg., Abt. I: Ref.* **215,** 545–546.

Slenczka, W., and Wolff, G. (1971). *In* "Marburg Virus Disease" (G. A. Martini and R. Siegert, eds.), pp. 105–108. Springer-Verlag, Berlin and New York.

Slenczka, W., Shu, H. L., Piepenburg, G., and Siegert, R. (1968a). *Dtsch. Med. Wochenschr.* **93,** 612–616.

Slenczka, W., Shu, H. L., Piepenburg, G., and Siegert, R. (1968b). *Ger. Med. Mon.* **13,** 524–529.

Slenczka, W., Siegert, R., and Wolff, G. (1970). *Arch. Gesamte Virusforsch.* **31,** 71–80.

Slenczka, W., Wolff, G., and Siegert, R. (1971). *Am. J. Epidemiol.* **93,** 496–505.

Smith, C. E. G., Simpson, D. I. H., Bowen, E. T. W., and Zlotnik, I. (1967). *Lancet* **2,** 1119–1121.

Stille, W., and Böhle, E. (1971). *In* "Marburg Virus Disease" (G. A. Martini and R. Siegert, eds.), pp. 10–18. Springer-Verlag, Berlin and New York.

Stille, W., Böhle, E., Helm, E., van Rey, W., and Siede, W. (1968a). *Dtsch. Med. Wochenschr.* **93,** 572–582.

Stille, W., Böhle, E., Helm, E., van Rey, W., and Siede, W. (1968b). *Ger. Med. Mon.* **13,** 470–478.

Strickland-Cholmley, M., and Malherbe, H. (1971). *In* "Marburg Virus Disease" (G. A. Martini and R. Siegert, eds.), pp. 195–202. Springer-Verlag, Berlin and New York.

Todorovitch, K., Mocitch, M., and Klasnja, R. (1971). *In* "Marburg Virus Disease" (G. A. Martini and R. Siegert, eds.), pp. 19–23. Springer-Verlag, Berlin and New York.

Wulff, H. (1977). In preparation.
Wulff, H., and Lange, J. V. (1975). *Bull. WHO* **52,** 429–436.
Zlotnik, I. (1969). *Trans. R. Soc. Trop. Med. Hyg.* **63,** 310–323.
Zlotnik, I. (1971). *In* "Marburg Virus Disease" (G. A. Martini and R. Siegert, eds.), pp. 129–135. Springer-Verlag, Berlin and New York.
Zlotnik, I., and Simpson, D. I. H. (1969). *Br. J. Exp. Pathol.* **50,** 393–399.
Zlotnik, I., Simpson, D. I. H., and Howard, D. M. R. (1968). *Lancet* **2,** 26–28.

Chapter 2

Diagnosis of Hepatitis Viral Infections

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I. INTRODUCTION

Many viruses may infect the liver of animals and man and may produce severe disease. Viruses that are important in animals include infectious canine hepatitis, duck and turkey hepatitis, Rift Valley Fever virus, ectromelia, and the group of mouse hepatitis viruses. In man, hepatitis A and hepatitis B viruses and yellow fever virus are the most important causes of acute inflammation and necrosis of the liver. Inflammation of the liver is also frequently associated with other common viral infections such as cytomegalovirus and Epstein-Barr (EB) virus. In addition, there are a number of viruses which do not normally cause liver damage but nevertheless display occasionally increased hepatotropism, resulting sometimes in jaundice and a clinical picture which may be primarily that of the systemic infection or of hepatitis. This group of viruses includes adenoviruses, rubella virus, paramyxoviruses, a number of enteroviruses, particularly coxsackie A and B viruses, and herpes simplex virus. The significance of these viruses in infections of the liver has been reviewed in detail elsewhere (Zuckerman, 1970a). However, the term viral hepatitis commonly refers to hepatitis caused by virus types A and B. In the following account these are considered in some detail.

Viral hepatitis is a major public health problem occurring endemically in all parts of the world. The existence of at least two distinct types of acute viral hepatitis in man, differing by their incubation periods and primary modes of transmission, has been recognized for some years. The predominant association of hepatitis A with an epidemic setting and the more sporadic nature of hepatitis B and its predominant association with a parenteral mode of transmission have also been long recognized. The most common route of transmission of hepatitis A is fecal–oral, primarily by person-to-person contact. The infection can also be transmitted by the parenteral and possibly other routes. The incubation period is between 3 and 5 weeks with a mean of 28 days. In the past, hepatitis type B was principally defined on the history of probable parenteral exposure to infection and a long incubation period of several months. Evidence is now available for inapparent parenteral transmission and, in addition, at least good circumstantial evidence of spread of hepatitis B by nonparenteral routes. The incubation period may vary within wide limits from about 14 up to 180 days.

The two clinically and pathologically similar forms of viral hepatitis can now be differentiated by specific laboratory techniques for antigens and antibodies associated with type A and type B infections. There is also recent epidemiological and some serological evidence that a hepatitis virus C may exist, but precise virological criteria are not yet available.

It should be noted that acute viral hepatitis is a generalized or systemic infection with particular emphasis on inflammation of the liver. The clinical picture of hepatitis ranges, therefore, in its presentation from inapparent or subclinical infection, slight malaise, mild gastrointestinal symptoms and the anicteric form of the disease, acute icteric illness, severe prolonged jaundice, chronic liver disease, to acute fulminant hepatitis. The incidence of individual symptoms and signs varies, therefore, both in different epidemics and in sporadic cases.

II. THE PATHOLOGY OF VIRAL HEPATITIS

Parenchymal cell necrosis and histiocytic periportal inflammation are the two constant features in acute viral hepatitis. In general, the reticulin framework of the liver is well preserved except in some cases of massive and submassive necrosis. The liver cells show various forms of necrotic changes. The necrotic areas are usually multifocal, but necrosis frequently tends to be zonal with the most severe changes occuring in the centrilobular areas. Individual hepatocytes are commonly swollen and may show ballooning, but they can shrink. Swollen cells typically show a granular "ground glass" appearance in the cytoplasm and shrunken cells give rise to acidophilic bodies. Dead or dying rounded liver cells are extruded into the perisinusoidal space. The variations in the size of the nuclei and in their staining quality is useful in diagnosis. Fatty changes in the liver are conspicuous by their absence.

A mononuclear cellular infiltration, which is particularly marked in the portal zones, is the characteristic mesenchymal reaction. This is also accompanied by some bile ductular proliferation. In some cases, during the early phases of the illness, polymorphonuclear leukocytes and eosinophils may be prominent. The mononuclear inflammatory changes are scattered throughout the sinusoids and in all parts of the lobules involved in focal necrosis. The lost hepatocytes are replaced mainly by mononuclear cells. The Kupffer cells and endothelial cells proliferate and are enlarged. The Kupffer cells often contain excess lipofuscin pigment. In the icteric phase of the average case of hepatitis, the wall of the tributaries of the hepatic vein may be thickened, frequently infiltrated, and the proliferation of the lining cells in the terminal hepatic veins would justify the term "endophlebitis."

Cholestasis may occur in the early stages of viral hepatitis and plugs of bile thrombi may be seen in the bile canaliculi. Occasionally, cholestatic features dominate the picture but spotty necrosis is almost invariable.

Necrosis with the associated inflammatory reaction is generally less

severe in anicteric hepatitis; whereas in fulminant hepatitis there is rapid massive hepatic cell necrosis.

At a later stage of the acute infection there is often a variable degree of collapse and condensation of reticulin fibers, and accumulation of ceroid pigment and stainable iron in large phagocytic cells, within the lobules and later also in the portal tracts.

Repair of the liver lobule occurs by regeneration of the hepatocytes. Frequent mitoses, polyploidy, atypical and binucleated cells are found. There is a gradual disappearance of the mononuclear cells from the portal tracts, but elongated histiocytes and fibroblasts may remain. The outcome of acute viral hepatitis may be morphologically complete resolution, recurrence, fatal massive necrosis, chronic persistent or aggressive hepatitis, resolution with scarring and cirrhosis. Cirrhosis may result from extensive confluent necrosis leading to passive septum formation, architectural distortion, and nodular hyperplasia, or it may follow chronic aggressive hepatitis or both processes may occur simultaneously. Chronic liver disease following acute viral hepatitis may thus be the result of necrosis, collapse of the reticulin framework, the formation of scars, and nodular hyperplasia. Other factors may include complex immunological processes and/or the persistence of the virus in the liver.

III. HEPATITIS B

Early studies of Australia antigen revealed the antigen to be a lipoprotein. The antigen was found relatively frequently in the serum of patients with acute leukemia, and it was suggested that its presence might be of value in the early diagnosis of this disease and that the antigen may be related to the postulated leukemia virus (Blumberg *et al.*, 1965). The later discovery of the close association between Australia antigen and type B hepatitis resulted in rapid and dramatic progress in the understanding of the epidemiology, and the clinical and immunopathological behavior of this disease. Australia antigen is now referred to as hepatitis B surface antigen (HB_sAg), one of at least three complex antigens associated with the hepatitis B virus.

A. The Epidemiology of Hepatitis B

The epidemiological concepts of hepatitis B infection have recently undergone a significant change. The importance of the parenteral and inapparent parenteral routes of transmission of hepatitis B virus is still recognized. However, the demonstration experimentally that hepatitis B virus

was infective by mouth and the finding that the infection was endemic in closed institutions, the prevalence of infection in adults in urban communities, the carrier rate and age distribution of hepatitis B surface antigen in different geographical regions, and the relatively high incidence in poor socioeconomic environments have altered the epidemiological dogma that hepatitis B was spread exclusively by blood and blood products through the parenteral route. Indeed, it has become increasingly difficult to attribute the entire spectrum of hepatitis B infection to parenteral transmission. Although various body fluids such as saliva, menstrual and vaginal discharges, semen, amniotic fluid, colostrum, and breast milk have been implicated in the spread of infection, infectivity appears to be essentially associated with blood and blood products. There is some evidence for the transmission of hepatitis B by intimate personal contact and considerable circumstantial evidence of spread of the infection by the venereal route. There are numerous reports of surface antigen and surface antibody findings in sexual partners, and a high prevalence of both antigen and antibody among patients attending venereal disease clinics. However, there is no information on the specific mechanism of transmission.

Although the modes of transmission of hepatitis B in the tropics are similar to those in other parts of the world, additional factors may be of importance, including traditional tattooing and scarification, ritual circumcision, and repeated biting by blood-sucking arthropod vectors. The reported detection of hepatitis B surface antigen in pools of mosquitoes of a variety of species caught in the wild in both warm climate and temperate regions remains controversial. Convincing evidence of replication of hepatitis B virus or persistence of the surface antigen after digestion of a blood meal has not been obtained. Nevertheless, mechanical transmission, for example, as a result of interrupted and consecutive feedings, is a possibility.

The importance of perinatal transmission of hepatitis B to babies born to asymptomatic carriers of the surface antigen appears to vary in different geographical areas. On the other hand, children born to women who contract acute hepatitis B infection during the last two trimesters of pregnancy or during the first 2 months postpartum run a much greater risk of neonatal infection. Most of these infants do not develop clinical hepatitis, but frequently display biochemical and histological evidence of subclinical infection and antigenemia that may be persistent. A proportion of these children have subsequently been found to suffer from chronic liver damage.

The introduction of advanced therapeutic procedures such as maintenance hemodialysis, transplantation, and treatment with immunosuppres-

sive and cytotoxic drugs has created new and potent sources of spread of hepatitis B virus. Further details on the epidemiology of hepatitis B infection may be found in recent reviews (World Health Organization, 1973, 1975; Zuckerman, 1975a).

B. The Antigenic Complexity of Hepatitis B

Careful serological studies and detailed analysis, discussed recently by Le Bouvier and Williams (1975), revealed that the particles bearing hepatitis B surface antigen activity share a common group specific antigen *a* and the particles generally carry at least two mutually exclusive subdeterminants either *d* or *y* or *w* or *r*. There is evidence that the subtypes are the phenotypic expressions of distinct genotype variants of hepatitis B virus. Four principal phenotypes have been generally recognized: *adw, adr, ayw,* and *ayr;* however, others are not precluded. Indeed, complex permutations of these subdeterminants and new variants have been described, all apparently on the surface of the same physical particles. A remarkable geographical pattern of distribution of hepatitis B subtypes has emerged with four global zones, where there is an excess of one subtype and regions where a mixture of subtypes is common. These subtypes provide valuable epidemiological markers and offer a method for distinguishing one of several sources of infection. These surface antigenic reactivities do not appear to be associated with particular clinical forms of liver disease.

C. The *e* Antigen–Antibody System

Magnius and Espmark (1972) described a new distinct precipitating antigen, which they termed *e*, in some sera containing hepatitis B surface antigen. This antigen differs immunologically from the previously described determinants of the surface antigen and it also has different physicochemical properties (Magnius, 1975). Paradoxically, antibody against *e* was found in serum specimens from healthy carriers. The *e* antigen seemed to be somehow intimately associated with the pathogenesis of liver damage. Nielsen *et al.* (1974) found the *e* antigen by the immunodiffusion technique to be significantly more common in patients with chronic liver disease with persistent hepatitis B antigenemia than in patients with acute viral hepatitis. Furthermore, the *e* antigen seemed to be a valuable prognostic marker since progression to chronic liver disease was recorded by serial liver biopsies in 11 out of 19 consecutive patients with surface antigen-positive acute hepatitis associated with *e*. The clinical significance of the *e* antigen was supported by differences in the clin-

ical, biochemical, and histological findings between the patients with the *e* antigen and those without this antigen during the initial phase of viral hepatitis. Magnius *et al.* (1975) also found the *e* antigen more frequently in surface antigen carriers with histological evidence of chronic persistent or chronic aggressive hepatitis. Other observations also linked the *e* antigen with infectivity. It was commonly found in patients receiving treatment by maintenance hemodialysis and the antigen also appeared in the serum early in the incubation period of acute hepatitis B, at about the time of appearance of the surface antigen and before elevation of the serum transaminases. Furthermore, most of twelve healthy surface antigen carriers who had donated blood without producing clinical hepatitis in recipients possessed anti-*e*, which suggests that when *e* antibody is present the carriers may no longer be infectious.

The association of *e* antigen with chronic liver disease has been confirmed more recently by a number of studies (El Sheikh *et al.*, 1975; Elefthériou *et al.*, 1975; Feinman *et al.*, 1975), whereas the presence of anti-*e* was associated with only minor histological changes in the liver.

The exact nature of the *e* antigen is uncertain. This antigen is known to exist free in the serum and it has been suggested as an antigen bound to the Dane particle and, in particular, to the core. Howard and Zuckerman (1975) proposed that *e* antibodies could be explained as an autoimmune response to an antigen normally sequestered within the liver cell mitochondria but released in large quantities in infection with hepatitis B virus as a result of extensive mitochondrial degradation. The possibility that the *e* antigen might be a host antigen synthesized by virus-infected liver cells was also suggested by Magnius *et al.* (1975).

D. The Morphology of Hepatitis B Antigens

Using the electron microscope, Bayer *et al.* (1968) examined hepatitis B surface antigen-rich fractions of serum that had been separated sequentially by rate-zonal centrifugation and electrophoresis. Serum containing this antigen was obtained from three different patients: one suffering from acute myelogenous leukemia, another with chronic reticuloendotheliosis, and the third patient with Down's syndrome and chronic hepatitis. Negative staining revealed spherical particles, with a diameter of 19 to 21 nm and 3 nm surface subunits, and elongated particles varying in length from 50 to 230 nm.

It is interesting to note that particles very similar, if not identical, to hepatitis B antigen, and consisting of three morphological entities—small spherical particles, rodlike structures, and double-shelled spheroidal particles—were previously described by Harris *et al.* (1966), after dif-

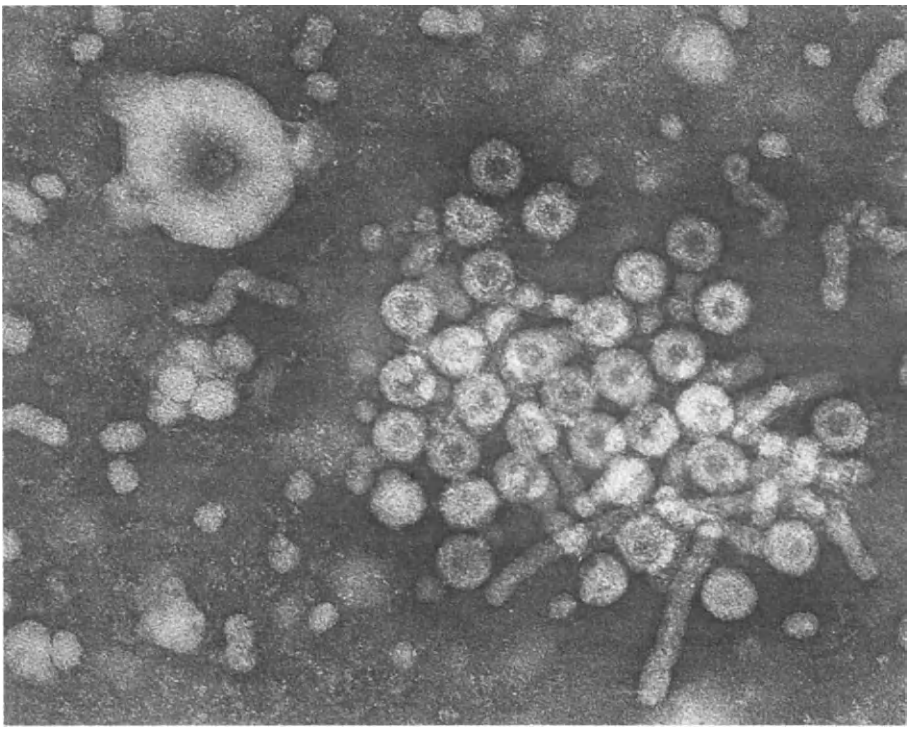

Fig. 1. Serum containing the three morphological entities of hepatitis B: small pleomorphic spherical particles (approximately 22 nm in diameter), tubular forms of varying lengths, and the double-shelled spheroidal Dane particles (approximately 42 nm in diameter). × 163,800. (Electron micrograph reproduced with permission from Zuckerman, "Human Viral Hepatitis." Am. Elsevier, New York, 1975.)

ferential centrifugation of plasma obtained from two patients, one with chronic lymphocytic leukemia, and the other with lymphocytic lymphosarcoma. These particles were recognized then under the electron microscope as unique viruslike structures, and in one of the patients the particles persisted over a period of months. Almeida *et al.* (1969) further characterized the antigen by applying the technique of immune electron microscopy using sera from a proved healthy carrier of hepatitis B virus, and two young drug addicts in the acute phase of typical hepatitis B infection. Dane *et al.* (1970) described the morphology of double-shelled 42-nm spheroidal particles in the serum of patients with antigen-associated hepatitis. These particles consisted of a core of about 27 nm in diameter with a 2-nm shell and an outer coat or envelope of about 7 nm in thickness. The small and large spherical particles and the tubules (Fig. 1) formed a mixed immune aggre-

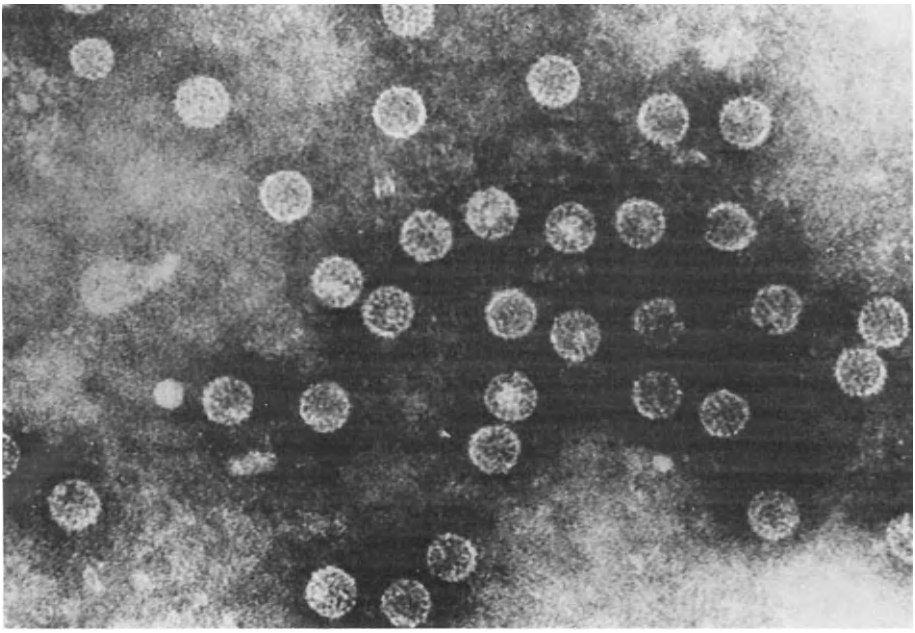

Fig. 2. The inner component or core of the Dane particle. × 182,000. (Electron micrograph kindly provided by Dr. June D. Almeida and reproduced with permission from "Human Viral Hepatitis" Am. Elsevier, New York, 1975.)

gate when antiserum to hepatitis B antigen was added, indicating that all three morphological entities share a common surface antigen. Dane and his colleagues suggested that the 42-nm particles represent the virus of type B hepatitis, whereas the small particles and the tubular forms of the antigen correspond to noninfectious surplus virus coat material.

After examination by electron microscopy, Almeida *et al.* (1971) described a second and distinct antigen–antibody system in hepatitis B virus infection. After detergent treatment of pellets of antigen obtained by ultracentrifugation of whole serum, the 42-nm particles separated into an outer coat of hepatitis B surface antigen and an inner component (or core), 27 nm in diameter (Fig. 2). Antibody in convalescent hepatitis B serum reacted with the core to yield immune aggregates (Fig. 3) resembling those seen in homogenates of liver taken postmortem from patients with type B hepatitis. The core antibody has a specificity entirely different from antibody against the surface antigen coat. The demonstration by immune electron microscopy that the core antigen (HB_cAg) and its antibody are immunologically distinct has been confirmed by immunofluorescence studies localizing the core antigen in the nuclei of hepatocytes, by

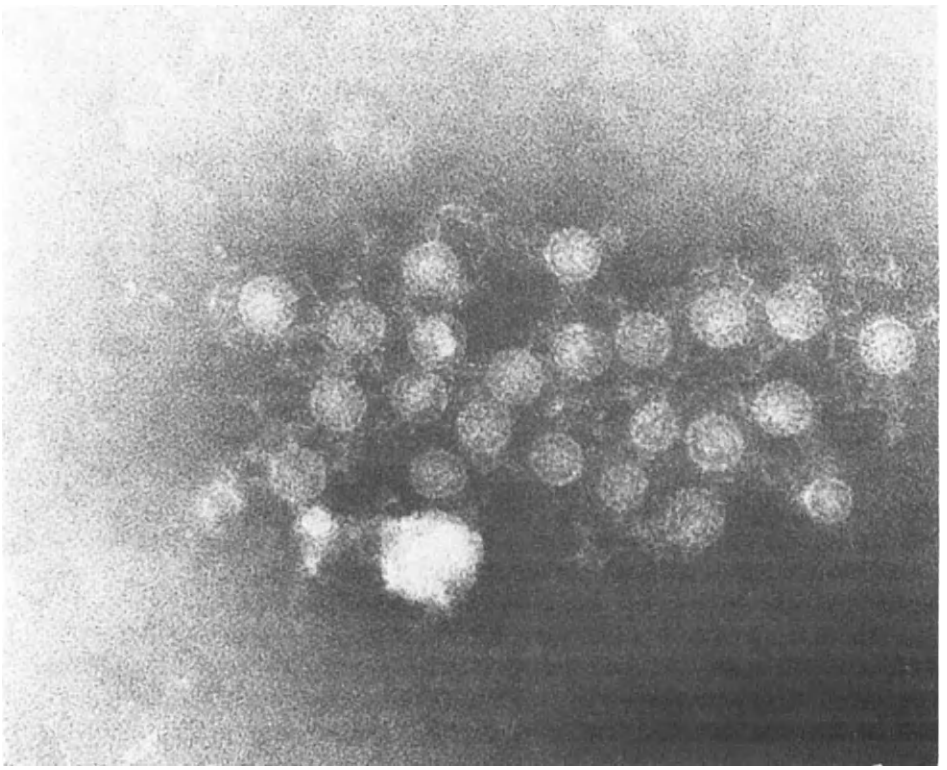

Fig. 3. An aggregate of the core particles. The particles are bridged by antibody molecules. × 300,000. (Electron micrograph kindly provided by Dr. June D. Almeida and reproduced by permission of the Editor of *Microbios*.)

complement fixation, by counterimmunoelectrophoresis, and by radioimmunoassay.

E. Properties of Hepatitis B Surface Antigen

The location of antigenic determinants on particles of various morphological forms has facilitated the isolation of this antigen from normal serum proteins to a standard of purity suitable for chemical and serological analysis. Early studies by flotation centrifugation and the staining properties of precipitin lines indicated that the surface antigen contains both lipid and protein. The lipoprotein nature of the surface antigen allows a partial separation from other serum proteins by virtue of its characteristic buoyant density. Antigenic activity is found at a density within the range defining one of the two major subclasses of serum high-density lipoproteins

(HDL_3: 1.08–1.21 g/ml), although the two species may be readily distinguished by the much smaller diameter of the HDL_3 fraction (8–14 nm). The exact buoyant density of the surface antigen varies between sera and the chemical employed in forming the density gradient. Centrifugation of serum in buffered cesium chloride results in the isolation of antigen at an average density of 1.20 g/ml, although immune complexes are thought to be represented by a second band of antigenic activity at 1.25 g/ml (Gerin *et al.*, 1969). Although the tubular forms are found in the same fraction, only a proportion of the 42-nm particles are recovered at this density. Full, or partially full 42-nm particles are recovered at the slightly higher density of 1.25 g/ml after equilibrium centrifugation in cesium chloride (Gerin *et al.*, 1975).

It should be noted that purified small particles, which comprise the bulk of the surface antigenic mass in most sera, have been the most common preparations used for chemical and serological analysis. All three major morphological forms may be resolved by rate zonal centrifugation, the relatively slowly sedimenting small particles possessing a mean sedimentation coefficient ($s_{20,w}$) in the range 33–54 S. The diffusion constant of the small particle was estimated by Le Bouvier and McCollum (1970) to be about 2×10^{-7} cm^2/sec by measuring the position of equivalence in immunodiffusion studies. Detailed analysis in the analytical ultracentrifuge has subsequently given a value of 2.278×10^{-7} cm^2/sec (Kim and Tilles, 1973). These values are compatible with an estimated molecular weight of 2.4×10^6, a value in good agreement with the estimate of 2.5×10^6 obtained by gel filtration (Skinhøj and Hansen, 1973).

The lipid content of purified small particles may account for up to 30% of its total weight. Analysis of a chloroform–methanol extract by thin-layer chromatography in silica gel revealed a predominance of polar lipids together with cholesterol and smaller quantities of nonpolar lipids (Kim and Bissell, 1973; Steiner *et al.*, 1974; Takahashi, 1975). Phosphatidylcholine, sphingomyelin, and lysophosphatidylcholine were found to be the major phospholipids present. Phosphatidylserine was noticeably absent and phosphatidylethanolamine could not be detected in one study (Steiner *et al.*, 1974).

The protein moiety of surface antigen-bearing particles has been extensively analyzed in several laboratories. Examination of purified small spherical particles by ultraviolet absorption spectroscopy produces an absorption spectrum typical of protein. A substantial tryptophan content of approximately 14% (Rao and Vyas, 1974) appears to account for the somewhat high extinction coefficient of this material ($E^{1\%}_{280\,nm} = 37.26$: Vyas *et al.*, 1972b), although other coefficients in the range of 25 to 30 have been reported (Ling and Overby, 1972; Takahashi, 1975). Surface antigen

is, in addition, rich in hydrophobic amino acids, particularly leucine, which would facilitate its close relationship with lipid. Optical rotatory dispersion and circular dichroism studies indicate that 70–80% of the total protein content exists as α-helix (Sukeno *et al.*, 1972b). It should be noted that proline,which contributes 11.6–13.6% of the total amino acids, does not take part in α-helix formation. A similar high α-helix content occurs in serum LDL and the filamentous bacteriophages, but other viruses, in general, possess 10–25% α-helix.

The antigen has also been subjected to extensive analysis of its protein polypeptide composition (Gerin, 1972; Dreesman *et al.*, 1972a; Rao and Vyas, 1973; Chairez *et al.*, 1974; Howard and Zuckerman, 1974; and others). Initial studies indicated the presence of two major polypeptide species with average molecular weights of 25,000 and 30,000. Other components of higher molecular weight, present in variable amounts at certain stages of purification, were assumed to be contaminating serum proteins which may have a stabilizing role in preserving antigenic activity. However, further studies have shown the presence of both larger and smaller polypeptide components. In addition, reproducible differences have been reported between surface antigen of *ad* and *ay* subtype, with *ay* material in several studies possessing additional minor components (Chairez *et al.*, 1973, 1975; Gerin, 1972). In another study, however, no fundamental qualitative difference was found between material of both subtypes (C. R. Howard, S. Washford, and A. J. Zuckerman, personal communication, 1975). At least some of the separated components are identifiable by the periodic acid-Schiff staining of acrylamide gels, revealing the presence of glycopeptides (Chairez *et al.*, 1973). The wide discrepancies between reported observations has hindered the determination of the size of the hepatitis B virus genome and its products. These findings are reviewed in detail by Howard and Burrell (1976).

Very little information is available on the quaternary structure of surface antigen particles and its importance in maintaining antigenic integrity. Some indication of its probable importance in the preservation of at least some of the surface antigenic determinants is illustrated by the reaction of the *t* determinant with its antibody (Le Bouvier and Williams, 1975). Immunodiffusion tests showed the presence of *t* on the surface of *adw* particles only. However, sera of other phenotypes, notably *ayw*, consumed *t* antibody without the formation of a precipitin line. This determinant was therefore regarded as being "cryptic" on the *ayw* particles.

Studies involving the treatment of purified surface antigen with organic solvents and dissociating reagents revealed that antigenic activity was remarkably stable in the presence of compounds promoting denaturation, in particular, diethyl ether, urea, sodium dodecyl sulfate, and various pro-

teolytic enzymes. Anan'ev *et al.* (1972) showed there was no loss of reactivity following treatment with 50% chloroform or diethyl ether. However, there was a complete loss after exposure to ethanol. A similar loss has also been reported after treatment with butanol (Jozwiak *et al.*, 1971).

Several reports have demonstrated the surface antigen to be stable for many hours at an acidic pH (Anan'ev *et al.*, 1972; Dreesman *et al.*, 1972b). Kim *et al.* (1971) found that treatment of a serum pool by fivefold dilution with 0.02 *N* HCl, pH 2.3, containing 0.02% pepsin provided antigen free of normal serum proteins. This preparation was suitable for the immunization of both guinea pigs and rabbits. However, it was noted by Kim and Bissell (1973) that pretreatment with sodium dodecyl sulfate or diethyl ether increased the susceptibility of the antigen to proteolytic enzymes. Superficial lipid may, therefore, exert a protective effect on antigenic determinants composed primarily of protein.

The reduction of disulfide bonds results in the complete loss of surface antigen reactivity (Vyas *et al.*, 1972a; Sukeno *et al.*, 1972a), although considerable antigenic activity may be regained by the alkylation of free sulfhydryl groups with iodoacetamide. Imai *et al.* (1974) were able to define by the use of immunodiffusion and hemagglutination-inhibition techniques reduction-sensitive and reduction-resistant components of the antigen. The group determinant *a* was destroyed by exposure to dithiothreitol at concentrations below 10 m*M*. At higher concentrations, resistance to reduction was serologically detected in all surface antigen preparations examined, regardless of the subtype determinants present.

The reactivity of the surface antigen is remarkably heat-stable. Anan'ev *et al.* (1972) found no loss of antigenic activity after exposing purified antigen for 10 hours at 60°C but heating for 5 minutes at 100°C completely abolished its affinity for antibody. Similarly, Millman *et al.* (1970) noted a total loss of antigenic activity following 60 minutes incubation at 85°C. In a detailed study, W. W. Bond, B. L. Murphy, M. S. Favero, J. E. Maynard and N. J. Peterson (personal communication, 1974) demonstrated that the *a* group-specific determinant was stable at 60°C for periods of up to 21 hours, whereas the *d* and *y* subtype reactivities were markedly reduced after only 3 hours of incubation at the same temperature.

The stability of the surface antigen at high temperatures together with its resistance to protease digestion strongly suggests the presence of carbohydrate. In addition, the precipitation of radiolabeled antigen by concanavalin A and a positive anthrone reaction indicate that carbohydrate may be present as well as lipid and protein. The possibility that carbohydrate may play a role in maintaining serological activity was investigated by Burrell *et al.* (1973). A 90% reduction in the serological activity of puri-

fied surface antigen particles was found after treatment with 0.1 *M* sodium periodate for 4 hours at 37°C. A significant amount of carbohydrate relative to the protein content was found in the same preparations by the phenol–sulfuric acid method. Chairez *et al.* (1973) estimated a 3.6–6.5% carbohydrate content by the same method. Further work has indicated that at least a proportion of this moiety may be present as glycolipid (Steiner *et al.*, 1974). However, the possibilities of carbohydrate either preserving the structural integrity of adjacent antigenic sites or possessing a novel haptenic specificity have yet to be distinguished.

There have been several recent attempts to raise specific antisera in animals using individual polypeptides separated by acrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. Dreesman *et al.* (1975) isolated from purified surface antigen of subtypes *adw* and *ayw* three glycopeptides of molecular weights 19,000, 24,000, and 27,000 and two larger nonglycosylated polypeptides of molecular weights 35,000 and 40,000. The 19,000 glycopeptide from subtype *ayw* together with the 27,000 molecular weight glycopeptides from both sources failed to elicit an antibody response in guinea pigs. The nonglycosylated polypeptides derived from both subtypes elicited antibodies which cross-reacted with intact surface antigen particles in a radioimmunoprecipitation assay. Both polypeptides were, therefore, assumed to contain at least the *a* group-specific determinant. However, the 24,000 molecular weight glycopeptide from both sources produced antibodies which reacted only with the homologous antigen subtype. Further studies demonstrated a cell-mediated immune response to the 24,000 and 40,000 molecular weight components (Cabral *et al.*, 1975). Peritoneal exudate cells from guinea pigs inoculated with the 40,000 molecular weight polypeptide showed a significant response when challenged with intact homologous and intact heterologous surface antigen particles. Exudate cells from animals immunized with the 24,000 molecular weight glycopeptide derived from subtype *adw* antigen responded to intact homologous antigen and its 24,000 and 40,000 molecular weight components. A poor response to surface antigen of subtype *ayw* was observed in these animals.

Shih and Gerin (1975) were also successful in raising antibodies to polypeptide components of the surface antigen. Antisera to seven polypeptides obtained by sodium dodecyl sulfate-acrylamide gel electrophoresis of surface antigen subtype *adw* were found to react with *ad* and *ay* coated red blood cells by passive hemagglutination assay, indicating that each of the seven polypeptides possessed at least one common group-specific determinant. Competition inhibition experiments with intact *ad* particles as the competing antigen resulted in parallel slopes for the antisera. The

displacement of the linear portion of the inhibition curve reflected a difference in binding affinity of these antisera for the intact surface antigen particle. Further characterization using the passive hemagglutination assay for antibody subtype analysis has shown that each polypeptide stimulated subtype-specific as well as group-specific antibodies (Gerin, 1975).

Although the surface antigen preparations in both of these studies contained no demonstrable normal human serum proteins, Cabral *et al.* (1975) demonstrated a positive cell-mediated immune response in guinea pigs immunized with normal human serum when challenged with the 24,000 molecular weight glycopeptide isolated by Dreesman *et al.* (1975). This finding suggests that the 24,000 molecular weight glycopeptide contains at least one antigenic determinant related to certain constituents of normal human serum.

Several workers have previously suggested that surface antigen particles may contain traces of normal serum components. Millman *et al.* (1971) found that purified antigen produced precipitin lines in immunodiffusion tests with antisera to several human serum components after treatment with 1% Tween 80. Howard and Zuckerman (1974) demonstrated that surface antigen reactivity in unfractionated serum is closely associated with many normal serum components. Recently, Neurath *et al.* (1974) reported that the surface antigen was specifically adsorbed to immunoadsorbent columns containing sheep anti-human plasma immunoglobulins covalently linked to Sepharose 4B. Prior treatment of purified surface antigen with proteases and nonionic detergents, in the presence or absence of diethyl ether, failed to prevent adsorption of the antigen to columns containing antisera to prealbumin, albumin, apolipoproteins C and D, and the γ chain of immunoglobulin G, indicating that antigenic determinants related to host proteins were integral components of surface antigen particles. Reduction and alkylation of the preparation abolished surface antigen reactivity but did not prevent its adsorption, indicating that the antigen-associated antigenic determinants related to plasma proteins were distinct from the group- and subtype-specific determinants of the surface antigen. Burrell (1975) also reported the presence of additional antigenic determinants in close association with surface antigen particles. Low affinity immunoprecipitation reactions with antisera to a range of normal human serum components were demonstrated. These determinants were not released by exposure to acid, Tween 80, or ether, but were removed by exposure of the surface antigen to trypsin or bromelain under conditions that otherwise preserved the structure of the small particles.

F. Properties of Hepatitis B Core Antigen

The description of the 42-nm double-shelled hepatitis B antigen particle by Dane *et al.* (1970) was closely followed by the demonstration of a unique antigenic specificity on the surface of the core component (hepatitis B core antigen). Jokelainen *et al.* (1970) had previously suggested that the core possessed a nucleoprotein following examination of the particles in the electron microscope after staining with 5% uranyl acetate. The other morphological forms of the antigen remained unstained. The suggestion that the core may represent the nucleocapsid of hepatitis B virus is strengthened by the finding of similar particles in liver homogenates obtained postmortem from cases of chronic hepatitis. It remains to be demonstrated, however, that particles obtained from liver homogenates and the core of the circulating 42 nm particles are indeed identical. Both types of particles carry similar antigenic determinants, since antisera to particles purified from liver have been shown to react with cores prepared from serum (Barker *et al.*, 1974; Moritsugu *et al.*, 1975).

Circulating core antigen has been closely identified with 42 nm particles separated by equilibrium centrifugation from the small spherical and tubular forms of the surface antigen by virtue of their slightly higher buoyant density of 1.24 to 1.27 g/ml in cesium chloride (Gerin, 1974; Chairez *et al.*, 1974). The buoyant density of the core may be estimated after the removal of the outer surface envelope by treatment with nonionic detergents, although the exact value may vary according to the completeness of removal of the surface antigen. After treatment of the 42-nm particles with 1% Nonidet P-40, Gerin (1974) recovered core particles with a density of 1.31 g/ml which appeared to be aggregated by small protein molecules. The latter were considered to be either core antibody or a "matrix" protein situated between the core antigen and the surface antigen and not removed by the nonionic detergent. Moritsugu *et al.* (1975) recovered core antigen at a similar density, but this material still contained traces of surface antigen, whereas a heavier population of 1.35 to 1.36 g/ml contained no detectable surface antigen. In this context, it should be noted that core antigen-reactive particles extracted from infected human liver by Hirschman *et al.* (1974a) had a buoyant density of 1.30 g/ml. In a similar study of particles obtained from the liver of an experimentally infected chimpanzee, Barker *et al.* (1974) found core antigen activity in fractions with densities of 1.30 to 1.33 g/ml.

Several studies have indicated that the core possesses icosahedral symmetry. Almeida *et al.* (1971) compared the symmetry of the core to that of the small icosahedral viruses. Traavik *et al.* (1973) found that treatment of the 42-nm particles with Tween 80 released a structure consisting of an

outer shell possessing capsomere-like units approximately 4 nm in diameter. This observation is consistent with the study of Yamada *et al.* (1973) who demonstrated by optical rotation electron microscopy the icosahedral symmetry of the core of the 42-nm particle. Examination by electron microscopy of particles purified from the liver of an experimentally infected chimpanzee suggested a subunit structure organized according to the principles of icosahedral symmetry (Barker *et al.*, 1974).

Additional circumstantial evidence that the 42-nm particle may represent the etiological agent of hepatitis B is provided by the finding of a core antigen-associated DNA-dependent DNA polymerase activity in close association with a DNA template. A preliminary report by Hirschman *et al.* (1971) suggested the existence of an endogenous activity contained in pelleted hepatitis B antigen prepared from four acute hepatitis sera. All the pellets contained some 42-nm particles under the centrifugation conditions adopted. A significant advance on these studies was the identification by Kaplan *et al.* (1973) of a DNA polymerase activity in close association with the core. In all the preparations examined, which had been selected on the basis of a high proportion of the 42-nm particles, a moderate rate of incorporation of ^{3}H-TTP into an acid-insoluble product was detected over a period of 6 hours of incubation at 37°C. No exogenous template was required. The reaction was stimulated by magnesium ions and an optimum pH of 7.7. The inclusion of the detergent Nonidet P-40 enhanced considerably the rate of incorporation of ^{3}H-TTP. The product remained firmly bound to the core as shown by the specific precipitation of trace label by core antibody (Greenman and Robinson, 1974). Detergent treatment, therefore, appears to be necessary for the activation of the polymerase reaction by removing the outer surface antigen envelope from the 42-nm particle. Exposure of the core antigen during this reaction results in trace labeled material suitable for use in radioimmune procedures for the detection of core antibody. Centrifugation studies demonstrated that the radiolabel cosediments with a rapidly sedimenting fraction of released core components possessing a sedimentation coefficient of 110 S.

The nature of the product was investigated further after phenol extraction of cores treated with sodium dodecyl sulfate and mercaptoethanol. Approximately 20% of the acid-precipitable label was recovered in the aqueous phase and it was subsequently found to possess a buoyant density typical of DNA, banding at 1.71 g/ml (Kaplan *et al.*, 1973). The double-stranded nature of this material was revealed by its resistance to degradation by single-stranded nuclease (s_1) and the demonstration that its sedimentation coefficient of 15 S remained unchanged over a wide range of salt concentrations (Robinson, 1974).

The failure to inhibit the synthesis of DNA *de novo* by either DNase or

RNase necessitated the disruption of purified cores in order to characterize the endogenous template. Double-stranded DNA has been isolated from circulating 42-nm particles (Robinson, 1974) and also from particles closely resembling cores from the nuclei of infected hepatocytes (Hirschman *et al.*, 1974b). Core particles isolated from plasma were concentrated more than 1000-fold before activation of the polymerase reaction and disruption with sodium dodecyl sulfate. The exposed extraparticulate DNA was examined by shadow casting electron microscopy and found to consist of circular nucleic acid molecules with a mean contour length of $0.79 \pm 0.09\ \mu m$, corresponding to a molecular weight of approximately 1.6×10^6. No single structures were seen after exposure to 40% formamide thereby confirming the double-stranded nature of this material. Similar observations were made if the polymerase reaction was omitted, indicating that the double-stranded circular form was not modified by, or was a product of, the polymerase reaction. In neither instance were supercoiled structures seen. Thermal denaturation studies indicated a G+C content of 48 to 49%, a value somewhat lower than the 56% approximation of Hirschman *et al.* (1974b) who similarly found double-stranded DNA in corelike particles obtained from the nuclei of infected hepatocytes.

Summers *et al.* (1975) confirmed that the 15 S DNA structure isolated from the 42-nm particles in serum is circular. However, fragmentation with the restriction enzyme endonuclease R. Hae III both before and after *in vitro* DNA replication suggested the existence of single-stranded gaps along 10–20% of the total molecule length. These single-stranded regions may have contributed to the wide range of molecular lengths previously observed in the electron microscope since the length of single-stranded DNA molecules is strongly dependent on ionic conditions. In that study, the endogenous polymerase reaction appeared to repair the single-stranded gap in the double-stranded circular DNA. Further, the ability of a polymerase obtained from avian myeloblastosis virus to synthesize a DNA product indicates that the specificity observed in the endogenous reaction appears to reside with the template DNA and not in the core antigen-associated DNA polymerase. The latter may, in fact, be host derived, a supposition supported by the failure to detect this enzyme activity in the corelike particles present in the nuclei of infected hepatocytes. Hirschman (1975) suggested that this activity may be acquired during passage of core particles through the cytoplasm of the hepatocyte.

A model of replication that does not require an exogenous primer has been proposed by Overby *et al.* (1975). Open double-stranded circles were seen by electron microscopic examination of detergent-treated Dane particles obtained by centrifugation of several liters of antigen-containing

plasma. These structures probably arise by a "nick" in one of the two strands, allowing elongation of the strand to take place from the exposed 3′-hydroxyl terminal nucleotide. This "rolling circle" mode of nucleic acid replication would be expected to produce short linear strands attached to the circular forms, such structures being visible in preparations where the polymerase reaction had occurred before nucleic acid extraction. It is difficult to envisage, however, how the product of the reaction, with a molecular weight of only 1.6×10^6, incorporates all the requisite information both for intracellular replication of the hepatitis B virus and for the expression of a wide variety of antigenic determinants expressed as hepatitis B antigens.

G. Laboratory Tests for Hepatitis B Surface Antigen and Antibody

A variety of laboratory techniques are now available for detecting hepatitis B surface antigen and its antibody (reviewed by Zuckerman, 1975b). The two-dimensional micro-Ouchterlony immunodiffusion test was the first method employed by Blumberg *et al.* (1965). Other more sensitive techniques including complement fixation, immune adherence, electron microscopy and immune electron microscopy, various procedures of counterimmunoelectrophoresis, passive hemagglutination and hemagglutination inhibition, radioimmunoassay, latex and charcoal particle agglutination, and reverse passive hemagglutination have since been described. Enzyme-linked immunosorbent assay methods are currently under evaluation.

1. Immunodiffusion

Immunodiffusion is based on the classical immunoprecipitation technique in fluid media but with the advantage that the precipitate is fixed in the supporting gel and appears as a discrete precipitin line. In general, in mixed antigen–antibody reactions, each antigen–antibody combination will form a separate line of precipitation, distinct from those of other antigen–antibody interactions. The two-dimensional micro-Ouchterlony immunodiffusion test, which allows direct comparisons to be made between reactions, is the simplest technique for detecting the surface antigen and the surface antibody (World Health Organization, 1970), but, in general, it is also the least sensitive. The sensitivity of the technique can be increased by preconcentration of the samples to be tested, by two or several fillings of the wells, or by reinforcement by placing known positive samples in wells adjacent to the specimens being tested. The use of agarose instead of agar also increases sensitivity. Staining the precipitin lines will allow the detection of weak reactions. Sensitivity can also be im-

proved by augmentation of reagent contact by rheophoresis or controlled evaporation of buffer from the surface (Jambazian and Holper, 1972), or by radial immunodiffusion in antibody-impregnated gel.

The immunodiffusion test, however, requires up to 3 days for completion, and although various modifications may marginally improve its sensitivity, the technique remains less sensitive than the more rapid electrophoretic and agglutination techniques.

After primary exposure to the antigen, hepatitis B surface antibody is not usually detected by precipitin methods, but a second or repeated exposure may result in the transient development of such antibody.

2. *Counterimmunoelectrophoresis*

The immunoelectrophoretic methods are essentially modifications of the electrosyneresis technique. Culliford (1964) described a form of gel electrophoresis, which has since been applied successfully to precipitin reactions in forensic tests. The basic principle of the method is that in an electrophoretic field, antigens which migrate at alkaline pH migrate according to their respective net mobilities. Serum albumin, α-, and β- globulins migrate toward the anode whereas the more basic globulins move toward the cathode. Under certain electrophoretic conditions in a suitable gel the two reactants will meet in optimal proportions to form a sharp precipitin line. The technique was first applied successfully for the detection of the surface antigen and antibody by Bedarida *et al.* (1969). A number of modifications of this method have since been introduced.

Counterimmunoelectrophoresis is a simple and rapid method for detection of hepatitis B surface antigen and antibody. This technique has been claimed by many investigators to be two to ten times more sensitive than immunodiffusion for the detection of the antigen but some have found no difference between the two methods. The principal advantage of immunoelectrophoresis lies in its rapidity since the results can be obtained within 2 hours. Strong positive reactions are easily read by direct examination of the slides using oblique illumination but washing and staining are recommended (Zuckerman and Taylor, 1970; Kohn and Morgan, 1971). Kohn and Morgan (1971) stressed that the main disadvantage of this technique is the use of a fixed antigen to antibody ratio and, consequently, no account is taken of the possibility of "false negative" results due to either antigen or antibody excess. It is important therefore, to determine the optimal size of wells for each antiserum by varying the sample volumes in adjacent wells of different size and shape. The use of more than one dilution of the serum being tested is also important. False positive results may also be obtained if tests are carried out simultaneously for both antigen and antibody along the same electrophoretic axis.

Bedarida *et al.* (1971) described a simple technique for measuring immune complexes in type B hepatitis sera by a modified technique of electrosyneresis. A proteolytic enzyme extracted from *Streptomyces griseus* is used to digest the antibody component, thus releasing the bound antigen. In electrosyneresis, agarose replaces the agar–agarose mixture in order to abolish electro-endosmotic flux. The pH is reduced from 8.6 to 5.9 in order to allow for the migration of antibody and, hence, its detection by electrosyneresis.

Dreesman *et al.* (1972c) found that dilution of hepatitis B surface antibody produced in goat in undiluted homologous (i.e., goat) normal serum resulted in enhanced sensitivity for detecting the antigen by counterimmunoelectrophoresis, using a discontinuous buffer system, compared to antibody reagent diluted in normal serum of heterologous species or in buffer solutions. It was postulated that homologous globulin stabilizes the hepatitis B globulin during migration through the agarose.

A sensitive indirect counterimmunoelectrophoretic method was described by Tripodi *et al.* (1973) for the detection of hepatitis B surface antibody whereby a "standard" antigen is inhibited by antibody in the serum being tested. An equal volume of surface antigen was added to the test serum and incubated. This serum was then tested by counterimmunoelectrophoresis for residual antigen against an animal hepatitis B surface antibody. The formation of a line of precipitation indicates the absence of the homologous antibody in the serum being tested. The migration of antigen is inhibited when antibody is present so that no precipitation can occur.

Counterimmunoelectrophoresis is, in many countries, still the most widely used technique for large-scale screening for hepatitis B surface antigen and antibody. A discontinuous buffer system increases the sensitivity and ease of reading of the precipitin lines. The technique has been employed for the simultaneous detection of antigen and antibody, but careful positioning of the wells is required. False positive reactions result from the crossing-over of one of the reagents leading to the formation of a precipitin line between the two reagents. Another source of false positive reactions is the presence of other precipitating antigen–antibody systems, such as antiruminant antibodies, and red cell and lipoprotein isoprecipitins. The sensitivity of the technique is influenced dramatically by the quality of the reagents and technical skill. Overall the method is perhaps up to three times more sensitive than immunodiffusion.

3. Complement Fixation

Attempts to utilize the complement fixation test for the diagnosis of viral hepatitis were described over 30 years ago but the results were not repro-

ducible. With the discovery of hepatitis B surface antigen, it soon became evident that as the antigen and its specific antibody became available, complement fixation could be readily employed in diagnostic tests. The technique has been described elsewhere (World Health Organization, 1970). Complement fixation has been found to be nearly 25 times more sensitive than immunodiffusion for measuring the surface antigen and three times more sensitive for the assay of surface antibody. The method is also more sensitive than counterimmunoelectrophoresis for antigen detection, but technically it is more difficult to execute. In addition, the antisera vary markedly in their suitability for detecting the antigen by complement fixation.

Complement-fixing surface antibody is usually detected after a second or repeated exposure to the antigen and is detectable for a period of days to weeks. The pattern of response of complement-fixing antibody roughly parallels that of antibody detected by immunodiffusion and counterimmunoelectrophoresis, but a number of precipitating antibodies that are detectable by the latter two techniques do not fix complement (World Health Organization, 1973).

Anticomplementary activity may be due to a number of unrelated causes, including antigen–antibody complexes. Anticomplementary activity has been observed following hepatitis A as well as hepatitis B, but the presence of anticomplementary activity should not be regarded as being specifically associated with viral hepatitis.

4. Inert Particle Agglutination

Detection of hepatitis B surface antigen by latex particles coated with surface antibody prepared in animals is a rapid and simple screening procedure (Leach and Ruck, 1971). The test requires a minimum of equipment and time, is relatively easy to interpret, and appears to be slightly more sensitive than complement fixation for detecting antigen (World Health Organization, 1973). Although nonspecific and false positive reactions are known to occur, the technique of latex particle agglutination appears to be useful in certain circumstances as a rapid preliminary screening test. Recent preparations of coated latex particles have been reported to have yielded more specific reactions. The technique can be readily automated. Hepatitis B surface antibody has been detected by its ability to inhibit latex agglutination.

Stevens *et al.* (1972) diluted and mixed purified hepatitis B surface antigen with an aqueous suspension of fine charcoal particles. These particle–antigen complexes were aggregated by the homologous antiserum and inhibition of this agglutination by serum containing the antigen constitutes the test. This test for the surface antigen is an adaptation of other

particle agglutination techniques such as the "reagin" card test for syphilis and the charcoal agglutination test for influenza virus antibody. Inhibition experiments, using purified hepatitis B surface antigen, revealed a sensitivity which was eighty times greater than the immunodiffusion technique and eight times that of counterimmunoelectrophoresis. It was noted that although the inhibition titers obtained with sera were always greater than the corresponding precipitin titers, the differences were not constant. Serum protein abnormalities, particularly the rheumatoid factor, may aggregate the particle suspension nonspecifically, or alternatively inhibit agglutination.

Kachani and Gocke (1973) described a simple and rapid agglutination-flocculation method for detection of the surface antigen involving simultaneous agglutination and flocculation in the presence of synthetic acrylic particles coated with high titer-globulin fractions of selected hepatitis B surface antibodies produced by hyperimmunization of rabbits. Approximately 30% of sera containing high titer rheumatoid factor produce a false positive reaction, which can be readily distinguished by retesting all positive reactions against acrylic particles coated with normal globulin prepared from the preimmunization serum of the same animals.

5. Passive Hemagglutination and Hemagglutination Inhibition

Passive hemagglutination provides a sensitive method for the detection of hepatitis B surface antibody (Vyas and Shulman, 1970). Red cells coated with highly purified antigen, using chromic chloride as a coupling agent, are agglutinated by very small amounts of antibody, and the results may be read within 2 hours. The test is very sensitive, being as much as 10,000 times more sensitive than the immunodiffusion technique.

The preparation of suitable antigen-coated red cells with chromic chloride is difficult. Different lots of cells vary considerably in their sensitivity. Nonspecific agglutination is frequently found with low dilutions of sera and control erythrocytes must be used. Nonspecific agglutinins can be absorbed with control erythrocytes before retesting and by heat inactivation. False negative results may be obtained with low dilutions of high titer antibody because of the formation of prozones and sera should, therefore, be tested at several dilutions.

Inhibition of hemagglutination by test sera as a result of neutralization by known antibody preparations may be used for the detection of the surface antigen. The sensitivity of hemagglutination inhibition is about the same as complement fixation.

Hopkins and Das (1972, 1973) tanned and sensitized with the surface antigen human group O, Rh-negative erythrocytes. A normal plasma-sensitized cell control was prepared in parallel. The sensitized cells were

used for the detection of antigen by two-stage hemagglutination inhibition. The sensitivity of the technique is reported to be similar to radioimmunoassay. A direct hemagglutination test was applied for the detection of hepatitis B surface antibody and the order of sensitivity, based on titration, was high. Leslie *et al.* (1975) modified with conventional hemagglutination plate procedure to a microcapillary system for testing sera for the surface antigen and antibody and the results can be read within 20 minutes. These techniques are being evaluated further.

Pert and Verch (1975) purified the surface antigen by adsorption to and elution from glass particles. The antigen was subsequently stabilized with dextran and adsorbed to the surface of freshly washed erythrocytes. Cells coated by this procedure were agglutinated by surface antibody at a final dilution as high as 1×10^9. The test can be readily modified for antigen detection by inhibition or by coating red cells with surface antibody.

6. Reverse Passive Hemagglutination

The technique of reverse passive hemagglutination for the detection of hepatitis B surface antigen was first described by Juji and Yokochi (1969). Purified surface antibody recovered from dissociated antigen–antibody complexes was attached to formalin-fixed erythrocytes. The hemagglutination technique was applied for the detection of the antigen. Hirata *et al.* (1973) treated human group O erythrocytes with pyruvic- and formaldehyde, and packed cells were resuspended in acetate buffer containing the 7 S fraction of surface antibody raised in guinea pigs. The coated red cells were used for the detection of surface antigen by the technique of reverse passive hemagglutination. This method, which has the obvious attraction of simplicity, has a limit of detection of antigen of 1.6 ng/ml, thus approaching the sensitivity of radioimmunoassay. Similar results have since been reported by many laboratories and a number of modifications have been introduced.

The overall finding is that erythrocytes from various species coated with the 7 S fraction of hepatitis B surface antibody provide a simple and sensitive technique for the detection of hepatitis B surface antigen. Tests on comparative sensitivity indicate considerably greater sensitivity than counterimmunolectrophoresis, complement fixation, and passive hemagglutination inhibition and a sensitivity that is somewhat less than a widely available commercial radioimmunoassay technique. The test can be performed rapidly, the results are generally easy to read, and the technique may be semiautomated with simple equipment. A number of nonspecific false positive results on screening are inherent in the method due to species-specific red cell agglutinins. Confirmatory tests are, therefore, required and appropriate reagents are available. Reverse passive hemag-

glutination would thus seem to be a practical compromise between the highly sensitive but technically complex radioimmunoassay and other simple but less sensitive techniques. This test is now recommended for use as a screening test for hepatitis B surface antigen (World Health Organization, 1975).

7. Radioimmunoassay

Radioimmunoassay, which combines the specificity of antigen–antibody reaction with the sensitivity of radioisotope detection, has provided extremely sensitive assays for the minute amounts of many of the peptide hormones present in plasma. All radioimmunoassay techniques depend upon the quantitation of non-antibody-bound antigen, non-antigen-bound antibody or the extent of antigen–antibody reaction. The most frequently used procedure has been the introduction of an isotope of iodine, ^{131}I or ^{125}I into the tyrosine residues of the peptides. Iodination with ^{125}I is more convenient due to its longer half-life and lower energy radiations with consequent increased ease of handling.

The various radioimmunoassay procedures differ mainly according to the technique of separation. The aim of separation is to resolve a reaction mixture into two portions containing free and bound reactants, respectively, at the end of the period of incubation. Numerous techniques of separation are now available based on the ability of paper, charcoal, and talc to adsorb small amounts of antigens more readily than the larger antibody molecules. Some methods utilize the size, charge, and differences in solubility between antigen and antibody. There are also methods in which the antibody is fixed to Sephadex or to polystyrene by chemical or physical bonding (Wide and Porath, 1966; Greenwood, 1967).

Walsh *et al.* (1970) were the first to describe a radioimmunoassay technique for the detection of hepatitis B surface antigen and antibody by the use of the surface antigen labeled with ^{125}I. The sensitivity of the technique was found to be some 20 to 100 times greater than complement fixation for detecting both antigen and antibody. Lander *et al.* (1971) described a radioimmunoassay adaptation of a double antibody precipitation method to a microtiter system for detecting the surface antibody. This technique combines specificity with extreme sensitivity. In addition, the procedure is relatively easy and rapid. The method can also be used for assaying the antigen by the competitive blocking of the reaction of ^{125}I-labeled antigen and antibody by the addition of unlabeled antigen. The sensitivity for measuring antigen is increased in this way by 100 to 1000 times when compared with complement fixation. Ling and Overby (1972) described a two-step, direct, noncompetitive, radioimmune technique (Ausria) using polypropylene tubes coated with hepatitis B surface anti-

body raised in guinea pigs and specific hepatitis B immune globulin labeled with ^{125}I. This technique was more sensitive than other serological methods in use at the time, including indirect radioimmune assays based on competition of unlabeled antigen with antigen labeled with ^{125}I. The sandwich-type radioimmunoassay technique has also been adapted for subtyping of the antigen using subtype-specific antisera. Considerable literature on the use of this commercially available method has now accumulated and recently the technique had been adapted for the detection of surface antibody.

A number of other radioimmunoassay techniques have been described (reviewed by Cameron and Dane, 1974). Purcell *et al.* (1973) developed a microsolid phase radioimmunoassay method for detection of hepatitis B antigen, using polyvinyl V-bottom plates as the solid phase for coating with guinea pig or human antiserum. The specificity of the reactions was confirmed by blocking. The microsystem prepared with guinea pig reagents yielded a proportion of false positive reactions but was nearly as sensitive as the Ausria technique. The microsystem prepared with human reagents was slightly less sensitive but did not give false positive results. Lambert *et al.* (1974) treated a guinea pig hepatitis B antiserum labeled with ^{125}I with papian to produce ^{125}I-Fab fragments. The Fab fragments were further purified by DEAE-cellulose chromatography. Polyethylene glycol was used to precipitate immune complexes. The advantages of the new technique include a reduced risk of interference by anti-Fc antiglobulins found in some sera and the technique could be applied to the detection of hepatitis B surface antigen in the presence of excess antibody.

Duimel and Brummelhuis (1975) recently described a competitive solid phase radioimmunoassay technique for detection of the surface antigen and antibody. The reproducibility of the test was found to be very good. False positive results may occur due to the presence of proteins which react or cross-react with the sheep antiserum employed in the test system. Specific inhibition of positive samples using human hepatitis B surface antibody is therefore required.

Simons (1973a) described a technique of radioelectrocomplexing for detection of hepatitis B surface antibody. The method depends on the interaction between the antigen labeled with ^{125}I and unlabeled antibody as a result of counterdirectional movement during electrophoresis. After electrophoresis the agar is cut into three zones and transferred in tubes to a gamma spectrometer. The percentage distribution of the radiolabeled antigen in the zone containing the antigen well, the antibody well zone, and the area between the wells is calculated. The radioelectrocomplexing reaction can be inhibited by unlabeled hepatitis B surface antigen so that

the technique can be utilized for detection of the antigen, as well as for subtyping if type-specific antisera are used (Simons, 1973b).

Aach *et al.* (1973) used double antibody radioimmunoassay for the determination of subtypes of the surface antigen. Their findings suggested that the number of *a* and *d* or *a* and *y* antigenic determinants per particle, or the number of antigenic sites sufficiently exposed to allow combination with antibody may vary considerably for any given population of antigen. The results also stress a potential problem which can be encountered, particularly when sensitive techniques are used for screening of sera for the surface antigen. Antisera may contain high titer, high affinity antibodies to one of the type specific *d* or *y* subdeterminants and yet fail to detect strongly positive sera of the other subtype. Furthermore, surface antigen markers may vary in their antigenic composition, thereby influencing the results of screening for antigen even with the same antisera. The double antibody radioimmunoassay procedure for hepatitis B surface antigen and antibody has been described in detail by Hollinger (1974).

Figenschau and Ulstrup (1974) described a rapid radioimmunoassay technique for the measurement of the surface antigen and surface antibody using the protein A of *Staphylococcus aureus* for the separation of bound antigen from free antigen. Radioimmunocompetition was used for testing. Antibody was measured by direct binding of radiolabeled hepatitis B surface antigen. The principle of the technique is as follows: protein A combines specifically with the Fc part of IgG, types 1, 2, and 4, which in normal sera comprises more than 90% of the total IgG. The reaction between protein A and the combining site on the IgG molecule is very rapid and there seems to be no difference in the combining properties to protein A between free and antigen-bound IgG. Radiolabeled antigen which reacted with its specific antibody develops, after attachment to protein A fixed to the staphylococcus, into a solid phase which is readily precipitated by light centrifugation. The pellet can be counted directly without washing, once the supernatant containing the unbound radiolabeled antigen has been removed. The sensitivity for detection of the antigen is similar to that of the sandwich-type radioimmunoassay technique, and the sensitivity for measurement of antibody is at least that of passive hemagglutination. Other techniques for the detection of surface antibody have been described more recently by Muller *et al.* (1975) and by Patil and Pert (1975).

In summary, radioimmunoassay techniques include assays in which antigen–antibody complexes are separated from unbound reagents by chromatoelectrophoresis, precipitation with antibody, attachment to a solid phase, or sandwhich methods. Double antibody, solid phase, and

sandwich systems are the most widely used techniques and currently provide the most sensitive methods for detecting hepatitis B surface antigen and antibody. Results should be confirmed as positive only if neutralization tests with the surface antibody show specific inhibition (World Health Organization, 1975).

The principal disadvantages include relative slowness of the test and the high cost of capital equipment and reagents. In addition, the test is tedious to perform on a large scale, the equipment is subject to breakdown and expensive maintenance, and there are hazards associated with the handling of radioactive isotopes.

8. Electron Microscopy

Viruses were among the first objects to be examined in the electron microscope over 30 years ago, but knowledge of viral fine structure was limited to bacteriophages, until the advent in 1959 of the negative staining contrast technique for high-resolution electron microscopy. The technique utilizes the principle of surrounding, within a rigid electron-dense material, particles such as viruses, isolated subcellular components, and other macromolecular structures. The principal advantages of negative staining are that the technique is relatively unaffected by impurities of small molecular size, which merge with the background, and, conversely, large sized impurities are clearly discernible from virus particles. There is often very good preservation of material under test with minimum distortion of structure. Negative staining yields clear images and many detailed and intricate features of particulate structures are revealed. Furthermore, only relatively small amounts of material to be tested are required. The main disadvantage of the negative staining technique is that a high concentration of particle suspension is usually required, often 10^9 particles/ml. However, an additional degree of sensitivity may be obtained by the technique of immune electron microscopy by looking for aggregates of antigen rather than individual particles by themselves.

The examination of sera and other tissue fluids for hepatitis B surface antigen by the techniques of electron microscopy and immune electron microscopy have been described in detail by Almeida *et al.* (1969), Zuckerman (1969, 1970b, 1972), and Dane *et al.* (1970). Immune electron microscopy has been especially useful for characterizing the different morphological forms of the surface and core antigens and for investigating antigen–antibody systems. Although sensitive and specific, electron microscopy is not suitable for large-scale testing (World Health Organization, 1970, 1973).

9. Other Tests for Hepatitis B Surface Antigen

The immune adherence hemagglutination technique is recognized as a sensitive method for the detection of antigen and antibody (Ito and Tagaya, 1966). This method has been applied by Okochi *et al.* (1970) and by Mayumi *et al.* (1971) for detection of hepatitis B surface antigen. Immune adherence was found to be forty times more sensitive than the complement fixation test for detection of the antigen in blood donors in Tokyo. This method, however, will neither detect antigen of low titer (1:2 to 1:4) nor antibody, especially when excess serum is present in the reac tion mixture. Furthermore, since immune adherence is a complement-dependent reaction, the same controls and precautions employed in the complement fixation test must be applied.

Enzyme-linked immunosorbent assay techniques developed by van Weemen and Schuurs (1971, 1972, 1974) and by Engvall and Perlmann (1971, 1972) have been applied successfully for measuring antigens and antibodies (Voller *et al.*, 1974, 1975a,b; Voller and Bidwell, 1975). More recently, solid phase enzyme immunoassay has been used for the detection of hepatitis B surface antigen (Wolters *et al.*, 1976). Preliminary results have revealed a sensitivity and specificity which compare very favorably with radioimmunoassay. More extensive evaluation of this method is currently in progress in various centers.

H. Laboratory Tests for Hepatitis B Core Antigen

1. Immunofluorescence

Since the original observations of Almeida *et al.* (1971), several laboratories have confirmed the existence of at least two distinct antigen–antibody systems associated with hepatitis B. Fluorescent antibody techniques clearly distinguish core antigen in the nuclei of infected hepatocytes. Prince and colleagues (1964) had previously described an antigen in the nuclei of parenchymal cells in a number of liver biopsies from patients with anicteric hepatitis. Antibody to this antigen was found in a serum pool from cases of acute viral hepatitis. Millman *et al.* (1969) and Coyne *et al.* (1970) detected fluorescent particles in or on the nucleus and occasionally in the cytoplasm of cells prepared from liver biopsy material by using fluorescein-conjugated rabbit antiserum to the surface antigen. Fluorescence was demonstrated in cells prepared from liver biopsies in every patient in whom hepatitis B surface antigen was also present in the serum. Nowoslawski *et al.* (1970) also observed specific immunofluorescence in the nuclei and on the cytoplasm of hepatic parenchymal cells in speci-

mens taken at necropsy from six patients with lymphoproliferative disorders who were seropositive for surface antigen. Edgington and Ritt (1971) described the immunofluorescent localization of the surface antigen in acute and chronic hepatitis. Competitive inhibition studies of hepatitis B surface antigen suggested that there were at least two antigenically and sterically independent determinants on the antigen particle. Brzosko *et al.* (1973) tested a large number of sera collected from patients with acute hepatitis and from patients with chronic hepatitis, using cryostat sections of a liver obtained at necropsy from a patient with chronic aggressive hepatitis as a substrate for the indirect immunofluorescent antibody technique. The presence of hepatitis B antigen in the nuclei and cytoplasm of the hepatocytes in this liver was established previously by direct staining with fluorescein-labeled human hepatitis B antibody. It was found that hepatitis B antibody from human sources, especially from patients with acute viral hepatitis and chronic aggressive hepatitis, reacted predominantly with antigen present in the nuclei of hepatocytes. Precipitating hepatitis B antibody from man and antibody raised by hyperimmunization of animals revealed almost exclusively cytoplasmic fluorescence. It was concluded that there were two immunochemically different specificities of hepatitis B antigen: nuclear and cytoplasmic. More recent studies, including electron microscopy, have confirmed that differential immunofluorescent staining identifies the surface antigen in the cytoplasm and the core antigen in the nuclei of infected cells.

The detection of hepatitis antigens in the nucleus and in the cytoplasm of cultured human liver cells by immunofluorescent techniques has also been reported from several laboratories (reviewed by Zuckerman, 1975c). Zuckerman and Earl (1973) demonstrated by immunofluorescence the core antigen in the nuclei of cultured human embryo hepatocytes and the surface antigen in the cytoplasm of the cells. Similar fluorescent changes, in the absence of cytocidal changes, were observed in cultured liver cells inoculated with the supernatant fluid passaged in culture three times.

2. *Complement Fixation and Immune Adherence*

Barker *et al.* (1973) successfully transmitted hepatitis B to chimpanzees. A human convalescent serum containing core antibody, in the absence of surface antibody, specifically stained the nuclei of infected hepatocytes whereas a hyperimmune surface antibody exclusively stained the cytoplasm of these cells. The isolation of core antigen from the liver of an infected, immunosuppressed chimpanzee enabled Hoofnagle *et al.* (1973) to characterize the pattern of antibody reactions in fifteen patients during the course of hepatitis B. Core antibody was assayed by a microtiter complement fixation method using hyperimmune antisera to the puri-

fied core antigen. Surface antibody reactivity was not detected in these sera. Examination of serial sera from these patients revealed core antibody during or immediately after surface antigenemia and within 3 weeks from the onset of jaundice. The presence of these antibodies did not signal the beginning of recovery from clinical infection, and preceded the appearance of surface antibody by a few weeks to several months. This finding suggests that core antibody is produced in response to virus replication. In a study of three cases of posttransfusion hepatitis, Kaplan *et al.* (1973) found core antigen production, as detected by DNA polymerase activity, to be at a maximum 3 weeks after the first appearance of surface antigen, but the level rapidly declined before the onset of liver dysfunction. The use of the DNA polymerase activity as an indicator of core antigen may be a clearer indication of the presence of the virus than the detection of surface antigen alone. In this context, Krugman *et al.* (1974) found that DNA polymerase activity was not present in two persons who were immunized against hepatitis with heat-inactivated MS-2 serum and in one who received hepatitis B immunoglobulin.

Although the titer of core antibody eventually declines to a low level during convalescence, the development of persistent carrier state of the surface antigen is closely associated with the presence of core antibody in high titer. Hoofnagle *et al.* (1973) found core antibody at titer 1:64 or higher by complement fixation regardless of the surface antigen subtype present in all 100 sera obtained from persistent carriers. Of considerable importance was the finding of core antibodies in 1% of 100 volunteer blood donors. The incidence of surface antibody was somewhat higher in the same group (4%). Further use of this technique demonstrated the presence of core antibody in 98% of 363 serum specimens containing the surface antigen and in each of 100 persistent carriers. Of particular interest was the observation of a donor serum that had core antibody in the absence of surface antigen and its antibody. The recipient of this donor's blood had evidence of hepatitis B infection. Retrospective testing of 16 donors who were implicated in cases of clinical posttransfusion hepatitis revealed significant titers of core antibody in 10 of these donors; surface antigen was detected in three of the sera and surface antibody in another three. However, the most interesting were four donors who were positive for core antibody but in whose sera surface antigen and antibody were not detected. These were donors who had, at the time of testing, no other evidence of being or having been infected with hepatitis B virus. Consequently, these are serious candidates for the type of donor who is capable of transmitting this infection without being identified by current tests for the surface antigen.

Tsuda *et al.* (1975) using immune adherence found a slightly higher pre-

valence rate of core antibody reactivity using human type O Rh-positive erythrocytes as the indicator cells. Preliminary experiments showed the technique to be highly specific and about ten times more sensitive than the microtiter complement fixation method. Core antibody was found in 36 of 215 (17%) serum samples obtained from healthy blood donors in Japan. Of these, 28 samples were found also to contain surface antibody and two others contained the surface antigen. There were six samples (3%) in which core antibody was detected in the absence of surface antigen, surface antibody, and core antigen. At the present time, experience with this technique remains limited.

Complement fixation together with counterimmunoelectrophoresis are currently the most widely used procedures for the detection of core antibody. While both methods are of comparable sensitivity and specificity, relatively large quantities of core antigen are required. The availability of these tests is, therefore, dependent upon a suitable source of standard antigen, such as the infected liver. The advent of radioimmunoassay procedures utilizes smaller quantities of core antigen which may be readily extracted from the serum of a proportion of persistent surface antigen carriers.

3. Radioimmunoassay

Purcell *et al.* (1974) described a microsolid phase radioimmunoassay technique for the detection of core antigen and core antibody. The wells of polyvinyl microtiter plates formed the solid phase to which was adsorbed a diluted human convalescent serum to hepatitis B. A standard core antigen preparation was prepared by the treatment of circulating 42-nm particles with Nonidet P-40, a nonionic detergent found previously to be more efficient than Tween 80 for complete removal of the outer surface antigen envelope. The detection of core antibody by its ability to inhibit the binding of radiolabeled core antibody to core antigen provided a means of measuring this antibody in sera with concomitant surface antibody. Similar results to those obtained by the complement fixation method were obtained. Core antibody was detected less frequently than surface antibody following acute hepatitis B infection. When present, core antibody is frequently associated with clinical hepatitis rather than inapparent infection. In addition, core antibody was found in eight chronically infected patients, whether or not there was evidence of liver damage.

The direct radiolabeling of core antigen by activation of the endogenous DNA polymerase activity offers an alternative radioimmune procedure. Moritsugu *et al.* (1975) described a radioimmune precipitation technique whereby antigen–antibody complexes were separated from unbound la-

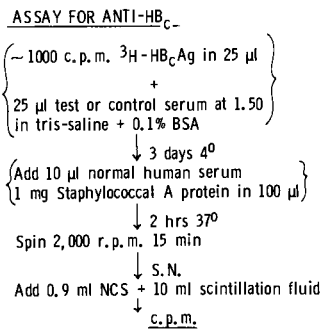

Fig. 4. Assay for hepatitis B core antibody. S.N., Supernatant; NCS, Nuclear Chicago solubilizer; c.p.m., counts per minute.

beled antigen by precipitation with anti-globulin. A similar procedure utilizing staphylococcal protein A for the removal of immune complexes is outlined in Fig. 4. Using this procedure, a rhesus antiserum at a dilution of 1:500,000 was found to precipitate 50% of added core antigen in comparison to a titer of 1:256 to 1:1024 obtained by complement fixation. For optimal sensitivity, radiolabeled core antigen is separated from both unlabeled core antigen and incompletely disrupted 42-nm particles by equilibrium centrifugation in cesium chloride. It is often necessary to adjust the amount of radiolabeled core antigen used when different batches of antigen are employed as the specific activity of each preparation varied according to the source of plasma.

A method involving simultaneous activation of core antigen-associated polymerase activity and incubation with antibody has been described by Greenman *et al.* (1975), but the technique appears to be a somewhat less sensitive procedure. Among the samples examined were two hepatitis B immune globulin preparations. Core antibody was detectable at a dilution of 1:20 but not at the routine test dilution of 1:50, indicating that both preparations contained a low titer of core antibody in addition to surface antibody. Five additional preparations previously tested by Purcell *et al.* (1974) were confirmed as being negative for core antibody.

More extensive use of radioimmune procedures for the detection of core antibody will almost certainly produce a closer estimate of the incidence of exposure to the core antigen in the blood donor population than has been hitherto possible using less sensitive techniques. In addition, radioimmunoprecipitation methods offer the advantage of allowing the estimation of core antibody levels in immunoglobulin preparations which are frequently anticomplementary when examined by complement fixation methods.

I. The Immune Response in Hepatitis B

The immune response after infection with hepatitis B virus is manifested by at least three antigenic systems, hepatitis B surface antigen, the core antigen, and the *e* antigen, resulting from replication of the virus in hepatocytes. The view that hepatitis B virus exerts its damaging effect on hepatocytes by direct cytopathic changes is inconsistent with the persistence of large quantities of surface and core antigen in liver cells of many apparently healthy persons who are chronic carriers of the surface antigen in their blood. On the other hand, evidence is accumulating to suggest that the pathogenesis of liver damage in the course of this infection is related to the immune response by the host (reviewed in the World Health Organization reports, 1973, 1975).

1. The Humoral Response

The surface antigen appears in the sera of most patients during the incubation period of the acute infection, as early as 4–6 weeks after infection and 2–8 weeks before biochemical evidence of liver damage or the onset of jaundice. The antigen persists during the acute illness and it is usually cleared from the circulation during convalescence. Free core antigen has not been detected in serum or plasma. Next to appear in the circulation is the associated DNA polymerase activity immediately prior to or at the time of raised serum transaminase elevations (Krugman *et al.*, 1974). The polymerase activity persists for days or weeks in acute cases and for months or years in approximately 10% of persistent surface antigen carriers (C. R. Howard, S. Thal, and A. J. Zuckerman, personal communication, 1975). Antibody to the core is found in the serum 2–10 weeks after the appearance of the surface antigen and is detectable for some time after recovery has taken place, but with declining titers (Hoofnagle *et al.*, 1975). In general, and at first sight, paradoxically, the highest titers of core antibody are found in chronic surface antigen carriers. Finally, antibody to the surface component appears. A late primary type of surface antibody response occurs in clinical cases of hepatitis B shortly after the disappearance of the surface antigen from the serum. The surface antibody response in most cases apparently resistant to parenteral infection is of the early, or secondary, anamnestic type. The surface antibody persists in most patients longer than core antibody, when sensitive assay techniques are employed. An anamnestic response to surface antigen only is seen on reexposure to surface antigen positive plasma, but core antibody levels remain unchanged.

Anticomplementary activity, which may be associated with surface antigen–antibody complexes, may be found in the sera of some patients

during the incubation period and the acute phase of illness. Such immune complexes have been seen by electron microscopy (Almeida and Waterson, 1969; Reed *et al.*, 1973). There is now additional evidence suggesting the importance of these complexes in the pathogenesis of syndromes characterized by severe damage of blood vessels in periarteritis nodosa (Gocke *et al.*, 1970; Trepo *et al.*, 1974, and others), in the renal glomeruli in some forms of chronic glomerulonephritis in children (Brzosko *et al.*, 1974), and in the Gianotti-Crosti syndrome (Gianotti, 1973; Ishimaru *et al.*, 1976). Surface antigen, surface antibody, core antibody, and surface antigen–antibody immune complexes have been identified in a proportion of patients with virtually all the presumed chronic sequelae of acute hepatitis. Serum anticomplementary activity and low serum complement levels may be found in patients with various forms of chronic hepatitis. Deposits of such immune complexes have also been demonstrated in the cytoplasm and plasma membrane of hepatocytes and on or in the nuclei. It is not clear, however, why circulating immune complexes are not found in a greater proportion of patients and why only a small proportion of patients with circulating complexes develop vasculitis or polyarteritis. It may be that complexes are critical pathogenic factors only if they are of a particular size and of a certain antigen–antibody ratio. Further studies are required to elucidate the role of immune complexes in the pathogenesis of liver damage.

2. Cellular Immune Responses

The cytopathic effect of viruses and the immune response of the host combine to produce the clinical syndrome of a particular infection, and variations in both these factors may account for the different clinical responses. Cellular responses are known to be of particular importance in determining the clinical manifestations and course of viral infections in man and in animals. Studies of cell-mediated immunity in liver disease initially showed depression of nonspecific responses. The occurrence of cell-mediated immunity to hepatitis B antigens has been demonstrated by lymphocyte transformation and more particularly by leukocyte migration inhibition. When partially purified surface antigen was used as the test antigen, leukocyte migration inhibition was found in most patients during the acute phase of hepatitis B. Inhibition becomes less pronounced during convalescence and disappears after recovery. Leukocyte migration inhibition has been demonstrated in a significant proportion of surface antigen-positive patients with chronic active hepatitis. However, lymphocyte transformation and leukocyte migration inhibition are invariably negative in asymptomatic chronic carriers of the surface antigen. These observations suggest that cell-mediated immunity may be involved in ter-

minating hepatitis B virus infection and, under certain circumstances, in promoting hepatocellular damage and in the genesis of autoimmunity. A normal T cell function would, therefore, be a prerequisite for the self-limited course of hepatitis, whereas a defective function or its absence would favor the development of chronic hepatitis and the asymptomatic carrier state, respectively. The subject has been discussed and reviewed extensively (Dudley *et al.*, 1972; Eddleston and Williams, 1974; Reed *et al.*, 1974; Thomson *et al.*, 1974; Lee *et al.*, 1975; and many others). As far as the core antigen is concerned, cell-mediated immunity to this antigen as measured by leukocyte migration inhibition has been found twice as frequently as inhibition to the surface antigen in patients with acute hepatitis B, in convalescent patients, and in patients with chronic hepatitis in whom surface antigen and/or core antibody have been found (World Health Organization, 1975). However, further studies of *in vitro* cell-mediated responses to hepatitis B antigens and to liver-specific lipoprotein are required to define the role of these responses in hepatitis B infection.

IV. HEPATITIS A

Hepatitis A (infectious or epidemic hepatitis) is a major public health problem, occurring endemically in all parts of the world with frequent reports of small and large outbreaks. Spread is usually by person-to-person contact and major outbreaks result most frequently from the fecal contamination of water and food. Subclinical cases are common; the disease has, in general, a low mortality but patients may be incapacitated for many weeks. Recent important advances in the laboratory studies of this infection include transmission to certain species of marmosets and susceptible chimpanzees and the identification of virus particles in fecal extracts. A number of serological tests for hepatitis A have been developed, including immune electron microscopy, immune adherence hemagglutination, complement fixation, and radioimmunoassay. Enzyme-linked immunosorbent techniques are currently under study.

A. Immune Electron Microscopy

The discovery of the association between an antigen found in blood in hepatitis B stimulated numerous efforts to find a similar antigenic marker in hepatitis A. A search for antigens in fecal extracts prepared from patients with hepatitis A was initiated in Melbourne. Ferris *et al.* (1970) reported the finding of an antigen by immunodiffusion in fecal extracts from

about 41% of patients with hepatitis A admitted to hospital and in only 3% of control patients. The fecal antigen was found early in the course of the disease and it usually disappeared within 3 weeks from the onset of dark urine. Examination by electron microscopy revealed that antigenic activity was associated with two types of spherical particles, most of which measured 18–25 nm and some measuring 40–45 nm in diameter. More detailed results of examination of the fecal antigen by electron microscopy were subsequently reported, and although antisera prepared against this fecal antigen in rabbits and guinea pigs gave encouraging results there were also many nonspecific reactions. Substantial and, indeed, dramatic progress was initiated by the findings reported by Feinstone *et al.* (1973) who examined by immune electron microscopy extracts of feces obtained before infection or during the acute illness from each of four adult volunteers who were infected orally or parenterally with the MS-1 strain of hepatitis A virus. Viruslike particles measuring 27 nm in diameter were found in fecal specimens from two of the four volunteers during the early acute phase of the illness. The particles were aggregated, and aggregates were composed of both "full" and "empty" particles which were heavily coated with antibody. These particles were not found in fecal specimens before infection. Fecal filtrates containing the 27-nm particles were used to examine by immune electron microscopy several groups of sera for the presence of antibody to this antigen. All six volunteers previously infected experimentally with hepatitis A developed serological evidence of infection, as judged by aggregation and antibody coating of the 27-nm particles. Similar results were obtained with sera from persons from two naturally occurring outbreaks of hepatitis A. None of the sera from the patients with hepatitis A contained detectable antibody to the 27-nm particles before exposure to the infection. There was no serological reaction between the hepatitis A fecal antigen and hepatitis B surface antigen and core antigen. Development of antibodies to these antigen particles has now been demonstrated in patients infected experimentally with the MS-1 strain, in patients from several different outbreaks of hepatitis type A infection, and in the serum of random cases of hepatitis A. Such antibodies were not found in sera collected before the onset of illness.

Maynard *et al.* (1974), Locarnini *et al.* (1974), Dienstag *et al.* (1975a), Gravelle *et al.* (1975), and others also reported the finding of antigenically similar 27-nm viruslike particles in fecal suspensions collected from patients with hepatitis A in different geographical regions. Almeida *et al.* (1974) also examined by immune electron microscopy fecal extracts from the same adult volunteer inoculated with the MS-1 strain examined by Feinstone *et al.* (1973), and found that the fecal material contained several small cubic viruslike particles which varied significantly in their diameter

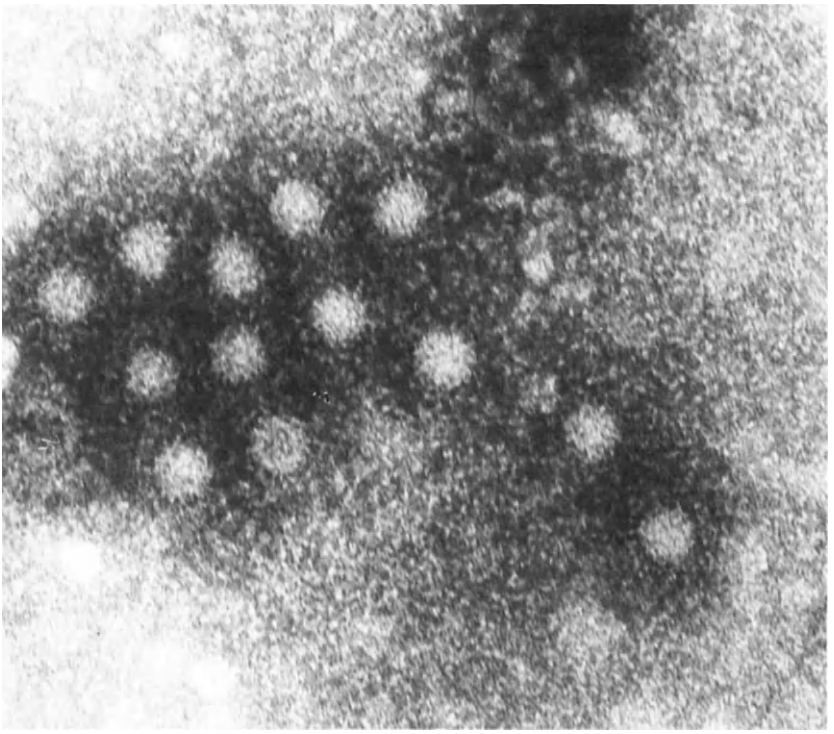

Fig. 5. Hepatitis A viruslike particles measuring 26–29 nm in diameter found in the feces of a patient incubating hepatitis A infection. The particles are heavily coated with antibody present in the convalescent serum of a chimpanzee successfully infected with human hepatitis A. ×214,000. (Electron micrograph from a series by Anthea Thornton and A. J. Zuckerman.)

size from 22 to 30 nm. Dienstag *et al.* (1975b) reported further results obtained by immune electron microscopic examination of serial specimens of feces from two volunteers who were infected experimentally in 1968 with the MS-1 strain of hepatitis A. The 27-nm viruslike particles were detected in fecal extracts at least 5 days before elevation of the serum transaminases but not later than the day of peak transaminase levels. The sensitivity threshold of immune electron microscopy for detection of enterovirus was $10^{3.5}$–10^5 infectious virus per milliliter and it was concluded, therefore, by analogy that the shedding of hepatitis A virus almost certainly begins earlier and lasts longer than detection of hepatitis A antigen in the feces by immune electron microscopy. The limitations of the cumbersome technique of immune electron microscopy for routine serological tests for antibody using the fecal antigen particles and, indeed, for searching for the antigen are obvious (Fig. 5).

B. Nonhuman Primates, Hepatitis A, and the Development of Serologic Techniques

In the past, the many attempts to transmit hepatitis A and B to various animal species, including nonhuman primates, failed or yielded inconsistent results. As regards hepatitis A, Deinhardt *et al.* (1967) were the first to provide evidence of the successful transmission of human hepatitis A to several species of marmosets, and the infection has been transmitted serially from animal to animal (Deinhardt *et al.*, 1972). Differences in susceptibility exist between the marmoset species, with *S. mystax* being the most susceptible (Lorenz *et al.*, 1970; Mascoli *et al.*, 1973). The MS-1 strain of hepatitis A was originally transmitted to marmosets by acute phase serum or plasma specimens from infected human volunteers, and the virus was neutralized *in vivo* in marmosets using convalescent sera but not preinoculation sera of the same volunteers (Holmes *et al.*, 1973). Mascoli *et al.* (1973) isolated the CR 326 strain of hepatitis A from naturally occurring family outbreaks in Costa Rica. The CR 326 strain of hepatitis A was neutralized by convalescent sera from patients in the same outbreak, convalescent sera from volunteers infected with the MS-1 strain, and pooled human immunoglobulin. Subsequently, Provost *et al.* (1975a) detected by electron microscopy virus particles, similar to those described by Feinstone *et al.* (1973) and others, in the sera and livers of *S. mystax* marmosets infected with the CR 326 strain.

Human hepatitis A has also been transmitted to chimpanzees shown to be free of the homologous antibody (Maynard *et al.*, 1975; Dienstag *et al.*, 1975a; Gravelle *et al.*, 1975; Thornton *et al.*, 1975). Thornton *et al.* (1975) inoculated intravenously a susceptible chimpanzee with 2 ml of 0.2% of suspension of human fecal material. The feces used were obtained from an adult human volunteer infected in the United States with the MS-1 strain of hepatitis A virus. Feces and serum were collected over a period of 8 weeks after inoculation. Serum enzyme levels became abnormal after 3 weeks and remained elevated until the sixth week. Examination of fecal suspensions by immune electron microscopy revealed the presence of a small cubic virus as early as 9 days after infection and persisting until the 28th day. The maximum number of particles was present on day 19. The particles themselves were distinctive and easy to identify. However, close examination revealed that unlike the majority of small cubic viruses, they showed considerable variation in size. Even within single aggregates, particle diameters ranged from 24 to 29 nm. There was also a suggestion that the larger particles possessed an additional rim which was lacking in those of smaller diameter.

The various reports show that the chimpanzee can be a realistic source

of hepatitis A virus for the development and application of serological tests. The nature of the illness in this species is so mild that the animals employed make a complete recovery and the experiment need not be a terminal one. This point is of importance in conserving a species that may be endangered. However, chimpanzees susceptible to hepatitis A are difficult to find (A. Thornton, J. D. Almeida, and A. J. Zuckerman, unpublished observations). At the same time, the detection of large numbers of virus particles only during the prodromal period of hepatitis A in the experimental model and indeed in natural infection in man accounts for the frequent failure to find virus in fecal extracts collected from patients after the onset of jaundice.

C. Complement Fixation

A specific complement fixation test for human hepatitis A antibody was described by Provost *et al.* (1975b) using as antigen liver extract of marmosets infected with the CR 326 strain of hepatitis A virus. The development of the complement fixing antibody against hepatitis A correlated well with the development of neutralizing antibody. In most cases, the highest titer of the complement fixing antibody was attained within the first month after the onset of the acute illness and this antibody persists for at least several years. It is interesting to note that anticomplementary activity appeared to be present shortly after the onset of illness and it has been suggested that the high anticomplementary activity was due to the presence of circulating antigen–antibody complexes. This was further complicated by the frequent presence of antibody reacting with normal marmoset liver antigen, although at lower titer and without significant increase except when anticomplementary activity was present. Krugman *et al.* (1975) used the same source of antigen for complement fixation and immune adherence hemagglutination for the measurement of hepatitis A antibody. The data obtained indicated that the immune adherence test was more specific, more sensitive, and simpler to perform than the complement fixation test.

D. Immune Adherence Hemagglutination

Miller *et al.* (1975) described a specific immune adherence assay for hepatitis A antibody. In general, this method provides clearly defined endpoint titrations. Selection of the human red cells is most important since these cells vary in their sensitivity and suitability for this assay. In addition, there are occasionally problems with specificity and purification of the antigen from feces (Moritsugu *et al.*, 1976); some preparations of the

antigen do not work satisfactorily. Nevertheless, this is a most useful technique for the serological diagnosis of infection with hepatitis A virus.

E. Radioimmunoassay

Hollinger *et al.* (1975) described a microtiter two-site immunoradiometric assay for hepatitis A antigen. The method involves the coupling of unlabeled antibody to an insoluble matrix. Antigen being tested reacts with the antibody and the specific antigen is detected after interaction with a second radiolabeled hepatitis A antibody, with the uptake of radioactivity being proportional to the concentration of antigen participating in the first antigen–antibody reaction. The effectiveness and specificity of this technique for rapid and quantitative detection of hepatitis A virus antigen was demonstrated in specimens from infected marmoset liver, and feces and serum from patients and chimpanzees. In addition, samples which were found negative by immune electron microscopy were found to contain significant levels of hepatitis A antigen by radioimmunoassay.

Purcell *et al.* (1976) used a blocking test in a microtiter solid-phase radioimmunoassay to measure hepatitis A antibody. Reduction in radioactivity of 40% or more compared with a negative serum is considered evidence for the presence of hepatitis A antibody. This technique appears to be the most sensitive method currently available for the assay of hepatitis A antigen and antibody.

The development of other diagnostic techniques such as enzyme-linked immunosorbent assay can be confidently predicted. The only limiting factor to new developments and to the widespread use of the techniques described above for hepatitis A is the scarcity of purified hepatitis A antigen.

V. IMMUNIZATION AGAINST HEPATITIS

A. Passive Immunization

1. Hepatitis A

Pooled human immunoglobulin, a 16% solution at 0.02 to 0.12 ml/kg body weight, administered intramuscularly before exposure to the virus or early during the incubation period prevents or attenuates a clinical illness (World Health Organization, 1964). The precise mode of action of immunoglobulin is not entirely clear but it is presumed that some degree of immunity against hepatitis A is obtained by globulin prepared from large pools of plasma. An inapparent or subclinical hepatitis which may

follow active-on-passive immunity may lead to prolonged immunity, but the possibility of inducing at least a temporary carrier state should be considered. The value of pooled immunoglobulin in controlling outbreaks of hepatitis A in situations such as infection in a nursery school or semi-closed communities in large institutions has been repeatedly demonstrated. However, the view has been expressed that the very large-scale and repeated use of immunoglobulin in the general school-age population is undesirable because unrecognized anicteric or subclinical cases may disseminate the virus in the community. In addition, the practice appears to be wasteful and repeated injections of immunoglobulin may be undesirable in, for example, healthy children (World Health Organization, 1976). The availability of techniques for titrating antibodies to hepatitis A provide means of assay of antibodies in pooled immunoglobulin.

2. Hepatitis B

Studies on passive immunization using immunoglobulin with a high titer of hepatitis B surface antibody prepared from the plasma of selected donors are in progress in several countries. Such preparations appear to offer some protection in a proportion of subjects exposed to infection with hepatitis B virus. Data from experimental transmission studies and from surveys (reviewed in World Health Organization reports, 1973, 1975) indicate a protective effect of surface antibody in recipients, presumably by neutralizing the virus, for example, in donor blood. Batches of such immunoglobulin preparations are now titrated for surface antibody in order to establish the levels of antibody required for protection. Hepatitis B core antibody, unlike the surface antibody, is not protective and its presence is not correlated with resistance to reinfection.

B. Active Immunization

1. Hepatitis A

At the present time, hepatitis A virus has not been propagated serially in tissue culture. However, since a candidate hepatitis A virus can now be identified morphologically and serologically, rapid progress in cultivation may be expected. Once this major step has been accomplished it should then be a relatively easy matter to produce a conventional killed or an attenuated live vaccine.

2. Hepatitis B

There is an urgent need for a hepatitis B vaccine for groups which are at an increased risk of acquiring this infection. These groups include health

care personnel and laboratory staff and staff and residents of institutions for the mentally retarded and other closed institutions. Other patients at high risk include patients on maintenance hemodialysis, those requiring repeated blood transfusions or the administration of blood products, patients requiring treatment with immunosuppressive or cytotoxic drugs, patients with malignant diseases and disorders associated with depression of the immune response, and chronic liver disease. Consideration will also have to be given to persons living in certain tropical areas where present socioeconomic conditions and sanitation are poor.

The repeated failure to passage hepatitis B virus serially in tissue or organ cultures has hampered progress toward the development of a safe and effective vaccine. Attention has therefore been directed toward the use of other preparations for active immunization against hepatitis B (reviewed by Zuckerman and Howard, 1973; Zuckerman, 1975d).

Active immunization was attempted by Krugman *et al.* (1971) using as the immunogen a known infective human serum (MS-2 serum) that contains hepatitis B virus. The serum was diluted 1 in 10 in distilled water and heated at 98°C for 1 minute. The serum treated in this manner was not infective and it successfully prevented or modified hepatitis B in 69% of susceptible persons inoculated with the heated serum and challenged with the original infective serum 4–8 months later. The results with the heat-inactivated serum were essentially the same after one, two, or three inoculations. In other studies carried out by Soulier *et al.* (1972), serum containing hepatitis B surface antigen obtained from a healthy carrier was heated at 60°C for 10 hours, but the infective agent was not completely inactivated as shown by the acquisition of antigen and elevated serum transaminase levels in a proportion of the recipients. Furthermore, this is a crude way of inducing immunity and it is unlikely to be accepted for general use. Nevertheless, the work of Krugman and his colleagues laid the foundations for unconventional hepatitis B immunogens that could be used as vaccines.

Isolated viral coat protein challenges the immune mechanism of the body in the same way as the intact infectious agent and the possibility of using purified small spherical 22-nm hepatitis B surface antigen particles, which are free of detectable nucleic acid, seems attractive. Such experimental vaccines have been prepared and a limited number of susceptible chimpanzees were shown to be protected by such immunogens (Purcell and Gerin, 1975; Hilleman *et al.*, 1975). These studies, although encouraging, were by no means comprehensive and some doubts linger regarding the possible harmful induction of immunological reactions to host proteins which may be closely associated with or incorporated in the small spherical particles of hepatitis B surface antigen. These host proteins may

include various preexisting structures of the liver cell. Several independent studies have shown a close association between the surface antigen and a number of serum proteins. These may be tightly bound as nonspecific contaminants during the purification procedure (Burrell, 1975) or as integral components of the surface antigen (Neurath *et al.*, 1974).

Demonstrable and significant levels of carbohydrate in purified fractions of the surface antigen have also been found. The carbohydrate might have a novel haptenic specificity which is either virus-coded or virus-induced host cell coded. Alternatively, the carbohydrate, and some lipoprotein components, might simply be derived from the host cell membranes as the mature virus particles are released. There may be some similarity between such a carbohydrate hapten, or the lipoprotein components, and those carbohydrate and lipoprotein antigens of normal cell surfaces, leading to a degree of tolerance because of a close antigenic resemblance between hepatitis B surface antigen and "self" antigens. Alternatively, an autoimmune reaction may be initiated. Such an autoimmune reaction may be induced by hepatitis B infection because of a change in antigenicity of the hepatocyte cell membranes due to alteration of existing antigens, or the appearance of viral determinants. T lymphocytes responsive to such new antigenic determinants could promote a B cell response to unaltered "self" antigens. The synthesis and release of the resulting autoantibody is subject, in turn, to control by suppressor T cells. These complex interactions between T and B cells could be of fundamental importance in the pathogenesis of chronic liver damage, as previously discussed.

Liver-specific lipoprotein is a macrolipoprotein which is thought to be a normal constituent of the hepatocyte plasma membrane. The isolation of two organ-specific proteins from human liver (Meyer zum Buschenfelde and Miescher, 1972) was followed by the demonstration of organ-specific antibodies in the sera of a proportion of patients with active chronic hepatitis. In addition, active chronic hepatitis has been induced in rabbits by repeated immunization with extracts containing these liver-specific proteins. Cellular hypersensitivity, as measured by leukocyte migration inhibition to these proteins was found in 69% of 16 patients with active chronic hepatitis, and 50% of 12 patients with primary biliary cirrhosis. More recently, evidence was found of cell-mediated sensitization to hepatitis B surface antigen in all patients with acute hepatitis B and transitory sensitization to liver-specific lipoprotein was detected in many of the patients (Lee *et al.*, 1975).

These findings are consistent with the hypothesis that a cell-mediated immune response to hepatitis B antigen, present early on at the onset of acute hepatitis, is the cause of acute liver damage by a cytotoxic effect on

virus-infected cells. If the response to liver-specific lipoproteins persisted, it could be responsible for progression to chronic liver damage. It is also postulated that the progressive liver damage of active chronic hepatitis is due to an autoimmune reaction directed against an hepatocyte surface lipoprotein which is initiated in most cases by infection with hepatitis B virus. Lee *et al.* (1975) found evidence of cell-mediated immunity to hepatitis B surface antigen in 62% of patients with antigen-negative chronic hepatitis, suggesting a high frequency of previous infection with hepatitis B virus. A cellular response to the antigen was found in the majority of hepatitis B antigen-positive patients. Evidence of sensitization to liver-specific lipoprotein was found in more than half of the patients, with a similar frequency in the two groups. These results are in agreement with the hypothesis that infection with hepatitis B virus is important in initiating the disease in many cases of active chronic hepatitis and that sensitization to the liver cell membrane antigen is responsible for the perpetuation of the liver injury. Thomson *et al.* (1974) demonstrated the killing of isolated rabbit hepatocytes *in vitro* when incubated with lymphocytes from 20 out of 22 patients with untreated active chronic hepatitis. Blocking experiments strongly suggest that the cytotoxicity is due to an immunological reaction directed at a cell surface antigen.

Thus immunological mechanisms and the presence of antibodies reacting with various tissue components may well be involved in the pathogenesis of liver damage. It may therefore be undesirable to employ preparations of hepatitis B surface antigen which may contain host cell components or host proteins for immunization against this infection.

The potential use of antigenic polypeptides for immunization against hepatitis B is under investigation in a number of laboratories. Dreesman *et al.* (1975) and Shih and Gerin (1975) recently demonstrated that polypeptides separated from purified surface antigen elicit a vigorous antibody response in guinea pigs. The use of these immunogenic polypeptides was validated by the finding of a cell-mediated immune response to intact hepatitis B surface antigen after inoculation with certain polypeptides. It should be noted that although there was no response to normal human serum, immunization with a low molecular weight component did elicit a cell-mediated response to normal human serum, suggesting that at least one integral component may contain an antigenic determinant related to a human serum protein (Cabral *et al.*, 1975). Work is now in progress to determine if such preparations could be used as hepatitis B vaccines (Zuckerman and Howard, 1975).

Another aspect for consideration in the design of a suitable immunogen is the need for stimulating an anti-*e* response (Neurath *et al.*, 1976). This antibody might be related in some way to protection, perhaps by aggregat-

ing the presumed hepatitis B virion, but such studies have yet to be carried out.

Perhaps one of the most interesting prospects for the future is the development of a synthetic vaccine. An immunochemical study of purified hepatitis B surface antigen is essential for this project. Analogous to the TMVP decapeptide, the primary sequency of the haptenic peptide of hepatitis B antigen would be the approach for developing a synthetic peptide, which, when coupled to a macromolecular carrier, could serve as a suitable immunogen. Once detailed data are available on the protein, peptide, and amino acid composition of this antigen, it should be possible to define by animal immunization the moiety responsible for the antigenic activity. Provided a sufficiently small fragment of the molecule would be immunogenic, a synthetic vaccine may then be feasible.

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REFERENCES

Aach, R. D., Hacker, E. J., and Parker, C. W. (1973). *J. Immunol.* **111,** 381.

Almeida, J. D., and Waterson, A. P. (1969). *Lancet* **2,** 983.

Almeida, J. D., Zuckerman, A. J., Taylor, P. E., and Waterson, A. P. (1969). *Microbios* **2,** 117.

Almeida, J. D., Rubenstein, D., and Stott, E. J. (1971). *Lancet* **2,** 1225.

Almeida, J. D., Deinhardt, F., and Zuckerman, A. J. (1974). *Lancet* **2,** 1083.

Anan'ev, V. A., Vorozhbieva, T. E., Naumora, V. K., Narskii, S. V., Gushcin, B. V., Braginskii, D. M., and Zhdanov, D. M. (1972). *Vopr. Virusol.* **5,** 544.

Barker, L. F., Chisari, F. V., McGrath, P. P., Dalgard, D. W., Kirschstein, R. L., Almeida, J. D., Edgington, T. S., Sharp, D. G., and Peterson, M. R. (1973). *J. Infect. Dis.* **127,** 648.

Barker, L. F., Almeida, J. D., Hoofnagle, J. H., Gerety, R. J., Jackson, D. R., and McGrath, P. P. (1974). *J. Virol.* **14,** 1552.

Bayer, M. E., Blumberg, B. S., and Werner, B. (1968). *Nature (London)* **218,** 1057.

Bedarida, G., Trinchieri, G., and Carbonara, A. (1969). *Haematologica* **54,** 591.

Bedarida, G., Zacchi, T., and Tassi, G. C. (1971). *Boll. Ist. Sieroter. Milan* **50,** 74.

Blumberg, B. S., Alter, H. J., and Visnich, S. (1965). *J. Am. Med. Assoc.* **191,** 541.

Brzosko, W. J., Madalinski, K., Krawczynski, K., and Nowoslawski, A. (1973). *J. Infect. Dis.* **127,** 424.

Brzosko, W. J., Krawczynski, K., Nazarewicz, T., Morzycka, M., and Nowoslawski, A. (1974). *Lancet* **2,** 477.

Burrell, C. J. (1975). *J. Gen. Virol.* **27,** 117.
Burrell, C. J., Proudfoot, E., Keen, G. A., and Marimion, B. P. (1973). *Nature (London), New Biol.* **243,** 260.
Cabral, G. A., Chairez, R., Marciano-Cabral, F., Suzrez, M., Dreesman, G. R., Melnick, J. L., and Hollinger, F. B. (1975). *Infect. Immun.* **12,** 564.
Cameron, C. H., and Dane, D. S. (1974). *Br. Med. Bull.* **30,** 90.
Chairez, R., Steiner, S., Melnick, J. L., and Dreesman, G. R. (1973). *Intervirology* **1,** 224.
Chairez, R., Hollinger, F. B., Melnick, J. L., and Dreesman, G. R. (1974). *Intervirology* **3,** 129.
Chairez, R., Hollinger, F. B., Brunschwig, J. P., and Dreesman, G. R. (1975). *J. Virol.* **15,** 182.
Coyne, V. E., Millman, I., Cerda, J., Gerstley, B. J. S., London, T., Sutnick, A., and Blumberg, B. S. (1970). *J. Exp. Med.* **131,** 307.
Culliford, B. J. (1964). *Nature (London)* **201,** 1092.
Dane, D. S., Cameron, C. H., and Briggs, M. (1970). *Lancet* **1,** 695.
Deinhardt, F., Holmes, A. W., Capps, R. B., and Popper, H. (1967). *J. Exp. Med.* **125,** 673.
Deinhardt, F., Wolfe, L., Junge, U., and Holmes, A. W. (1972). *Can. Med. Assoc. J.* **106,** 468.
Dienstag, J. L., Feinstone, S. M., Kapikian, A. Z., and Purcell, R. H. (1975a). *Lancet* **1,** 102.
Dienstag, J. L., Feinstone, S. M., Kapikian, A. Z., Purcell, R. H., Boggs, J. D., and Conrad, M. E. (1975b). *Lancet* **1,** 765.
Dreesman, G. R., Hollinger, F. B., McCombs, R. M., and Melnick, J. L. (1972a). *Infect. Immun.* **5,** 213.
Dreesman, G. R., Hollinger, F. B., Suriano, J. R., Fujioka, R. S., Bruwschwig, J. P., and Melnick, J. L. (1972b). *J. Virol.* **10,** 469.
Dreesman, G. R., Hollinger, F. B., and Melnick, J. L. (1972c). *Appl. Microbiol.* **24,** 1001.
Dreesman, G. R., Chairez, R., Suarez, M., Hollinger, F. B., Courtney, R. J., and Melnick, J. L. (1975). *J. Virol.* **16,** 508.
Dudley, F. J., Fox, R. A., and Sherlock, S. (1972). *Lancet* **1,** 723.
Duimel, W. J., and Brummelhuis, H. G. J. (1975). *Vox Sang.* **29,** 1.
Eddleston, A. L. W. F., and Williams, R. (1974). *Lancet* **2,** 1543.
Edgington, T. S., and Ritt, D. J. (1971). *J. Exp. Med.* **134,** 871.
Elefthériou, N., Thomas, H. C., Heathcote, J., and Sherlock, S. (1975). *Lancet* **2,** 1171.
El Sheikh, N., Woolf, I. L., Galbraith, R. M., Eddleston, A. L. W. F., Dymock, I. W., and Williams, R. (1975). *Br. Med. J.* **4,** 252.
Engvall, E., and Perlmann, P. (1971). *Immunochemistry* **8,** 871.
Engvall, E., and Perlmann, P. (1972). *J. Immunol.* **109,** 129.
Feinman, S. V., Berris, B., Sinclair, J. C., Wrobel, D. M., Murphy, B. L., and Maynard, J. E. (1975). *Lancet* **2,** 1173.
Feinstone, S. M., Kapikian, A. Z., and Purcell, R. H. (1973). *Science* **182,** 1026.
Ferris, A. A., Kaldor, J., Gust, I. D., and Cross, G. (1970). *Lancet* **1,** 243.
Figenschau, K. J., and Ulstrup, J. C. (1974). *Acta Pathol. Microbiol. Scand., Sect. B* **82,** 422.
Gerin, J. L. (1972). *In* "Hepatitis and Blood Transfusion" (G. N. Vyas, H. A. Perkins, and R. Schmid, eds.), pp. 205–219. Grune & Stratton, New York.
Gerin, J. L. (1974). *In* "Mechanisms of Virus Disease" (W. S. Robinson and C. F. Fox, eds.), pp. 215–223. Benjamin, New York.
Gerin, J. L. (1975). *Am. J. Med. Sci.* **270,** 115.

Gerin, J. L., Purcell, R. H., Hoggan, M. D., Holland, P. V., and Chanock, R. M. (1969). *J. Virol.* **4,** 763.
Gerin, J. L., Faust, R. M., and Holland, P. V. (1975). *J. Immunol.* **115,** 100.
Gianotti, F. (1973). *Arch. Dis. Child.* **48,** 793.
Gocke, D. J., Morgan, C., Lockshin, M., Hsu, K., Bombardieri, S., and Christian, C. L. (1970). *Lancet* **2,** 1149.
Gravelle, C. R., Hornbeck, C. L., Maynard, J. E., Schable, C. A., Cook, E. H., and Bradley, D. W. (1975). *J. Infect. Dis.* **131,** 167.
Greenman, R. L., and Robinson, W. S. (1974). *J. Virol.* **13,** 1231.
Greenman, R. L., Robinson, W. S., and Vyas, G. N. (1975). *Vox Sang.* **29,** 77.
Greenwood, F. C. (1967). *In* "Modern Trends in Endocrinology" (H. Gardner-Hill, ed.), pp. 288–300. Butterworth, London.
Harris, W. W., Anderson, N. G., Bartlett, T. W., Rutenberg, E. L., McCauley, L. L., and Kniseley, R. M. (1966). *Natl. Cancer Inst., Monogr.* **21,** 389.
Hilleman, M. R., Buynak, E. B., Roehm, R. R., Tytell, A. A., Bertland, A. U., and Lampson, G. P. (1975). *Am. J. Med. Sci.* **270,** 401.
Hirata, A. A., Emerick, A. J., and Boley, W. F. (1973). *Proc. Soc. Exp. Biol. Med.* **143,** 761.
Hirschman, S. Z. (1975). *Lancet* **2,** 436.
Hirschman, S. Z., Vernace, S. J., and Schaffner, F. (1971). *Lancet* **1,** 1099.
Hirschman, S. Z., Gerber, M., and Garfinkel, E. (1974a). *Proc. Natl. Acad. Sci. U.S.A.* **71,** 3345.
Hirschman, S. Z., Gerber, M., and Garfinkel, E. (1974b). *Nature (London)* **251,** 540.
Hollinger, F. B. (1974). *In* "Manual of Clinical Microbiology" (E. H. Lennette, E. H. Spaulding, and J. P. Truant, eds.), pp. 819–833. Am. Soc. Microbiol., Washington, D.C.
Hollinger, F. B., Bradley, D. W., Maynard, J. E., Dreesman, G. R., and Melnick, J. L. (1975). *J. Immunol.* **115,** 1464.
Holmes, A. Q., Deinhardt, F., Wolfe, L., Froesner, G., Peterson, D., Casto, B., and Conrad, M. E. (1973). *Nature (London)* **243,** 419.
Hoofnagle, J. H., Gerety, R. J., and Barker, L. F. (1973). *Lancet* **2,** 869.
Hoofnagle, J. H., Gerety, R. J., and Barker, L. F. (1975). *Am. J. Med. Sci.* **270,** 179.
Hopkins, R., and Das, P. C. (1972). *J. Clin. Pathol.* **25,** 832.
Hopkins, R., and Das, P. C. (1973). *Br. J. Haematol.* **25,** 619.
Howard, C. R., and Burrell, C. J. (1976). *Prog. Med. Virol.* **22,** 36.
Howard, C. R., and Zuckerman, A. J. (1974). *Intervirology* **4,** 31.
Howard, C. R., and Zuckerman, A. J. (1975). *Lancet* **2,** 774.
Imai, M., Gotoh, A., Nishioka, K., Kurashina, S., Miyakawa, Y., and Mayumi, M. (1974). *J. Immunol.* **112,** 416.
Ishimaru, Y., Ishimaru, H., Toda, G., Baba, K., and Mayumi, M. (1976). *Lancet* **1,** 708.
Ito, M., and Tagaya, I. (1966). *Jpn. J. Med. Sci. & Biol.* **19,** 109.
Jambazian, A., and Holper, J. C. (1972). *Proc. Soc. Exp. Biol. Med.* **140,** 560.
Jokelainen, P. T., Krohn, K., Prince, A. M., and Finlayson, N. D. C. (1970). *J. Virol.* **6,** 685.
Jozwiak, W., Koscielak, J., Madalinski, K., Brzosko, W. J., Nowoslawski, A., and Kloczewiak, M. (1971). *Nature (London), New Biol.* **229,** 92.
Juji, T., and Yokochi, T. (1969). *Jpn. J. Exp. Med.* **39,** 615.
Kachani, Z. F., and Gocke, D. J. (1973). *J. Immunol.* **111,** 1564.
Kaplan, P. M., Greenman, R. L., Gerin, J. L., Purcell, R. H., and Robinson, W. S. (1973). *J. Virol.* **12,** 995.
Kim, C. Y., and Bissell, D. M. (1973). *J. Infect. Dis.* **123,** 470.
Kim, C. Y., and Tilles, J. G. (1973). *J. Clin. Invest.* **52,** 1176.

Kim, C. Y., Spano, J., and Clark, G. E. (1971). *J. Infect. Dis.* **124,** 411.
Kohn, J., and Morgan, J. R. (1971). *J. Clin. Pathol.* **24,** 673.
Krugman, S., Giles, J. P., and Hammond, J. (1971). *J. Am. Med. Assoc.* **217,** 41.
Krugman, S., Hoofnagle, J. H., Gerety, R. J., Kaplan, P. M., and Gerin, J. L. (1974). *N. Engl. J. Med.* **290,** 1331.
Krugman, S., Friedman, H., and Lattimer, C. (1975). *N. Engl. J. Med.* **292,** 1141.
Lambert, P. H., Tribollet, E., Knoepfel, M., Madalinski, K., and Miescher, P. A. (1974). *Schweiz. Med. Wochenschr.* **104,** 128.
Lander, J. J., Alter, H. J., and Purcell, R. H. (1971). *J. Immunol.* **106,** 1166.
Leach, J. M., and Ruck, B. J. (1971). *Br. Med. J.* **4,** 597.
Le Bouvier, G. L., and McCollum, R. W. (1970). *Adv. Virus Res.* **16,** 357.
Le Bouvier, G. L., and Williams, A. (1975). *Am. J. Med. Sci.* **270,** 165.
Lee, W. M., Reed, W. D., Mitchell, C. G., Galbraith, R. M., Eddleston, A. L. W. F., Zuckerman, A. J., and Williams, R. (1975). *Br. Med. J.* **1,** 705.
Leslie, M., Hopkins, R., and Das, P. C. (1975). *Med. Lab. Technol.* **32,** 121.
Ling, C. M., and Overby, L. R. (1972). *J. Immunol.* **109,** 834.
Locarnini, S. A., Ferris, A. A., Stott, A. C., and Gust, I. D. (1974). *Intervirology* **4,** 110.
Lorenz, D., Barker, L., Stevens, D., Peterson, M., and Kirchstein, R. (1970). *Proc. Soc. Exp. Biol. Med.* **135,** 348.
Magnius, L. O. (1975). *Clin. Exp. Immunol.* **20,** 209.
Magnius, L. O., and Espmark, A. (1972). *J. Immunol.* **109,** 1017.
Magnius, L. O., Lindholm, A., Lundin, P., and Iwarson, S. (1975). *J. Am. Med. Assoc.* **231,** 356.
Mascoli, C. C., Ittensohn, D., Villarejos, V. M., Arguedas, J. A., Provost, P. J., and Hilleman, M. R. (1973). *Proc. Soc. Exp. Biol. Med.* **142,** 276.
Maynard, J. E., Bradley, D. W., Gravelle, C. R., Hornbeck, C. L., and Cook, E. H. (1974). *Lancet* **2,** 1207.
Maynard, J. E., Bradley, D. W., Gravelle, C. R., Ebert, J. W., and Krushak, D. H. (1975). *J. Infect. Dis.* **131,** 194.
Mayumi, M., Okochi, K., and Nishioka, K. (1971). *Vox Sang.* **20,** 178.
Meyer zum Buschenfelde, K. H., Kossling, F. K., and Miescher, P. A. (1972). *Clin. Exp. Immunol.* **11,** 99.
Miller, W. J., Provost, P. J., McAleer, W. J., Ittensohn, O. L., Villarejos, V. M., and Hilleman, M. R. (1975). *Proc. Soc. Exp. Biol. Med.* **149,** 254.
Millman, I., Zavatone, V., Gerstley, B. J. S., and Blumberg, B. S. (1969). *Nature (London)* **222,** 181.
Millman, I., Loeb, L. A., Bayer, M. E., and Blumberg, B. S. (1970). *J. Exp. Med.* **131,** 1190.
Millman, I., Hutanen, H., Merino, F., Bayer, M. E., and Blumberg, B. S. (1971). *Res. Commun. Chem. Pathol. Pharmacol.* **2,** 667.
Moritusgu, Y., Gold, J. W. M., Wagner, J., Dodd, R. Y., and Purcell, R. H. (1975). *J. Immunol.* **114,** 1792.
Moritsugu, Y., Dienstag, J. L., Valdesuso, J., Wong, D. C., Wagner, J., Routenberg, J. A., and Purcell, R. H. (1976). *Infect. Immun.* **13,** 898.
Muller, R., Stephan, B., Kramer, R., and Deicher, H. (1975). *Vox Sang.* **29,** 330.
Neurath, A. R., Prince, A. M., and Lippin, A. (1974). *Proc. Natl. Acad. Sci. U.S.A.* **71,** 2663.
Neurath, A. R., Trepo, C., Chen, M., and Prince, A. M. (1976). *J. Gen. Virol.* **30,** 277.
Nielsen, J. O., Dietrichson, O., and Juhl, E. (1974). *Lancet* **2,** 913.
Nowoslawski, A., Brzosko, W. J., Madalinski, K., and Krawczynski, K. (1970). *Lancet* **1,** 494.

Nowoslawski, A., Krawcyznski, K., Brzosko, W. J., and Madalinski, K. (1972). *Am. J. Pathol.* **68,** 31.
Okochi, K., Mayumi, M., Haguino, Y., and Saito, N. (1970). *Vox Sang.* **19,** 332.
Overby, L. R., Hung, P. P., Mao, J. C.-H., Ling, C. M., and Kakefuda, T. (1975). *Nature (London)* **255,** 84.
Patil, J. R., and Pert, J. H. (1975). *J. Immunol. Methods* **7,** 169.
Pert, J. H., and Verch, R. L. (1975). *Vox Sang.* **29,** 157.
Prince, A. M., Fuji, H., and Gershon, R. K. (1964). *Am. J. Hyg.* **79,** 365.
Provost, P. J., Wolanski, B. S., Miller, W. J., Ittensohn, O. L., McAleer, W. J., and Hilleman, M. R. (1975a). *Proc. Soc. Exp. Biol. Med.* **148,** 532.
Provost, P. J., Ittensohn, O. L., Villarejos, V. M., and Hilleman, M. R. (1975b). *Proc. Soc. Exp. Biol. Med.* **148,** 962.
Purcell, R. H., and Gerin, J. L. (1975). *Am. J. Med. Sci.* **270,** 395.
Purcell, R. H., Wong, D. C., Alter, H. J., and Holland, P. V. (1973). *Appl. Microbiol.* **26,** 478.
Purcell, R. H., Gerin, J. L., Almeida, J. D., and Holland, P. V. (1974). *Intervirology* **2,** 231.
Purcell, R. H., Wong, D. C., Moritsugu, Y., Dienstag, J. L., Routenberg, J. A., and Boggs, J. D. (1976). *J. Immunol.* **116,** 349.
Rao, K. R., and Vyas, G. N. (1973). *Nature (London), New Biol.* **241,** 240.
Rao, K. R., and Vyas, G. N. (1974). *J. Gen. Virol.* **24,** 571.
Reed, W. D., Eddleston, A. L. W. F., Cullens, H., Williams, R., Zuckerman, A. J., Peters, D. K., Williams, D. G., and Maycock, W. d'A. (1973). *Lancet* **2,** 1347.
Reed, W. D., Mitchell, C. G., Eddleston, A. L. W. F., Lee, W. M., Williams, R., and Zuckerman, A. J. (1974). *Lancet* **1,** 581.
Robinson, W. S. (1974). *In* "Mechanisms of Virus Disease" (W. S. Robinson and C. F. Fox, eds.), pp. 225–241. Benjamin, New York.
Shih, J. W.-K., and Gerin, J. L. (1975). *J. Immunol.* **115,** 634.
Simons, M. J. (1973a). *Bull. W. H. O.* **48,** 499.
Simons, M. J. (1973b). *Bull. W. H. O.* **49,** 107.
Skinhøj, P., and Hansen, J. F. (1973). *Scand. J. Clin. Lab. Invest.* **32,** 199.
Soulier, J. P., Blatix, C., Courouce, A. M., Bensmon, D., Amouch, P., and Drouet, J. (1972). *Am. J. Dis. Child.* **123,** 429.
Steiner, S., Huebner, M. T., and Dreesman, G. R. (1974). *J. Virol.* **14,** 572.
Stevens, R. W., Ziegler, F. D., and Kelly, J. H. (1972). *J. Immunol.* **108,** 1123.
Sukeno, N., Shirachi, R., Yamaguchi, J., and Ishida, N. (1972a). *J. Virol.* **9,** 182.
Sukeno, N., Shirachi, R., Shiraishi, H., and Ischida, N. (1972b). *J. Virol.* **10,** 157.
Summers, J., O'Connell, A., and Millman, I. (1975). *Proc. Natl. Acad. Sci. U.S.A.* **72,** 4597.
Takahashi, T. (1975). *J. Jpn. Med. Assoc.* **73,** 225.
Thomson, A. D., Cochrane, A. M. G., McGarlane, I. G., Eddleston, A. L. W. F., and Williams, R. (1974). *Nature (London)* **252,** 721.
Thornton, A., Zuckerman, A. J., and Almeida, J. D. (1975). *Lancet* **2,** 226.
Traavik, T., Kjeldsberg, E., and Siebke, J. C. (1973). *Acta Pathol. Microbiol. Scand., Sect. B* **81,** 37.
Trepo, C. G., Zuckerman, A. J., Bird, R. G., and Prince, A. M. (1974). *J. Clin. Pathol.* **27,** 863.
Tripodi, D., Hawk, J., Gocke, D. J., Redeker, A., and Starkovsky, N. A. (1973). *Am. J. Clin. Pathol.* **59,** 549.
Tsuda, T., Takahashi, T., Takahashi, K., Miyakawa, Y., and Mayumi, M. (1975). *J. Immunol.* **115,** 834.
van Weemen, B. K., and Schuurs, A. H. W. M. (1971). *FEBS Lett.* **15,** 232.

van Weemen, B. K., and Schuurs, A. H. W. M. (1972). *FEBS Lett.* **24,** 77.
van Weemen, B. K., and Schuurs, A. H. W. M. (1974). *FEBS Lett.* **43,** 215.
Voller, A., and Bidwell, D. E. (1975). *Br. J. Exp. Pathol.* **56,** 338.
Voller, A., Bidwell, D., Huldt, G., and Engvall, E. (1974). *Bull. W. H. O.* **51,** 209.
Voller,A., Draper, C., Bidwell, D. E., and Bartlett, A. (1975a). *Lancet* **1,** 426.
Voller, A., Huldt, G., Thors, C., and Engvall, E. (1975b). *Br. Med. J.* **1,** 659.
Vyas, G. N., and Shulman, N. R. (1970). *Science* **170,** 332.
Vyas, G. N., Rao, K. R., and Ibrahim, A. B. (1972a). *Science* **178,** 1300.
Vyas, G. N., Williams, E. W., Klaus, G. C. B., and Bond, H. E. (1972b). *J. Immunol.* **108,** 1114.
Walsh, J. H., Yalow, R., and Berson, S. A. (1970). *J. Infect. Dis.* **121,** 550.
Wide, L., and Porath, J. (1966). *Biochim. Biophys. Acta* **130,** 257.
Wolters, G., Kuijpers, L. Kacaki, J., and Schuurs, A. (1976). *J. Clin. Pathol.* **29,** 873.
World Health Organization. (1964). *W. H. O., Tech. Rep. Ser.* **285.**
World Health Organization. (1970). *Bull. W. H. O.* **42,** 957.
World Health Organization. (1973). *W. H. O., Tech. Rep. Ser.* **512.**
World Health Organization. (1975). *W. H. O., Tech. Rep. Ser.* **570.**
World Health Organization. (1976). "Report on a Working Group on Viral Hepatitis," World Health Organ., Copenhagen.
Yamada, G., Kobayashi, T., Tsuji, T., Ohta, Y., and Kosaka, K. (1973). *Tohoku J. Exp. Med.* **111,** 93.
Zuckerman, A. J. (1969). *Nature (London)* **223,** 569.
Zuckerman, A. J. (1970a). *In* "Virus Diseases of the Liver," pp. 120–127. Butterworth, London.
Zuckerman, A. J. (1970b). *Bull. W. H. O.* **42,** 975.
Zuckerman, A. J. (1972). *Am. J. Med. Technol.* **38,** 339.
Zuckerman, A. J. (1975a). *In* "Human Viral Hepatitis," pp. 33–65. Am. Elsevier, New York.
Zuckerman, A. J. (1975b). *In* "Human Viral Hepatitis," pp. 155–182. Am. Elsevier, New York.
Zuckerman, A. J. (1975c). *Am. J. Med. Sci.* **270,** 197.
Zuckerman, A. J. (1975d). *Nature (London)* **255,** 104.
Zuckerman, A. J., and Earl, P. M. (1973). *Vox Sang.* **24,** Suppl., 123.
Zuckerman, A. J., and Howard, C. R. (1973). *Nature (London)* **246,** 445.
Zuckerman, A. J., and Howard, C. R. (1975). *Bull. N.Y. Acad. Med.* [2] **51,** 491.
Zuckerman, A. J., and Taylor, P. E. (1970). "Shandon Monographs on Instrument Applications," No. 32.

Chapter 3

Slow Virus Infections: Comparative Aspects and Diagnosis

RICHARD F. MARSH

I. INTRODUCTION

Since Pasteur's proposal of the germ theory in the 1860's, our knowledge of how microorganisms produce disease has progressed rapidly. We have learned to control most infectious diseases and by so doing have increased the quality of life enormously. We now enter into a new era of virological research, that of the chronic or persisting infection. Technical

advancements now make it possible to examine the virus–host relationship in greater detail than ever before. This is an exciting time to be an investigator, and one of the most challenging new areas of study is slow virus disease.

Slow virus infections were first described by Sigurdsson (1954) who set forth a number of criteria, some of which are subject to different interpretations today. It is easy to get caught up in semantics when attempting to define slow virus disease. In usage, the term usually refers to a fatal illness with insidious onset resulting from a slowly progressive virus infection. More important than constructing definitions is to realize that slow infection is not an absolute property of any virus which can be used for taxonomic classification, but rather the result of the intricate interaction between a virus and its host. For example, rabies virus produces an acute disease if experimentally inoculated intracerebrally into a suscep-

TABLE I
Some Characteristics of Virus or Host Which May Contribute to the Establishment of a Slow Infection

Virus	Host	Possible mechanisms
Defective	Normal	Favors cell-associated infection; may lack surface antigens required for neutralization
Incomplete	Normal	Presence of many incomplete virus particles may interfere with an effective immune response to infectious virus, or suppress acute infection
Temperature-sensitive mutant	Normal	Favors defectiveness, especially in CNS
Poor antigen	Normal	Insufficient stimulation of immune system favoring persistence
	Inadequate immune response (T & B systems)	Unable to eliminate infected cells enhancing virus spread
		Formation of unneutralized, disease-producing virus–antibody complexes
		Activate latent infections
	Genetic anomaly	May work through above mechanisms via *Ir* genes
		Complement deficiency
		Reduced synthesis of biological mediators, e.g., lymphotoxin, interferon
		Alteration of target cell population

tible animal, whereas, natural rabies often results in a slow infection producing disease after an incubation period of several months.

There are many ways slow infections may be initiated with the course of infection determined for the most part by the immune status of the host (see Table I). Much of our knowledge on possible mechanisms of infection is theoretical, based on test tube models of interaction. Some information comes from inadvertent experiments where patients have been immunosuppressed for transplantation or have impaired immune responses due to lymphoid malignancies. It is certainly a field of study that requires inputs from many disciplines before any real understanding of disease processes can be reached. This chapter will review three slow virus diseases of the central nervous system (CNS) emphasizing their pathogenesis, comparative aspects, and epidemiology, including possible means of detection and control.

II. PROGRESSIVE MULTIFOCAL LEUKOENCEPHALOPATHY

Progressive multifocal leukoencephalopathy (PML) was first described by Åstrøm and associates (1958) as a complication of chronic lymphatic leukemia and Hodgkin's disease. Characteristic clinical features of this neurologic disorder include dementia, aphasia, hemiparesis, ataxia, and visual disturbances. The course of illness is usually less than 12 months, although there is one report of a patient surviving for as long as 19 years (Stam, 1966). The major gross pathological findings are focal areas of demyelination, usually found at the junction of white matter with cerebral cortex, which may coalesce to form large softenings. Microscopically, early lesions contain oligodendrocytes with enlarged, hyperchromatic nuclei. As the lesions mature, oligodendrocytes are replaced by mononuclear phagocytes and astrocytes, some of which have large, pleomorphic, deeply basophilic nuclei (Fig. 1).

A. Viral Etiology

Shortly after the first report of PML, Cavanagh *et al.* (1959) and Richardson (1961) suggested that the disease might be caused by an opportunistic virus infection in patients with impaired immune responses. This hypothesis was partially substantiated several years later when oligodendrocytes were found to contain virions of a papovavirus (ZuRhein and Chou, 1965; Silverman and Rubinstein, 1965). Padgett and co-workers (1971) were then successful in isolating a new human papovavirus from the brain of a PML patient with Hodgkin's disease. Almost simulta-

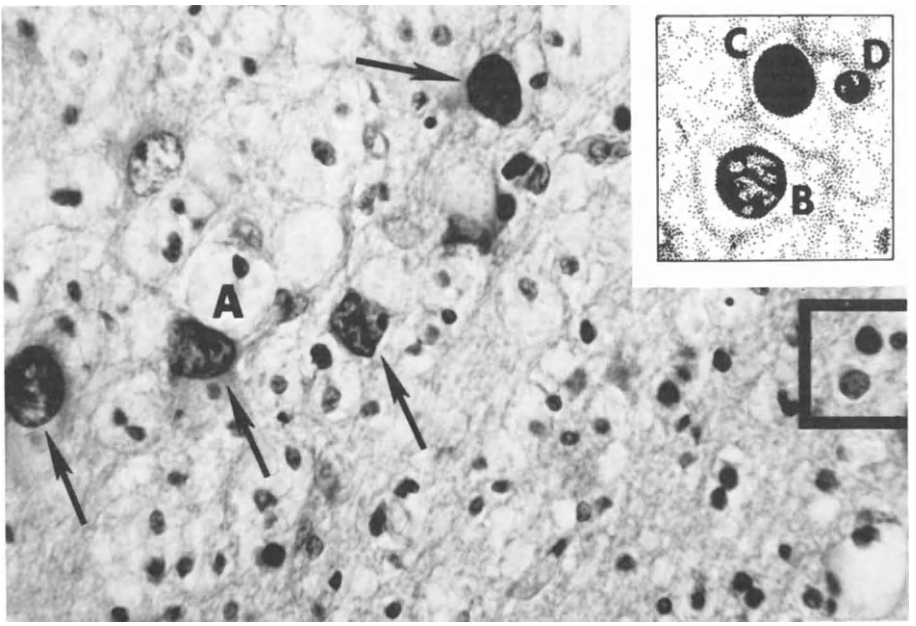

Fig. 1. Brain lesion demonstrating major cell types present in progressive multifocal leukoencephalopathy. Phagocytes (A) have begun to clear an area of myelin breakdown containing several astrocytes with enlarged, basophilic nuclei (arrows). Area outlined in black is diagramatically illustrated in upper right hand corner to show an unaffected astroglial nucleus (B), unaffected oligodendroglial nucleus (D), and the enlarged, basophilic nucleus of a virus-containing oligodendrocyte (C). Hematoxylin and eosin; ×460. Pathology specimen courtesy of Dr. G. M. ZuRhein.

neously, Weiner *et al.* (1972) also reported the recovery of papovaviruses from two cases of PML. These later two isolates were indistinguishable from the simian virus, SV40.

These recent virus isolations from PML, together with numerous reports on the recovery of papovaviruses from the urine of patients immunosuppressed after receiving renal allografts (Gardner *et al.*, 1971; Lecatsas *et al.*, 1973), have created a flury of activity in human papovavirus research (see Chapter 3, Volume I, and also an excellent review by Padgett and Walker, 1976). Papovaviruses have now been isolated from over 20 cases of PML. All but the two isolates by Weiner *et al.* (1972) have been similar to the original virus recovered by Padgett *et al.* (1971) and designated JC virus. There can be no doubt that JC virus is the major cause of PML. However, progress has been slowed by difficulty in detecting this virus in cell culture. JC virus grows only in primary human fetal glial cell cultures with CPE often not appearing on initial passage for 6 to 9 months.

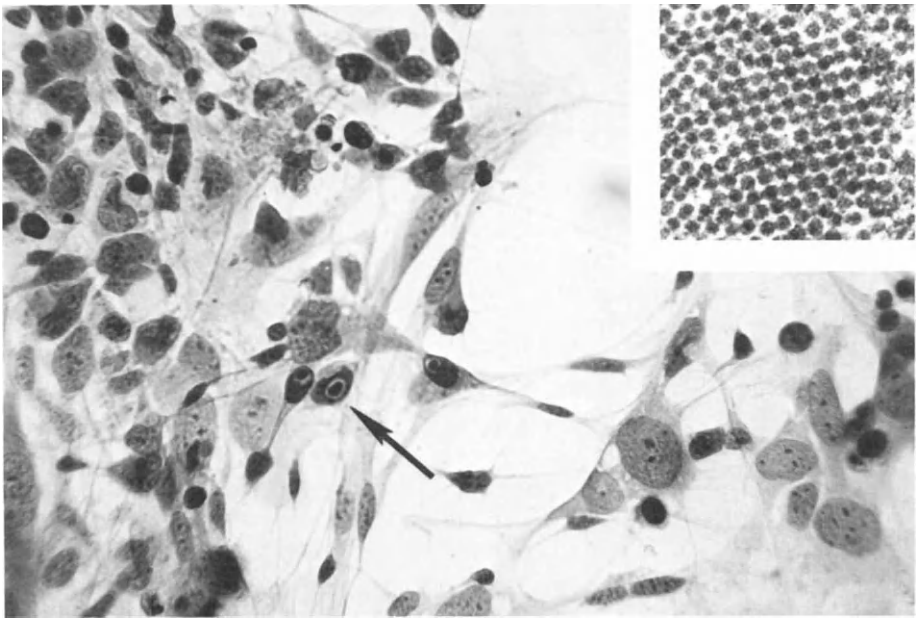

Fig. 2. Primary human fetal glial cell culture containing spongioblasts, some of which have Cowdry type A inclusions (arrow), undergoing a cytopathic infection with JC virus. Hematoxylin and eosin; × 460. Courtesy of Dr. Billie Padgett. Inset: electron micrograph of JC virus crystal, × 11,500. Courtesy of Dr. G. M. ZuRhein.

This long period, however, may be shortened by a series of blind passages using sonicated cells (Padgett and Walker, 1976). In cell culture, the virus has a preferential effect on spongioblasts, thought to be primordial stem cells differentiating into either oligodendrocytes or astrocytes. The spongioblasts slowly enlarge, losing their spindle shape, and ultimately are destroyed completely. These cells often contain Cowdry type A inclusions demonstrated by hematoxylin and eosin staining (Fig. 2). Astrocytes are not destroyed, but sometimes become enlarged with bizarre, multilobulated nuclei (B. L. Padgett, personal communication).

B. Pathogenesis

In their original report describing virus particles in PML brain tissue, ZuRhein and Chou (1965) astutely observed that this disease would be seen in increasing numbers in persons requiring immunosuppressive therapy. PML has subsequently been reported in three individuals following kidney transplant (Manz *et al.*, 1971; Legrain *et al.*, 1974; ZuRhein and Varakis, 1974), and in a patient treated for systemic lupus erythematosus

(Weiner *et al.*, 1972). It is apparent that the virus requires some help from its host before attaining full pathogenic potential. Most cases of PML are encountered secondary to a debilitating illness, most commonly lymphoid malignancies and leukemia. There have been cases of primary PML reported where no underlying disease was detected (Fermaglich *et al.*, 1970; Bolton and Rozdilsky, 1971; Faris and Martinez, 1972), however, no immunological studies were performed on these patients. Cases of PML in which the immune status has been evaluated have yielded inconclusive results on humoral immunoglobulins, but there has been a consistent deficiency in cell-mediated immune responses (Ellison, 1969; Knight *et al.*, 1972; Narayan *et al.*, 1973; Mathews *et al.*, 1976). It would appear that whether the immune deficiency is naturally produced by disease or is iatrogenic in origin, it is the alteration of cellular immunity which is the most important prerequisite for induction of PML.

Studies on the pathogenesis of PML have been limited by the lack of development of a suitable animal model. Since most papovaviruses are restricted in their host range, it should be anticipated that this model will likely need to be a subhuman primate. Inoculation of JC virus into newborn rhesus monkeys, newborn marmosets, and normal or immunosuppressed adult owl monkeys have yet to produce any signs of disease after observation periods of up to 3 years (Padgett and Walker, 1976). Recently, a spontaneous PML-like disease has been reported in macaques (Gribble *et al.*, 1975) and further study of this entity may well be applicable to the human disease.

One can only speculate on the progression and cellular effects of JC virus in host tissues. PML is a demyelinating disease and virus can be detected in oligodendrocytes, the cells principally responsible for the formation and maintainance of myelin sheaths. The simplest explanation is that there is interference in myelin synthesis due to the direct cytotoxic effect of virus on oligodendroglia. However, because some virus-containing oligodendrocytes do not appear to be undergoing a cytopathic infection, it has been suggested that myelin breakdown may be initiated by an autoimmune mechanism (Kepes *et al.*, 1975). This question can be resolved by examining PML sera and cerebrospinal fluid for a myelinotoxic effect on organizing brain explant cultures. A more important problem is to determine if JC virus can produce latent infections and, if so, in which tissues of the body. We do not yet know if PML is caused by activation of a latent infection, or due to an aberrant response to primary or recurrent exposure to the virus. If the virus is latent, does it reside in CNS tissue or a reservoir organ? Unidentified papovaviruses have been seen in brain tissue from persons with disorders other than PML (Koprowski *et al.*, 1970; Vernon *et al.*, 1970; Payne and Sibley, 1975). However, this does not nec-

essarily imply that these viruses are latent in brain, or that they have a significant effect on other disease processes. Gardner has recovered JC virus from the urine of two patients receiving renal allografts (see Chapter 3, Volume I). It is therefore possible that the virus may be harbored in tissues other than brain and spread to the CNS as an opportunistic infection in a compromised host. The fact that PML brain tissue has, on occasion, been found to be concomitantly infected with nocardia (Weiner *et al.*, 1973), listeria (Mathews *et al.*, 1976), or cryptococcus (Mathews *et al.*, 1976), would indicate that the CNS is subject to outside invasion in these susceptible individuals.

C. Epidemiology

Seroepidemiological studies of normal persons 10–14 years of age has shown a prevalence of HI antibody to JC virus of about 65%, rising to 70–75% by middle age (Padgett and Walker, 1973). Infection with this virus is therefore common, occurring relatively early in life. Nothing is known of the means of transmission. If JC virus behaves similarly to SV40, it is likely to be shed from infected individuals in urine or feces and to enter the body via oral or respiratory routes. There is no evidence of an animal reservoir.

There is as yet no convincing evidence that JC virus participates in disease processes other than PML. Examination of 70 patients with various neurological illnesses detected JC antibody in 72% (Brown *et al.*, 1975), a level comparable to normal populations. However, there are a number of observations which suggest that this virus should be carefully evaluated for a possible oncogenic effect in humans. First, the large, pleomorphic astrocytes found in PML brain tissue resemble malignant astrocytes in glioblastomas (ZuRhein, 1969) and, in fact, multiple gliomas have been recognized in an 18-year-old patient with PML (Castaigne *et al.*, 1974). Second, intracerebral inoculation of JC virus into newborn Syrian hamsters has resulted in the development of highly malignant brain tumors in 83% of the animals over a 6-month period (Walker *et al.*, 1973). This need not infer that the virus is oncogenic for humans, especially since papovaviruses rarely, if ever, produce malignant tumors in their natural hosts. It does indicate, however, an immediate need to examine human tumors for viral-induced antigens. To date, limited studies have not detected JC virus-induced T antigen in human tumor tissues (B. L. Padgett, personal communication).

Diagnosis of PML is based on clinical features of the disease together with a history of debilitating illness or use of immunosuppressive agents. Brain scans, detecting large areas of demyelination, have occasionally

yielded positive results (Smith, 1959; Mosher *et al.*, 1971; Mathews *et al.*, 1976), but the most definitive means of confirmation is by brain biopsy and demonstration of papovavirus virions in affected oligodendroglial cells. Early recognition of the disease syndrome has become more important because of reports on remission of PML following treatment with cytarabine (Bauer *et al.*, 1973; Conomy *et al.*, 1974). Control of this disease is likely to be difficult because of the high prevalence of infection. If PML is caused by primary or recurrent infection, strict isolation of patients receiving immunosuppressive therapy may be helpful. But if the disease is produced by activation of a latent infection, the seeds of destruction are already present. It should be obvious that immunosuppressive agents should never be used indiscriminately, and that benefits of such treatment be carefully weighed against possible sequelae.

III. SUBACUTE SCLEROSING PANENCEPHALITIS

Subacute sclerosing panencephalitis (SSPE) is a rare disease occurring primarily in children older than 4 years and in adolescence. Clinical signs are characterized by a gradual loss of cerebral functions beginning with a diminution of intelligence, then progressing to amentia, spastic paralysis, coma, and death. Histopathologic lesions are found in both white and

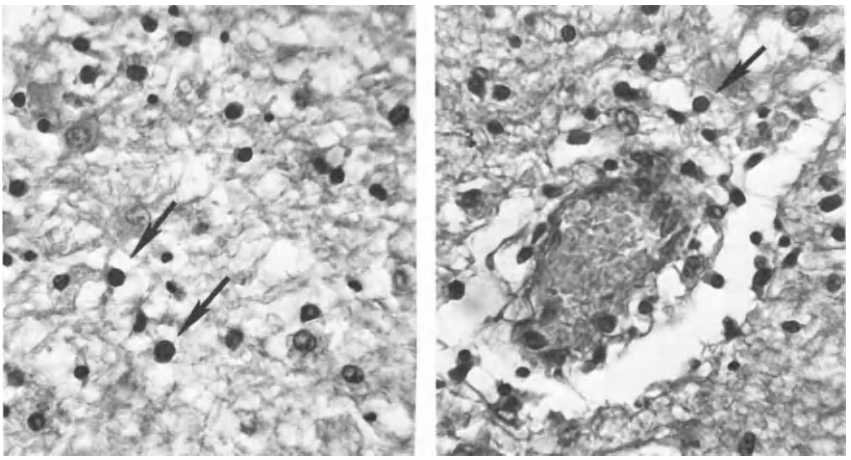

Fig. 3. Brain lesions from a 9-year-old girl with subacute sclerosing panencephalitis. Cells with nuclei containing hemogeneous, amphophilic inclusions (arrows) are interspersed in an affected area which, in this case, also shows an accompanying anoxic encephalopathy due to modern, life-support machines. Hematoxylin and eosin; ×391. Pathology specimen courtesy of Dr. G. M. ZuRhein.

gray matter and consist of inflammation, necrosis, demyelination, and astrocytosis. The major hallmark of the disease is the presence of intranuclear inclusion bodies in neurons and glia. The inclusions are quite pleomorphic; some appear as homogeneous masses (Fig. 3), while others are more classic Cowdry type A inclusions. SSPE was first described by Dawson (1933), who called the disease "inclusion body encephalitis" and suggested a viral etiology. However, it was 30 years before electron microscopic studies were able to confirm the presence of nucleocapsids of a paramyxovirus (Fig. 4) in affected brain cells (Bouteille *et al.*, 1965). This was followed relatively shortly by the isolation of measles virus from SSPE brain tissue (Horta-Barbosa *et al.*, 1969; Payne *et al.*, 1969). There

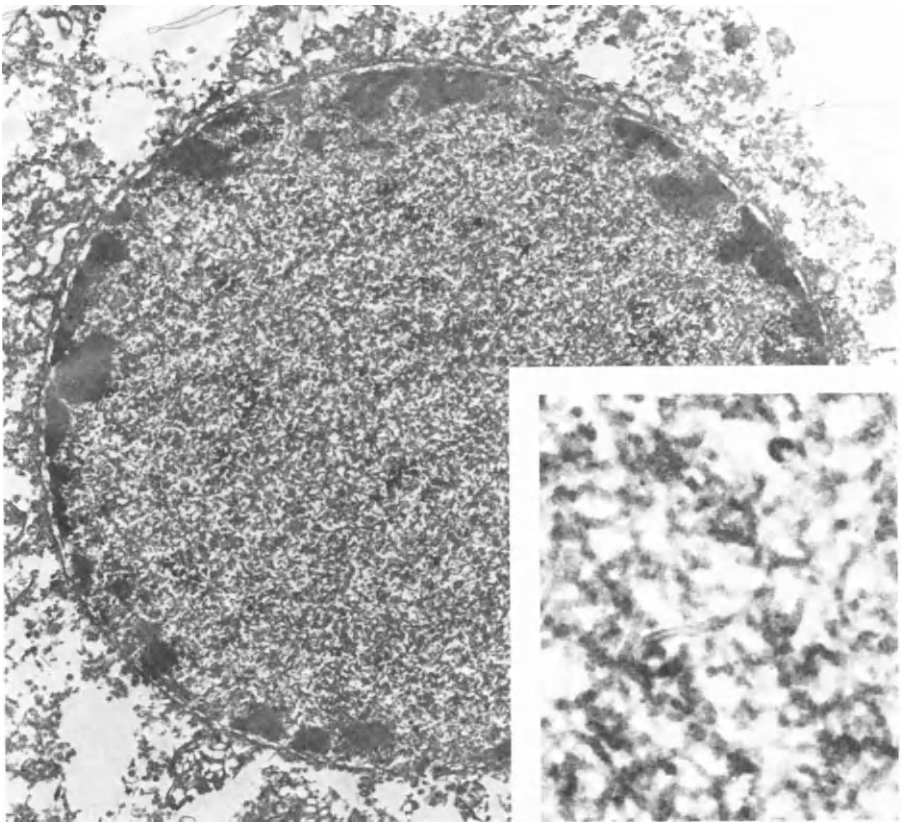

Fig. 4. Subacute sclerosing panencephalitis. Ultrastructure of brain cell nucleus containing viral nucleocapsid material, ×12,000. Inset: ×100,000. Courtesy of Dr. John Varakis.

is no doubt that the disease results from a slowly progressive measles virus infection in certain susceptible, but as yet undefined, children.

A. Pathogenesis

Natural infection with measles virus can take several courses. Most commonly the virus produces an acute, febrile illness with exanthem followed by complete recovery and life-long immunity. However, there are three recognized complications to infection; (1) individuals with impaired cell-mediated immune responses to measles virus may develop fatal giant cell pneumonia, (2) postinfectious encephalomyelitis may result from an abnormally vigorous immune response in which there is likely some element of hypersensitivity, and (3) SSPE. Acute encephalitis, caused by direct invasion of the CNS by measles virus, is rarely seen, although electroencephalographic studies show a high incidence of subclinical involvement (Pampiglione, 1964; Gibbs *et al.*, 1959), indicating that the virus infects brain tissue more commonly than generally appreciated.

It is during this initial interaction of measles virus with the CNS that SSPE distinguishes itself. The infection is incomplete, e.g., there is no infectious measles virus produced, or the small amount which is synthesized is not detectable using conventional virological techniques. Efforts which have been successful in recovering measles virus from SSPE tissues have employed procedures of cocultivation of brain (Horta-Barbosa *et al.*, 1969; Payne *et al.*, 1969) or lymphoid (Horta-Barbosa *et al.*, 1971) cells with permissive cells allowing viral rescue. Once rescued, isolates from SSPE tissues are immunologically indistinguishable from wild or attenuated measles viruses. They do vary in their biological properties; but none of the characteristics are unique, or consistently found from one SSPE virus to another. Similar properties can be acquired by various strains of measles viruses after adaptation to nervous tissue (Janda *et al.*, 1971; Raine *et al.*, 1971; Webb *et al.*, 1971; Bather *et al.*, 1973; Nakamura *et al.*, 1975). It would appear, therefore, that examination of the viral nature of SSPE isolates has not been particularly rewarding in explaining the unusual course of infection.

Although there is little or no intact measles virus produced extra- or intracellularly, the infection slowly progresses throughout the brain leaving few areas unaffected. The spread of replicating nucleocapsid material is by cell-to-cell transmission, possibly facilitated by fusion of plasma membranes (Iwasaki and Koprowski, 1974). The pathological mechanisms of tissue injury and the anomaly which allows the infection to persist are poorly understood. Patients with SSPE have high titers of measles anti-

body in their serum and cerebrospinal fluid (Connolly *et al.*, 1967). The antibody is produced mainly by immune competent cells residing in the CNS (Connolly, 1968; Norrby *et al.*, 1974) in response to the locally intense immunization by measles antigens. The antibody response is predominantly directed against nucleocapsids, which is also true in regular measles infections (Norrby and Gollmar, 1972). *In vitro* experiments have shown that SSPE brain cells can be lysed in an autologous antibody–complement system (Oldstone *et al.*, 1975). However, lysis was dependent on expression of measles virus antigens on the cell surface, a phenomenon greatly augmented by passage in culture. Whether or not this is a significant means of cell destruction *in vivo* remains to be seen.

It should not be surprising that this slow measles infection is able to progress in the presence of specific humoral antibody. Children with congenital agammaglobulinemia have been found to undergo a normal course of infection and are subsequently immune to measles (Good and Zak, 1956). It is the cell-mediated immune response to measles virus which is most likely to be deficient in SSPE, as has been postulated by Burnet (1968). Although it may seem paradoxical that the two immune responses would not parallel each other, studies have shown discrepancies between serum antibody and cellular responses to measles virus (Labowskie *et al.*, 1974). There have, however, been conflicting reports on cell-mediated immunity in patients with SSPE. Some studies have found minor abnormalities (Kolar, 1968; Vandvik, 1970; Gerson and Haslam, 1971; Klajman *et al.*, 1973), while others have found cellular immunity normal (Jabbour *et al.*, 1969; Moulias *et al.*, 1971; Mizutani, 1972; Lischner *et al.*, 1972). Both heat-labile (Allen *et al.*, 1973; Ahmed *et al.*, 1974) and heat-stable (Swick *et al.*, 1976) blocking factors, which inhibit lymphocyte transformation, have been identified in SSPE sera. Characterization of the heat-labile inhibitor has indicated that it may be an immune complex (Ahmed *et al.*, 1974). Complement-binding immune complexes containing measles-specific antibody have been found in serum and cerebrospinal fluid from patients with SSPE (Oldstone *et al.*, 1975). If these complexes appear only intermittently in sera, this could explain the discrepancies in studies on cellular immunity as well as the waxing and waning course of illness. However, if this is the mechanism by which measles virus persists, why is SSPE not seen more commonly? One explanation would be that the incomplete infection is antigenically distinct. No studies have shown a qualitative difference in measles antibodies in SSPE patients, but there does appear to be a substantial difference in the concentration of antibody produced to different structural components of measles virus (Norrby *et al.*, 1975). This quantitative variation may serve to modulate

the infection as well as contribute to the formation of immune complexes. This, however, does not answer the question of why the infection becomes suppressed in the first place.

B. Animal Models

Chronic encephalitides resembling SSPE have been experimentally produced in ferrets (Katz *et al.*, 1970), hamsters (Byington *et al.*, 1970), calves (Thein *et al.*, 1972), and lambs (Thein *et al.*, 1972) inoculated with SSPE measles isolates or infected cells. In addition, several laboratory-adapted strains of measles virus have been shown to become neurotropic and produce SSPE-like disease in hamsters (Wear and Rapp, 1971), mice (Griffin *et al.*, 1974), and rhesus monkeys (Albrecht *et al.*, 1972). By manipulating these animal models, it has been possible to define conditions most favorable for producing a suppressed, defective, cell-associated infection causing chronic measles encephalitis. Two important factors have emerged: the age of the animal when exposed to measles, and the existing immune condition at the time of exposure. Studies in non-immune animals have shown measles virus to produce an acute encephalitis in newborn hamsters (Burnstein *et al.*, 1964; Johnson and Byington, 1971), mice (Griffin *et al.*, 1974), and rats (Byington and Burnstein, 1973). Weanling hamsters (Byington and Johnson, 1972) and mice (Griffin *et al.*, 1974) were found to be more resistant with a high percentage of inoculated animals developing suppressed infections. Griffin and associates (1974) found that the immune response appeared to play no role in determining the course of infection, and they have suggested that it is the maturity of CNS cells at the time of measles infection which determines whether defective replication occurs. Other studies, on immunosuppressed adult hamsters (Byington and Johnson, 1975), indicate that the immune response is an important regulator of measles CNS infections. In support of this latter observation, Wear and Rapp (1971) have reported latent measles virus infection of the CNS in inoculated suckling hamsters born to immune mothers.

There is one natural disease of animals which bears a striking resemblance to SSPE, old dog encephalitis (ODE). ODE was originally described by Cordy (1942) who suspected a viral origin but failed in attempts to transmit the disease to puppies, ferrets, guinea pigs, or mice. ODE is a disease of mature dogs featuring progressive impairment of mental and motor abilities. The clinical course lasts several months and inevitably ends in death. Histopathologically, there is disseminated encephalomyelitis with perivascular infiltrations and intranuclear inclusion bodies. Studies on the etiology of ODE have indicated that the disease is

caused by an atypical infection with canine distemper virus (Lincoln *et al.*, 1971, 1973). Many of the findings are similar to SSPE; elevated serum antibody to distemper, paramyxovirus nucleocapsid material within inclusion bodies, and failure to isolate infectious virus from affected brain tissue. These observations, together with recent studies on a strain of distemper virus which consistently produces a chronic, demyelinating encephalitis in dogs (Koestner *et al.*, 1974), serve to emphasize the kinship between human and animal diseases (Leader, 1967).

C. Epidemiology

SSPE occurs in children 4–18 years of age with a peak incidence between 7 to 10 years. The disease is twice as common in boys and seems to be seen more in children from rural backgrounds (Pettay *et al.*, 1971; Detels *et al.*, 1973). Most SSPE patients have a history of exposure to measles virus, many within the first 2 years of life (Pettay *et al.*, 1971). This is especially interesting in light of experiments on animal models indicating age of exposure as a critical factor in determining whether the infection becomes suppressed or not. Diagnosis of SSPE is based on clinical signs, electroencephalogram readings, and elevated measles antibody in serum and cerebrospinal fluid. If these studies are inconclusive, diagnosis can be confirmed by brain biopsy and demonstration of measles nucleocapsids by electron microscopy. Many therapeutic approaches have been attempted in SSPE (Sever, 1972), but none have been consistently successful. Zeman and Kolar (1968) reported the beneficial effect of immunosuppressive agents in some cases, and there is one report of remission following thymectomy (Kolar *et al.*, 1967). Vandvik *et al.* (1973) observed clinical improvement with reduced levels of measles antibody in a patient treated with transfer factor. Control of SSPE will need to be based on knowledge of the complex factors interacting to produce this defective measles virus infection.

IV. CREUTZFELDT–JAKOB DISEASE

In the early 1920's, two German neuropathologists almost simultaneously described a degenerative brain disorder which has become known as Creutzfeldt–Jakob disease (Creutzfeldt, 1920; Jakob, 1921). This presenile dementia has a devastating effect causing rapid deterioration of nervous function over a course seldom exceeding 1 year. The disease has several recognized clinicopathological syndromes (Roos *et al.*, 1973), a feature causing some confusion as illustrated by sixteen syn-

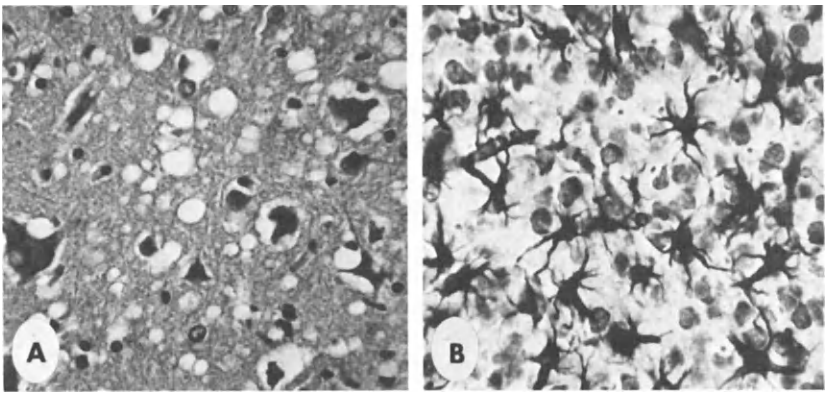

Fig. 5. Creutzfeldt–Jakob disease. (A) Microvacuolation and neuronal degeneration in parietal cortex. Hematoxylin and eosin, ×313. (B) Astrocytic hypertrophy. Cajal's gold sublimate; ×313.

onyms for the condition (Herzberg *et al.*, 1975). The most consistent histopathological changes are spongiform degeneration of gray matter (Fig. 5A), neuronal degeneration and astrocytic hypertrophy (Fig. 5B).

A. Animal Models and Viral Etiology

Gibbs *et al.* (1968) were successful in experimentally producing a neurological syndrome similar to Creutzfeldt–Jakob disease in chimpanzees inoculated with affected brain tissue. The disease has since been transmitted to twelve species of monkeys (Gajdusek and Gibbs, 1975), domestic cats (Gibbs and Gajdusek, 1973), and guinea pigs (Manuelidis, 1975; Manuelidis *et al.*, 1976). In addition to these animal models, Creutzfeldt–Jakob disease is similar to scrapie, a disease of sheep and goats which has been recognized for over 200 years. This similarity can be taken advantage of since scrapie is much better characterized and this information can be used to complement the paucity of experimental findings on the human disease. There is as yet no reason to believe that these transmissible agents behave differently in humans than in animals.

Creutzfeldt–Jakob disease, kuru, scrapie, and transmissible mink encephalopathy (TME) comprise a group of diseases known as the subacute spongiform encephalopathies (recent reviews include Asher *et al.*, 1976; Marsh, 1976). These diseases have the following general characteristics (Marsh, 1974):

1. The natural or experimental disease has an insidious onset following a long incubation period of several months or years.

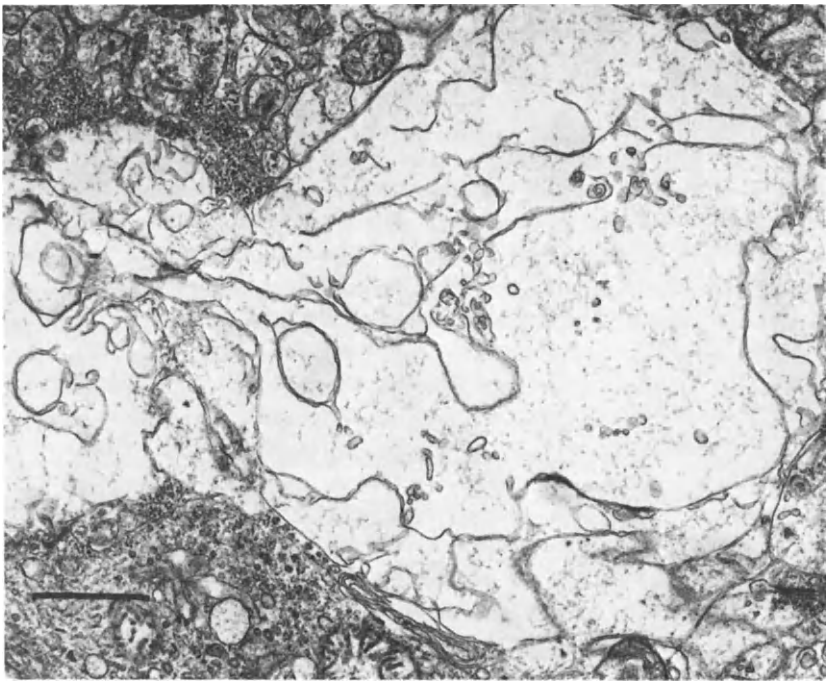

Fig. 6. Electron micrograph of vacuolar lesion in cerebral cortex from an animal with transmissible mink encephalopathy. Scale = 1 μm, × 15,000. From Marsh (1972) and published with permission of the Editorial Board of the *American Journal of Pathology*.

2. The clinical course of illness is protracted and relentlessly progressive leading to complete mental and motor deterioration, debilitation, and death.

3. Pathology is limited to the CNS and is microscopic in nature. Astrocytic hypertrophy is always present and usually accompanied by spongiform degeneration of the gray matter, which is a secondary effect of the disease process (Marsh *et al.*, 1976). Microvacuolation is produced by focal cytoplasmic swellings, usually containing disrupted plasma membranes, found most commonly in nerve cells and their processes (Fig. 6).

4. All are caused by nonconventional viruses which have never been positively visualized in infected tissues, nor have they been shown to produce recognizable cytopathology in a variety of tissue culture systems.

5. They elicit no inflammatory reactions, or any detectable immune response.

Little is known of the viral properties of the transmissible agent(s) causing Creutzfeldt–Jakob disease. Limited studies do not convincingly

distinguish them from scrapie agents. The plural is used because scrapie is now known to be a composite rather than a single entity, and can be produced by microorganisms having similar characteristics but separable by their differential biological properties in inbred strains of mice (Dickinson, 1976). This variability in subpopulations of scrapie is an important factor in determining host susceptibility (Marsh and Hanson, unpublished), and may also influence physicochemical properties of individual agents (A. G. Dickinson, personal communication). Studies on the "Chandler" scrapie agent show that it has an operational size of about 30 nm, or greater than 5×10^7 daltons, as measured by exclusion filtration (Kimberlin *et al.*, 1971), but a radiation target size of only 2×10^5 daltons (Alper *et al.*, 1966). These results, indicating that the agent contains only a small amount of nucleic acid, stimulated Diener (1972) to hypothesize that scrapie may be similar to a viroid, e.g., a low molecular weight RNA producing disease in plants. Subsequent studies have shown that, contrary to plant viroids, nucleic acids extracted from scrapie or TME-infected brains do not produce disease and there is no amplification of viroid-like RNA species in polyacrylamide gels (Marsh *et al.*, 1974). While some have concluded that scrapie can, therefore, not be a viroid (Ward *et al.*, 1974), recent findings demonstrating an association of the citrus exocortis viroid with a component of the endomembrane system (Semancik *et al.*, 1976b) encourages a further examination of this analogy. One of the unequivocal physicochemical properties of scrapie infectivity is its integral association with cell membrane (Millson *et al.*, 1971; Semancik *et al.*, 1976a). It is possible that viroids of animals may require a membrane carrier for protection or recognition of susceptible cells. This may not be necessary in plants which do not have as elaborate a system of cellular differentiation. Plant and animal viruses have been shown to share pathogenic mechanisms before, therefore, it is not inconceivable that some day there may exist "plant models of human disease." Suckling mouse cataract agent, once believed to produce a slow virus disease (Hotchin, 1967), is now known to be a spiroplasma (Tully *et al.*, 1976), a group of mycoplasmas thought previously to only infect plants and insects.

B. Pathogenesis

Almost nothing is known of the progress and effect of these agents in tissues. It is quite clear that after a peripheral injection the scrapie agent first replicates in lymph nodes and spleen before appearing in the CNS (Eklund *et al.*, 1967). We do not know which cells, if any, of the lym-

phoid system are infected. Circulating lymphocytes in TME are not infected (Marsh *et al.*, 1973) and altering populations of lymphoid cells by thymectomy plus irradiation (McFarlin *et al.*, 1971), or by treatment with cyclophosphamide (Worthington and Clark, 1971), does not appreciably effect the course of scrapie in the mouse. It has been suggested that these agents may replicate in nerve endings (Marsh *et al.*, 1973), progressing to the CNS via nerve fibers as in rabies (Murphy *et al.*, 1973) and herpes simplex (Cook and Stevens, 1973) infections. An experiment initiated by a report on transmission of Creutzfeldt–Jakob disease by corneal transplant (Duffy *et al.*, 1974) supports this suggestion. Corneal epithelium from hamsters with TME was found to contain a high level of infectivity which was not maintained after tissue culture (Marsh and Hanson, 1975). This would indicate that infectivity is mainly associated with a noncellular component, possibly nerve fibers, since corneal epithelium is richly innervated. The high titer in spleen can be explained simply by the fact that this tissue contains more adrenergic nerves than any other visceral organ based on noradrenaline content (von Euler, 1971). An alternative hypothesis is one suggested by Notkins (1974), albeit not particularly referring to scrapie agents. He speculated that since certain viruses will only replicate in lymphocytes undergoing blastogenesis, it is possible that there may exist viruses which infect and destroy the very clone of cells responsible for producing antibodies against them. If such a mechanism was operable in scrapie, it would explain lymphoid affinity and also the lack of a specific immune response while retaining responsiveness to other antigens (Gardiner and Marucci, 1969). This theory need not be exclusive of progression in nerve fibers for the two may well complement each other.

These are neurotropic agents; pathology is limited to the CNS, highest titers of infectivity are in brain tissue, and the effects of any manipulation of lymphoid tissues can be bypassed by intracerebral inoculation. But we know little of how they produce their lethal effects. Neuronal degeneration and astrocytic hypertrophy are constant findings. Spongiform degeneration is secondary and may be due to lysosomal enzymes of reactive astrocytes causing a breakdown in tissue integrity. Studies on biochemical indexes have failed to show any changes suggestive of a primary pathologic process (Kimberlin and Marsh, 1975). There is no evidence to suggest that cell injury may be due to an immune mechanism (Marsh *et al.*, 1970; Porter *et al.*, 1973). The lack of cytopathic changes in various *in vitro* cell systems indicates that the effects of these agents must be quite selective and likely only elicited in nerve cells. Neurons are distinct in their specialized functions and their inability to replicate. How these unique properties contribute to their apparent susceptibility remains to be shown.

C. Epidemiology

Creutzfeldt–Jakob disease occurs worldwide, affecting men and women almost equally during their middle ages. One epidemiological study in Israel reported an incidence of the disease of 0.4 to 1.9 cases per million people (Kahana *et al.*, 1974). A similar level of prevalence is thought to exist in the United States, however, there is disagreement on which criteria best constitute a diagnosis of Creutzfeldt–Jakob disease. In one form of the disease, that described by Heidenhain (1929), diagnosis is based on the presence of a rapidly fulminating dementia with characteristic electroencephalogram readings. Confirmation is by brain biopsy and the appearance of spongiform degeneration in the occipitoparietal cortex. Other forms of the disease, such as the amyotrophic and transitional types, are not as easily recognized. This variability in pathologic syndromes has stimulated speculation that more common degenerative brain diseases may be caused by similar agents. Studies on scrapie-infected animals have shown that some mouse–agent combinations consistently produce amyloid plaques (Bruce and Fraser, 1975), which are indistinguishable from those found in Alzheimer's disease (Wiśniewski *et al.*, 1975). The concept that these transmissible agents may produce different pathologic syndromes in humans is supported by experiments on the differential effects of purified subpopulations of scrapie on mice (Dickinson, 1976) and squirrel monkeys (R. F. Marsh, R. P. Hanson, and A. G. Dickinson, unpublished). The results in squirrel monkeys are perhaps most relevant to Creutzfeldt–Jakob disease for they present a range of pathological reactions from severe to undetectable spongiform degeneration, with the distribution of lesions varying markedly between gray and white matter.

Creutzfeldt–Jakob disease usually occurs sporadically, but there have also been reports of two or more cases within a single family (Kirschbaum, 1968; May *et al.*, 1968). This familial form of the disease, occurring over several generations, effects more than 25% of the siblings and is, therefore, consistent with autosomal dominant inheritance (Asher *et al.*, 1976). These cases are also transmissible to animals (Ferber *et al.*, 1974), indicating that the disease itself is not hereditary but only the tendency to contract the infection or express its degenerative effect. Genetic predisposition of sheep to scrapie is well documented but the processes are poorly understood. Since treatment and prophylaxis are ineffective or unavailable, control of Creutzfeldt–Jakob disease will need to be based on epidemiological considerations on breaking the chain of transmission. Kuru has been controlled and almost eliminated by ceasing ritual cannibalistic practices. Although the means of transmission is not as readily

discernible in Creutzfeldt–Jakob disease, control seems possible if the source of infection can be identified.

V. CONCLUDING REMARKS

It should be apparent that diagnosis of slow virus diseases is difficult and often involves procedures no more sophisticated than removing a chunk of brain and looking for pathological changes. The state of the art will improve as we refine techniques to distinguish virus from host, and as we expand our knowledge on how viruses interact with immune mechanisms.

REFERENCES

Ahmed, A., Strong, D., Sell, K., Thurman, G., Knudsen, R., Wistar, R., and Grace, W. (1974). *J. Exp. Med.* **139,** 902.

Albrecht, P., Shabo, A. L., Burns, G. R., and Tauraso, N. M. (1972). *J. Infect. Dis.* **126,** 154.

Allen, J., Oppenheim, J., Brody, J. A., and Miller, J. (1973). *Infect. Immun.* **8,** 80.

Alper, T., Haig, D. A., and Clarke, M. C. (1966). *Biochem. Biophys. Res. Commun.* **22,** 278.

Asher, D. M., Gibbs, C. J., Jr., and Gajdusek, D. C. (1976). *Ann. Clin. Lab. Sci.* **6,** 84.

Åstrøm, K. E., Mancall, E. L., and Richardson, E. P., Jr (1958). *Brain* **81,** 93.

Bather, R., Furesz, J. M., Fanok, A. C., Gill, S. D., and Yarosh, W. (1973). *J. Gen. Virol.* **20,** 401.

Bauer, W. R., Turel, A. P., Jr., and Johnson, K. P. (1973). *J. Am. Med. Assoc.* **226,** 174.

Bolton, C. F., and Rozdilsky, B. (1971). *Neurology* **21,** 72.

Bouteille, M., Fontaine, C., Vedrenne, C. L., and Delarue, J. (1965). *Rev. Neurol.* **118,** 454.

Brown, P., Tsai, T., and Gajdusek, D. C. (1975). *Am. J. Epidemiol.* **102,** 331–340.

Bruce, M. E., and Fraser, H. (1975). *Neuropathol. Appl. Neurobiol.* **1,** 189.

Burnet, F. M. (1968). *Lancet* **1,** 610.

Burnstein, T., Jensen, J. H., and Waksman, B. H. (1964). *J. Infect. Dis.* **114,** 265.

Byington, D. P., and Burnstein, T. (1973). *Exp. Mol. Pathol.* **19,** 36.

Byington, D. P., and Johnson, K. P. (1972). *J. Infect. Dis.* **126,** 18.

Byington, D. P., and Johnson, K. P. (1975). *Lab. Invest.* **32,** 91.

Byington, D. P., Castro, A. E., and Burnstein, T. (1970). *Nature (London)* **225,** 554.

Castaigne, P., Rondot, P., Escourolle, R., Dumas, J., Cathala, F., and Hauw, J. (1974). *Rev. Neurol.* **130,** 379.

Cavanagh, J. B., Greenbaum, D., Marshall, A. H. E., and Rubinstein, L. J. (1959). *Lancet* **2,** 524.

Connolly, J. H. (1968). *Neurology* **18,** 87.

Connolly, J. H., Aller, I. U., Hurwitz, L. J., and Millar, J. H. D. (1967). *Lancet* **1,** 542.

Conomy, J. P., Beard, S., and Matsumoto, H. (1974). *J. Am. Med. Assoc.* **229,** 1313.

Cook, M. L., and Stevens, J. G. (1973). *Infect. Immun.* **7,** 272.

Cordy, D. R. (1942). *Cornell Vet.* **32,** 11.

Creutzfeldt, H. G. (1920). *Z. Gesamte Neurol. Psychiatr.* **57,** 1.

Dawson, J. R., Jr. (1933). *Am. J. Pathol.* **9,** 7.
Detels, R., McNew, J., Brody, J. A., and Edgar, A. H. (1973). *Lancet* **2,** 11.
Dickinson, A. G. (1976). *In* "Slow Virus Diseases of Animals and Man" (R. H. Kimberlin, ed.), pp. 209–241. Elsevier, Amsterdam.
Diener, T. O. (1972). *Nature (London)* **235,** 218.
Duffy, P., Wolf, J., Collins, G., DeVoe, A. G., Streeten, B., and Cowen, D. (1974). *N. Engl. J. Med.* **290,** 692.
Eklund, C. M., Kennedy, R. C., and Hadlow, W. J. (1967). *J. Infect. Dis.* **117,** 15.
Ellison, G. W. (1969). *J. Neuropathol. Exp. Neurol.* **28,** 501.
Faris, A. A., and Martinez, J. A. (1972). *Arch. Neurol. (Chicago)* **27,** 357.
Ferber, R. A., Wiesenfeld, S. L., Roos, R. P., Bobowick, A. R., Gibbs, C. J., Jr., and Gajdusek, D. C. (1974). *In* "Proceedings of the Xth International Congress for Neurology" (A. Subirana and J. M. Burrows, eds.), pp. 358–380. Elsevier, Amsterdam.
Fermaglich, J., Hardman, J. M., and Earle, K. M. (1970). *Neurology* **20,** 479.
Gajdusek, D. C., and Gibbs, C. J., Jr. (1975). *Adv. Neurol.* **10,** 291–317.
Gardiner, A. C., and Marucci, A. A. (1969). *J. Comp. Pathol.* **79,** 233.
Gardner, S. D., Field, A. M., Coleman, D. V., and Hulme, B. (1971). *Lancet* **1,** 1253.
Gerson, K. L., and Haslam, R. H. A. (1971). *N. Engl. J. Med.* **285,** 78.
Gibbs, C. J., Jr., and Gajdusek, D. C. (1973). *Science* **182,** 67.
Gibbs, C. J., Jr., Gajdusek, D. C., Asher, D. M., Alpers, M. P., Beck, E., Daniel, P. M., and Matthews, W. B. (1968). *Science* **165,** 388.
Gibbs, F. A., Gibbs, E. L., Carpenter, P. R., and Spies, H. W. (1959). *J. Am. Med. Assoc.* **171,** 1050.
Good, R. A., and Zak, S. J. (1956). *Pediatrics* **18,** 109.
Gribble, D. H., Haden, C. C., Schwartz, L. W., and Henrickson, R. (1975). *Nature (London)* **254,** 602.
Griffin, D. E., Mullinix, J., Narayan, O., and Johnson, R. T. (1974). *Infect. Immun.* **9,** 690.
Heidenhain, A. (1929). *Z. Gesamte Neurol. Psychiatr.* **118,** 49.
Herzberg, L., Gibbs, C. J., Jr., Asher, D. M., Gajdusek, D. C., and French, E. L. (1975). *In* "Transmissible Disease and Blood Transfusion" (T. J. Greenwalt and G. A. Jamieson, eds.), pp. 197–220. Grune & Stratton, New York.
Horta-Barbosa, L., Fuccilo, D. A., London, W. T., Jabbour, J. T., Zeman, W., and Sever, J. L. (1969). *Proc. Soc. Exp. Biol. Med.* **132,** 272.
Horta-Barbosa, L., Hmilton, R., Wittig, B., Fucillo, D. A., Sever, J. L., and Vernon, M. L. (1971). *Science* **173,** 840.
Hotchin, J. (1967). *Curr. Top. Microbiol. Immunol.* **40,** 33.
Iwasaki, Y., and Koprowski, H. (1974). *Lab. Invest.* **31,** 187.
Jabbour, J. T., Roane, J. A., and Sever, J. L. (1969). *Neurology* **19,** 929.
Jakob, A. (1921). *Z. Gesamte Neurol. Psychiatr.* **64,** 147.
Janda, Z., Norrby, E., and Marusyk, H. (1971). *J. Infect. Dis.* **124,** 553.
Johnson, K. P., and Byington, D. P. (1971). *Exp. Mol. Pathol.* **15,** 373.
Kahana, E., Alter, M., Braham, J., and Sofer, D. (1974). *Science* **183,** 90.
Katz, M., Rorke, L. B., Masland, W. S., Barbanti-Brodano, G., and Koprowski, H. (1970). *J. Infect. Dis.* **121,** 188.
Kepes, J. J., Chou, S. M., and Price, L. W., Jr. (1975). *Neurology* **25,** 1006.
Kimberlin, R. H., and Marsh, R. F. (1975). *J. Infect. Dis.* **131,** 97.
Kimberlin, R. H., Millson, G. C., and Hunter, G. D. (1971). *J. Comp. Pathol.* **81,** 383.
Kirschbaum, W. R. (1968). "Jakob-Creutzfeldt Disease." Am. Elsevier, New York.
Klajman, A., Sternbach, M., and Ranon, L. (1973). *Acta Paediatr. Scand.* **62,** 523.
Knight, A., O'Brien, P., and Osoba, D. (1972). *Ann. Intern. Med.* **77,** 229.

Koestner, A., McCullough, B., Krakowka, G. S., Long, J. K., and Olsen, R. G. (1974). *In* "Slow Virus Diseases" (W. Zeman and E. H. Lennette, eds.), pp. 86–101. Williams & Wilkins, Baltimore, Maryland.
Kolar, O. (1968). *Lancet* **2,** 1242.
Kolar, O., Vobrucnik, M., Behounkova, L., Musil, J., and Psenickoa, V. (1967). *Br. Med. J.* **3,** 22.
Koprowski, H., Barbanti-Brodano, G., and Katz, M. (1970). *Nature (London)* **225,** 1045.
Labowskie, R. J., Edelman, R., Rustigian, R., and Bellanti, J. A. (1974). *J. Infect. Dis.* **129,** 233.
Leader, R. W. (1967). *Sci. Am.* **216,** (No. 1), 110.
Lecatsas, G., Prozesky, O. W., Van Wyk, J., and Els, H. J. (1973). *Nature (London)* **241,** 343.
Legrain, M., Graveleau, J., Brion, S., Mikol, J., and Kuss, R. (1974). *J. Neurol. Sci.* **23,** 49.
Lincoln, S. D., Gorham, J. R., Ott, R. L., and Hegreberg, G. A. (1971). *Vet. Pathol.* **8,** 1.
Lincoln, S. D., Gorham, J. R., Davis, W. C., and Ott, R. L. (1973). *Vet. Pathol.* **10,** 124.
Lischner, H. W., Sharma, M. K., and Grover, W. D. (1972). *N. Engl. J. Med.* **286,** 787.
McFarlin, D. E., Raff, M. C., Simpson, E., and Nehlsen, S. H. (1971). *Nature (London)* **233,** 336.
Manuelidis, E. E. (1975). *Science* **190,** 571.
Manuelidis, E. E., Kim, J., Angelo, J. N., and Manuelidis, L. (1976). *Proc. Natl. Acad. Sci. U.S.A.* **73,** 223.
Manz, H. J., Dinsdale, H. B., and Morrin, P. A. F. (1971). *Ann. Intern. Med.* **75,** 77.
Marsh, R. F. (1972). *Am. J. Pathol.* **69,** 209.
Marsh, R. F. (1974). *Adv. Vet. Sci. Comp. Med.* **18,** 155–178.
Marsh, R. F. (1976). *In* "Slow Virus Diseases of Animals and Man" (R. H. Kimberlin, ed.), pp. 359–380. Elsevier, Amsterdam.
Marsh, R. F., and Hanson, R. P. (1975). *Science* **187,** 656.
Marsh, R. F., Pan, I. C., and Hanson, R. P. (1970). *Infect. Immun.* **2,** 727.
Marsh, R. F., Miller, J. M., and Hanson, R. P. (1973). *Infect. Immun.* **7,** 352.
Marsh, R. F., Semancik, J. S., Medappa, K. C., Hanson, R. P., and Ruckert, R. R. (1974). *J. Virol.* **13,** 993.
Marsh, R. F., Sipe, J. C., Morse, S. S., and Hanson, R. P. (1976). *Lab. Invest.* **34,** 381–386.
Mathews, T., Wisotzkey, H., and Moossy, J. (1976). *Neurology* **26,** 9.
May, W. W., Itabashi, H. H., and DeJong, R. (1968). *Arch. Neurol. (Chicago)* **19,** 137.
Millson, G. C., Hunter, G. D., and Kimberlin, R. H. (1971). *J. Comp. Pathol.* **81,** 255.
Mizutani, H. (1972). *N. Engl. J. Med.* **286,** 786.
Mosher, M. B., Schall, G. L., and Wilson, J. (1971). *J. Am. Med. Assoc.* **218,** 226.
Moulias, R. L., Reinert, P. H., and Goust, J. M. (1971). *N. Engl. J. Med.* **285,** 1090.
Murphy, F. A., Bauer, S. P., Harrison, A. K., and Winn, W. C., Jr. (1973). *Lab. Invest.* **28,** 361.
Nakamura, K., Homma, M., and Ishida, N. (1975). *Infect. Immun.* **12,** 614.
Narayan, O., Penney, J. B., Jr., Johnson, R. T., Herndon, R. M., and Weiner, L. P. (1973). *N. Engl. J. Med.* **289,** 1278.
Norrby, E., and Gollmar, Y. (1972). *Infect. Immun.* **6,** 240.
Norrby, E., Salmi, A. A., Link, H., Vandvik, B., Olsson, J., and Panelius, M. (1974). *In* "Slow Virus Diseases" (W. Zeman and E. H. Lennette, eds.), pp. 72–85. Williams & Wilkins, Baltimore, Maryland.
Norrby, E., Enders-Ruckle, G., and ter Meulen, V. (1975). *J. Infect. Dis.* **132,** 262.
Notkins, A. L. (1974). *Hosp. Pract.* **9,** 65.

Oldstone, M. B. A., Bokisch, V. A., Dixon, F. J., Barbosa, L. H., Fucillo, D., and Sever, J. L. (1975). *Clin. Immunol. Immunopathol.* **4,** 52.
Padgett, B. L., and Walker, D. L. (1973). *J. Infect. Dis.* **127,** 467.
Padgett, B. L., and Walker, D. L. (1976). *Prog. Med. Virol.* **22,** pp. 1–35.
Padgett, B. L., Walker, D. L., ZuRhein, G. M., Eckroade, R. J., and Dessel, B. H. (1971). *Lancet* **1,** 1257.
Pampiglione, G. (1964). *Br. Med. J.* **2,** 1296.
Payne, C. M., and Sibley, W. A. (1975). *Acta Neuropathol.* **31,** 353.
Payne, F. E., Baublis, J. V., and Itabashi, H. H. (1969). *N. Engl. J. Med.* **281,** 585.
Pettay, O., Donner, M., Halonen, H., Palosuo, T., and Salmi, A. (1971). *J. Infect. Dis.* **124,** 439.
Porter, D. D., Porter, H. G., and Cox, N. A. (1973). *J. Immunol.* **111,** 1407.
Raine, C. S., Feldman, L. A., Sheppard, R. D., and Bornstein, M. R. (1971). *J. Virol.* **8,** 318.
Richardson, E. P., Jr. (1961). *N. Engl. J. Med.* **265,** 815.
Roos, R., Gajdusek, D. C., and Gibbs, C. J., Jr. (1973). *Brain* **96,** 1.
Semancik, J. S., Marsh, R. F., Geelen, J. L. M. C., and Hanson, R. P. (1976a). *J. Virol.* **13,** 993–996.
Semancik, J. S., Tsuruda, D., Zaner, L., Geelen, J. L. M. C., and Weathers, L. G. (1976b). *Virology* **69,** 669–676.
Sever, J. L. (1972). *Science* **175,** 220.
Sigurdsson, B. (1954). *Br. Vet. J.* **110,** 341.
Silverman, L., and Rubinstein, L. J. (1965). *Acta Neuropathol.* **5,** 215.
Smith, J. L. (1959). *Arch. Ophthalmol.* **62,** 828.
Stam, F. C. (1966). *Psychiatr., Neurol., Neurochir.* **69,** 453.
Swick, H. M., Brooks, W. H., Roszman, T. L., and Caldwell, D. (1976). *Neurology* **26,** 84.
Thein, P., Mayr, A., ter Meulen, V., Koprowski, H., Kackell, M., Muller, D., and Meyermann, R. (1972). *Arch. Neurol. (Chicago)* **27,** 540.
Tully, J. G., Whitcomb, R. F., Williamson, D. L., and Clark, H. F. (1976). *Nature (London)* **259,** 117.
Vandvik, B. (1970). *Acta Neurol. Scand.* **43,** 232.
Vandvik, B., Frøland, S. S., Høyeraal, H., Stein, R., and Degré, M. (1973). *Scand. J. Immunol.* **2,** 367.
Vernon, M. L., Horta-Barbosa, L., Fuccillo, D., Sever, J., Baringer, J., and Birnbaum, G. (1970). *Lancet* **2,** 964.
von Euler, U. S. (1971). *Science* **173,** 202.
Walker, D. L., Padgett, B. L., ZuRhein, G. M., Albert, A. E., and Marsh, R. F. (1973). *Science* **181,** 674.
Ward, R. L., Porter, D. D., and Stevens, J. G. (1974). *J. Virol.* **14,** 1099.
Wear, D. J., and Rapp, F. (1971). *J. Immunol.* **107,** 1593.
Webb, H. E., Illavia, S. J., and Laurence, G. D. (1971). *Lancet* **2,** 4.
Weiner, L. P., Herndon, R. M., Narayan, O., Johnson, R. T., Shah, K., Rubinstein, L. J., Preziosi, T. J., and Conley, F. K. (1972). *N. Engl. J. Med.* **286,** 385.
Weiner, L. P., Narayan, O., Penney, J. B., Jr., Herndon, R. M., Feringa, E. R., Tourtellotte, W. W., and Johnson, R. T. (1973). *Arch. Neurol. (Chicago)* **29,** 1.
Wiśniewski, H. M., Bruce, M. E., and Fraser, H. (1975). *Science* **190,** 1108.
Worthington, M., and Clark, R. (1971). *J. Gen. Virol.* **13,** 349.
Zeman, W., and Kolar, O. (1968). *Neurology* **18,** 1.
ZuRhein, G. M. (1969). *Prog. Med. Virol.* **11,** 185–247.
ZuRhein, G. M., and Chou, S. (1965). *Science* **148,** 1477.
ZuRhein, G. M., and Varakis, J. (1974). *N. Engl. J. Med.* **291,** 798.

Chapter 4

Human Proliferative Diseases and Viruses

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I. INTRODUCTION

All proliferative diseases of man and animals represent new growths of various types of tissues and cells. These new growths differ from normal tissue growth or from inflammatory reactions by certain characteristics of which the most important are their progressive growth and, on occasion, tendency to escape the regulatory mechanisms of the so-called homeostasis. The progressive growth of proliferating tissues results in their extension and may eventually lead to invasion of other tissues of the host. Multiplication of the cells in the newly formed tissues may be self-limiting or unrestricted and may be rapid as in melanomas or slow as in most leukemias (Gallo *et al.*, 1975), but it always leads to formation of tumors which may assume either a benign, self-limiting character or a malignant, unrestrained behavior. The difference between benign and malignant behavior appears frequently to be arbitrary or even artificial.

Among the many etiological factors such as genetic, hormonal, nutritional, and environmental now known to be implicated in the origin of proliferative disorders and tumors, viruses have, during the last three decades, gained considerable interest and indeed a position of preeminence as a result of extensive studies on neoplasia in animals.

There are at present three groups of viruses known to induce tumors in animals of a number of species extending from amphibians to mammals: oncornaviruses, herpesviruses, and papovaviruses. In man (at present) only three tumors are known to be caused by viruses. The so-called benign tumors are the common wart, condyloma acuminatum (also called genital wart), both caused by small DNA viruses, and molluscum contagiosum, caused by a large, DNA-containing poxvirus (Dmochowski, 1963; Oriel, 1971; Powell, 1972). Transformation of these tumors into malignant neoplasms is exceptional although it does occur (Kovi *et al.*, 1974).

When the rapid progress of animal tumor virology in the past few dec-

ades is considered, the lack of conclusive results in the quest for viruses causing cancer in humans may appear surprising. At present, the technological means of detecting virus particles or their biochemical and immunological expression in infected cells include a host of sophisticated methods ranging from electron microscopy, radioimmunoassays to hybridization of nucleic acids. There is, at present, a trend of thought that failure to discover a cancer-causing virus in man by using these highly sophisticated methods may simply mean that cancers in man may not have a viral etiology.

However, it may be more accurate to say that the quest for cancer-inducing viruses in man is just at its beginning, and that the bulk of the work to get at the heart of the problem still lies ahead. Human virus studies have lagged behind animal virus studies for many well-known reasons, such as impossibility of bioassays in humans, lack of sufficiently inbred human populations, excessive life-span of man in comparison to that of experimental animals, hormonal and environmental factors differing on many important points from those of animals, great difficulties encountered in growing human tumor cells *in vitro,* and many others. While in experimental animals the same virus-induced tumors can be obtained in unlimited numbers of specimens by inoculation and transplantation into isologous or even homologous recipients, tumor specimens from each individual patient available for study are generally unique and are frequently, if not always, different in many ways from similar tumor specimens from other patients. Other factors have also intervened. It has to be remembered that, after all, most of the biochemical tools available for study of the possible presence of covert viruses in tumor cells (which is a frequent case even in animals) are derived from a 6-year-old discovery of reverse transcriptase or RNA-dependent DNA polymerase (RTase) in oncornaviruses by Temin and Mizutani (1970) and by Baltimore (1970). Techniques for extraction from cells and purification of specific viral components and antigens are fairly recent and, in addition, are time-consuming. Before the recent biochemical techniques became available, electron microscopy and biological assays *in vivo* and *in vitro* were, with all their drawbacks, nearly the only ways of approaching the human cancer virus problem. Finally, it must also be kept in mind that it took almost half a century to convince investigators to attack the problem of possible viral implication in the origin of human proliferative diseases long after repeated evidence that viral induction of neoplasia in some animals was already at hand.

Thanks to the newly developed biochemical and immunological techniques, the studies in human tumor virology are now advancing rapidly

and appear to be catching up with animal studies. Because of the many techniques presently available, the problem of viral causation of human proliferative diseases can be approached in a rational way, utilizing a multifaceted approach which has appropriately been described by Dmochowski and Bowen (1973), Sanders (1974), Spiegelman (1975), and others.

This approach is based on (a) electron microscopic detection of virus or viruslike particles in human tumor tissues and in their tissue cultures; (b) attempts to activate latent viruses in tissue cultures by various methods (cocultivation, inoculation of tumor extracts on indicator cells, use of chemicals such as halogenated pyrimidines, corticosteroids, cycloheximide, carcinogens, radiations, or the use of "helper viruses," etc.); (c) search in tumor cells of DNA or RNA homologous to RNA or DNA products of known animal oncorna or DNA viruses by molecular hybridization or of oncornavirus-like enzymatic activities; (d) immunological detection of virus-related antigens in tumor cells; and (e) whenever possible, bioassays.

The purpose of this chapter is to relate the methods that have been applied to the search for human tumor viruses, and of the results so far obtained. The results indicate that viruses similar in their biophysical, immunological, and biochemical properties to those known to induce malignancies in animals may be indirectly if not directly involved in the origin of human neoplasia. For a better understanding of the rather complex topic of the relationship of viruses to human cancer, it may be useful first to describe the properties of RNA and DNA viruses already found associated with animal tumors.

It is impossible within the limited scope of the present chapter to review and discuss in detail all the methods and results that have led not only to the demonstration of the viral etiology of many types of neoplasia in animals of most diverse species from molluscs to apes, but also to clues indicatory of a possible involvement of viruses in the origin of human tumors. The aim of this chapter is to present some general but updated characteristics of RNA and DNA viruses known to cause tumors in animals and suspected of being implicated, indirectly if not directly, in the development of at least some types of human neoplasia. These viruses include the group of RNA viruses called "Retroviridae" because they carry a special enzyme (reverse transcriptase), and viruses belonging to the broad family of herpesviruses which contain DNA. A summary description of molecular biological techniques presently available for analysis and characterization of these viruses has been included for a better understanding of the difficult problems faced in the search for human tumor viruses.

II. VIRUSES ASSOCIATED WITH NEOPLASIA OF ANIMALS

Many types of animal neoplasia are now classic examples of virus-induced tumors: leukemia and sarcoma of chickens, mice, rats, cats, hamsters, and guinea pigs, induced by so-called type C RNA viruses; mammary tumors of mice induced by so-called type B RNA virus (or MMTV); kidney tumors of frogs and lymphoid tumors of domestic fowl, both induced by herpesviruses; and a whole series of tumors induced in animals of several species by small DNA (papova and SV40) viruses. A detailed review of these tumors and of their causative viruses may be found in the classic treatise of Gross (1970) and a number of major publications (see Dmochowski and Grey, 1958; Dmochowski, 1960a,b, 1963, 1973; Biggs *et al.*, 1972; McAllister, 1973; Tooze, 1973; Bowen *et al.*, 1974).

Besides these well-established examples of viral oncogenesis in animals, recent findings of RNA and DNA viruses in tumors or normal tissues of some mammalian species, especially of primates, have greatly contributed to a better understanding of the different biological behavior of oncogenic RNA and DNA viruses, and to an approach of viral carcinogenesis in man with more sophisticated means and strategies than ever before. These findings have also revealed complexity of the problems of the isolation of viruses apparently harmless to hosts of the species of origin, but pathogenic and sometimes tumorigenic in heterologous hosts. First the RNA viruses and then the DNA viruses known to be associated with animal tumors will be described.

A. RNA Viruses

RNA viruses found to be associated with or to induce neoplasia in animals of many species belong to a group of viruses with the singular property, suspected by Temin more than 10 years ago, of replicating in infected cells via DNA copies of their single-stranded RNA. Transcription of viral RNA into complementary DNA strands is mediated by an enzyme called RNA-dependent DNA polymerase (RDDP) packaged in the core of the virions (Temin and Mizutani, 1970; Baltimore, 1970).

Classification of oncogenic RNA viruses originally termed "oncornaviruses" by Nowinski *et al.*, (1970) is presently based on morphological, biochemical, and immunological criteria (see Dalton, 1972a,b; Bowen *et al.*, 1974; Dmochowski *et al.*, 1975; Dalton *et al.*, 1975). These criteria are not necessarily determined by the host of origin, since the host range of most oncornaviruses may include a broad spectrum of animal species.

There are also some RDDP-containing RNA viruses which have not been found, at least at this point, to be endowed with a tumorigenic effect. For these reasons, oncogenic as well as nononcogenic RDDP-containing viruses have been included in a family of Retroviridae, subdivided into genera and subgenera according to morphological and biological characteristics (see Dalton *et al.*, 1975). In this context, Retroviridae comprise six genera: *Cisternavirus* A, *Oncornavirus* B, *Oncornavirus* C, *Oncornavirus* D, *Lentivirus* E, and *Spumavirus* F. Genus *Oncornavirus* C has been subdivided into avian, reptilian, and mammalian type C viruses.

The morphological, biochemical, immunological, and biological characteristics common to this family of viruses will first be described.

1. Structural and Biophysical Properties of Retroviridae

Oncornaviruses have been most extensively studied. The morphology and internal structure of oncornaviruses have been examined by a variety of electron microscopic procedures (reviewed by Seman and Dmochowski, 1976), and described in a series of major publications (see Dmochowski and Grey, 1958; Bernhard and Guérin, 1958; Bernhard, 1960; Nermut *et al.*, 1972; Dalton, 1972a,b; Dmochowski, 1960a,b, 1973; Dalton and Haguenau, 1973; DeHarven, 1974; Bowen *et al.*, 1974; Sarkar *et al.*, 1975; Hall and Schidlovsky, 1976; Dmochowski *et al.*, 1976).

Oncornaviruses and other retroviruses appear in thin sections as rounded or oval particles 80–110 nm in diameter. Internal structure of fully formed, extracellular virions is composed of an electron-dense core (containing mostly nucleoprotein) surrounded by a core shell. The core and core shell are often referred to as the nucleoid. The nucleoid is surrounded by a unit membrane, the envelope, from which it is separated by an electron-lucent space. The envelope is mostly derived from the cell membrane. Oncornaviruses are released from the cells by a process of budding, a process during which the core appears, according to the virus type, either as a horseshoe- or as a doughnut-shaped double-walled structure with an electron-lucent center. Of great interest is the fact that oncornaviruses acquire their definitive so-called "mature" internal structure only after their release from the cell. This extracellular "maturation" apparently corresponds to a rearrangement of nucleoproteins in the core (East *et al.*, 1973). However, in spite of many studies, the fine structure of oncornaviruses is as yet only partially known.

Spumaviruses (or so-called foamy viruses) differ from other Retroviridae by the absence of morphological changes in extracellular virions. Their core retains a ring-shaped structure and an electron-lucent center similar to that observed in budding particles.

Virions of retraviruses sediment at a characteristic buoyant density of

1.14 to 1.17 g/cm^3 in gradients. Their core can be isolated by treatment with detergents (see review by Seman and Dmochowski, 1976). It is more dense than the virions and sediments at 1.20 to 1.35 g/cm^3 (Sarkar *et al.*, 1971; Stromberg, 1972; Lange *et al.*, 1973). As will be described later, methodologies for the extraction of cores from virions and their centrifugal characteristics have been used in many studies aimed at detecting oncornavirus-like biochemical activity in human tumor cells.

2. Morphological Classification of Retroviridae

Knowledge of the morphological appearance of oncogenic and other RDDP-containing viruses found in animals is of obvious importance in a search for viruses in human tumors by ultrastructural methods. Bernhard's (1960) original classification of type A, B, and C oncornaviruses has been retained in the newest classification of Retroviridae (see Dalton *et al.*, 1975).

Intracisternal type A viruses (genus *Cisternavirus* A, Fig. 1) are double-shelled particles with no known extracellular forms. These par-

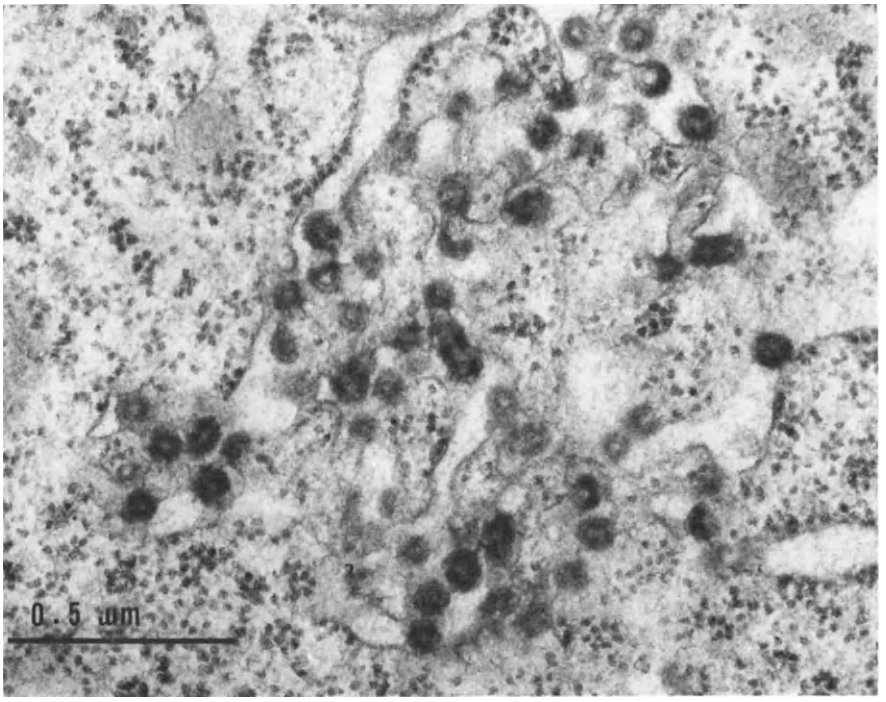

Fig. 1. Intracisternal type A virus particles in cell of mammary tumor of a BALB/c mouse. × 60,000.

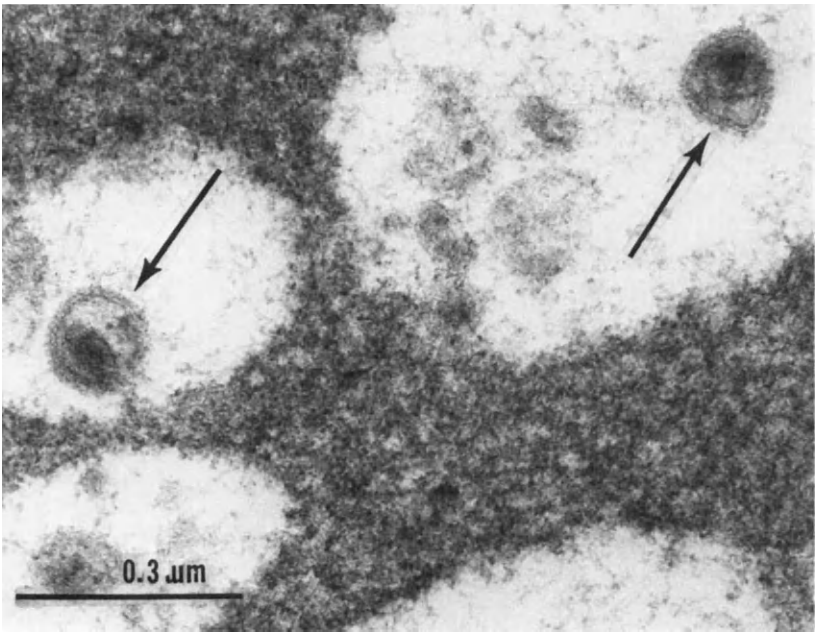

Fig. 2. Type B virus particles (arrows) in stomach milk of a newborn $C_3H/He/J/Dm$ mouse taken away from his mother for 2 hours. Type B particles are still well preserved, protected as they are for several hours by the coagulum. × 100,000.

ticles have been observed in most tissues, benign or malignant of mice of many strains, and in cells of hamsters and guinea pigs. Their oncogenic potential is unknown, but their existence alone even on a passenger virus basis is of importance in biochemical and immunological studies of murine, hamster, or guinea pig cells.

Type B viruses (genus *Oncornavirus* B, Fig. 2) are characterized by an eccentric location of their nucleoid and the presence of spikes covering the surface of their envelope. The prototype is the mouse mammary tumor-inducing virus or MMTV. Similar particles have been observed in guinea pigs (Fong and Hsiung, 1976). Immature intracellular forms of MMTV are represented by the intracytoplasmic type A particles (Fig. 3) with a double shell and an electron-lucent center.

Type C viruses (genus *Oncornavirus* C, Fig. 4) are by far the largest group of known oncogenic RNA viruses. These viruses are known to cause leukemias and sarcomas in chickens, mice, rats, hamsters, guinea pigs, cattle, and monkeys. They are characterized by a central location of their nucleoid and short spikes covering the surface of their envelope. According to the host of origin, type C viruses have been divided into three

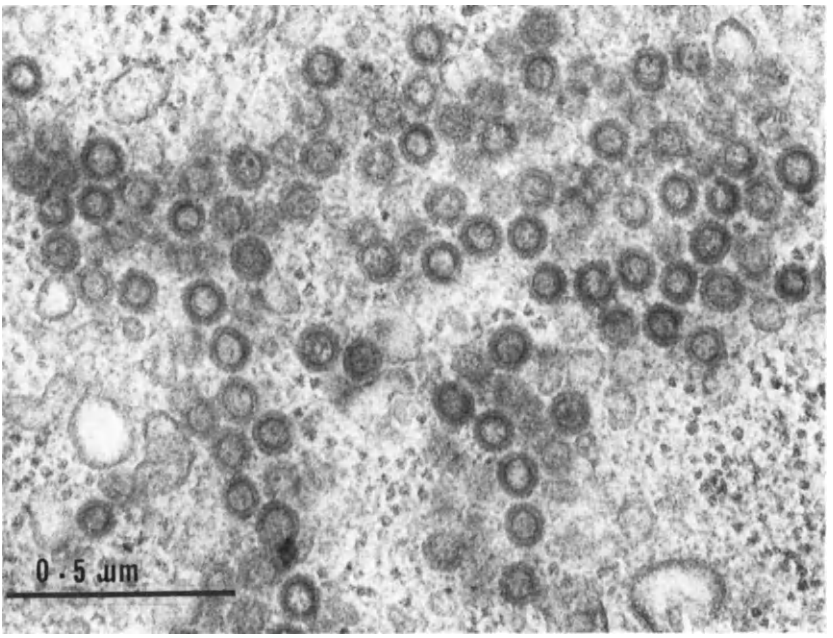

Fig. 3. Intracytoplasmic type A particles in tumor cell of reticulum sarcoma in a high mammary cancer strain RIII/Dm mouse. × 60,000.

subgenera of avian, mammalian, and reptilian type C viruses. However, the group of type C viruses is likely to extend, in the future, to most animal classes. A great number of recent discoveries of particles resembling type C particles, especially in primate and human tissues, have clearly shown variations in their ultrastructural aspects (see Dalton, 1972a,b; Bowen *et al.*, 1974). These variations may be circumstantial or systematic according either to the origin of the viruses or the fixation methods employed. Based on fixation methods, which included chrome-osmium (Dalton, 1972a), Hall and Schidlovsky (1976) have insisted that "typical" type C virus particles include the presence, in "typical" particles, of a distinctly outlined electron-lucent interval separating the envelope from the outer portion of the nucleoid. A particular and still unresolved problem concerns bovine leukemia virus (BLV), which, although described as a type C virus (Calafat *et al.*, 1974), differs in some morphological details and above all in biochemical and immunological properties from other mammalian type C viruses (Gilden *et al.*, 1975). These points only underline the fact that morphological definition of "type C" viruses is probably too abstract and that differential diagnosis of type C viruses

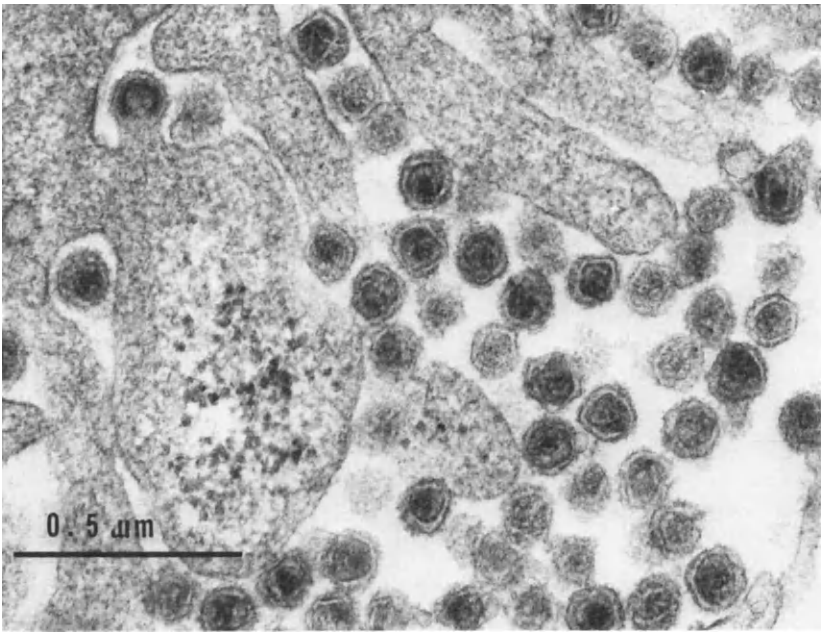

Fig. 4. Type C virus particles in tissue culture of spleen cells of an RIII/Dm mouse with reticulum cell sarcoma. A budding particle is indicated by an arrow. × 60,000.

requires a combination of morphological, biochemical, and immunological criteria.

Type D viruses (genus *Oncornavirus* D, Fig. 5) belong to a recently discovered group of Retroviridae. The prototype is a virus isolated from the mammary tumor of a rhesus monkey (Chopra and Mason, 1970), and designated as Mason-Pfizer monkey virus (MPMV). Mature, extracellular virions of MPMV have an eccentric, bar-shaped nucleoid, giving the virions the appearance of the greek letter theta. There are no spikes on the surface of the envelope. Like type B particles, MPMV has intracytoplasmic precursor particles, somewhat similar to intracytoplasmic type A of MMTV. As will be discussed later, particles identical to MPMV have been isolated from a series of established human cell lines.

Lentiviruses (or type E viruses) are represented by viruses causing chronic pulmonary diseases in sheep (visna, maedi, and progressive pneumonia). These viruses are very similar to each other (see Thormar, 1965; Takemoto *et al.*, 1971). Viruses of the same type have been isolated from buffy coat cells of cattle with lymphocytosis (Boothe and Van der Maaten, 1974). Virions of lentiviruses have an eccentric, often bar-shaped nucleoid as in type D viruses. Whether the envelope has spikes is not

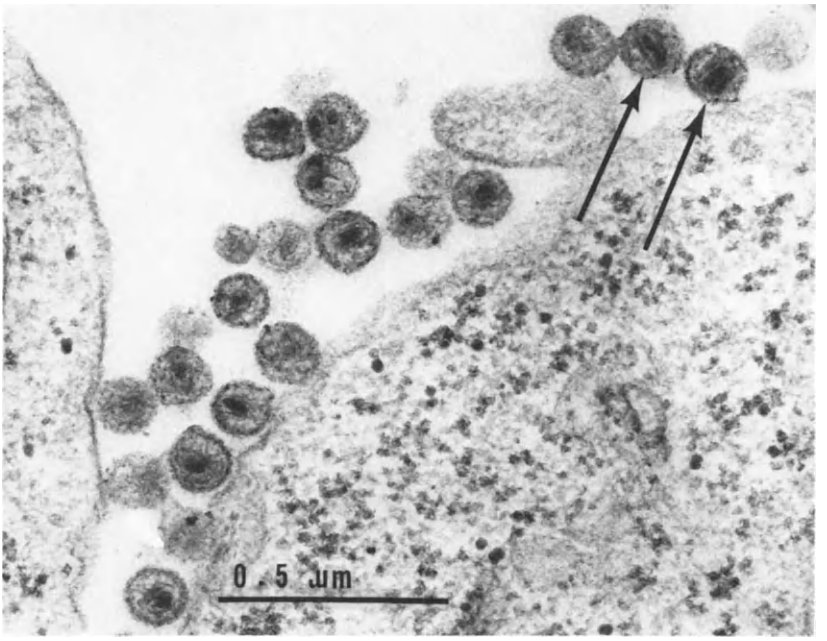

Fig. 5. Example of type D virus particles. This figure illustrates the appearance of Mason-Pfizer monkey virus (MPMV) grown in the human lymphoblastoid cell line NC-37. Arrows show bar-shaped nucleoid in particles resembling the greek letter theta.

clear, because there seems to be some confusion in the literature, at least on a morphological basis, between type C and visna viruses (see, for instance, Takemoto *et al.*, 1973; Calafat *et al.*, 1974). There is no doubt, however, that type E viruses have intracytoplasmic precursor particles similar in many respects to those of MMTV or MPMV. The morphogenesis of visna viruses, especially of bovine visna virus, has as yet not been completely elucidated (see Boothe and Van der Maaten, 1974).

Spumaviruses (or foamy viruses) are a morphologically homogeneous group of viruses isolated from tissue cultures of bovine (Boothe *et al.*, 1970), feline (Riggs *et al.*, 1969), and simian (Clarke *et al.*, 1969; Chopra *et al.*, 1972) cells, derived from tumor or normal tissues. Foamy viruses have also been isolated from a lymphoblastoid culture derived from a specimen of human nasopharyngeal carcinoma (Epstein *et al.*, 1974). These isolates, designated as bovine syncytial virus (or BSV), feline syncytial virus (FSV), and simian foamy virus (SiFV) for viruses of animal sources, and the human isolate are serologically distinct. Foamy viruses produce a characteristic cytopathic effect in cells infected *in vitro* (Parks and Todaro, 1972). Foamy viruses have an icosahedral capsid. Budding

and extracellular virions are covered with long, characteristic spikes. As mentioned earlier, the core of extracellular virions does not undergo maturation, and resembles the intracytoplasmic 65-nm precursor particles observed in infected cells.

3. Biochemical Properties of Retroviridae

The major biochemical properties of Retroviridae, and, above all, of oncornaviruses (the latter having been the most extensively studied) are defined by their nucleic acid, their protein and glycoprotein makeup, and their enzymes, of which reverse transcriptase is of critical importance.

The nucleic acid of the viruses is essentially single-stranded RNA sedimenting at 60 to 70 S. This RNA is, in fact, an aggregate of a few (2–4) subunits sedimenting with a coefficient of 20 to 35 S (Duesberg, 1968; Erikson and Erikson, 1971; Faras *et al.*, 1972; Mangel *et al.*, 1974). The native 60–70 S RNA has a molecular weight of 10 to 12 $\times$ 10^6 daltons (Granboulan *et al.*, 1966), while the 20–35 S subunits weigh around 2.5–3.5 $\times$ 10^6 daltons (Duesberg, 1968; Maisel *et al.*, 1973). The presence of several subunits has suggested that the genome of oncornaviruses is in fact polyploid (see Duesberg and Vogt, 1973; Bishop and Varmus, 1975). The 60–70 S RNA of oncornaviruses has been shown to contain sequences of polyadenylic acid (poly(A) regions) (Gillespie *et al.*, 1972).

There are also in the virions of oncornaviruses nucleic acids of low molecular weight, with sedimentation coefficients of 4 and 5 S and which probably are of cellular origin (see Faras *et al.*, 1973). Duesberg and Vogt (1970) and later several authors (see Bishop and Varmus, 1975) have observed that viruses with transforming properties had 10% heavier 35 S subunits (class "a" subunits) than 35 S subunits (class "b" subunits) of nontransforming viruses.

Proteins, polypeptides, and glycoproteins of oncornaviruses have been extensively analyzed in recent years by acrylamide gel electrophoresis, gel filtration, electrofocusing, and other methods, and a number of components with characteristic molecular weight have been isolated. According to a nomenclature suggested by August *et al.* (1974), proteins are designated by the prefix p and glycoproteins by the prefix gp, followed by their molecular weight in thousands. The study of biochemical components of oncornaviruses is of extreme importance not only for biochemical and immunological characterization of the various virus strains, but also for detection of these components in cells in the absence of complete virus. The biochemical components of oncornaviruses have been studied by Duesberg *et al.* (1970), Fleissner (1971), Bolognesi *et al.* (1972a,b), Robinson and Robinson (1972), and Stromberg *et al.* (1974) for avian viruses; by Nowinski *et al.* (1971b), Dickson and Skehel (1974), Teramoto *et al.*

(1974), and Sarkar and Dion (1975) for the mouse mammary tumor virus; and for the various presently known mammalian type C viruses by Gregoriades and Old (1969), McDugald *et al.* (1970), Nowinski *et al.* (1972), Strand and August (1971, 1974), Moroni (1972), Schafer *et al.* (1972), Bolognesi *et al.* (1973), Green *et al.* (1973), Lange *et al.* (1973), Panem *et al.* (1973), Green and Bolognesi (1974), Panem and Kirsten (1975), Ihle *et al.* (1975), Schafer *et al.* (1975), Yeh *et al.* (1975), and Gilden *et al.* (1975). These studies have shown that each type of virus was characterized by a set of proteins or glycoproteins present in their envelope and their core. There are at least eleven major proteins in avian type C viruses, of which the best characterized reside mostly in the core: p27, p19, p15, p12, and p10. Mammalian type C viruses contain at least four major proteins: p30, p15, p12, and p10, and two major glycoproteins, located at their surface: gp69–71 and gp32. Type B virus particles of MMTV contain at least eleven protein components of which p28, p18, p12, and gp52 and gp34 are apparently the more important. Interestingly enough, intracytoplasmic type A particles of mice contain five major proteins: p52, p43, p37, p28, and p15, of which only p28 is shared by MMTV virions. The major proteins of the Mason-Pfizer virus are p27 and p12 core proteins. Recently, bovine leukosis virus, a rather unusual type C virus, was found to contain a p24 major protein, not found in any other mammalian type C virus (Gilden *et al.*, 1975).

The most important enzymes carried by oncornaviruses are ribonuclease H, that destroys RNA of DNA–RNA hybrid molecules (Moelling *et al.*, 1973; Baltimore and Smoler, 1972; Baltimore *et al.*, 1974) and above all, reverse transcriptase (RT or RDDP), which functions as an RNAse sensitive, RNA-directed DNA polymerase. Reverse transcriptase which, as mentioned above, characterizes the group of Retroviridae, has been discovered simultaneously in 1970 by Temin and Mizutani in chicken sarcoma virus and by Baltimore in Rauscher mouse leukemia virus. Because of the great importance of reverse transcriptase in the search for putative human tumor RNA viruses, the properties of reverse transcriptase will be briefly described.

4. Properties of Reverse Transcriptase

The properties of reverse transcriptase have been described in detail in a number of reviews (Temin and Baltimore, 1972; Gallo, 1972; Sarin and Gallo, 1973; Green and Gerard, 1974; Bishop and Varmus, 1975; Sarngadharan *et al.*, 1976).

Reverse transcriptase is a polypeptide which, like all polymerases, makes DNA by adding deoxyribonucleotide triphosphates to each other in the 5′→3′ direction, under the direction of an RNA template which de-

termines the base sequence of the produced DNA. To initiate DNA synthesis, reverse transcriptase requires a primer molecule with a 3′-OH end (primer end), which, in the case of oncornaviruses, is provided by a 4 S RNA molecule attached to the genome of the viruses and has the properties of a transfer RNA.

When viral RNA of disrupted virions is used *in vitro* to synthetize DNA in the presence of deoxyribonucleotides, the reaction is called an endogenous reaction. When instead of viral RNA, the enzyme is made to copy synthetic templates, we have an exogenous reaction.

The requirements for the endogenous reaction of the various oncornaviruses have been extensively studied (see Summary Table in Green and Gerard, 1974, pp. 204–205). Virions have to be disrupted by nonionic detergents (Nonidet P-40, Sterol-XL, Triton X-100, and Tweens 40, 60, or 80) to release reverse transcriptase and viral RNA, at concentrations between 0.01 and 0.02%. All four deoxyribonucleotides (dA, dT, dC, dG) have to be added in the form of triphosphates. The reaction has an absolute requirement for cations, Mg^{2+} or Mn^{2+}, which explains the fact that it can be stopped by addition of EDTA. The addition of reducing agents such as dithiothreitol or mercaptoethanol improves the rate of reaction, but is not absolutely required. The optimum pH was found to be 8, and optimum temperatures 37°C for mammalian, and 40°C for avian oncornaviruses. Preincubation of disrupted virions with pancreatic ribonuclease characteristically inhibits the endogenous reaction.

The early products of the endogenous reaction are small single-stranded DNA molecules. They are attached by covalent bonds to the 3′-terminus of a 4 S RNA primer derived by endonucleolytic cleavage from the viral 70 S RNA. They are also attached by hydrogen bonds to the 70 S RNA template. This DNA is a heteropolymer of all four dn TP's added to the system *in vitro* (Spiegelman *et al.*, 1970a; Fujinaga *et al.*, 1970; Verma *et al.*, 1971; D. H. L. Bishop *et al.*, 1971; Roy-Burman, 1971; Robinson and Robinson, 1971; J. M. Bishop *et al.*, 1973). This early single-stranded DNA attached to its RNA template in turn serves as template for further synthesis of double-stranded DNA free of RNA. The DNA products, early or late, are rather small molecules which, although containing copies of all the nucleotide sequences present in viral 70 S RNA, represent only a limited portion of the viral genome, transcribed preferentially (Gelb *et al.*, 1971, Varmus *et al.*, 1971; Garapin *et al.*, 1973). During the reaction an important part of the viral 70 S RNA is degraded.

It has been possible by purification of reverse transcriptase of avian viruses to study the characteristics of the so-called reconstructed reaction in which purified reverse transcriptase is made to react *in vitro* with puri-

fied viral 70 S RNA. Requirements for this reaction are the same as for the endogenous reaction. Native 70 S viral RNA is also the best template among natural RNA's. However, the amount of DNA synthesized in a reconstructed reaction is very low, and the size of the DNA molecules synthesized is as small as that of DNA synthesized in the endogenous reaction—120–150 nucleotides (Leis and Hurwitz, 1972; Faras *et al.*, 1972).

Since the finding by Spiegelman *et al.* (1970b) that reverse transcriptase was capable of using very efficiently synthetic ribohomopolymers attached to a complementary oligodeoxyribonucleotide primer, many studies have been devoted to determining the template specificity of the reverse transcriptase of oncornaviruses. This problem is of major importance in a search for viral footprints in animal, and above all in human cells, because it is now well known that RNA → DNA transcription is not unique to reverse transcriptase but can also be performed by a number of DNA polymerases of bacteria or cells. It has been found that the most efficient synthetic templates for viral reverse transcriptase were the combinations poly(rA)·oligo(dT) (Goodman and Spiegelman, 1971) and above all the combination poly(rC)·oligo(dG) (Lewis *et al.*, 1974). Reverse transcriptase works better in the presence of Mn^{2+} when poly(rA)·oligo(dT) is used, and with Mg^{2+} when poly(rC)oligo(dT) is used. It appears also that the 2′-*O* methylated homopolymer poly(Cm) is a highly specific template for reverse transcriptase when complexed to oligo(dG) (Gerard *et al.*, 1974).

The ability to transcribe into DNA natural heteropolymeric RNA's, above all 70 S RNA, and to transcribe with high fidelity the synthetic complexes poly(rA)·oligo(dT), poly(rC)·oligo(dG) may be used to distinguish oncornaviral RNA-directed DNA polymerase activity from that of other DNA polymerases found in normal or malignant cells. There are several known DNA polymerases (Lewis *et al.*, 1974; Srivastava, 1974,1976; Gallo *et al.*, 1975). DNA polymerase I (or alpha) is a polyribosyladenyl acid-dependent DNA polymerase sedimenting at 6 to 7 S. DNA polymerase II (or beta) sediments at 3 to 4 S and is a DNA-directed DNA polymerase. DNA polymerases I and II prefer both poly(dA)·oligo(dT) to poly(rA)·oligo(dT) and are unable to transcribe natural heteropolymeric RNA's. DNA polymerase III [poly(rA)-dependent DNA polymerase] found in HeLa cells and phytohemagglutinin-activated lymphocytes transcribes poly(rA)·oligo(dT), but not natural RNA templates. None of the three polymerases transcribes poly(rC)·oligo(dT). Finally there is a terminal deoxynucleotidyltransferase which has no template preferences and adds deoxynucleotidyl residues from dnTPs to the 3′-hydroxyl group of preformed polynucleotide chains.

It has been possible to obtain reverse transcriptase of sufficient purity from most known oncornaviruses to determine their approximate molecular weight and more importantly to study their immunological relatedness by using specific antisera to each of them. Techniques to purify reverse transcriptase from virus or cells are briefly described in Section III. The reverse transcriptase of avian type C viruses consists of two subunits of 69,000 and 110,000 molecular weight (Duesberg *et al.*, 1971; Faras *et al.*, 1972). Reverse transcriptase of murine and feline viruses (Hurwitz and Leis, 1972) and of woolly monkey virus (Abrell and Gallo, 1973) have an estimated molecular weight of 70,000. Reverse transcriptase of hamster leukemia virus has a molecular weight of 120,000 but is actually composed of two subunits, one weighing 68,000 and the other 53,000 (Verma *et al.*, 1974). Type B mammary tumor virus (MMTV) has a reverse transcriptase with a molecular weight of 100,000 (Dion *et al.*, 1974) slightly lower than the molecular weight of the reverse transcriptase of Mason-Pfizer virus (Abrell and Gallo, 1973) and that of reptilian type C virus (Twardzik *et al.*, 1974), which weigh 110,000 and 109,000 respectively.

By measuring the neutralizing effect of specific antibodies to a reverse transcriptase on DNA-synthesizing activity of other reverse transcriptases *in vitro*, the serological relatedness of the reverse transcriptase of most oncornaviruses has been outlined. The reverse transcriptases of avian-type C viruses cross-react with each other, as do reverse transcriptases of murine, rat, hamster, and cat type C viruses (Parks *et al.*, 1972). Reverse transcriptases of mouse mammary tumor virus, of Mason-Pfizer monkey virus, and of visna viruses share no common antigenic determinants (Ahmed *et al.*, 1973; Yaniv *et al.*, 1974; Nowinski *et al.*, 1971a; Tronick *et al.*, 1974). Gibbon ape and woolly monkey type C virus polymerases are closely related, but differ from all other mammalian polymerases (Scolnick *et al.*, 1972; Long *et al.*, 1973) and from polymerase of the baboon endogenous virus M-7 (Mayer *et al.*, 1974).

The endogenous reaction has been of great importance for determination of the nucleic acid homologies between RNA's of oncornaviruses, by hybridizing radioactive ^{3}H-DNA with viral 70 S RNA.

Extensive studies have already been carried out on possible inhibitors of reverse transcriptase or of reverse transcription by reverse transcriptase. These studies have been reviewed in detail by Green and Gerard (1974). A great number of compounds have been found to inhibit in some way or another the reverse transcriptase activity. The most interesting of them include antibiotics, drugs, and antitumor agents: derivatives of rifamycin, actinomycin D, daunomycin, and ethidium bromide, etc. The most efficient inhibitors are those which bind to duplex DNA by intercalation between base-paired dinucleotide sequences. Actinomycin D is of

particular interest, because it does not inhibit the early formation of RNA–DNA hybrid in endogenous or exogenous reverse transcriptase reactions, but blocks the subsequent formation of duplex DNA.

5. *Antigenic Properties of Retraviridae and of Their Subcomponents*

By using the classic immunologic methods of neutralization, complement fixation, immunofluorescence, etc., and the more sensitive, recently developed techniques of immunoelectron microscopy (Kurstak and Kurstak, 1974; Dmochowski *et al.*, 1974; Kurstak *et al.*, 1975) and of radioimmunoassay, it has been possible to divide the antigenic properties of oncornaviruses into three categories. These categories include type-specific, group-specific, and interspecies antigenicities. In many cases, especially with type C viruses, it has been possible to correlate these antigenic specificities with particular protein components of the virions (see review by Bauer, 1974; also Geering *et al.*, 1970; Gilden and Oroszlan, 1972; Bowen *et al.*, 1974; Aoki and Sibal, 1976). For instance, the major antigen shared by mammalian type C viruses corresponds to the p30 protein, also designated as gs-3 determinant. Type specificities are mostly determined by surface glycoproteins. The study of the antigenic specificity of type C oncornaviruses is made difficult by the fact that protein components of each virus type may carry all three antigenic specificities (type, group, and interspecies) simultaneously. However, at the same time, antigenic analysis has demonstrated important differences between oncogenic RNA viruses of animals which are very significant from a biologic point of view. Thus, type C oncornaviruses of avian, mammalian, and reptilian origin share no common antigens; neither does MMTV with MPMV or any other Retraviridae. Lentiviruses and spumaviruses also form two separate groups.

Mostly on the basis of their immunologic characteristics, type C oncornaviruses have been divided into three subgroups already mentioned: avian, mammalian, and reptilian. However, each subgroup, again on the basis of antigenic properties, presently includes several virus strains (Dalton *et al.*, 1975). Seven strains (from A to G) have been identified in the group of avian type C viruses. Murine type C viruses include three major strains: the FMR (Friend-Moloney-Rauscher) strain of murine leukemia virus (MuLV), the G(Gross) strain of MuLV, and the murine sarcoma virus (MSV). Subtypes of the FMR strain comprise F (Friend)-MuLV, M (Moloney) MuLV, and R (Rauscher)-MuLV. The MSV strain exists in six substrains, always mixed to MuLV: M-MSV (Moloney), Ki-MSV (Kirsten), SD-MSV (Soehner-Dmochowski), FJB-MSV (Finkel), H-MSV (Harvey), and GZ-MSV (Gazdar). Feline type C viruses

comprise feline leukemia virus (FeLV) and feline sarcoma virus (FeSV), antigenically divided into three subgroups A, B, and C. Type C viruses of hamsters probably include a nontransforming leukemia virus found *in vitro* (HaLV), and a sarcoma virus (HaSV) derived from MSV-induced sarcomas in hamsters. HaSV is considered as having an MSV genome and an HaLV envelope. Type C viruses of primates comprise the simian sarcoma virus (SiSV) of the woolly monkey, and the simian lymphosarcoma virus (GaLV) of gibbon ape. SiSV is associated with a nontransforming leukemia virus (WLV) not known to be leukemogenic *in vivo*.

B. Oncornaviruses of Animals

A brief summary of the most recent, or less known, type C oncornaviruses found in animals will be presented because (like the extensively investigated murine type C viruses) they may have an indirect bearing on human tumor virology.

1. C-Type Virus of Snakes

In 1967, typical type C virus particles were observed in a tissue culture (VSW) derived from the spleen of an Asian pit viper with myxofibroma (Zeigel and Clark, 1969, 1971), but not in the original tumor. Type C particles have also been found by electron microscopy in the mesenchymal tumor of a corn snake (*Elaphe guttata*) (Lunger *et al.*, 1974; Lunger and Clark, 1974). Both viper and corn snake virus (VSV) have been propagated subsequently in a number of reptilian cell lines, but their oncogenic potential is unknown.

2. Avian Type C Viruses

Type C viruses, which are the agents of Rous sarcoma, avian myeloblastosis, and avian erythroblastosis have been extensively studied (Dmochowski, 1963; Gross, 1970). They have been classified into avian sarcoma viruses (strains RSV, SR, PR, MH, CZ, and B77), avian leukemia viruses (strains RAV, RIF, and AMV), and avian reticuloendotheliosis (duck infectious anemia) virus.

3. Mouse Type C Viruses

The various strains of murine type C viruses have been mentioned in Section II, A,5. Of interest are type C viruses isolated from wild mice (*Mus musculus musculus*) of southern California, which present an incidence of lymphomas, associated with lower limb paralysis, that increase with age (Gardner *et al.*, 1973a,b). Another type C virus has been isolated from Japanese wild mice (*Mus musculus molossinus*) by Bedigian and

Meier (1975). These viruses have the particular property of being unable to replicate or infect within cells of their own species, and, therefore, represent endogenous (genetically transmitted) but xenotropic (replicating in other species) viruses, a property which, in fact, is shared by most type C viruses of laboratory mice (see Dalton *et al.*, 1975).

4. Rat Type C Viruses

A type C virus of interest is a virus designated R-35, discovered and isolated in 1970 from a transplantable mammary tumor of rats (Chopra *et al.*, 1970). Type C particles have also been observed by electron microscopy in another rat mammary tumor (Engle *et al.*, 1969). R-35 virus contains reverse transcriptase (Schlom and Spiegelman, 1971). Inoculation of newborn Sprague-Dawley rats with virus produced *in vitro* by cells derived from R-35 tumor induced, around an age of 4 to 5 months, an incidence of mammary tumors higher than the incidence of spontaneous mammary tumors in controls (Bogden *et al.*, 1974). Interestingly enough, virus-induced tumors contained virus detectable by electron microscopy and immunofluorescence test, while virus and antigens were absent from tumors in controls (Bogden *et al.*, 1974).

5. Feline Type C Viruses

Lymphosarcomas and/or leukemias are the most common neoplasms in cats (Gross, 1970). Disseminated lymphosarcoma is more frequent than overt leukemia. The disease is nearly always associated with anemia varying in degree, and often severe. The first indication of a viral etiology of cat leukosis came from Jarrett *et al.* (1964) who induced the disease in newborn kittens by cell-free extracts of the tumor of a field cat. Cell-free transmission of the disease was successfully carried out in newborn animals by a number of other investigators (Kawakami *et al.*, 1967; Rickard *et al.*, 1967; Laird *et al.*, 1968). These authors have also demonstrated by electron microscopy the presence of type C virus particles in tissues and organs of leukemic cats. Type C viruses have been observed budding from the plasma membrane of erythroblasts in cats with leukemia or lymphoma (Oshiro *et al.*, 1972; Gardner *et al.*, 1971). Cat leukemia has been successfully transmitted to marmosets (Deinhardt *et al.*, 1970) and to dogs (Rickard *et al.*, 1973). As in mice, type C leukemia viruses can be found in tissues of normal animals, for instance, in salivary glands of domestic cats (Gardner *et al.*, 1971).

Another type C virus, designated as ST-virus, then FeSV, was isolated in 1969 by Snyder and Theilen (1969) from the tumor of a cat with spontaneous fibrosarcoma. The virus induces sarcomas not only in cats (McKissick and Lamont, 1970; Gardner *et al.*, 1971; McDonough *et al.*, 1971;

Snyder, 1971) but also in heterologous species such as dogs and marmosets (Deinhardt *et al.*, 1970).

Type C virus particles have been observed by electron microscopy in feline mammary tumors (Feldman and Gross, 1971) and more recently in the tumor of a cat with angioma (Feldman *et al.*, 1974), together with small numbers of intracytoplasmic type A virus particles morphologically identical to intracytoplasmic type A virus particles of mice. The significance of the latter findings remains to be determined. However, type C or intracytoplasmic type A viruses may perhaps behave as passenger viruses in cats with anemia of undetermined origin or with infectious peritonitis (Gardner *et al.*, 1971). On the other hand, type C virus particles may, at least in certain instances, be responsible for inflammatory diseases and in others for proliferative and malignant diseases (Dmochowski, 1968).

6. Type C Viruses of Guinea Pigs

Lymphoblastic leukemia L2C of guinea pigs is transmissible through whole blood or spleen homogenates of leukemic animals (Sarma *et al.*, 1970). The possible viral etiology of guinea pig leukemia has been suggested by the finding of typical type C virus particles in tissues of leukemic animals (Opler, 1967; Nadel *et al.*, 1967). Later (Gross, 1970) type C particles were found not only in cultures of leukemic tissues (spleen, kidney, thymus) but also in tissue cultures of spleen or kidney of normal guinea pigs of strain 2, following exposure of the cultures to BUdR (Hsiung, 1972; Hsiung *et al.*, 1973). A cell line designated LGPS carrying type C virus has thus been derived from normal spleen fibroblasts (Hsiung, 1972). The virus does not appear to be leukemogenic and behaves as an endogenous virus.

7. Type C Viruses of Pigs

There is now a growing number of porcine cell lines which release spontaneously, after long-term propagation *in vitro*, type C virus particles. Release of type C particles has also been induced in cells of some of the lines by treatment with BUdR. The cell lines have been established from a variety of porcine tissues. Cell lines PK-15 (Breese, 1970; Lieber *et al.*, 1973; Woods *et al.*, 1973; Todaro *et al.*, 1974), MPK (minipig kidney), and PORC were derived from normal kidney tissues. Cell line 38A-1 (Strandstrom *et al.*, 1974; Moennig *et al.*, 1974) was derived from lymph node tissue of a pig with lymphoma. A cell line ST-MO was established (Lieber *et al.*, 1975) and a cell line PFT from oviduct of an adult sow (Bouillant *et al.*, 1973, 1975). Type C virus from lines PFT and PK-15 are apparently not infectious to cells of many other species and its genome shows no relatedness to other mammalian type C viruses, which indicates the en-

dogenous character of this virus (Lieber *et al.*, 1973). DNA of normal pig cells contain sequences homologous to RNA sequences of PK-15 virus (Benveniste and Todaro, 1974).

8. Bovine Type C Viruses

Leukemia and lymphosarcoma are the most common neoplasms in cattle. Thymic lymphosarcoma, generalized lymphadenopathies, and skin and heart infiltration by leukemic cells are the prevalent clinical symptoms in the diseased animals. The blood picture is not always characteristic of leukemia, being within normal limits at least in 50% of the cases. In the other cases the major symptom is hyperlymphocytosis of varying degrees (Weber, 1963; Ritter, 1965). The incidence of leukosis in cattle is variable but generally low (0.01–0.06%). In the United States the disease seems to be increasing (Baumgartner *et al.*, 1975). It also shows a definite clustering in herds or families of cattle (Marshak *et al.*, 1963; Croshaw *et al.*, 1963). Animals at higher risk of developing the disease show an increased number of peripheral blood lymphocytes (Bendinxen, 1965). The clustering of leukosis in cattle suggests transmission of a viral agent from parents to offspring. Early attempts to demonstrate by electron microscopy the presence of a virus in milk of cows from herds with high incidence of leukosis (Dutcher *et al.*, 1964; Jensen and Schidlowsky, 1964) or in tissues of leukemic animals (Dutcher *et al.*, 1964) were, for technical reasons, not conclusive. A clearer indication of a possible viral etiology came from the discovery, by electron microscopy, of typical type C virus particles in cultures of peripheral blood cells of cattle with persistent lymphocytosis (Miller *et al.*, 1969; Kawakami *et al.*, 1970; Dutta *et al.*, 1970; Stock and Ferrer, 1972; Calafat *et al.*, 1974). The possibility of growing the virus in tissue culture has made available at least three different tests for characterization of the bovine type C virus (BLV): gel immunodiffusion (Miller and Olson, 1972), immunofluorescence (Ferrer *et al.*, 1974), and complement fixation (Miller and Van der Maaten, 1974). These tests proved useful for screening cattle for natural or experimental infection with BLV. Recent surveys have shown that dairy cattle are more frequently infected with the virus (about 10%) than beef cattle (about 1.5%) (Olson *et al.*, 1973; Baumgartner *et al.*, 1975). Reasons for this difference are unknown. BLV has no common group-specific (*gs*) antigens with feline leukemia or murine leukemia viruses (Kawakami *et al.*, 1970; Gilden *et al.*, 1975).

The monitoring of cattle for BLV is complicated by the fact that animals with the juvenile, thymic form of lymphosarcoma have no demonstrable virus or antibodies to the virus, yet their tumor tissues can infect test calves and sheep (Miller *et al.*, 1972). Another complication has

arisen from the discovery in the buffy coat cells of cattle with persistent lymphocytosis or in tumor tissues of animals with lymphosarcoma, of two cytopathic viruses: the bovine syncytial virus or BSV (Malmquist *et al.*, 1969) and of a virus resembling visna virus of sheep (Van der Maaten *et al.*, 1972; Boothe and Van der Maaten, 1974). A herpes-like virus has also been isolated from tissues of lymphomatous cattle (Van der Maaten and Boothe, 1972). The relationship of the three viruses to the etiology of cattle leukosis remains undetermined.

9. Simian Type C Viruses

Discoveries of type C particles which are of great interest have recently been made in monkeys, in particular, in subhuman primates. In 1969, Theilen *et al.*, observed by electron microscopy budding and mature type C virus particles in bone marrow and in tumor tissues of a woolly monkey (*Lagothrix* sp.) with spontaneous fibrosarcoma. This virus, now designated as SiSV (simian sarcoma virus) was successfully isolated from the tumor and propagated in muscle cell cultures of woolly monkey (Wolfe *et al.*, 1971a). Virus extracted from the cultures showed transforming activity on fetal marmoset lung cells and induced fibrosarcomas in newborn marmosets (Wolfe *et al.*, 1971b).

Another type C virus was isolated by Kawakami *et al.* (1972) from tissues of a gibbon (*Hylobates lar*) with generalized lymphosarcoma. This virus, designated as GALV (gibbon ape leukemia virus) was propagated successfully in a lymphoblastoid cell line derived from timor tissue. GALV replicates well in simian, bovine, and even human cells grown *in vitro*.

At about the same time as type C virus particles were accidently discovered by electron microscopy in human placentas (Kalter *et al.*, 1973a), examination by electron microscopy of simian placentas proved rewarding. Type C particles were easily found in all baboon (*Papio cynocephalus*) placentas examined (Kalter *et al.*, 1973b, 1975a). Although less frequently, type C virus particles were also found by electron microscopy in placentas of rhesus monkeys (Schidlovsky and Ahmed, 1973), cynomologous monkeys (*Macaca fascicularis*), marmosets (*Saguinus fascicollis*), chimpanzees (Kalter *et al.*, 1975b), and of cotton-top marmosets (*Saguinus oedipus*) (Seman *et al.*, 1975).

In baboons, type C particles have been found in follicular and tubal ova (Kalter *et al.*, 1975a). A type C virus designated as M-7 (Benveniste *et al.*, 1974) has been isolated from the placenta of a normal baboon (*Papio cynocephalus*), after cocultivation of placental cells with dog, bat, rhesus monkey, and human rhabdomyosarcoma (A-204) cells. The virus has been detected in cocultures by electron microscopy and by biochemical

methods after a few weeks of cultivation, at a time when baboon placental cells have already been replaced by heterologous cells.

Another type C virus has been isolated from tissues of a lymphomatous baboon by the same cocultivation methods using dog thymus (Fcf 2th) and human A-204 indicator cell lines. The virus, designated as BILN (Goldberg *et al.*, 1974) replicated well in canine, bat, mink, rhesus monkey, and human cells, but not in mouse, rat, cat, or rabbit cells. Interestingly enough the baboon has been inoculated on several occasions with whole blood of another baboon with lymphadenopathy and splenomegaly following injections of whole blood of several leukemic human patients. It should be pointed out that the whole experiment lasted for about 5 years.

The biochemical and immunological properties of the various simian type C virus particles recently isolated will be examined together with those of human type C isolates (see Section IV).

10. Type B Oncornaviruses of Animals

The properties of mouse mammary tumor virus (MMTV) are well known (see Moore, 1963; Dmochowski *et al.*, 1967a) and will not be examined further here. Of interest is the observation of type B-like particles in guinea pigs (Fong and Hsiung, 1976) and of intracytoplasmic type A virus particles in cells of cat with angiosarcoma (Feldman *et al.*, 1974). Whether these particles seen by electron microscopy are of feline origin is not known, in view of the particular impossibility of infecting heterologous cells with mouse MMTV. Recently successful infection of feline cells and of mink cells with MMTV has been reported (Lasfargues *et al.*, 1974, 1976).

11. Type D Oncornaviruses of Monkeys

An oncornavirus different from type C has been discovered by electron microscopy in breast tumor of an 8-year-old rhesus monkey (*Macaca mulatta*) undergoing chemotherapy with phenesterin (Chopra, 1970; Chopra and Mason, 1970). The virus (also designated as MPMV or Mason-Pfizer monkey virus) appears to resemble the type B virus of mouse mammary tumors (MMTV). It buds from the cell membrane and has precursor particles somewhat similar to, but not identical to, the type A intracytoplasmic particles of MMTV. However, unlike MMTV, MPMV particles do not have spikes on the outer side of their envelope (Jensen *et al.*, 1970). Ultrastructural differences between MPMV and MMTV and MuLV have recently been described (Dmochowski *et al.*, 1976). The MPMV has a characteristic nucleoid resembling the Greek letter theta. MPMV has been successfully propagated from the original tumor in a cell line designated CMMT (Mason *et al.*, 1972). The virus shows trans-

forming potential on rhesus foreskin cells *in vitro* (Fine *et al.*, 1974). MPMV-like viruses have also been isolated from breast (virus X-381) and placenta (virus FTP-1) of healthy rhesus monkeys (Ahmed *et al.*, 1974). Although not demonstrated so far to be oncogenic *in vivo*, MPMV is a virus of great interest because a number of established human cell lines have been found to synthesize a very similar virus (see below). MPMV has been maintained in a human lymphoblastoid cell line (NC-37) which at present is the usual source of the virus for many studies. As already mentioned (see Section II,A,5), MPMV is completely unrelated to the mouse mammary tumor virus, and, for that matter, to bovine visna virus which seems morphologically very close to MPMV.

C. Oncogenic Herpesviruses of Animals

The relationship of RNA oncogenic viruses to human neoplasia has been up to now much more difficult to evaluate than that of herpesviruses, which in recent years have acquired a particularly high status as candidate oncogenic viruses in humans. This is why the properties of herpesviruses will be examined in a later section dealing with human herpesviruses (see Section VII).

We will briefly describe the presently known herpesviruses which appear to have a close relationship with some malignant proliferative diseases in animals. Like Retroviridae, herpesviruses have been found in many animal species, including frogs and oysters. There are essentially two neoplasias of animals which are induced by herpesvirus: the Lucké frog adenocarcinoma, and neurolymphomatosis of chickens (Marek's disease). There have also been a number of recent isolations of simian herpesviruses of great interest because, unlike Retroviridae which appear to be an inhomogeneous family of viruses, herpesviruses form a family of viruses very similar in many of their properties.

1. Lucké Frog Tumor and Frog Herpesviruses

Lucké tumor (see review by Granoff, 1973) is an adenocarcinoma of the kidneys of *Rana pipiens*, a frog species found in northeast and north central parts of the United States (Lucké, 1938). The incidence of tumors among the frogs may be very high, up to 50 and even 100%. The tumors contain a virus (LHV) typical of the herpes group. Synthesis of the virus by tumor cells is temperature dependent. Tumors of hibernating frogs or of frogs maintained between 4° and 9°C contain extractable herpesvirus in degenerating tumor cells showing classic Cowdry type A inclusions. Tumors of frogs captured during summer, or maintained between 20° and

25°C, do not contain inclusions or virus. Upon cooling, tumor explants begin virus synthesis *in vitro* (Skinner and Mizell, 1972). Virus extracted from "cold" tumors induce typical kidney tumors when inoculated into frog embryos (Tweedell, 1967).

Urine of tumor-bearing frogs was shown to contain a herpesvirus with characteristics different from those of LHV and apparently not oncogenic (Gravell, 1971).

2. Marek's Disease (MD) of Domestic Fowl

This disease (see review by Nazerian, 1973), first described by Marek in 1907, is characterized by a lymphoid infiltration of peripheral nerves, and lymphoid tumors of visceral organs, muscle, and skin. The disease is highly infectious, especially in young chickens. The causative agent was found to be a herpesvirus (MDV) synthesized in infected chickens by cells of the epithelial layer of feather follicles (Calnek *et al.*, 1970), leading to airborne propagation of the virus. There are several strains of MDV with varying degrees of infectivity and pathogenicity. Strains JM and B14 produce mostly nerve lesions, and strains HPRS-16, GA, and RPL produce essentially visceral tumors. Most of the time, infection of chickens with MDV remains latent. Mortality from Marek's disease may vary from 1 to 50% during outbreaks. Chicks hatched from infected hens have natural antibodies to the virus, which disappear gradually in 3 weeks.

Although the exact nature of the target cells of MDV is not clear (thymus-dependent or bursa-dependent lymphoid cells), Payne (1972) has suggested that the thymus of susceptible chickens may contain MDV target cells which transform and develop tumors, and also immunologically active cells which react against MDV-infected target cells. In resistant chickens, but not in susceptible animals, this immune response can lead to destruction of MDV-transformed cells.

MDV is not a vertically transmitted virus and can, therefore, be prevented by genetic selection, by isolation of sick chickens, and primarily by vaccination (Purchase, 1976). Flocks of turkeys carry a herpesvirus (HVT) very similar immunologically to MDV (Witter *et al.*, 1970) and harmless to turkeys, chickens, or primates. Inoculation of chickens with HVT-carrying cell cultures (Purchase *et al.*, 1972) is very effective in preventing Marek's disease. The vaccine, however, does not prevent synthesis of MDV by feather follicles.

3. Herpesvirus of Guinea Pigs

Hsiung *et al.* (1971) and Nayak (1971) have isolated a herpesvirus (GPHV) from leukemic lymphoblasts of strain 2 guinea pigs. The virus

persists in infected animals, in spite of the presence of neutralizing antibodies, and is detectable in cultured lymphoblasts only, but not in fresh leukemic tissues.

4. Herpesvirus of Rabbits

A herpesvirus (*Herpesvirus sylvilagus*) has been isolated from the kidneys of wild cottontail rabbits (Hinze and Wegner, 1973). Infection of rabbits with the virus may produce lymphocytosis, lymphoid hyperplasia, and sometimes lymphoma. Virus production can be induced *in vitro* by cocultivation of infected leukocytes with rabbit kidney cells, although infected leukocytes (unlike EBV-infected human cells) do not appear capable of indefinite proliferation.

5. Simian Herpesviruses

At present time, some thirty-seven herpesvirus strains have been identified and named after the primate species from which they were isolated (Plummer, 1967; Hunt and Melendez, 1969; Kalter and Heberling, 1971; Hull, 1973; Falk, 1974; McCarthy and Tosolini, 1975). Most of these strains have been isolated only recently and knowledge of their host range, latency, pathogenicity and serological relatedness is still fragmentary. The viruses have been found in both Old and New World monkeys. As a rule, they are harmless to their host of origin, but may be highly pathogenic to monkeys of other species, to rabbits, or even to man.

Some herpesvirus isolates of monkeys are of great importance because of their oncogenicity. One is *Herpesvirus saimiri,* isolated in 1968 by Melendez *et al.,* from the kidney of a healthy squirrel monkey (*Saimiri sciureus*). Adult squirrel monkeys are natural carriers of the virus, as demonstrated by cocultivation of the monkey's lymphocytes with permissive cells (such as vero cells) or by indirect immunofluorescence tests (Falk *et al.,* 1972; Rabin *et al.,* 1973). *Herpesvirus saimiri* is not pathogenic to *Saimiri sciureus,* but produces leukemia, lymphomas, or lymphoproliferative diseases in a large variety of monkeys of other species (Falk, 1974), and also in rabbits (Daniel *et al.,* 1970). Surprisingly, viral inclusion bodies, or viral antigens, or particles visible by electron microscopy cannot be detected in fresh tumor tissues of inoculated animals. Infectious virions or viral antigens are found only following maintenance of lymphoid tumor cells *in vitro.* Lymphoblastoid cell lines established from tumors tend to lose their ability to synthesize virions, but still show the presence of viral antigens (Rabson *et al.,* 1971). Antibodies to HVS in natural or experimental hosts can be detected by neutralization, complement fixation, or immunofluorescence tests. As with other herpesviruses, viral antigens synthesized *in vitro* can be distinguished into early antigen (EA)

which is formed independently of DNA synthesis, and into late antigen (LA), the formation of which requires a full replicative cycle. Infected monkeys produce antibodies to both antigen types (Klein *et al.*, 1973).

Another herpesvirus isolate of interest is *Herpesvirus ateles* (HVA-810) isolated in 1972 by Melendez *et al.* (1972b), from a healthy spider monkey (*Ateles geoffroyi*) kidney culture. Most spider monkeys examined had neutralizing antibodies to the virus. HVA-810 has a broad host range *in vitro* (Melendez *et al.*, 1972a) and induces lymphoblastosarcomas in cottontop marmosets. Falk *et al.* (1976) have also isolated *Herpesvirus ateles* strains 73, 93, and 94 from spider monkeys by cocultivation of their lymphocytes with permissive cells; all three strains induce lymphomas in marmosets. Interestingly enough, heat and formaldehyde-inactivated *Herpesvirus ateles* gave efficient protection against lethal doses of *Herpesvirus ateles* in marmosets (Laufs and Steinke, 1976). A similar protection of marmosets against herpesvirus simplex by killed virus was previously demonstrated by the same authors.

III. BIOCHEMICAL AND MOLECULAR METHODS IN STUDIES OF VIRAL INVOLVEMENT IN ANIMAL AND HUMAN NEOPLASIA

As mentioned in Section I, recent biochemical techniques in studies of viral involvement in neoplasia have been at the source of a rapid advance in human tumor virus research. A brief description of the most fundamental of these techniques will help to better understand how this advance has been achieved. Details on most of these techniques will be found in reviews, such as those of Green and Gerard (1974), Bishop and Varmus (1975), Church (1973), and Spiegelman (1975).

A. Preparation of Reverse Transcriptase from Virions and from Corelike Cytoplasmic Particles of Human Cells

Preparation of reverse transcriptase from virions of Retroviridae is a standard procedure involving the following steps (Kacian *et al.*, 1971; Faras *et al.*, 1972; Waters and Yang, 1974): (a) purification of virus particles extracted from tissue culture fluid, from milk, or from tumor tissue, using essentially sucrose gradient procedures (Seman and Dmochowski, 1976); (b) disruption of virions in Tris-NaCl buffer, after addition of $MgCl_2$ or $MnCl_2$, dithiothreitol, and a nonionic detergent (usually Nonidet P-40); (c) high-speed centrifugation of virus lysates in glycerol gradients and recovery of the fraction corresponding to the expected molecular

weight of the reverse transcriptase (65,000–70,000 and 105,000–110,000 for mammalian oncornaviruses); (d) further purification of the enzyme fraction by column chromatography—first on hydroxyapatite, then on a phosphocellulose column.

Another methodology, derived from animal oncornavirus studies, has been applied to a search for reverse transcriptase activity in particulate components of breast tumors (Michalides *et al.*, 1975), of human milk (Feldman *et al.*, 1973), or of human leukemic cells (Sarngadharan *et al.*, 1972; Gallo *et al.*, 1975; Mondal *et al.*, 1975). This methodology is based on the assumption that human tumor cells, milk, or particulates from tissue culture medium of human cell cultures may contain particles with virus-related reverse transcriptase activity, particles difficult to detect by electron microscopy but accessible to biochemical analysis ("corelike" structures). There are several technical variations in this methodology, the simplest being the one published by Gallo *et al.* (1975). This technique comprises the following steps: (a) preparation of a postmitochondrial fraction from homogenized cells; (b) solubilization of components of the fraction with detergents; (c) removal of nucleic acids by DEAE-cellulose chromatography; and (d) chromatography of the remaining fraction on phosphocellulose to separate reverse transcriptase-like enzyme from DNA polymerases I and II. The first peak corresponds to viral polymerase, which is concentrated and characterized by its molecular weight, velocity sedimentation in glycerol gradient, and by its enzymatic activity. The oncornavirus-like reverse transcriptase activity of the polymerase is ascertained by its absence of activity with oligo(dT)·poly(dA), highest activity with oligo(dT)·poly(rA) and oligo(dG)·poly(rC), and preference for Mn^{2+} ions or Mg^{2+} ions when oligo (dT)·poly(rA) is the template primer (Gallo *et al.*, 1975).

B. Analytical Methods in Examination of the DNA Product of the Endogenous Reaction

There are a number of classic procedures of molecular biology for analysis of the DNA product of the endogenous reaction.

1. Cesium sulfate (Cs_2SO_4) equilibrium density gradient centrifugation (Szybalski, 1968), which characterizes the 70 S RNA–DNA hybrid by its density of 1.68 g/cm^3 (Fujinaga *et al.*, 1970).

2. Rate-zonal (velocity) gradient centrifugation in neutral sucrose or glycerol. This method separates components according to sedimentation coefficients. Thus, DNA product complexed to 70 S RNA can be separated from the slower moving DNA products, which sediment at 10 to

15 S, or less (Faras *et al.*, 1971). If the RNA of early RNA–DNA hybrid is destroyed, it can be shown that the early DNA complexed to RNA is small, around 6–7 S (Fanshier *et al.*, 1971).

3. Treatment of the DNA product by specific nucleases allows for determination of the amount of single- or double-stranded DNA, and for dissociation of it from viral single-stranded RNA. Ribonucleases A and T digest single-stranded RNA. Nuclease from *Neurospora crassa* digests all single-stranded, but not double-stranded, nucleic acid (Fraser *et al.*, 1970). Exonuclease I from *E. coli,* and exonuclease S_1 of *Aspergillus oryzae* (Sutton, 1971) specifically digest single-stranded DNA.

4. Hydroxyapatite (HA), a crystalline form of calcium phosphate, has the interesting property of fixing double-stranded, but not single-stranded, DNA (Walker and McLaren, 1965; Britten and Kohne, 1968). This property is widely used for separation of the two types of DNA strands contained in a mixture of both applied to hydroxyapatite columns. Hydroxyapatite chromatography has shown that DNA produced late in an endogenous reaction is double stranded. It has also shown that the amount of double-stranded DNA produced is proportional to the logarithm of the concentration of deoxythymidine triphosphate (Stromberg, 1972) and that synthesis of single-stranded DNA is a prerequisite to the synthesis of double-stranded DNA (Faras *et al.*, 1971).

5. For base-composition analysis, ^{32}P-labeled DNA strands are broken down into 3′-mononucleotides by micrococcal nuclease, and into 5′-mononucleotides by pancreatic DNase. The products are separated by paper electrophoresis or ion-exchange chromatography (Sebring and Salzman, 1964).

C. Characterization of the DNA Product by Molecular Hybridization Techniques

Molecular hybridization techniques have played an important part not only in recent studies of the molecular biology of oncogenic viruses, but also in attempts to determine the presence of RNA virus-related nucleic acid sequences in human tumor cells. Hybridization reactions between complementary nucleic acid strands are highly specific, but require careful interpretation of the results, because of the limits of resolution inherent to the various techniques employed and of the various parameters involved (length and relative concentrations of the complementary strands, temperature, cation concentration, nature of the paired or non-paired base sequences, etc.) (McCarthy and Church, 1970). It is impossible here to review the various techniques of molecular hybridization.

They have been described in detail in a number of reviews (McCarthy and Church, 1970; Church, 1973; Gallo *et al.*, 1975).

1. Hybridization of the DNA Product with Viral 70 S RNA in Excess (DNA–RNA Hybridization)

If the DNA product is a genuine transcript of viral RNA it should be possible to hybridize it to the viral RNA template. To that effect, radiolabeled, double-stranded ^{3}H-DNA product is denatured by heating or by formamide, and hybridized (annealed) with homologous RNA in vast excess. The extent of hybridization is measured by cesium sulfate density gradient analysis (Duesberg and Canaani, 1970; Coffin and Temin, 1972). Hybridization is assumed to be specific when more than 60% of the DNA product is hybridized to the homologous RNA.

This type of hybridization is of great importance for two reasons. First, by challenging the radiolabeled ^{3}H-DNA products synthesized by the reverse transcriptase of various oncornaviruses with homologous or nonhomologous viral RNA, the genetic relatedness of these oncornaviruses can be established. Second, using these ^{3}H-DNA products as probes, it is possible to search for virus-related RNA in the RNA of human tumor cells. After annealing the DNA probe with cellular RNA, the result of the reaction is examined by Cs_2SO_4 density gradient centrifugation. Uncomplexed DNA is found at densities around 1.45, while DNA–RNA complexes are found around a density of 1.65 (Spiegelman *et al.*, 1970a). Next, fidelity of base pairing is examined by temperature-melting analysis (see below).

2. Analysis of the Sequence Content of Viral DNA Transcript

Since the DNA product must be an intermediate in virus replication, it must contain copies of parts of the entire viral 70 S RNA genome. Whether this is the case is examined by two different procedures: by RNA–DNA hybridization or by studying the reassociation kinetics of denatured double-stranded DNA product (DNA–DNA hybridization). The latter technique is widely used because it can be applied to study the reassociation of single-stranded DNA strands with complementary single-stranded RNA strands.

In RNA–DNA hybridization, radioactive 70 S viral RNA is hybridized with product DNA in excess (at least tenfold). The fraction of the viral genome actually transcribed into DNA is measured by the resistance of the RNA–DNA hybrid to digestion by RNase in high ionic strength buffer (Duesberg and Canaani, 1970; Stephenson and Aaronson, 1972). A resistance of 60 to 85% is indicative of a specific transcription.

The study of reassociation kinetics of denatured DNA product (Varmus *et al.*, 1971; Gelb *et al.*, 1971), or DNA–DNA hybridization, is based on

the widely used procedure of Britten and Kohne (1968). The double-stranded DNA product is denatured into single strands by heating (100°C) or treatment with formamide. Then the single strands are allowed to reassociate (or anneal) in precise conditions of salt concentration (which must be high), temperature (which must be at least 25°C below the dissociation temperature of native DNA), and duration (up to 18 hours). The sequence complexity of double-stranded DNA is determined in these conditions by its direct proportionality to the C_0t value (where C_0t = concentration of nucleotides in moles per liter, usually measured by absorbance at 260 μm, and t = time in seconds) at which one-half of the denatured DNA has reassociated.

The fidelity of base pairing which follows reassociation of single strands into double strands is then examined by establishing a melting profile. The procedure is based on the simple fact that upon heating mismatched DNA strands dissociate at lower temperature than better base-paired strands (about 1°C lower for every 1.5% of mismatched base pairs). The melting profile is established by fixing double-stranded, reassociated DNA on hydroxyapatite at 50°C, washing it free from single-stranded DNA with buffer, then increasing the temperature by 5°C steps. At each step, dissociated DNA is collected and quantitated by the number of radioactive counts. Measurements are stopped when 95°C is reached.

Reassociation studies of DNA to DNA (or RNA to DNA) can be done on nitrocellulose filters (Gillespie and Spiegelman, 1965).

Technical details of these procedures and the necessary controls are described in detail by Church (1973).

D. Applications of Molecular Techniques to the Search for Human RNA Tumor Viruses

Spiegelman (1975) has pointed out there is a high probability that if human tumor viruses exist, they should exhibit biochemical homologies with known mammalian tumor viruses. In the absence of unquestionable virus isolates, at least at the present time, the properties of animal oncornaviruses could be used advantageously to probe for viral footprints in human tumors of various types.

1. Simultaneous Detection of Reverse Transcriptase and High Molecular Weight RNA

It has been shown (Section II) that the early DNA product of the endogenous reaction is complexed to the high molecular weight, virus-specific 70 S RNA. On this basis, it is possible to detect simultaneously both reverse transcriptase and its template (Schlom and Spiegelman,

1971) in an endogenous reaction using radioactive ^{3}H-dTTP, because by Cs_2SO_4 density gradient or glycerol velocity gradient or acrylamide gel electrophoresis, the radioactive DNA product will be detected at the same place as 70 S viral RNA. The mere existence of a radioactive DNA product proves the existence of reverse transcriptase using 70 S RNA as a template.

Based on this principle, Gulati *et al.* (1972) have devised a simultaneous detection assay which allows detection of 70 S RNA and reverse transcriptase in human tumor cells. Tumor cells are homogenized, nuclei removed by centrifugation, and the resulting supernatant fluid centrifuged at high speed. The resulting pellet is banded on a sucrose gradient and the fraction banding between 1.16 to 1.19 g/ml is collected. This fraction is assumed to be composed of particles of a density similar to that of oncornaviruses. After treatment with detergent, applied in the same way as that for preparation of oncornavirus cores, material of the fraction is made to react in a short-term endogenous reaction (15 minutes at 25°C), in the presence of radioactive dTTP, and of actinomycin D in order to stop DNA-directed DNA synthesis. After the reaction, the nucleic acids are extracted with phenol-cresol and sized on a glycerol gradient. A peak of radioactivity in the 70 S zone indicates that, indeed, the fraction contained 70 S RNA and reverse transcriptase which synthesized DNA from the 70 S RNA template. A control reaction in the presence of RNase T and RNase T1 showing the absence of DNA synthesis proves that the positive reaction was RNA-instructed. Another control reaction, from which dATP, dCTP, or dGTP are omitted, showing the absence of RNA–DNA complex formation, indicates that the positive result was not due to nontemplated end addition reactions (for details, see Spiegelman, 1975).

The simultaneous assay has also been widely used to detect oncornaviruses or oncornavirus-like activity in pellets prepared from tissue culture fluids of human cells grown *in vitro,* from human milk, and from human tumor cell extracts (see below).

2. *Hybridization Tests between Viral DNA Probes and RNA of Human Tumor Cells*

Radioactive ^{3}H-DNA product of known oncornaviruses can be used as probes to detect complementary RNA in human tumor cells. As we have seen, this can be done by annealing denatured DNA product with single-stranded RNA extracted from tumor cells. DNA–RNA hybrid formation is then detected by gradient centrifugation in cesium sulfate. A positive reaction is indicated by a peak of radioactivity at a density of 1.65

g/ml. Fidelity of base pairing, when possible, is determined by temperature-melt analysis.

This type of test has been widely used by Spiegelman and his co-workers to probe for the presence of murine oncornavirus-related RNA, and by Gallo and his associates to probe for simian type C-related RNA in human tumor cells (see below).

3. *Hybridization of DNA Product Synthesized by Human Cell Particulates with RNA of Known Oncornaviruses*

This test is done to check the homology of DNA product obtained during an endogenous reaction with human cell particulates to the RNA of known oncornaviruses. It is in a way complementary to experiments where DNA probes generated by known oncornaviruses are tested with RNA of human cells, as described above.

4. *Recycling Experiments*

As will be discussed later (Section V), the problem of viral oncogenesis could be linked to the existence in normal cells of virus-related genetic information in the form of a complete DNA copy of the viral genome. A direct demonstration of the existence of such a copy is apparently technically not feasible (for discussion, see Spiegelman, 1975). However, information of great interest may be obtained by trying to find in malignant cells viral-related DNA sequences which are absent in normal, counterpart cells.

This approach requires a rather sophisticated experimental design (Baxt *et al.*, 1973; Kufe *et al.*, 1973a,b; Spiegelman, 1975): a tritiated DNA probe is prepared by an endogenous reverse transcriptase reaction of human tumor cell particulates (from cell fractions sedimenting at 1.16 g/ml). This purified probe is then used to detect complementary DNA sequences in normal and in tumor cells. If the result is positive in both normal or tumor cells, the sequences found in normal cells are removed by exhaustive hybridization to normal cell DNA, and the remaining hybridized to DNA of tumor cells.

Protocols and conditions for these experiments have been represented diagrammatically by Spiegelman (1975) and will not be described here.

An obvious application of such a methodology was to search for viral-related DNA sequences in cells of normal and leukemic identical twins. Two sets of identical twins, of which one was leukemic, the other normal, have been tested by recycling experiments by Baxt *et al.* (1973).

Care was taken to hybridize the DNA product not with DNA of the normal twins, but with that of normal blood donors. Indeed, the leukemic twins possessed in their leukemic cells particle-related sequences which could not be detected in their healthy siblings. These experiments, if valid, demonstrate that the onset of leukemia was not linked to the inheritance of a complete viral genome, and that the viral information present in leukemic cells was acquired after zygote formation. This is in accordance with what is known from the results of animal experimentation, namely that oncornaviruses, following *in vivo* or *in vitro* infection of host cells, insert the viral information into the genome of these cells (Goodman *et al.*, 1973; Sweet *et al.*, 1974).

5. Viral RNA–Cellular DNA Hybridization

This type of hybridization is of great importance for the detection in cellular DNA of sequences homologous to the single-stranded RNA of oncornaviruses, since (according to the DNA provirus theory of Temin, 1971) DNA copies of viral RNA should be integrated in the DNA of infected cells. RNA–DNA hybridization has been extensively used to characterize endogenous viruses (''vertically'' transmitted) as opposed to exogenous (''horizontally'' transmitted) RNA viruses. Radioactive viral ^{3}H-RNA is annealed with DNA extracted from cells by pronase–SDS digestion, phenol–chloroform–cresol extraction, precipitation of the aqueous phase with ethanol, alkaline hydrolysis, and shearing of the precipitated DNA by sonication and hybridization at 66°C in phosphate buffer with radioactive 70 S RNA extracted from the virus. The mixture is then analyzed for percentage of hybrid formation. An application of the technique for study of carcinoma of the prostate can be found in Dmochowski *et al.* (1975).

6. Cytohybridization in Situ

This rather recent procedure (Gall and Pardue, 1971; Huang *et al.*, 1973; Pagano and Huang, 1974) has the great advantage of allowing a localization of RNA–DNA or DNA–DNA hybrid formation in whole cells of tissue cultures, peripheral blood cells, or frozen sections. Basically the procedure (Pagano and Huange, 1974) consists of treating ethanol–acetic acid-fixed cells on a microscope glass with radioactive RNA complementary to DNA of DNA viruses (Epstein-Barr virus, cytomegalovirus, herpes simplex virus), or with radioactive DNA synthesized by reverse transcriptase of an oncornavirus, or with radioactive oncornaviral RNA. In all cases, hybrid formation will be detected and localized in cytoplasm or nucleus of test cells with fairly good accuracy, using autoradiographic procedures.

IV. SEARCH FOR HUMAN TUMOR VIRUSES

A. Search for Human Tumor Viruses by Electron Microscopy

1. Search for Oncornaviruses (Retroviridae)

As soon as good commercial electron microscopes became available, about two decades ago, and thanks to improved methods of fixation, embedding, and thin sectioning, investigators in increasing numbers began to examine human tumor material for the possible presence of viral particles. Because of the early discoveries by electron microscopy of type C virus particles in leukemic chickens (Benedetti *et al.*, 1956; Dmochowski, 1965b) and leukemic mice (Dmochowski and Grey, 1957, 1958; Dmochowski *et al.*, 1962; Feldman and Gross, 1966), of type B virus particles in mouse mammary tumors, and of type B and type C virus particles in milk of mice (Passey *et al.*, 1950; Dmochowski and Passey, 1952; Dmochowski, 1954; Bernhard *et al.*, 1956), the main efforts were first concentrated on finding similar viruses in tumor tissues, plasma, and milk of patients with leukemia, lymphoma, and breast cancer.

Problems and limitations specific to electron microscopy became rapidly apparent during the studies of human material. The numbers of viral particles or of viruslike particles (i.e., with morphological characteristics similar to those of known viruses) turned up to be nowhere as high as in animal tumor material (Seman, 1974). The low probability of detecting virus particles below a certain concentration of the particles in the specimens examined was compounded by the difficulty of correctly identifying occasional structures resembling viruses. We now know that this identification involves a "recognition factor" which can be estimated on a mathematical basis (M. F. Miller, 1974). It has also been demonstrated that in tumor tissues or cell cultures *in vitro,* only certain clones or certain types of cells synthesize virus particles at any one time, causing a clustering of the particles in limited portions of the specimens examined. Because of the necessarily small size of blocks submitted to thin sectioning, these portions can be missed, constituting what is called the "sampling error."

Nevertheless, the systematic electron microscopic screening of human tumor material and of milk for the presence of oncornaviruses has produced results of great interest. While examination of leukemic plasma pellets (Braunsteiner *et al.*, 1960; Burger *et al.*, 1964; Porter *et al.*, 1964; Prince and Adams, 1966; Levine *et al.*, 1967; Seman and Seman, 1968) and of human milks (Jensen and Schidlovsky, 1964; Lunger *et al.*, 1964; Moore *et al.*, 1969; Sarkar and Moore, 1972) by the negative staining procedure produced results difficult to interpret, more substantial results have been

gained by the thin sectioning method. In plasma pellets or in tumor biopsies of patients with leukemia, lymphoma, and Hodgkin's disease, particles resembling C-type particles have been observed by Dmochowski and his associates (Dmochowski and Grey, 1957; Dmochowski, 1960a,b, 1963, 1965a, 1971; Dmochowski and Bowen, 1973, 1974; Dmochowski *et al.*, 1967a,b, 1968; Yumoto and Dmochowski, 1968) and by other investigators (Porter *et al.*, 1964; Levine *et al.*, 1967; Hirshaut *et al.*, 1974a,b). Particles resembling type A particles have repeatedly been observed by Schumacher *et al.* (1973) and by Szekely *et al.* (1976) in mitochondria of human leukemic cells. Very recently, budding of oncornavirus-like particles have been seen in platelets of leukemic patients (Brodsky and West, 1976).

An intensive search for particles resembling types B and C oncornaviruses in human breast cancer biopsies and in human milk by thin sectioning or negative staining has also yielded positive results. Several groups of investigators have found particles resembling B (Fig. 6) and C particles in this material (Feller *et al.*, 1967; Feller and Chopra, 1968, 1969; Moore *et al.*, 1969; Dmochowski *et al.*, 1968, 1969; Seman *et al.*, 1969, 1971; Sarkar and Moore, 1972; Dmochowski, 1972).

These systematic investigations have led also to observations of viruslike particles of unknown classification, differing from type B and C viruses. This is the case of particles observed in blast cells of two patients with Chediak-Higashi syndrome (White, 1966), and of cytoplasmic viruslike structures (Fig. 7) found in blood cells of patients with acute myeloblastic or lymphoblastic leukemia, in about 10 to 20% of the cases examined (Seman and Seman, 1968; Seman, 1968, 1969; Cawley and Karpas, 1974; Foá *et al.*, 1976). Sun *et al.* (1972) have also observed viruslike particles in cytoplasmic inclusions in bone marrow and peripheral blood cells of patients with lymphoblastic leukemia.

In breast cancer and in milk of patients with breast cancer and of normal women, small 30 nm particles, aggregating in clusters and sedimenting at a density of 1.11 g/cm^3 have been described by Feller and Chopra (1968) and by Seman *et al.*, (1969). In breast cancer, intracisternal viruslike particles of two different types, one 160–200 nm in size (Fig. 8) and the other 60–80 nm in size (Fig. 9), have been found in a biopsy specimen of comedocarcinoma (Seman and Dmochowski, 1973b).

Leukemias, lymphomas, and breast cancer, of course, have not been the only targets of this screening for virus particles in human tumors. Biopsies of bone tumors and other sarcomas have, on occasion, revealed particles resembling C-type viruses (Dmochowski, 1971; Bowen *et al.*, 1974). In tumors of the urinary tract, clusters of 100 nm viruslike particles have been observed in inclusions of the tumor cells (Elliott *et al.*, 1973a,

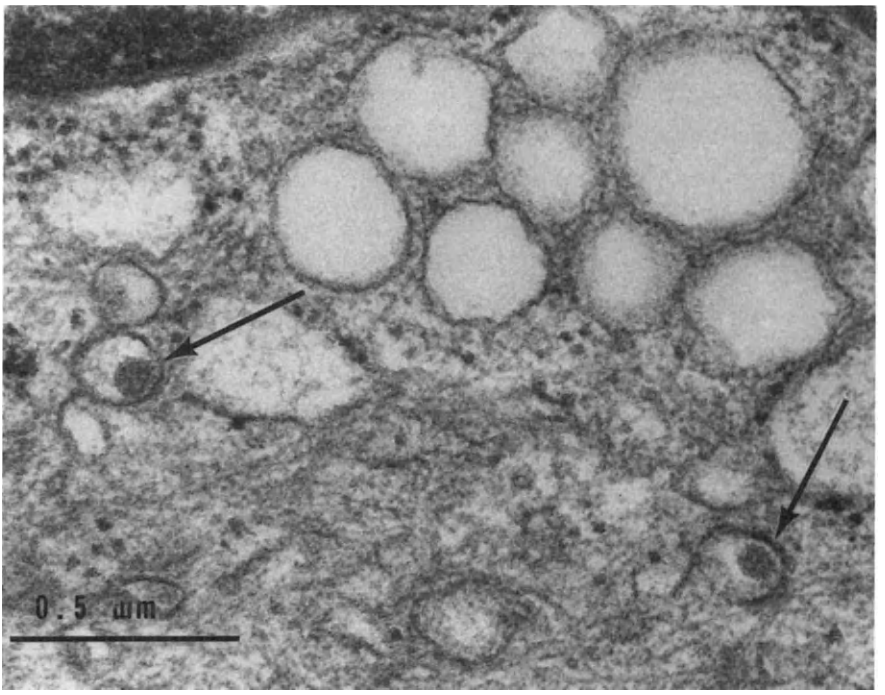

Fig. 6. Two particles resembling B virus in degenerating tumor cell of a human breast cancer biopsy (arrows). The particles are very similar to those observed in human milk. These particles contain structures corresponding to the "core" particles present in density gradient fractions from breast tumor tissue or human milk in which 70 S RNA and reverse transcriptase have been demonstrated. × 60,000.

1974) and propagated for some time in tissue culture (Elliott *et al.*, 1973b). More recently, systematic examination of human prostate tumors has revealed small numbers of particles resembling type C virus in some of the tumor cells and also of large and small intracisternal viruslike particles identical to those previously observed (Seman and Dmochowski, 1973b) in human breast cancer (Dmochowski *et al.*, 1975; Ohtsuki *et al.*, 1976).

These findings clearly indicate that, in spite of negative results reported in some screening studies, for instance, in leukemia (Bessis and Thiery, 1962) and breast cancer (Haguenau, 1959), there are virus particles in human tumors. However, these screening studies by electron microscopy have not resulted in the isolation of any human oncornavirus and, worst of all, could not efficiently be correlated with a screening of normal tissues. The failure to characterize *in vitro* viruses seen by electron microscopy in human tumors, and the usually small number of the particles observed, acted as deterrents to pursue a systematic screening of normal

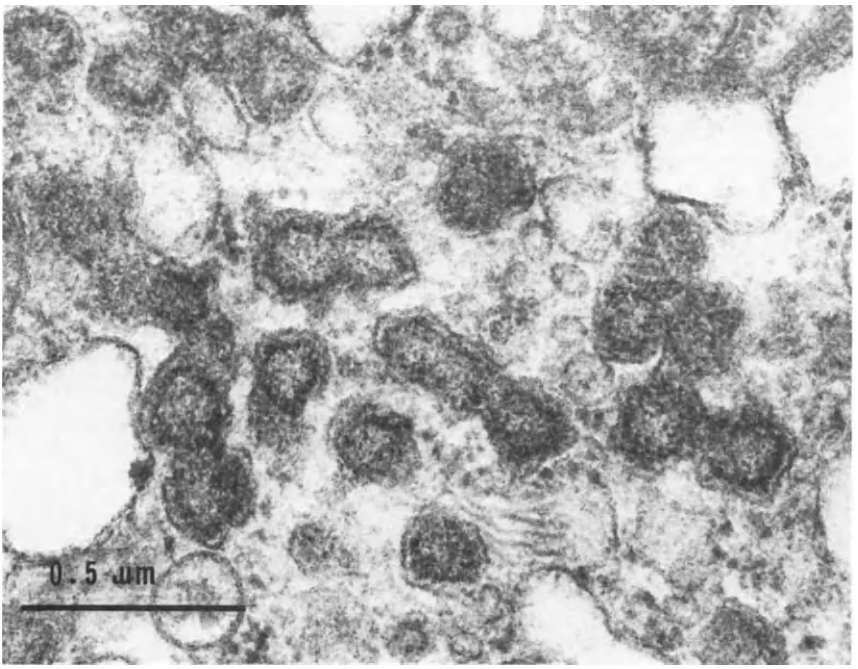

Fig. 7. Viruslike particles observed in the cytoplasm of a leukemic cell of a patient with acute myelogenous leukemia. Such structures are probably aggregates of smaller subunits, and are located inside cytoplasmic channels. × 60,000.

tissues. It was assumed by many microscopists that any virus conspicuous by its morphology and by its number, present in specimens of normal or pathological human tissues examined by the hundreds of investigators worldwide for various purposes, would at any rate have been reported. However, this type of indirect control system is not at all reliable, because most of the time an electron microscopist who is not searching for viruses will not see viruses, unless they are observed accidentally. Such an accident was the discovery of characteristic type C virus particles in human placentas during studies on the physiology of that organ (Kalter *et al.*, 1973a,b). This observation and the previous observation of a few type C virus particles seen budding from human embryonic cells from biopsies or tissue cultures (Chandra *et al.*, 1970) prompted a systematic search for oncornaviruses in mammalian placentas. Characteristic type C particles have been observed in trophoblastic cells not only of primate placentas, but in about 10% of the many human placentas examined by several workers (Vernon, 1974; Schidlovsky and Ahmed, 1973; Kalter *et al.*, 1974; Bierwolf *et al.*, 1975). Type C particles were much more

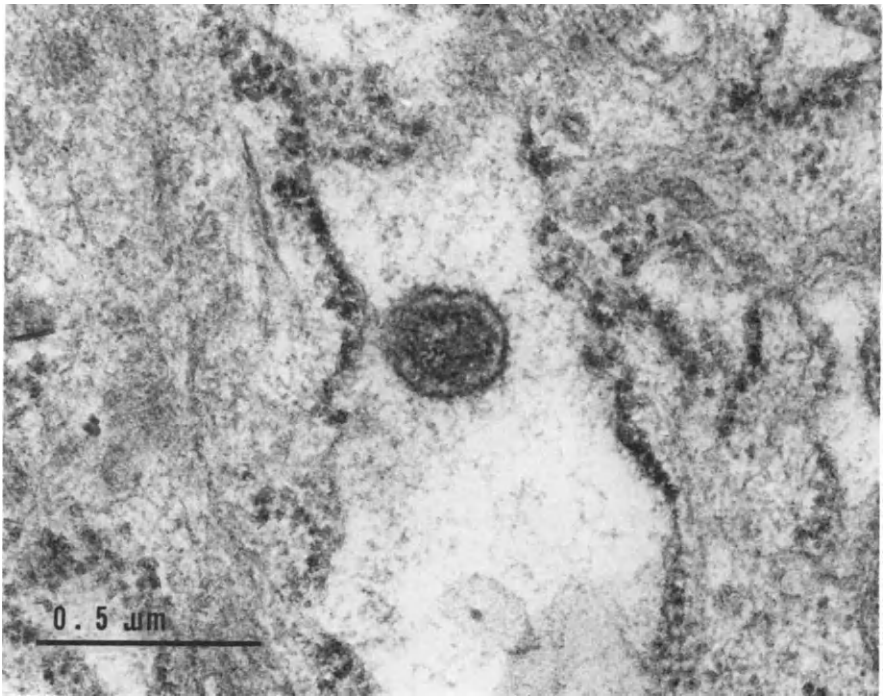

Fig. 8. Large intracisternal viruslike particles in tumor cell of comedocarcinoma of the human breast. × 60,000.

difficult to detect in human than in primate placentas. In baboons, for instance, all the placentas examined were found to be positive for the presence of type C particles. The presence of type C particles in human placentas is probably of far-reaching importance. Although no virus particles have so far been found in placentas of dogs or rabbits, type C particles have been observed in placentas of mice (Seman *et al.*, 1974). Placentas of mice also contained intracisternal type A particles in all strains examined (Smith *et al.*, 1975). By correlating these observations with those of type C particles in human embryo cells, in reproductive organs of baboons, mice, and guinea pigs, one reasonably concludes that the placenta of mammals is the place where oncornaviruses of fetal and of material origin can cross-contaminate the mother and/or the fetus.

In parallel to electron microscope screening of tumors, many experiments have been undertaken to isolate oncornaviruses by growing human tumor or embryonal cells *in vitro* with or without attempts to induce virus synthesis by chemical agents, cocultivation, etc. Morton *et al.* (1969) have observed in some late passages of a culture derived from liposar-

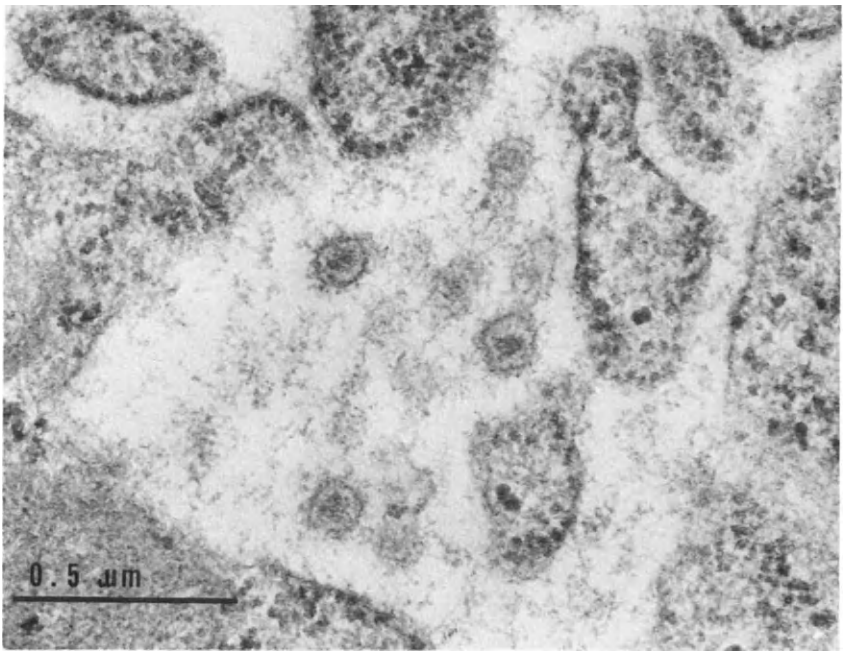

Fig. 9. Same biopsy specimen as for Fig. 8. Several 60 to 80 nm viruslike particles are seen in the endoplasmic cavity of a tumor cell. × 60,000.

coma intracisternal particles similar to intracisternal type A particles of mice and guinea pigs (Hall and Schidlovsky, 1976). By treating tissue culture cells of human sarcomas with halogenated pyrimidines, Stewart *et al.* (1972a,b) have induced temporary synthesis of intracisternal C-type particles. These particles have not been characterized, however. Kotler *et al.* (1973) have maintained buffy coat cells of patients with acute and chronic leukemia in arginine-deprived medium, and observed the release of virus-like particles resembling oncornaviruses by electron microscopy. The particles also had biochemical characteristics of oncornaviruses, but were not propagated in tissue culture.

The first two type C viruses to be isolated from human cell cultures were ESP-1 and RD-114. The ESP-1 virus was discovered by electron microscopy in a low passage number of pleural effusion cells from a young male patient with Burkitt's lymphoma, American type (Priori *et al.*, 1971a,b). RD-114 virus has been isolated from tissue culture of rhabdomyosarcoma cells after passage of the tumor cells in the brain of a kitten *in utero* (McAllister *et al.*, 1972). The true nature of ESP-1 and RD-114 viruses has been very hotly debated on morphological (Dalton, 1972a,b),

immunological, and biochemical grounds. ESP-1 virus is very close but not identical in its properties to Rauscher virus (Scolnick *et al.*, 1973; Eckner *et al.*, 1974), while RD-114 virus has been classified as a feline endogenous virus (Gallo *et al.*, 1975).

An intriguing series of demonstrations of virus particles by electron microscopy has occurred during routine examination of HeLa cell lines in Germany, and of several established human cell lines in the USSR and the United States. Virus particles similar in every respect to the Mason-Pfizer virus of rhesus monkeys have been observed by Gelderblom *et al.* (1972, 1974) in the Dusseldorf strain of HeLa cells (HeLa-D). At about the same time, virus particles identical in appearance have been found in a series of established human cell lines (HEp-2, AO, Detroit 6, KB, DAPT, J-96, CaOV, Tg, and HeLa lines) by Russian investigators (Zhdanov *et al.*, 1972; Bykovsky *et al.*, 1973; Ilyin *et al.*, 1973; Miller *et al.*, 1974). Immunological and biochemical characterization of the virus particles (designated as HeLa virus) have shown that they were identical to the Mason-Pfizer monkey virus (Parks *et al.*, 1973; Gelderblom and Schwartz, 1976). The origin of the HeLa virus has raised serious problems. MPMV replicates with no difficulty in human cells, and is presently maintained in a human lymphoblastoid cell line, NC-37, established at the Pfizer laboratories. In addition, there have been claims, supported by chromosome banding and enzymatic studies, that most, if not all, of the cell lines containing HeLa viruses were actually HeLa clls (Nelson-Rees and Flandermeyer, 1975). A similar problem has arisen with a tissue culture derived by Hooks *et al.* (1972) from human brain producing virus particles resembling MPMV particles. Whether HeLa virus is a monkey virus contaminating human cells, which, in turn, are perhaps all HeLa cell "contaminants" or whether MPMV is a human virus which has contaminated the rhesus monkey from which MPMV is derived, is an urgent problem requiring a solution. Hybridization of ^{3}H-DNA product of MPMV to the DNA of uninfected NC-37 human cells, or to the DNA of rhesus monkey liver have produced negative results (Parks *et al.*, 1973). These results indicate that MPMV is not endogenous to either humans or rhesus monkeys. However, MPMV has been detected in normal tissues of rhesus monkeys (Ahmed *et al.*, 1974).

Two recent human type C particle isolates have an interesting background. The first isolate is derived from late culture passages of a fibroblastic cell line derived from human fetal lung. The virus, designated as HEL-12, has been discovered in 6-month-old subcultures during routine electron microscopic monitoring (Panem *et al.*, 1975). The other isolate has a more complicated history. It has been derived from peripheral blood cells of a patient with acute myelogenous leukemia, first by cocultivating

the cells for a week with human embryo cells, then by growing the floating cells with the addition of filtered tissue culture medium of the human embryo culture (conditioned medium). The procedure, repeated on three occasions using freshly collected cells from the patient (designated by the initials HL-23), has led on all three occasions to the finding of type C particles in the suspension cultures of leukemic cells (Gallagher and Gallo, 1975; Gallagher *et al.*, 1975). Results of the characterization of HEL-12 and HL-23 virus are reported in Section V.

2. Search for Herpesviruses

The difficulties in detecting herpesviruses in human tumors have been similar, if not worse, to those encountered in the systematic search for human oncornaviruses by electron microscopy. Herpesviruses, being assembled in fairly visible structures in cell nuclei, and being usually cytopathic, are much less likely to be missed during ultrastructural examination of human tumors. It has to be stressed, however, that no herpesvirus particles have at present been seen in animal tumors known to be induced by herpesviruses, either naturally or experimentally (Marek's disease of fowl, "cold" kidney tumors of frogs, and lymphomas of marmosets induced by *Herpesvirus saimiri* or *ateles*). Similarly, electron microscopy has failed to show the presence of intra- or extracellular virions in human tumors presently suspected to have some association with herpesviruses, i.e., Burkitt's lymphoma, nasopharyngeal carcinoma, and cervical carcinoma. A few sightings of herpeslike viruses have been reported in Burkitt's lymphoma (Griffin *et al.*, 1966), in cervical carcinoma (Herrera *et al.*, 1974), and in prostatic cancer (Kaufman *et al.*, 1973), but they did not amount to decisive findings. Yet, as will be discussed later, herpesviruses have been raised in recent times, through seroepidemiological and experimental studies, to the status of first-choice candidates as etiological agents in certain types of human neoplasia.

V. MOLECULAR AND BIOCHEMICAL STUDIES IN HUMAN NEOPLASIA

A. Background

The guiding lines of recent biochemical studies on the involvement of oncornaviruses in human neoplasia, mostly based on molecular hybridization procedures, have been, as Spiegelman (1975) has pointed out, based on some fundamental concepts of the mechanism of viral on-

cogenesis, and on the assumption that, until a genuine human oncogenic RNA virus isolate is found, oncornaviruses of animals could be used to probe human tumors by molecular methods.

The present fundamental concepts of viral oncogenesis are contained in two different hypotheses. The first hypothesis assumes that oncogenic RNA viruses insert their genetic information into the genome of infected cells. This information is inserted in the form of a newly formed DNA species added to the host genome, as a result of horizontal transmission (host to host) of the viruses. While for DNA viruses the biochemical mechanism of such a process was not a problem, it was necessary to assume that at one point of the replication of oncornaviruses the genome of the viruses was transcribed into a "proviral" DNA inserted into the host genome. This is the provirus theory of Temin (1964), which was established following the discovery of reverse transcriptase, the enzyme responsible for this transcription (Temin and Mizutani, 1970; Baltimore, 1970). Activation of the proviral DNA, by some mechanism still to be discovered, may lead to either replication of virus particles or to transformation of the infected cells.

The second hypothesis, proposed by Huebner and Todaro (Huebner and Todaro, 1969; Todaro and Huebner, 1972), postulates that animal cells (probably most or all animal cells) carry RNA viral genomes (virogenes) inherited and transmitted through the germinal line. The virogene includes a portion responsible for malignant transformation (oncogene) usually unexpressed because of repressor systems. The oncogene, however, can be activated (switched on) by external factors (irradiation, carcinogenesis, autoantibodies, perhaps other viruses, etc.), leading to cell transformation. The vertical transmission of RNA virus information has also been postulated for the mouse mammary tumor virus by Bentvelzen *et al.* (1970).

While horizontal transmission of oncogenic RNA viruses (provirus theory) is supported by data showing that in several animal and human systems infected and transformed cells contain new viral DNA sequences not found in noninfected cells (see below), vertical transmission of viral genes (virogeneoncogene theory) is also supported by data showing that normal, uninfected cells may carry viral genetic information (for instance, of MMTV and MuLV), and above all, that synthesis of oncornaviruses can be activated in cells of many vertebrates by various induction methods.

No hypothesis can be fully explanatory and it is quite plain that both the provirus theory and the virogene–oncogene theory contain part of the truth. There is no doubt that attempts will be made to reconcile them on

the basis of some compromise. One such attempt, the protovirus theory (Temin, 1971), tries to combine both theories. It is a rather complicated way for which there is as yet no solid experimental support. A simple and practical assumption may be to imagine that, at some time or another, a horizontally transmitted virus becomes integrated into the genome of allogeneic or heterologous host cells and from then on is transmitted through the germ line of the host (Benveniste and Todaro, 1974). This concept leads directly to the problem of endogenous viruses.

The present views on viral oncogenesis are strongly influenced by the notion of endogenous viruses. The fact that in animal systems nonproducer of normal cells can be induced *in vitro* to synthesize oncornavirus antigens or virus particles, following prolonged cultivation, cocultivation, treatment of the cells with chemicals, hormones, or irradiation (Hehlmann, 1976), is a clear indication that animal cells may contain in latent form the genetic information coding for virus replication. A recent example is the finding, by Ahmed *et al.* (1976) of type C virus in MPMV-replicating human cells. Experience has shown that animal cells may even contain information necessary to the synthesis of more than one type of oncornavirus (Aoki and Todaro, 1973; Benveniste and Todaro, 1974), a possibility well illustrated by the simultaneous observation of type B, type C, and, frequently, of intracisternal type A particles, in mammary tumors of mice and their cultures (Dmochowski *et al.*, 1968; Hilgers *et al.*, 1971), in reticulum cell sarcoma of mice (Seman and Dmochowski, 1973a), or in normal prostate tissues of mice of several strains (Ohtsuki *et al.*, 1976).

Studies of the properties of murine, feline, and baboon oncornaviruses induced *in vitro* have shown that such viruses are not oncogenic to the host of origin, although they are immunogenic (Kawakami *et al.*, 1973; Aaronson and Stephenson, 1974; Nowinski and Kaehler, 1974). These studies have also shown that RNA of such viruses exhibits extensive homology to the DNA of the host of origin.

D. Gillespie and R. C. Gallo (personal communication, 1975) and Gallo *et al.* (1975) have proposed a classification of RNA tumor viruses into two classes. Class I viruses are those that show more than 70% homology between their RNA and the DNA of the uninfected host cell (endogenous viruses). Examples of endogenous viruses are the AKR mouse virus, the baboon placental virus (M-7), the feline RD-114 virus, and the porcine PK-15 virus. These viruses are probably nononcogenic to their homologous hosts, and are transmitted vertically. Class II viruses show less than 30% homology of their RNA to the host's DNA, are transmitted horizontally, and are generally oncogenic. Class II viruses include the avian

(AMV, RSV), murine (Rauscher-Moloney), feline (Rickard and Gardner strains), and simian (GALV, SiSV) leukemia-sarcoma viruses. These viruses insert into the host cell DNA genetic information which is not present in uninfected cells. Gallo *et al.* (1975) assume that Class II viruses evolved from Class I viruses by horizontal transmission. However, this relatively simple situation is complicated by the fact that some endogenous viruses cannot replicate in homologous cells but only in heterologous ones (xenotropic viruses) (Levy, 1973; Todaro *et al.*, 1973; Stephenson *et al.*, 1974). Further, the tissue or the types of cells involved in the production of viral genome may be of great importance. Fibroblastic cells of mice are apparently unable to replicate MMTV; yet, according to Bentvelzen *et al.* (1970), the MMTV genome is inscribed in the genome of every murine cell. Another example of interest is that of the baboon placental (endogenous) M-7 virus. A ^{3}H-DNA probe made by this virus hybridizes fully with DNA extracted from baboon placenta and from liver, but hybridizes only with the cytoplasmic RNA of placenta but not with RNA of liver (Benveniste *et al.*, 1974). This appears to be a clear indication that the viral genome is produced only by placental cells. Great caution should, therefore, be exercised before any systematic generalization is made. Nevertheless, it is reasonable to assume that horizontally transmitted viruses may become harmless to heterologous hosts by becoming endogenous in them, and that this process may also work in the other direction. That is, under certain conditions, endogenous viruses may become harmful to the heterologous hosts, to which they are exogenous or, stated more simply, the oncogenic potential of an oncornavirus is related to the degree of genetic disparity between the host cell DNA and the viral genome.

Animal oncornaviruses have extensively been used in the past few years in attempts to demonstrate in human tumors the presence of RNA or of RNA-containing particles with characteristics of viruses. These attempts were based on the assumption that a putative human leukemia-sarcoma virus was likely to resemble mammalian leukemia-sarcoma viruses (notably the murine viruses), and that a putative human breast cancer virus was probably close to the mouse mammary tumor virus (Spiegelman, 1975). The discovery of simian leukemia sarcoma viruses provided better tools than the murine viruses to probe human leukemic and sarcomatous tissues, because of the phylogenetic relationships between the human and simian species.

Biochemical and molecular studies undertaken to discover viral information in human neoplasia, essentially in leukemias, lymphomas, and breast cancer, have been conducted along several lines of attack.

B. Biological and Molecular Approaches of Various Types

1. Use of Radioactive DNA Probes

The basis of this approach is to prepare radioactive ^{3}H-DNA product using the endogenous reaction of a known oncornavirus, and to hybridize it with cytoplasmic RNA of human tumor cells. The hybrid is examined by centrifugation in cesium sulfate gradient. Uncomplexed DNA bands at 1.45 g/cm^3, while complexed radioactive DNA bands at the density of RNA (1.65), which has to be in large excess. The fidelity of the pairing is then determined by a temperature-melt analysis (Section III).

Breast cancer (Axel *et al.*, 1972a,b; Schlom *et al.*, 1972), acute and chronic leukemia cells (Hehlmann *et al.*, 1972a,b), sarcomas including fibrosarcomas, osteosarcomas, and liposarcomas (Kufe *et al.*, 1972), and lymphomas including Hodgkin's disease, Burkitt's tumor, reticulum cell sarcomas, and lymphosarcomas (Hehlmann *et al.*, 1972a,b) showed a significant amount of hybrid formation, 67% for breast cancer (using an ^{3}H-DNA probe of MMTV), and more than 90% for the other neoplasia (using an ^{3}H-DNA probe of Rauscher virus). No hybridization was observed with the ^{3}H-DNA probe of avian viruses, or with RNA of control, adult, or fetal human tissues or cells. Leukemias and lymphomas did not contain MMTV-related information. Gallo *et al.* (1973) have, in addition, shown the high amount of hybridization of RNA of leukemic cells to the ^{3}H-DNA probe of simian sarcoma virus. By RNA–RNA hybridization, Vaidya *et al.* (1974) found in 5 out of 17 breast tumors sequences homologous (18–76%) to MMTV-RNA, while Colcher *et al.* (1974) found, in the same type of material, RNA sequences homologous to the RNA of Mason-Pfizer virus.

2. The Simultaneous Detection Assay

The principle of the method devised by Schlom and Spiegelman (1971) has been described in Section II. The method has been adapted to detect oncornavirus-like particles in human milk (Schlom *et al.*, 1972) and in tumor tissue homogenates using mouse mammary tumors as the experimental model (Gulati *et al.*, 1972; diagrammatic representation by Spiegelman, 1975).

The simultaneous detection procedure revealed in 79% of 38 specimens of breast cancer the presence of a 70 S RNA–DNA complex with RNA-instructed DNA-synthesizing activity in a particulate fraction sedimenting between 1.16–1.19 g/ml (Axel *et al.*, 1972a,b). Interestingly enough, the ^{3}H-DNA synthesized by 70 S RNA-containing milk particulates were found to hybridize with RNA of breast cancer cells (Das *et al.*, 1972). In two studies of human milks, about 70% of the samples were

found to contain reverse transcriptase activity associated with a 70 S RNA (Schlom *et al.*, 1972; Gerwin *et al.*, 1973). Feldman *et al.* (1973) and Michalides *et al.*, 1975) successfully isolated "core" structures from human milks, sedimenting at a density of 1.24–1.27 g/ml, and showing reverse transcriptase activity. Attempts to correlate the presence of enzymatically active particles in human milks and family histories of breast cancer, however, were not successful (Schlom *et al.*, 1972; McCormick *et al.*, 1974).

In correlative electron microscopic and biochemical studies on milk from women with breast cancer, a partial correlation has been observed between the presence of particles resembling type B particles (MMTV) and reverse transcriptase (Miller *et al.*, 1973).

The simultaneous detection assay has been extensively used to study human leukemias and lymphomas. Baxt *et al.* (1972) have found that peripheral leukocytes of 22 patients out of 23 with chronic or acute leukemias, lymphocytic or myelogenous, contained particulate material with 70 S RNA and reverse transcriptase activity. Leukocytes of normal donors were inactive. The reaction product was homologous to Rauscher virus RNA. These results were confirmed by Hehlmann *et al.* (1973) with other cases of leukemia. Kotler *et al.* (1973) detected, in short-term cultures of leukemic cells in arginine-deprived medium, particles resembling oncornaviruses by electron microscopy and showed that these particles contained 70 S RNA and reverse transcriptase activity. Mak *et al.* (1974a,b) have shown that bone marrow cultures of leukemic patients in active phase or in remission also contained enzymatically active particles with 70 S RNA, resembling oncornaviruses. Of great interest are recent observations by Viola *et al.* (1976) that leukocytes of leukemic patients in remission, although morphologically normal, still contained 70 S RNA-associated reverse transcriptase. Similar particles with oncornavirus properties were found by Weinmann *et al.* (1975) in tissue culture of bone marrow cells from a patient with polycythemia vera, and by Witkin *et al.* (1975) in spleens of leukemic patients.

The simultaneous detection assay was also applied to other human malignancies. Brain tumors, gastrointestinal tumors, and lung cancer were found by Cuatico *et al.* (1973, 1974) to contain 70 S RNA and reverse transcriptase. However, no homology was found between the particles of these tumors and other animal oncornaviruses, including visna virus. Enzymatically active particles have also been observed in melanomas (Birkmayer *et al.*, 1974; Parson *et al.*, 1974).

Viruslike particles with biochemical properties of oncornaviruses have been reported by McGrath in an established human cell line MCF-7 derived from breast cancer (McGrath *et al.*, 1974) cells grown *in vitro*. It

should, however, be pointed out that electron microscopy has failed to provide evidence of morphologically discernible viruslike particles. This may or may not detract from the possible importance of the reported findings (see also DeHarven, 1974). Activation of oncornavirus-like particles in cultures of fibrosarcoma desmoides after inoculation of aspirate of bone marrow from a patient with acute leukemia has been reported by Bowen *et al.* (1974).

3. Search for Virus-Specific New DNA in Tumor Cells

Infection and transformation of a cell by an oncornavirus should result in the addition to the cell DNA of new DNA sequences homologous to viral RNA. This is the basis of Temin's provirus theory. The new sequences should not be detectable in uninfected cells. Experiments by Baluda (1972), Varmus *et al.* (1974), Goodman *et al.* (1973), and others confirmed the presence of new, virus-related DNA sequences in rat and marmoset cells transformed by the Rous sarcoma chicken virus.

To test the hypothesis in human systems, Baxt *et al.* (1972) have designed a rather complex protocol, diagrammatically represented by Spiegelman (1975) and described as "recycling experiment" by which the ^{3}H-DNA product of cellular particulates containing 70 S RNA is first annealed with DNA of normal cells, then with DNA of malignant cells.

The recycling procedure has been applied to human leukemia cells by Baxt *et al.* (1972) and to Hodgkin's disease and Burkitt's lymphoma by Kufe *et al.* (1973b). The DNA of leukemic cells of Burkitt's tumor cells and of spleens of Hodgkin's patients all contained DNA sequences absent from normal leukocytes or spleens. The leukemia-specific sequences were found by Baxt (1974) to be related to the RNA of Rauscher murine leukemia virus. Interestingly enough, Burkitt tumors contained DNA sequences detected by the ^{3}H-DNA from Hodgkin's spleens and vice versa. Blood cells and spleen cells of a patient with infectious mononucleosis, known to contain Epstein-Barr virus-related DNA sequences, gave negative results in recycling experiments with probes from Burkitt's tumor and Hodgkin's disease (Spiegelman, 1975) thus demonstrating that EBV-related DNA has nothing to do with the 70 S RNA of Burkitt's or Hodgkin's particles.

Another proof for the existence of leukemia-specific oncornavirus-like DNA sequences in leukemic cells has been obtained from the study of two sets of identical twins (Baxt *et al.*, 1973). Cells of the leukemic twin contained sequences not found in cells of the normal twin.

Recycling and other hybridization experiments in animal systems (Sweet *et al.*, 1974; Shoyab *et al.*, 1974; Varmus *et al.*, 1974; Lowy *et al.*, 1974) have confirmed that DNA of virus-infected cells or cells of leukemic

chickens or mice contained virus-specific sequences either absent from normal cells, or much higher DNA equivalents of the viral genome.

4. Human Leukemia and Simian Viruses

Reverse transcriptases of simian sarcoma virus (SiSV), gibbon ape leukemia virus (GALV) (Abrell and Gallo, 1973; Mondal *et al.*, 1975), and of the baboon so-called endogenous virus (BEV or M-7 virus) isolated from placenta have a molecular weight of about 70,000. Reverse transcriptase of SiSV and GALV are immunologically very similar and differ from that of BEV (Scolnick *et al.*, 1972a,b; Todaro and Gallo, 1973; Sherr *et al.*, 1974). Reverse transcriptase was also extracted from baboon placenta from the postmitochondrial fraction, according to the procedure described by Sarngadharan *et al.* (1972), and found to be related immunologically to the reverse transcriptase of BEV, but not of SiSV or GALV (Mayer *et al.*, 1974). By back-hybridization of the DNA product of SiSV and GALV to viral RNA, the SiSV was found to contain additional sequences not found in the RNA of GALV, a situation similar to that of avian and murine sarcoma viruses which contain sequences not found in leukemia viruses (Stephenson and Aaronson, 1972).

Following the discovery of reverse transcriptase activity in the cytoplasmic particulate fraction of human leukemic cells (Sarngadharan *et al.*, 1972; Gallo *et al.*, 1974), the human enzyme was found to have a molecular weight of some 135,000 (Mondal *et al.*, 1975), and by immunological criteria to be inhibited by antibodies to reverse transcriptase of SiSV and GALV, but not by that of BEV (Gallagher *et al.*, 1975). The biochemical properties and behavior of the reverse transcriptase of human leukemic cells have been extensively studied by Gallo *et al.* (1975). This enzyme is different from other cellular DNA polymerases. Whether leukemic cells acquire RNA tumor virus information by infectious process has not been determined thus far (Gallo *et al.*, 1975). However, the relationship of the human leukemic cell polymerase to that of polymerases of simian viruses isolated from tumors and probably not endogenous (contrary to the BEV virus, which is endogenous to the baboon and apparently not tumorigenic) is of obvious importance. These still fragmentary observations clearly show that primates are probably the best source of oncornaviruses closely related to putative oncogenic RNA viruses of humans. This is illustrated by the finding of the HEL-12 and HL-23 isolates.

5. The HEL-12 and HL-23V Type C Isolates

As previously mentioned (Section IV), HEL-12 type C virus has been isolated from a 6-month-old subculture of human embryonic fibroblasts

derived from an 8-week-old embryo (Panem *et al.*, 1975). HEL-12 virus particles have the morphological and biochemical characteristics of type C oncornaviruses. Preliminary immunological and biochemical characterization of the virus (Panem *et al.*, 1975) has shown a strong relatedness to the simian sarcoma virus (SiSV). Among antisera to p30 interspecies antigen of SiSV, of Rauscher leukemia virus, and of feline leukemia virus (Rickard strain) only antiserum to p30 of SiSV precipitated the HEL-12 virus preparations. By comparing the inhibitory effect of antisera to the reverse transcriptase of gibbon ape leukemia virus (GALV) and of SiSV on the reverse transcriptase of HEL-12 virus, murine Gross virus (G-MuLV), and Rous chicken sarcoma virus (RSV), maximum inhibition of the reverse transcriptase of HEL-12 virus was observed with antiserum to reverse transcriptase of SiSV, and to a lesser extent with antiserum to reverse transcriptase of GALV. In further attempts to characterize HEL-12 virus (Prochownik and Kirsten, 1976), it was found that 7 S IgG from 2 out of 8 patients with various types of leukemia significantly inhibited reverse transcriptase not only of HEL-12 virus, but also the reverse transcriptase of SiSV and that of the baboon M-7 endogenous virus. Reasons for the presence of antibodies to reverse transcriptase in sera of leukemic patients have not as yet been described.

HL-23V type C virus has been isolated from leukocytes of a patient with acute myelogenous leukemia (Gallagher and Gallo, 1975). The leukocytes were first cocultivated with human embryonic cells (WHE-1) and then grown in conditioned medium (containing filtrate of WHE-1 cell cultures). Virus particles have been reisolated, on different occasions, from frozen cells of the first culture (Gallagher *et al.*, 1975) and from cells of the patient obtained 14 months after the first isolation (strains HL23V-1 and HL-23V-5, respectively). Type C particles have not been observed in fresh leukocytes. Both the reverse transcriptase of HL-23 virus and of 70 S RNA-containing cytoplasmic particles prepared from fresh leukocytes have been found to be inhibited by antiserum to reverse transcriptase of simian sarcoma virus (SiSV). According to Prochownik and Kirsten (1976), the IgG serum fraction of a patient that inhibited reverse transcriptase of HEL-12 virus did not inhibit reverse transcriptase of HL-23 virus, in spite of the close similarity of both isolates to SiSV. This observation may just be the result of happenstance.

The HL-23 virus has further been characterized in extensive studies by Teich *et al.* (1975), Okabe *et al.* (1976), and Chan *et al.* (1976). The virus has been successfully propagated in human cells (A-204 cells, derived

from rhabdomyosarcoma and WHE-2 whole human embryo cells) and heterologous cells (canine thymus A-7573 cell line and NRK normal rat kidney cell line). HL-23 virus contains proteins binding to antibodies to p30 proteins of SiSV, GALV, and baboon BEV-M7 virus, but not to those of p30 of rat or mouse leukemia viruses. The presence of two viruses in the HL-23 isolates, one resembling the SiSV and the other the BEV-M7, was confirmed in human and canine cultures using ^{3}H-DNA cellular RNA hybridizations (Okabe *et al.*, 1976). Type-specific radioimmunoassays have shown, using antibodies to p30, p15, p12, and gp70 of monkey (SiSV, GALV, and BEV-M7) viruses, that HL-23 virus was a mixture of two viruses, one indistinguishable from woolly monkey-gibbon ape leukemia virus, the other indistinguishable from the baboon endogenous placental virus (Okabe *et al.*, 1976). Reciprocal hybridization experiments using ^{3}H-DNA made by HL-23 virus grown in A-204 human rhabdomyosarcoma cells and RNA of SiSV and BEV-M7 have shown 37% hybridization to BEV-M7 RNA and 44% hybridization to SiSV RNA, again indicating that HL-23 virus contains the genomes of both woolly monkey sarcoma and baboon endogenous placental viruses (Chan *et al.*, 1976).

Immunodiffusion tests using antisera to p30 of woolly monkey virus and to p28 of baboon virus have also detected both antigens in HL-23 virions.

These findings, combined with Prochownik and Kirsten's (1976) observation that the human embryo-derived HEL-12 virus was also probably a similar mixture of woolly monkey (sarcoma) and baboon (endogenous) viruses, and with the repeated finding in human leukemic cells of RNA related to DNA of SiSV and BEV-M7 (Gallo *et al.*, 1974; Sherr and Todaro, 1974), suggest that these simian viruses are widespread in human populations. However, even if HEL-12 and HL-23V are not laboratory contaminants, the relationship of these viruses to the etiology of human leukemia remains to be demonstrated.

6. Other Isolates

Nooter *et al.* (1975) have reported the isolation from bone marrow cultures of a child with lymphoblastic leukemia of type C particles. These particles could be replicated by human fibroblasts (FB 289) when exposed to cell-free supernatants of human embryonic kidney cultures. These virus particles seem to possess MuLV antigens as shown by immunofluorescence tests. Antigenic properties of this isolate have not as yet been fully determined.

VI. IMMUNOLOGICAL APPROACHES TO THE STUDY OF VIRUSES IN HUMAN NEOPLASIA

A. General Remarks

In this section the most difficult aspect of immunologic studies on the detection of viruses in human neoplasia will be presented, that is, attempts to detect antigens of or antibodies to oncornaviruses in human tumor cells and in human sera. The situation differs widely from that found with herpesviruses. Although herpesviruses or herpesvirus antigens have been difficult to demonstrate in fresh tumors, large-scale seroepidemiological studies have already been conducted in human populations with well-defined herpesviruses grown *in vitro,* namely, EB virus, herpes simplex 1 and 2, and cytomegalovirus (Section VII).

There are, of course, several lines of attack that can be used to detect oncornaviruses by immunologic means in human neoplasia. In one approach, heterologous antisera to purified oncornavirus antigens (structural or nonvirion) are used to probe human tumor cells. This type of approach is rather recent and has not been used in systematic screenings. In a second type of approach, sera of patients are tested with animal or human cell cultures known to contain a well-characterized oncornavirus or with purified viral antigens. Finally, a third type of approach consists in testing sera of cancer patients with homologous tumor cells hoping that, after appropriate absorptions, a virus-related antigen can be demonstrated. The third type of approach has been very extensively applied to fresh human tumor cell preparations or to human tumor cells grown *in vitro,* because of the interest in finding tumor-specific, possibly virus-induced, antigens in human tumors.

It is impossible within the scope of this chapter to discuss the basic problems relating to immunity in human neoplasia. The nature of these problems has been outlined in several recent publications (Dmochowski and Bowen, 1974; Bowen *et al.,* 1974). Viral involvement in human tumors can be investigated on an immunological basis by searching for the presence in tumor cells of specific viral antigens (structural and nonstructural proteins and glycoproteins), and for new antigens (embryonal, Forssman, tumor-specific, etc.) which accompany malignant transformation of cells by viruses at least in certain systems (Fogel and Sachs, 1964; Gold and Freedman, 1965; Old and Boyse, 1965; Klein, 1969; Maruyama *et al.,* 1968; Hirshaut *et al.,* 1974a; Priori and Dmochowski, 1974). The methodologies involved have included the whole spectrum of immunological procedures, but recently much more importance has been given to certain techniques such as radioimmunoprecipitation, now universally

employed, and the more cumbersome but very informative techniques of immunoelectron microscopy (Kurstak and Morisset, 1974). In addition, the recently refined techniques of gel filtration and gel electrophoresis have at last provided the adequate means to prepare antigens and antibodies of reasonable purity.

B. Search for Oncornavirus Antigens in Human Tumor Cells by Using Heterologous Antisera

On theoretical basis, attempts to detect RNA tumor virus antigens in human cells should be the best way to approach the problem of oncornavirus involvement in human tumors. These attempts are very recent and data are far too scarce to arrive at any conclusions about the value of this type of approach. Kim and Spiegelman (1975, quoted by Hehlman, 1976) have reported that the 70 S RNA-containing particulate fraction of human leukemic cells competed in competitive radioimmunoassays with the p30 structural protein of Mason-Pfizer virus. A study by Zurcher *et al.* (1975) using antisera to p30 of Rauscher virus and p28 of SiSV, showed positive immunofluorescence reactions with cells of several human cell lines derived from bone tumors, although virus particles have not been observed in the cells by electron microscopy.

In a recent study by Louie *et al.* (1976), polypeptides with a molecular weight of 70,000, 45,000, and 30,000 have been found in a media of short-term cultures of leukemic human bone marrows. These polypeptides were precipitated by antiserum to Rauscher virus. Interestingly enough, more than 50% of the sera of 42 leukemic patients and of 45 normal donors also contained antibodies to these proteins, the viral nature of which has as yet not been determined.

These results are of interest in view of previous findings obtained by antisera to Rauscher virus reacting with human leukemic cells (Fink *et al.*, 1964; Bates *et al.*, 1969) and with human tumor cells *in vitro* (Schafer *et al.*, 1971).

C. Search for Antibodies to Oncornavirus Antigens in Human Sera

1. Leukemia-Lymphomas

The possibility of a widespread distribution of antibodies in human sera to animal oncornaviruses is further suggested by the recent observation of Vosika *et al.* (1976) of antibodies present in eleven sera of 20 apparently

normal donors to major polypeptides (gp69/70, gp45, and p15) of the baboon endogenous M-7 virus.

With respect to human antibodies to oncornaviruses, two groups of observations are of great interest. A systematic screening by immunofluorescence test of sera of many patients with leukemia, lymphomas, and other malignancies, and of 180 normal donors, against the ESP-1 cell line, have shown a high proportion (more than 50% on the average) of positive reactions with sera of patients, against 1% positivity with sera of normal donors (Priori and Dmochowski, 1974). The ESP-1 virus is closely related but not identical to Rauscher virus, since it contains the interspecies *gs*-3 antigen, but not the type-specific *gs*-1 antigen of Rauscher virus (Eckner *et al.*, 1974). Obviously the nature of "natural" antibodies to Rauscher virus in humans appears in this context more complicated than at first sight and many more investigations will be required to clarify the problem. The second group of observations pertains to studies of antibodies in human sera (patients or normals) to mouse mammary tumor virus antigens.

2. Antibodies to MMTV and Breast Cancer

The finding by electron microscopy of particles resembling MMTV in human breast tumors and milks (Dmochowski *et al.*, 1968, 1969; Seman *et al.*, 1971; Moore *et al.*, 1971; Dmochowski, 1972; Dmochowski and Bowen, 1973) has generated many studies in an attempt to demonstrate the presence of antibodies to MMTV in sera of breast cancer patients, of their relatives, and of normal women. This approach was justified both on the basis of many similarities in the natural history of mouse and human mammary tumors (Dmochowski, 1958), of the finding by Schlom *et al.* (1971, 1972) of reverse transcriptase and 70 S RNA in particulate material of certain human milks, and of particles with similar activity in breast tumors (Axel *et al.*, 1972a,b).

One way of testing human sera for antibodies to MMTV was to test the sera by immunofluorescence with mouse cells producing type B oncornaviruses. This type of experiment has been carried out by Priori *et al.* (1972) with cells of a mouse mammary tumor cell line producing type B virus. Sera of about 50% of the breast cancer patients, of 40% of their relatives, and 15% of normal donors gave positive reactions with this cell line. A similar result has been observed by Bowen *et al.* (1976a) in tests of breast cancer patients sera with type B virus-producing cell line MMT-1.

The specificity of these immunofluorescence reactions has been demonstrated by absorptions of human sera with Forssman antigens, with human embryonal cells, with mouse cells not producing type B virus, with mouse milk, and with purified preparations of MMTV. These absorptions

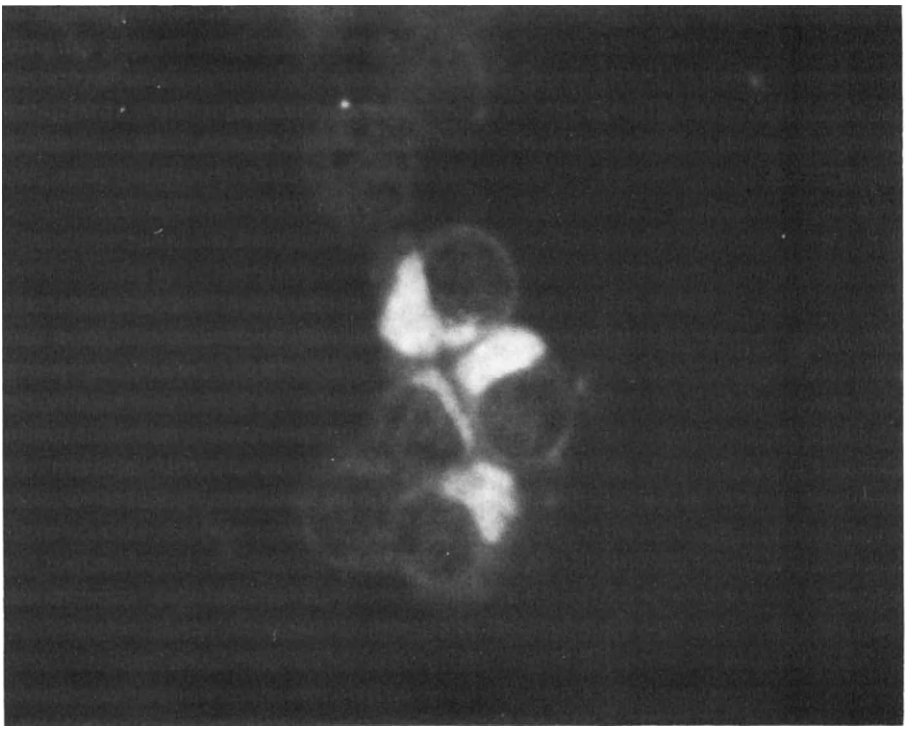

Fig. 10. Indirect immunofluorescence reaction with serum of a breast cancer patient and acetone-fixed mouse mammary tumor cells, smeared on a microscope slide. The positive reaction is clearly located in cytoplasmic region, corresponding to inclusions containing intracytoplasmic type A virus particles seen by electron microscopy. × 520.

have shown that at least in 20% of the cases, sera of breast cancer patients only reacted with type B-producing cells (Bowen *et al.*, 1976a,b).

A very similar approach has been taken in Germany by Müller and his associates (Müller *et al.*, 1971; Müller and Grossman, 1972) who have observed positive immunofluorescence reactions with sera of 5 of 45 patients with breast cancer and cells of mouse mammary tumors in frozen sections. Localization of the reaction was very precise, suggesting a reaction with intracytoplasmic type A virus-containing inclusions, as was also observed by us in our laboratory (Fig. 10). Müller *et al.* (1973) have later confirmed by ferritin labeling and thin sectioning that the reaction was given by type A particles and not by mature type B virus particles.

Attempts to visualize the reaction of breast cancer patients sera with type B particles using immunoelectron microscopy (Hoshino and Dmochowski, 1973; Dmochowski *et al.*, 1974) have given results of great interest. Some sera of breast cancer patients have been found by the immuno-

peroxidase technique, to label type B (mature and immature) virus particles, and sometimes intracytoplasmic type A particles (Dmochowski *et al.* 1974; Bowen *et al.*, 1976a,b). The reactions appeared specific enough after sequential absorptions to conclude that women with breast cancer (and with fibrocystic disease also as shown by Müller *et al.*, 1973) had in their sera antibodies to an antigen similar to that of type B virions. The significance of such a reaction, in regard to the viral etiology of human breast cancer, is, of course, still an open question which cannot be answered before seroepidemiological studies on a large scale are completed or a candidate human breast cancer virus isolated.

3. Search for Virus-Induced Tumor Antigens

A considerable number of investigations have been devoted during the past decade to detect tumor-specific and tumor-associated (transplantation or viral-induced) antigens. For various reasons little progress has been made in finding oncornavirus-related antigens in human tumors. Difficulties in growing human tumor cells *in vitro* and the absence of a human "reference" oncornavirus have been among the major obstacles in such studies. It is noteworthy that, as will be seen later, much more progress has been made in finding herpesvirus-associated antigens in a broadening variety of human neoplasia, because human herpesviruses are well known and because, contrary to oncornaviruses, all herpesviruses share common antigens (Evans *et al.*, 1973).

The results of many immunological studies have shown that sera of patients with different types of neoplasia contain antibodies directed against their own and their homologous tumors, and often to tumors of a different type than their own. Such antibodies have been demonstrated in leukemias, sarcomas, breast cancer, melanoma, etc. (Morton and Malmgren, 1968; Priori *et al.*, 1972; Dmochowski *et al.*, 1973; Bowen *et al.*, 1974, 1976a,b), but have not been found to be directly related to oncornaviruses. This type of investigation may not be well suited to detect virus-related antigens in human tumor cells because they rely on too complex absorption protocols with antigens difficult to purify or to quantitate. In addition, the usual absorption with so-called "normal tissues" may often be a fallacy, since they may contain, as in mice, viral information or antigens.

VII. HERPESVIRUSES AND HUMAN NEOPLASIA

The concept of an association of herpesviruses with the etiology of some human neoplasia has progressively emerged in the past 15 years

from a vast number of observations both in animals and humans. Some of these observations were literally accidental, for instance, the discovery by electron microscopy of Epstein-Barr virus in tissue cultures of Burkitt lymphoma cells in 1964 (Epstein *et al.*, 1964). Other observations were derived from systematic and purposeful investigations of a possible association of herpesviruses with human neoplasia, as in the case of cervical carcinoma. As of today there is already an extensive literature on the topic of herpesviruses and human cancers which the interested reader will find in monographs, such as the book edited by Biggs *et al.* (1972), and the collected papers of a series of important symposia published in *Cancer Research* (Symposia, 1973, 1974, 1976).

Two human cancers, the African Burkitt's lymphoma and nasopharyngeal carcinoma, show a very consistent association with a herpesvirus, the Epstein-Barr virus (EBV), which is also related to the etiology of a nonneoplastic disease, infectious mononucleosis. The association of herpes simplex virus (HSV) with cervical carcinoma has been inferred from cytopathological and seroepidemiological studies conducted on a large scale in several countries by a number of investigators.

For a better understanding of this aspect of the relationship of viruses to human neoplasia, first, the main clinicopathological features of the incriminated diseases will be described, followed by a brief discussion on the properties of herpesviruses, in general, and of EBV and HSV, in particular. In a final section the facts associating these viruses to human neoplasms will be presented in a summarized form.

A. Clinicopathological Features of Herpesvirus-Related Human Diseases and Neoplasms

1. Burkitt's Lymphoma

Burkitt's lymphoma is the most common neoplasm among children in equatorial Africa. The disease was known long before, although it is only in 1958 that Burkitt described it as an entity (Burkitt, 1958; Burkitt and Wright, 1970). Burkitt's lymphoma has a peak incidence between the ages of 5 to 9. Its most striking feature is represented by facial tumors developing unilaterally in relation to the molars and premolars of the jaw or of the maxilla. The tumor is caused by a destructive invasion of bone trabeculae by deposits of lymphoma cells. It frequently invades the orbit. Although tumors of the face are the most conspicuous, there are always other tumors which develop in the abdomen, invading the ovaries, the kidneys, the adrenals, and the retroperitoneal spaces. Skull and other bones are frequently invaded as well as the nervous system, causing paraplegia.

Although Burkitt's lymphoma has been most extensively investigated in Africa, there are clinically and histopathologically similar tumors in New Guinea (Booth *et al.*, 1967) and in the United States (Cohen *et al.*, 1969; O'Conor *et al.*, 1965; Levine and Cho, 1974). African and American Burkitt's lymphoma differ by the higher incidence of abdominal tumors in American cases at the time of diagnosis.

Burkitt's lymphoma tumors are characterized by histological and cytological monomorphism. Histological sections show a "starry sky" pattern due to the presence of large histiocytes, containing lipids and cell debris, interspersed with tumor cells. Burkitt's lymphoma tumor cells are lymphoid cells very similar in size (10–12 nm) with a hyperbasophilic cytoplasm. By light and by electron microscopy, they closely resemble antigen-stimulated lymphocytes *in vitro*. Burkitt's lymphoma cells are probably derived from monoclonal proliferation of the B (bone marrow) lymphocytic series (Klein *et al.*, 1970; Jondal and Klein, 1973). Burkitt lymphoma cells in suspension or grown in tissue culture synthesize all classes of immunoglobulins (Van Furth *et al.*, 1972).

Detailed studies of Burkitt's lymphoma will be found in books (Burkitt and Wright, 1970) and reviews (Wright, 1972; O'Sullivan and McLaughlin, 1975).

2. *Nasopharyngeal Carcinoma*

Nasopharyngeal carcinoma is a tumor prevalent among Cantonese Chinese. It is also found in some populations of Africa, for instance, those of Kenya and Tunisia (Ho, 1972; Shanmugaratnam, 1971; de Thé and Geser, 1974). The tumor is usually unilateral and originates from the walls of either the right or the left side of the nasopharyngeal cavity. Histologically some 60% of nasopharyngeal carcinomas are undifferentiated. Other histological types include squamous cell, clear cell or pleomorphic cell carcinomas.

There is always a characteristic admixture of apparently normal lymphocytes among the tumor cells. However, this histological feature is not specific for nasopharyngeal carcinoma (Goldenberg and Brandes, 1976). Ultrastructural studies have demonstrated that the tumor cells are of epithelial origin (Svoboda *et al.*, 1965; Gazzolo *et al.*, 1972). The neoplasm spreads by lymphatic and hematogenous routes to various parts of the head, frequently infiltrating the skull, the cranial nerves, and cervical lymph nodes. Bones, liver, and lungs are the most frequent places of distant metastases.

Nasopharyngeal carcinoma is nearly three times more frequent in males than females, and is essentially seen in adults. Besides obvious ethnic

factors, some predisposing genetic factors, such as increased frequency of HL-A2 antigens, have been singled out (Simons *et al.*, 1974). None of the many studies of possible environmental carcinogens (household fumes, incense smoke, tobacco, eating habits, etc.) has conclusively linked any of the environmental factors with the origin of nasopharyngeal carcinoma (Shanmugaratnam, 1971).

3. Infectious Mononucleosis

Infectious mononucleosis is a sporadic disease of young adults characterized by fatigue, fever, pharyngitis, enlarged lymph nodes, and spleen. The incubation period is from 5 to 8 weeks. The disease seems to be transmitted via the oral route by patients who recovered from the disease. The duration of uncomplicated infectious mononucleosis is 4–6 weeks with complete recovery. Recurrences are very rare and immunity to the disease is permanent (Worlledge and Dacie, 1969; Evans and Niederman, 1972; Chervernick, 1974).

Hematological and serological findings in infectious mononucleosis are of great interest. Most of the time there is a relative and absolute lymphocytosis due to the presence in the blood of atypical, hyperbasophilic lymphocytes of various sizes and shapes (Gallbraith *et al.* 1963; Mathé and Seman, 1962). By ultrastructural and biochemical criteria these cells resemble antigen-stimulated lymphocytes *in vitro* (Epstein and Brecher, 1965). They actively synthetize RNA and DNA and have been shown to be thymus-dependent T lymphocytes (Sheldon *et al.*, 1973).

Paul and Bunnel (1932) have found that sera of infectious mononucleosis patients contained unusually elevated titers of agglutinin to sheep red blood cells (SRBC). This agglutinin is a heterophil antibody of the IgM class which can be detected, although at lower titers, in sera of normal individuals and of patients with leukemia or Hodgkin's disease. The Paul-Bunnel-Davidson test is based on the fact that this heterophil antibody is not completely removed by Forssman antigen (guinea pig kidney) but is readily removed by beef erythrocytes. Peak titers of the antibody are found by the end of the third week following the onset of clinical symptoms. Elevated titers may persist from 6 to 12 months, but usually disappear in 8 to 12 weeks. Sera of patients with infectious mononucleosis are also found with increased levels of IgM, and, to a lesser degree, of IgG and IgA. Finally, there is also a cold-reacting antibody against human red cell antigen i (present in fetal red blood cells) in sera of some 70% of the patients. The relationship of infectious mononucleosis to leukemia-lymphoma is as yet unknown.

4. Genital Herpes and Cervical Carcinoma

It is now well known that the vast majority of herpes simplex virus infections of the upper part of the human body are caused by herpesvirus type 1 (HSV-1) while those below the umbilicus, especially the genital area, are caused by herpesvirus type 2 (HSV-2) (Nahmias and Roizman, 1973; Josey *et al.*, 1972). Primary genital lesions, most frequently seen in teenage girls and unmarried women, are usually extensive, involving the ectocervix, the vaginal mucosa, and the vulva (Kaufman *et al.*, 1970, 1973), and are accompanied by discomfort, itching, cystitis, fever, and lymphadenopathies. Recurrent lesions occur at the same sites as the primary lesions, but are much less severe and may even go unnoticed. In most cases, the presence of detectable lesions leads to the isolation of herpes simplex virus type 2 in cultures (Aurelian, 1974a,b).

Following the demonstration in 1963 by Stern and Longo that cellular changes (giant cells, nuclear inclusions, etc.) sometimes observed in vaginal smears for cancer screening were caused by herpes simplex virus, Naib *et al.* (1966) were the first to suspect an association of herpes genitalis with cervical anaplasia (anaplasia denoting dysplasia, carcinoma *in situ*, or invasive carcinoma). The same authors have extensively studied the cellular alterations of cervical epithelium during herpesvirus infection (Naib *et al.*, 1973). They found by systematic screenings that 16% of the cases of detected genital herpes were associated with anaplasia, while two-thirds of the detected cases of anaplasia were associated with herpetic alterations. However, this association, obviously not fortuitous, did not exclude the fact that cervical tumor tissue is simply more susceptible to herpes simplex virus infection. Only seroepidemiological studies, which will be described later, have provided sufficient background to link herpes genitalis to cervical cancer.

B. Properties of Herpesviruses

1. Classification of Herpesviruses

Herpesviruses have been classified in several ways, either according to the species of origin (simian herpesviruses, equine herpesviruses, etc.) or to the disease they may cause (herpes simplex, Marek's disease viruses, pseudorabies, pink eye, frog adenocarcinoma, etc.), or after their discoverers (Epstein-Barr virus). A proposal was also made to classify herpesviruses into group A and group B viruses (Melnick and McCombs, 1966), group A virus being infective in cell-free form, group B being always cell-associated. However, none of the classifications has been found entirely satisfactory.

2. *Structure and Composition of Herpesviruses*

Ultrastructural investigations (Morgan *et al.*, 1959, 1968; Epstein, 1962; Schwartz and Roizman, 1969; Epstein and Achong, 1970; Roizman and Spear, 1973) (Fig. 11) have shown that herpes virions were 140–160 nm in diameter, and were composed of a DNA core, surrounded by a capsid (core and capsid forming the nucleocapsid), and an envelope consisting of two layers. The capsid is an aggregate of 162 subunits (capsomers) arranged according to an icosahedral symmetry, which gives the virion a characteristic hexagonal appearance in thin sections. The DNA in the core is coiled around a cylindrical protein body in a toroidal fashion.

The DNA of herpesviruses has, on the average, a molecular weight of 100×10^6 daltons. Epstein-Barr virus and herpes simplex viruses have similar DNA's (Becker *et al.*, 1968) and proteins (Olshevsky and Becker, 1970). The base composition of the DNA of herpesviruses varies from 43 to 73 moles % of guanine and cytosine. Although there does not seem to be a correlation between base composition and biological properties of

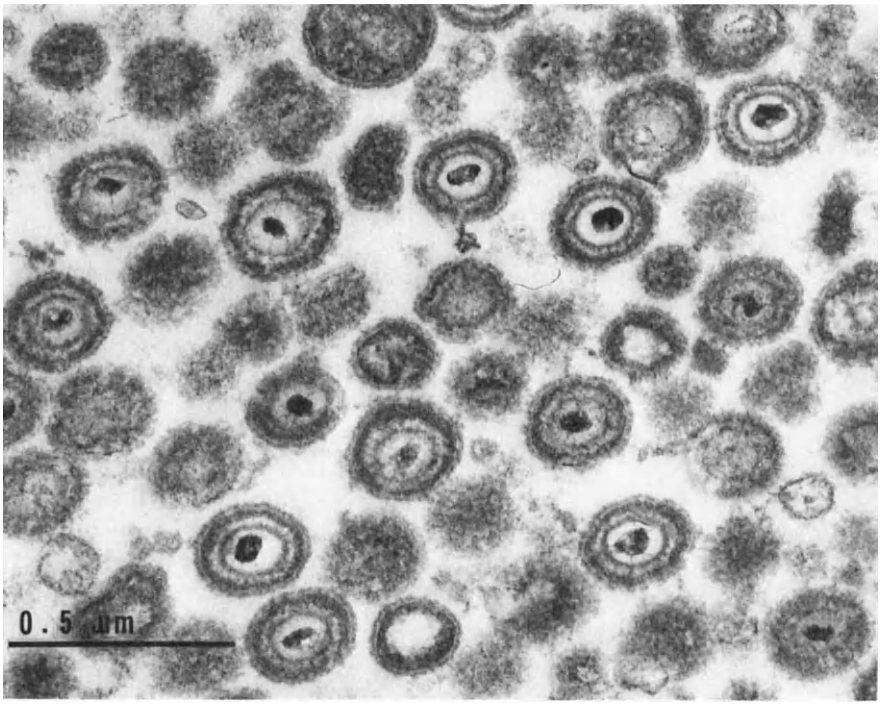

Fig. 11. Appearance of herpesviruses in thin section. The viruses have been found in centrifugal pellet of cyst fluids taken from skin lesions of a cancer patient suffering from intestinal obstruction. × 60,000.

herpesviruses (Roizman, 1972), some differences have been found between the DNA of HSV-1 and HSV-2 (Roizman and Frenkel, 1973). DNA of HSV-1 contains 67 moles % of G + C, while that of HSV-2 contains 69 moles %. At least 95% of the sequences of HSV-1 DNA are unique; in HSV-2 DNA, at least 16% of the sequences are repetitive. Hybridization of HSV-1 DNA with DNA of HSV-2 shows that the viruses share 47% of their sequences (Kieff *et al.*, 1971).

Biochemically, herpesviruses are composed of lipids, proteins, polyamines, and DNA. HSV-1 and HSV-2 virions contain at least 24 specific proteins ranging from 16,000 to 275,000 daltons in molecular weight (Spear and Roizman, 1972; Honess *et al.*, 1974; Aurelian and Strnad, 1976). The envelope contains at least eleven glycosylated proteins and spermidine (Heine *et al.*, 1972). While immunodiffusion, immunoelectrophoresis, and immunofluorescence experiments have demonstrated that human and animal herpesviruses share common antigens (Evans *et al.*, 1973), it is known that type specificity of herpesviruses is determined by the amount and nature of glycoproteins in the envelope (Savage *et al.*, 1972).

3. Herpesvirus–Host Cell Interaction

Interactions between herpesviruses and their host cells are of great importance in determining the possible oncogenicity of some viruses of this group. These interactions have been summarized in a number of publications (Tooze, 1973; Roizman and Frenkel, 1973; Kaplan, 1973; Aurelian, 1974a,b).

It is generally accepted that only enveloped virions are infectious. Naked nucleocapsids extracted from nuclei of infected cells or obtained by detergent treatment of whole virions are apparently unable to penetrate into cells. Cellular infection with herpesviruses may lead to two different results: productive or nonproductive infection. In productive infection, complete virions are synthesized but virus-producing cells are ultimately destroyed, liberating the virus. In nonproductive infection the cells survive infection but do not synthetize virions; instead, they perpetuate the viral genome. An extremely important aspect of herpesvirus infection is that infection can be propagated from cell to cell in the absence of cell-free virions.

Herpesviruses penetrate rapidly into permissive cells, which include cells of many mammalian and nonmammalian species. Once in the cell, virions are uncoated and the free DNA–protein complex enters the nucleus.

The biochemical events that follow the entry of herpesviruses, especially of HSV-1 and HSV-2, into a cell are fairly well known (Roizman

and Frenkel, 1973). Immediately after infection, there is a progressive decrease of cellular DNA and protein synthesis; synthesis comes to a halt in about 7 to 8 hours. Meanwhile, increasing amounts of viral DNA and viral proteins appear in the cell. About 50% of input viral DNA is transcribed in the nucleus into RNA of relatively large size which is later cleaved into smaller messenger RNA molecules. The RNA transcribed from viral DNA (perhaps by an RNA polymerase of viral origin) has been divided into three categories: immediate-early RNA, early RNA (transcribed before the onset of viral DNA synthesis), and late RNA (transcribed only if cellular protein or DNA synthesis are still continuing). The RNA transcripts migrate from the nucleus into the cytoplasm where they bind to free or membrane-bound polyribosomes. At that time synthesis of structural and nonstructural proteins of the virus is initiated. All the synthesized proteins migrate back into the nucleus, except the virion envelope proteins which bind to cell membranes and are glycosylated *in situ*.

The mechanism of viral DNA synthesis is not well understood, but it is known that this synthesis requires continuing active protein synthesis. Viral proteins and viral DNA are aggregated in the nucleus into naked capsids. It would appear that only a minor part of these components are used to build capsids; the remainder may be found in the various types of nuclear inclusions that frequently accompany herpesvirus infection. Capsids are next extruded through the nuclear membrane into perinuclear spaces and into the endoplasmic reticulum, at which time they acquire an envelope. Complete virions are released following disintegration of the nuclear membrane, then of the whole cell.

The way herpes simplex virions are released appears to be of great importance. It is known that only a fraction of the released virions are infectious. With some herpesviruses (herpes zoster, cytomegaloviruses, and Epstein-Barr virus) cell-free transmission of infection is impossible or very difficult, which indicates that some degree of cell-association is necessary for successful transmission. This cell association may depend not on the virus but on the cells which synthesize it. For instance, Marek's disease virus of chickens is strongly cell associated in *in vitro* systems, yet *in vivo* virus produced by feather follicles appears highly infectious.

The mechanism of nonproductive infection has not been completely elucidated. However, it is known that virus replication stops following penetration of the virus into the cell (Aurelian and Roizman, 1965; Lowry *et al.*, 1971). The block seems to occur at the level of protein synthesis. The problem of nonproductive infection is relevant to the problem of latency of herpes simplex virus *in vivo*, and to that of the transforming ability of herpes simplex virus. Several lines of evidence suggest that HSV-1 is maintained in the latent state in sensory neurons, in animals as well as

in humans (Baringer, 1974). HSV-2 seems to persist in a latent state in the male genitourinary system (Centifanto *et al.*, 1972) and in sacral ganglia (Baringer, 1974). Roizman (1965) has hypothesized that the persistence of recurrent herpes simplex virus infection can be explained either by a low rate of replication of the virus between recurrences in some limited parts of the body (dynamic state theory) or by its persistence in a noninfectious form in previously infected tissues (static state theory). There are arguments in favor of both theories, but the static state theory appears to better explain the problem of latency (Aurelian and Strnad, 1976). HSV-1 DNA is infectious in itself and can be transcribed by the cells in the absence of protein synthesis by the intermediary of a host cell polymerase. Transcription may either be repressed or inhibited in the static state. Exposure of the cells to stress of varying nature may reactivate the transcription mechanism and lead to virus synthesis (Aurelian, 1974a,b).

Infection of cells with herpesviruses, such as Epstein-Barr virus and herpes simplex virus, elicits the appearance in the cells of a group of antigens detectable by a variety of immunological methods using heterologous antisera to the virions. These antigens are either intracellular antigens, like the viral capsid antigen (Henle and Henle, 1966) and the early antigen, synthesized earlier than the viral capsid antigen and produced even in the presence of inhibitors of DNA synthesis (Gergely *et al.*, 1971), or surface antigens, called membrane antigens (Klein *et al.*, 1966, 1968; Klein, 1971). Virus-infected cells also contain a soluble, complement-fixing antigen (Old *et al.*, 1968). Early antigens have been divided into EA-R and EA-D (Henle *et al.*, 1971a,b) on the basis of their detectability by immunofluorescence following acetone or methanol fixation of the test cells. Methanol destroys R antigen, and preserves D antigen, while acetone does the reverse. Finally, virus-infected cells contain three types of antigens designated as A, B, and C (Kirkwood *et al.*, 1972; Evans *et al.*, 1973), which can be analyzed by immunodiffusion to determine the type or group specificities of herpesviruses. Melnick *et al.* (1976) have analyzed by polyacrylamide gel electrophoresis the HSV-1- and HSV-2-induced cellular proteins and prepared antisera to four of them (VP 175, an early nonstructural protein; VP 154, which is the major nucleocapsid polypeptide; VP 134, an early nonstructural polypeptide; and VP 123, the major envelope glycoprotein). Immunofluorescence and neutralization tests have shown that only anti VP 123 serum was type specific. Type specificity related to envelope glycoproteins was also shown by Powell *et al.* (1974). HSV-1 and HSV-2 infected cells also acquire nonvirion new antigens (Hollinshead *et al.*, 1973, 1974, 1976) which are type-specific. These antigens can be demonstrated by skin tests, by complement fixa-

tion tests, and by leukocyte migration inhibition tests with extracts of virus-infected cells. These antigens have played an important part in attempts to relate certain human carcinomas to herpesviruses (see below).

C. Epstein-Barr Virus, Burkitt's Lymphoma, Infectious Mononucleosis, and Nasopharyngeal Carcinoma

There is already a considerable amount of literature concerning the relationship of Epstein-Barr virus to the origin of infectious mononucleosis, Burkitt's lymphoma, and nasopharyngeal carcinoma, since the discovery of the virus in cultures of Burkitt's lymphoma biopsy tissues (Epstein *et al.*, 1965). A series of seroepidemiological studies using immunofluorescence, complement fixation, immunoprecipitation, and other methods have shown that Epstein-Barr virus was not only associated with Burkitt's lymphoma (Klein, 1971) but also with nasopharyngeal carcinoma (Henle *et al.*, 1970; de Thé, 1972; Henle and Henle, 1972) and was the causative agent of infectious mononucleosis (Henle *et al.*, 1968; Henle and Henle, 1970). Epstein-Barr virus has also been found in cultures of lymphoblastoid cells derived from patients with leukemia and from normal donors (Klein, 1971). It soon appeared that Epstein-Barr virus was a virus that infected human populations of all countries (Klein, 1975). Epstein-Barr virus is transmitted horizontally. Newborns carry antibodies of maternal origin to the virus, replaced later by antibodies acquired through positive seroconversion. As shown by seroepidemiological studies and the epidemiological behavior of infectious mononucleosis, primary infection of young adults or children with Epstein-Barr virus is strongly dependent on socioeconomic conditions. The poorer the conditions, the earlier the infections. Most of the time primary infection with Epstein-Barr virus is clinically silent.

The association of Epstein-Barr virus to Burkitt's lymphoma, nasopharyngeal carcinoma, and infectious mononucleosis has been studied by testing human sera with Epstein-Barr virus-producing or nonproducing lymphoblastoid cell lines derived from Burkitt's lymphoma, nasopharyngeal carcinoma, and infectious mononucleosis cells, and with fresh tumor cells. These studies using a variety of immunological techniques (immunofluorescence has been predominantly employed) have led to the establishment of an antibody–antigen system for screening purposes (Henle and Henle, 1974). The detectable antigens comprise: a viral capsid antigen present in virus-producing cells; an early antigen synthesized before the viral capsid antigen in infected cells or in abortively infected cells (nonproducer cells); membrane antigens in live cells producing the virus; a nuclear antigen detectable in nonproducer cells; a complement-fixing,

soluble antigen detectable in extracts of nonproducer cells; and a neutralizing antigen present in centrifugal concentrates of producer cells. As already mentioned, early antigen is distinguishable as D and R antigens. Viral capsid antigens, membrane antigens, and early antigens are detectable by immunofluorescence techniques; soluble antigen is detectable by complement fixation, neutralizing antigen by inhibition of colony formation, and Epstein-Barr neutralizing antigen by anticomplementary immunofluorescence. Cultured cells from Burkitt's lymphoma and nasopharyngeal carcinoma have been shown to contain all of these antigens, but biopsy tissues have been found negative not only for virus particles by electron microscopy, but also for viral capsid antigens and early antigens (Henle and Henle, 1974). Sera of Burkitt's lymphoma patients reacted with early antigen R of tissue culture cell lines, while sera of patients with infectious mononucleosis and nasopharyngeal carcinoma reacted with early antigen D (Henle and Henle, 1972). This difference has suggested the possibility that there might be more than one type of Epstein-Barr virus. However, a series of studies to that effect have been inconclusive. Immunofluorescence tests with heterologous antisera have shown that at least three membrane antigens were present in virus-producing cell lines (Pearson *et al.*, 1971), but sera of Burkitt's lymphoma, nasopharyngeal carcinoma, and infectious mononucleosis patients reacted with all three types of membrane antigens. This showed that no type differences existed between Epstein-Barr virus of cells derived from Burkitt's lymphoma, nasopharyngeal carcinoma, or infectious mononucleosis. Nishioka *et al.* (1971) have distinguished on the cell membranes of lymphoblastoid cell lines derived from Burkitt's lymphoma or nasopharyngeal carcinoma an "IgM receptor" for sheep erythrocytes sensitized with IgM antibody and fractions 3 and 4 of complement, and an "IgG receptor" for sheep erythrocytes sensitized with IgG antibody. Lymphoblastoid cell lines derived from nasopharyngeal carcinoma, but not of Burkitt's lymphoma, contained the IgM receptors. Using radioimmunoassays, Greenland *et al.* (1972) have found antigenic determinants on Epstein-Barr virus-producing cell lines, determinants of which one ("C" determinant) was present only in the nasopharyngeal cell lines. The minor antigenic differences between cell lines derived from Burkitt's lymphoma, nasopharyngeal carcinoma, and infectious mononucleosis probably do not represent differences between different Epstein-Barr virus types. It may be concluded that, at least on immunological grounds, Epstein-Barr virus is a unique virus type (Pearson, 1974).

Molecular hybridization experiments have greatly contributed to the detection of Epstein-Barr virus DNA in Burkitt's lymphoma and naso-

pharyngeal carcinoma cells (Zur Hausen *et al.*, 1970; Nonoyama and Pagano, 1971). An interesting difference has been observed between African and American Burkitt lymphoma tumors (Nonoyama *et al.*, 1974). Using DNA-DNA reassociation kinetics and DNA-complementary cellular RNA hybridizations, the authors were able to detect 5 to 100 genomes of Epstein-Barr virus in the DNA of every cell of African Burkitt lymphoma tumors, but none in cells of American Burkitt lymphoma cases, and also none in cellular DNA of Hodgkin's disease. No satisfactory explanation has as yet been found for this intriguing difference between African and American cases of Burkitt's lymphoma. The lack of association between Epstein-Barr virus and American Burkitt's lymphoma has also been observed on serological grounds (Levine, 1972). However, the recent finding of one European and one American Epstein-Barr virus positive case (Klein, 1975) will probably help to explain the difference. In nasopharyngeal carcinoma, Pagano and Huang (1974) have found some differences in the DNA sequences of Tunisian cases of nasopharyngeal carcinoma, using Burkitt's lymphoma-derived Epstein-Barr virus DNA probe, but the significance of these differences is not clear.

It is now well established that the target cell of Epstein-Barr virus is the B lymphocyte (Greaves *et al.*, 1975). Epstein-Barr virus can convert normal lymphocytes into permanently growing cells *in vitro* which carry multiple Epstein-Barr virus genomes per cell and express Epstein-Barr virus-specific nuclear antigen (Reedman and Klein, 1973). It is of interest that Epstein-Barr virus-carrying lymphoblastoid cell lines can be established only from peripheral blood or lymph nodes of seropositive donors (Nilsson *et al.*, 1971).

In contrast to Burkitt's lymphoma, only poorly differentiated tumors have been found to carry the Epstein-Barr virus genome in nasopharyngeal carcinoma. In nasopharyngeal carcinoma, viral genomes are not carried by the infiltrating lymphocytes (which are T lymphocytes). The viral genomes have been found in the epithelial cells of the tumors which have also been shown to contain Epstein-Barr neutralizing antigen (Klein *et al.*, 1974).

The oncogenicity of Epstein-Barr virus is perhaps linked to its effect on chromosomes of Epstein-Barr virus-carrying cells. Malonov and Malonova (1972), Jarvis *et al.* (1974), and Zech *et al.* (1976) have found that Burkitt tumor cells contain a specific marker (translocation) on chromosome 14. This translocation was also found in cell lines derived from Burkitt's lymphoma, but not in Epstein-Barr virus-transformed cells derived from normal donors or from patients with infectious mononucleosis. The exact meaning of such an observation in terms of oncogenesis by

Epstein-Barr virus is not clear because a minority of African Epstein-Barr virus-carrying Burkitt's lymphoma do not show this translocation.

The oncogenicity of Epstein-Barr virus has recently been demonstrated by bioassays in monkeys. Epstein *et al.* (1973a,b) have inoculated Epstein-Barr virus-containing Burkitt's lymphoma cells in culture into owl monkeys (*Aotus trivirgatus*) and observed lymphoproliferative disease in one of the animals. Werner *et al.* (1975) and Shope and Miller (1974) have reported the successful induction of lymphosarcoma in cottontop marmosets using Epstein-Barr virus from cultures of Burkitt's lymphoma and infectious mononucleosis. Virus recovered from tumorous lymph nodes was capable of transforming lymphocytes of normal marmosets into Epstein-Barr virus-carrying cell lines with characteristics of B lymphocytes. These observations have confirmed a series of previous findings on the transforming activity of Epstein-Barr virus for lymphocytes of nonhuman primates: gibbon, squirrel monkeys, cottontop, and white-lipped marmosets, and cinnamon ring-tail and owl monkeys (Werner *et al.*, 1975).

There have been many serological studies designed to determine the levels of antibodies to early antigens and viral capsid antigens of Epstein-Barr virus in patients with Hodgkin's disease and leukemia (Gotlieb-Stematsky *et al.*, 1975). All the patients with Hodgkin's disease, and most patients with leukemia, had higher levels of antibodies to viral capsid antigens than healthy controls. Some patients with leukemia had no antibodies to either viral capsid antigens or early antigens. Around 30% of all patients, but none of the controls, had antibodies to early antigens of Epstein-Barr virus. These results indicate that seroepidemiological studies may lead to results difficult to interpret or to use as a demonstration of the oncogenicity of Epstein-Barr virus in humans. Because of the ubiquity of Epstein-Barr virus and the widespread occurrence of primary infection of humans in their early life, the occurrence of lymphomas or carcinomas, if caused by Epstein-Barr virus, must represent an unusual, late event in virus carriers (Henle and Henle, 1974). Nevertheless, important clues to the relationship of Epstein-Barr virus to Burkitt's lymphoma and nasopharyngeal carcinoma are provided by correlations between the clinical history of the patients and the changes in their serological reactions. Burkitt's lymphoma patients in long-term remission show a decline of their anti-membrane antigen—antibody titers; patients with no early antigen R antibodies are also those who survive longer. Nasopharyngeal carcinoma patients show a gradual increase in their antiviral capsid antigen titers with aggravation of their disease (Henle and Henle, 1974). Anti-D antibody also becomes gradually detectable in their sera, and, conversely, declines following treatment.

D. Herpesvirus Type 2 and Cervical Carcinoma

As mentioned earlier, herpesvirus type 2 (HSV-2) has been implicated in the etiology of cervical carcinoma not only on the basis of cytopathological findings, but also (and mainly) on the basis of extensive seroepidemiological studies. These studies have been conducted by using a number of immunological techniques. A brief reminder of the immunology of herpesvirus infection in humans and of the various techniques which have been used may be appropriate.

1. Immunology of Herpes Simplex Virus (HSV) Infection

Knowledge of the mechanisms of immune responses to HSV-infection is of great importance in attempts to find the relevance of HSV infections to cervical carcinoma. A brief mention will be made of the salient facts which have been analyzed in more detail by Nahmias *et al.* (1976) and Aurelian and Strnad (1976).

After onset of a primary infection with HSV-1 or HSV-2, IgM antibodies appear first, followed 2–3 weeks later by IgG and IgA antibodies. Complement-dependent neutralizing antibodies are present very early, while the cell-mediated response, as tested by lymphocyte stimulation *in vitro*, is manifest sometime between the second and fourth week after infection.

Newborn children frequently suffer from HSV (mostly HSV-2) infection. They are protected against visceral propagation of the virus by maternal, transplacental antibodies for some 2–3 weeks, after which protection is supplied by their own IgM antibodies. These antibodies may persist for a year.

Primary exposure to HSV-1 occurs early in life in at least 85% of people, while primary exposure to HSV-2 seems to be more frequent around puberty (Nahmias and Roizman, 1973). Exposure to HSV-2 is much less frequent than to HSV-1.

Many immunological studies have been devoted to the immune response in recurrent HSV infections (Nahmias *et al.*, 1976). Individual suffering from recurrences have high titers of neutralizing antibodies, however, differences between patients with primary infection and those with recurrences have not been found significant enough to be of clinical value.

The role of immunity to HSV is well demonstrated by the severity of recurrences in immunologically compromised hosts (patients with drug-induced immunosuppression, with cancers or leukemias, with burns, or children suffering from malnutrition or measles, etc.). The immunological defect in these cases is not so much humoral as it is cellular, a point that

shows the importance of cell-mediated immunity in protection against HSV.

The mechanism(s) by which humoral and cell-mediated immunity interplay in protection against the spread of latent HSV is not known, especially in the case of HSV-2. It may be that, at the humoral level, IgA antibodies, which cannot fix complement, attach to virus-infected cells and protect the cells against cytotoxic IgG, thus preventing the spread of infective virus (Aurelian and Strnad, 1976).

2. *Titration of Antibodies to HSV-1 and HSV-2 in Human Sera*

In view of the importance of serological techniques in demonstration of the association of HSV with cervical carcinoma, a brief summary of the methodologies involved will be presented. In spite of the close immunological relationship of HSV-1 to HSV-2, these techniques allow for distinguishing of the antibodies directed against either HSV-1 or HSV-2 with fairly good accuracy (Plummer, 1973).

In neutralization kinetics tests (Skinner *et al.*, 1971; Rawls *et al.*, 1969), a diluted test serum is incubated for a given period of time with a given concentration of virus. The mixture is tested on baby hamster kidney (BHK-1) cells for plaque formation. Since plaques of HSV-1 can be differentiated morphologically from those of HSV-2, antibodies to both virus types can be titrated.

The plaque reduction assay (Plummer *et al.*, 1970; Royston and Aurelian, 1970a) consists of incubating the tested serum in doubling dilutions with constant doses of virus for a given period of time. The mixture is assayed on baby hamster kidney cells for plaque formation. Neutralization end points are expressed in 50% titers.

In microneutralization assays, serum in doubling dilutions and the virus preparation are tested in microplates on small numbers of cells (Sever, 1962; Rosenbaum *et al.*, 1963). Results are expressed as a function of presence or absence of cytopathic effect.

Human sera can also be titrated by indirect immunofluorescence tests on membranes of HSV-infected cells *in vitro* (Geder and Skinner, 1971; Nahmias *et al.*, 1971). The end point is taken as the highest dilution that causes fluorescence of 50% of the cells.

Sheep red blood cells coated with antigens of HSV-1 or HSV-2 can be titrated in microtests with human sera (Fucillo *et al.*, 1970). Titration can also be performed after absorption of sera with the same antigens (inhibition-agglutination test; Schneveis and Nahmias, 1971).

In cytotoxic tests using ^{51}Cr release (Smith *et al.*, 1972), ^{51}Cr-labeled HSV-infected cells are exposed to test serum and complement. The release of radioactive chromium in the medium is a measure of the amount

of antibodies to HSV. The special interest of this technique resides in the fact that preabsorption of the serum with nonradiolabeled cells infected with HSV-1 will not prevent the cytotoxic effect of antibodies to HSV-2, and vice versa. It is known that the cytotoxic effect of human sera against HSV-infected cells is related to IgM antibodies in the first 3 weeks that follow exposure to HSV, and to IgG antibodies thereafter.

3. Cervical Carcinoma as a Venereal Disease

A number of diseases are regarded today as venereally transmitted in humans: syphilis, gonorrhea, herpes simplex, lymphogranuloma venereum, condyloma acuminatum, and some parasitic diseases. There seems to be little doubt that genital herpes with which HSV-2 is associated behaves as a venereally transmitted disease. The studies of Nahmias *et al.* (1969) and of Rawls *et al.* (1971) have demonstrated direct transmission of herpes genitalis through penile or cervical lesions. The direct transmission of herpetic infection has been also demonstrated in studies of patients or prostitutes seen in diagnostic clinics (Kessler, 1976). The successful isolation of HSV-2 in primary genital lesions, more often than in recurrent lesions (Rawls *et al.* 1971), indicates that the reservoir of virus is more likely to be found in younger populations. Statistical studies on populations at higher risk of having cervical carcinoma have shown many parallels between the epidemiology of genital herpes and the factors influencing the risk of cervical carcinoma. A clear relationship has been found between factors governing a higher incidence of genital herpes and of cervical carcinoma: low socioeconomical status, prostitution, multiplicity of sexual partners, early sexual intercourse, and aggravating effects of syphilis and gonorrhea (Goldberg and Gravell, 1976; Kessler, 1976). These statistics have been paralleled by seroepidemiological studies of antibody titers to virion and nonvirion antigens of HSV-2 in various populations. These studies have demonstrated that antibodies to HSV-2 are more prevalent in patients with cervical carcinoma (Rawls *et al.*, 1969, 1973, 1976; Kessler *et al.*, 1974; Pacsa *et al.*, 1975).

4. Molecular Studies

As in the case of Epstein-Barr virus, Burkitt's lymphoma, and nasopharyngeal carcinoma, seroepidemiological studies of antibodies to HSV-2 cannot provide a direct proof of the involvement of HSV-2 in cervical cancer. The elevated antibody titers to HSV-2 antigens in patients with cervical anaplasia or cancer may simply indicate a higher susceptibility of cancer patients to the virus. Therefore, considerable efforts have been devoted to substantiate the hypothesis of an association of HSV-2 to cervical cancer by more elaborate techniques.

Molecular hybridization techniques had obvious applications in helping to solve the problem. Frenkel *et al.* (1972) and Roizman and Frenkel (1973) have found one cervical carcinoma tumor containing cytoplasmic RNA complementary to radioactive DNA of HSV-1 and HSV-2, as well. DNA-DNA hybridization of viral DNA to DNA of tumor cells have shown (in the same tumor) the presence in cellular DNA of some sequences homologous of HSV-2 DNA. Using RNA probes made from HSV-1 and HSV-2 DNA by *E. coli* RNA polymerase, Zur Hausen *et al.* (1974) failed to find complementary, virus-related DNA in some 40 specimens of cervical carcinoma. These workers have reached the temporary conclusion that HSV-2 was not etiologically related to cervical carcinoma (Zur Hausen, 1976).

5. *HSV-Related Antigens in Cervical Tumors and Their Tissue Cultures*

It is important to note that until now infectious viral structural antigens of HSV-2 and cytopathic changes typical of herpesvirus infection have not been detected directly in biopsies of cervical neoplasia. Apparently, HSV-2-specific antigens have been detected by immunofluorescence in exfoliated tumor cells of vaginal smears (Aurelian, 1972, 1973; Royston and Aurelian, 1970a,b; Aurelian *et al.*, 1972). However, electron microscopy has not shown the presence of virus particles in these cells.

Two cell lines have been established by Aurelian and co-workers, one (S332G) (Aurelian *et al.*, 1971) from carcinoma *in situ*, the other (614) from invasive tumor (Aurelian, 1974a,b). Spontaneous degeneration of S332G cells was observed after a number of transfers and HSV-2 virus was detected in the cells by electron microscopy and by immunological means. The virus was transferred into HEp-2 cells (isolate S-1). Virus expression was inducible in S332G cells by submitting the cells to increased pH. Cells of line 614 showed spontaneous appearance of HSV-2-specific membrane antigens in a few percentage of the cells, but virus could not be isolated or induced. Aurelian and co-workers (Aurelian, 1973) have prepared a crude extract of HEp-2 cells exposed to S-1 virus for 4 hours. They found by microcomplement fixation tests that 90% of women with invasive carcinoma, 67% of women with carcinoma *in situ*, 35% of women in cervical atypia, and 10% of control women had antibodies reacting with the extract, designated as AG-4. The authors also concluded that AG-4 antigen was tumor-specific, because neutralizing antibodies to HSV-2 in control women were much more frequently (68%) found than antibodies to AG-4 (10%). Aurelian and Strnad (1976) have further analyzed the properties of AG-4 antigen by preparing purified, [^{35}S]methionine-labeled virus from infected HEp-2 cells. Twenty-four virion proteins were found by acryla-

mide gel analysis of virion preparations (proteins VP-1 to VP-24) from 16,000 to 250,000 daltons in molecular weight. These proteins were challenged by AG-4-positive and -negative human sera. AG-4 antigenic activity was apparently related to virion protein VP-4, which has a molecular weight of 161,000 daltons. Aurelian *et al.* (1976), by using various immunological assays, have concluded that AG-4 was an antigen expressed on the cell surface. This interesting approach will have to be further explored by finding some biochemical links between the antigen present in HSV-2-infected HEp-2 cells and similar antigens in either cervical cancer cells or in cervical cells following episodes of herpes genitalis.

E. Herpesviruses and Squamous Carcinomas of the Head and Neck

Hollinshead *et al.* (1976) have studied by complement fixation test and by leukocyte migration inhibition tests (LMI), the reactivity of patients with a variety of squamous cell carcinomas of the head and neck to herpesvirus tumor-associated antigens (HSV-TAA). They have observed that sera of 90% of patients with squamous carcinomas, compared with 11% of patients with nonsquamous cancers (melanomas, sarcomas, adenocarcinomas) and with 4% normal controls, have CF antibodies to HSV-TAA prepared from HSV-infected human embryonic kidney cells. Results with leukocyte migration inhibition tests showed a similar pattern of cell-mediated response. The PHA-stimulation test with lymphocytes of the patients and the delayed hypersensitivity test have shown that most patients with squamous carcinomas were immunodepressed for life, contrary to patients with nonsquamous cancers who show a return to normal immune activity after removal and treatment of their tumors. Age, race, and sex had no apparent influence on the results. The significance of these observations may be multiple. As in the case of Epstein-Barr virus or herpes simplex virus in cervical carcinoma, these observations may indicate either an etiological role of HSV or a higher susceptibility of cancer patients to herpes simplex virus infections.

VIII. CONCLUDING REMARKS

The preceding sections have covered in broad outline the various properties of RNA and DNA viruses suspected at present to play a part in the origin of human proliferative diseases. Human tumors have been found to contain the "footprints" or "fingerprints" of RNA viruses apparently very similar to those of murine and simian viruses but unrelated

to each other, and of DNA viruses belonging to the family of herpesviruses. These observations naturally lead to the question: How many viruses, if any, are involved in human neoplasia? Some viruses have already been excluded from the family of potential human oncogenic viruses. For instance, great interest was generated a decade ago by the finding that human adenoviruses types 12, 18, and 31 were highly oncogenic in hamsters. However, extensive serological, biochemical, and immunological investigations have failed to demonstrate an association of adenoviruses with human tumors (McAllister, 1973).

It is of great importance to note that demonstration by electron microscopy, biochemistry, and immunology of the association of a virus with tumors or other cells (sometimes of several different viruses at the same time) does not allow for differentiation of a causative virus from a so-called "passenger" virus, simply because the presence of a virus in a cell is not synonymous with malignancy—it only indicates viral infection. Even the finding of virus-related nucleic acid sequences absent from normal cells but present in tumor cells may not mean more than a particular susceptibility of tumor cells to infection by certain viruses such as oncornaviruses (Retroviridae) or herpesviruses. Electron microscopic observations in human leukemic cells, in breast cancer, and in prostate cancer cells of viruslike particles, some resembling type C or type B particles, and some of a structure apparently unrelated to that of presently known viruses, is an additional proof that tumor cells may harbor and replicate viruses with yet unknown biological properties. A classic example in this field is the widespread occurrence in cells of normal as well as cells of malignant tissues of mice of practically all strains examined of the so-called intracisternal type A virus particles which have resisted until now all attempts to ascertain their biological activity.

It seems pretty obvious that demonstration of the oncogenic potential of a virus has to be based on biological and epidemiological observations and, whenever possible, on bioassays. The causative relationship of viruses to proliferative diseases can only be ascertained by demonstration that the suspected virus was present in the host before the clinical onset of the disease, and that the virus, once isolated, can induce the same type of disease in various hosts, including humans. The case of herpesviruses is particularly well-suited for this type of demonstration for the very reason that, contrary to putative human oncornaviruses, a number of human herpesviruses have already been well-characterized and their behavior traced in human populations by extensive seroepidemiological studies. The studies of herpesvirus-induced proliferative diseases, such as Marek's disease of chickens or lymphoid tumors of monkeys, which are induced by several strains of herpesviruses, have greatly contributed to

herpesviruses being suspected as tumorigenic agents in humans. Particularly illustrative of the oncogenic potential of herpesviruses is the induction in a marmoset of malignant lymphoproliferative disease by Epstein-Barr virus extracted from a human lymphoblastoid cell line derived from a patient with infectious mononucleosis (Werner *et al.*, 1975). Even more instructive is the establishment of an Epstein-Barr virus-carrying lymphoblastoid cell line from a lymph node tumor of the marmoset, and the demonstration that the Epstein-Barr virus produced by this cell line was capable of transforming lymphocytes of healthy marmosets. These experiments have shown how close the behavior of Epstein-Barr virus appears to be to that of simian herpesviruses, which are generally harmless to the host of origin, but pathogenic to heterologous hosts (McCarthy and Tosolini, 1975). Epstein-Barr virus has also been shown to be directly oncogenic in owl monkeys (G. J. Miller, 1974). In this respect, it may be of interest to speculate how Burkitt's lymphomas and nasopharyngeal carcinomas are related to the oncogenic effect of Epstein-Barr virus which is unquestionably a human virus and, therefore, should not (as in the case of simian herpesviruses) induce malignant proliferative diseases in homologous, human hosts. The absence of Epstein-Barr virus in other than African Burkitt's lymphomas (Henle *et al.*, 1971b; Zur Hausen *et al.*, 1970) perhaps indicates that there may be different strains of Epstein-Barr virus with varying malignant potential, related to the genetic differences in human populations. Nasopharyngeal carcinoma also appears to be strongly linked to genetic factors (Ho, 1972) which may render Epstein-Barr virus more pathogenic.

Herpesviruses and oncornaviruses share a number of biological properties. They are extremely difficult to find as ultrastructural entities in tumors *in vivo*. Both require for their retrieval *in vitro* manipulations of tumor or tumor-derived cells. Both herpesviruses and oncornaviruses have been demonstrated to be endowed with cell-transforming potential *in vitro*. Both virus families comprise viruses which are endogenous and/or apparently harmless to random-bred individuals of the species of origin. Another parallelism between the biological behavior of herpesviruses and that of oncornaviruses is illustrated by the results of experiments on monkeys. In this respect, the results of attempts to induce proliferative diseases in monkeys with human tumor material appear of great interest. Lapin (1975) and Lapin *et al.* (1975) have inoculated intraperitoneally blood cells and plasma filtrates of leukemic patients into stump-tail monkeys and hamadryas baboons. Most monkeys developed splenomegaly and adenopathies with periodic appearance of blast cells in peripheral blood which, however, remained aleukemic. This wasting disease was observed following inoculation of either blood cells or plasma

filtrates of leukemic patients, especially of patients with chronic lymphocytic leukemia. Type C virus particles have been found by electron microscopy in various organs of 32 out of 42 monkeys inoculated. The type C virus particles after extraction induced lymphomas in recipient monkeys. These virus particles are apparently not related immunologically to any known mammalian oncornaviruses, including the simian sarcoma virus. These provocative experiments remain to be completed by demonstration that the type C virus was a genuine human virus and that no DNA virus has been transmitted to the monkeys. Most recently, herpes-type virus particles have been observed in the induced lymphomas of baboons (Yakovleva *et al.*, 1975). It should be pointed out that these experiments led to the apparent demonstration of both horizontal and vertical transmission of leukemia among the hamadryas baboons in close contact with baboons inoculated with human leukemic blood (Lapin, 1975; Lapin *et al.*, 1975).

Because of the obvious impossibility of bioassays in humans, no human candidate tumor virus can be demonstrated to have an oncogenic effect in man other than by accident or conceivably by exhaustive, seroepidemiological studies. However, there may be an indirect way to prove that a virus is pathogenic. This is to prevent its harmful effect by a vaccine. A viral vaccine against at least some human malignant proliferative diseases is still in the far future, because besides other problems the number and nature of possible viruses involved is as yet unknown. However, the prevention of Marek's disease in chickens by using herpesvirus of a heterologous species (turkeys) as immunizing agents (Purchase, 1976) is of great scientific interest and of considerable economic value. This accomplishment represents perhaps a breakthrough toward active protection against oncogenic viruses. Monkeys of some species have also been efficiently protected against lymphoma induced by *Herpesvirus saimiri* and *Herpesvirus ateles* by inoculation of attenuated (Falk *et al.*, 1976) or killed virus (Ablashi and Easton, 1976; Laufs and Steinke, 1976). It has to be stressed that this protection was obtained against a challenge by highly infectious virus isolated in the laboratory. This may not correspond to a "natural" situation. *Herpesvirus saimiri* has been isolated from owl monkeys with an apparently spontaneous lymphoma (Rabin *et al.*, 1975). Successful vaccination of primates against "naturally" occurring lymphoproliferative diseases may lead to attempts to immunize human populations at high risk against Epstein-Barr virus, a task of great magnitude comprising many difficulties and subject to numerous reservations.

Prevention of oncornavirus-induced proliferative diseases appears promising in certain animal systems. It is now known that cats exposed to feline leukemia or sarcoma virus under field or laboratory conditions

develop a definite immunological resistance to leukemias or sarcomas (Essex *et al.*, 1976). Lymphoma and lower limb paralysis caused by type C virus in wild mice can be prevented by specific viral antisera (Henderson *et al.*, 1974). So far, no efficient prevention of spontaneous lymphomas, leukemia, and mammary tumors has been obtained in inbred mice by using specific antisera to MuLV or MMTV or by using attenuated or killed viruses. Mice develop natural antibodies to MuLV (Aoki and Sibal, 1976) or MMTV (Bowen *et al.*, 1976a,b), but these antibodies have apparently no tumor preventive effect. However, comparisons between murine proliferative diseases and human proliferative diseases, which show many common pathological features, cannot be extended to the level of viruses until such time when a type C or type B virus with well-defined biochemical and immunological properties is isolated from tissues of human proliferative diseases. The surprising simian characteristics of two recent type C viruses demonstrated in apparently authentic human cells (viruses HEL-12 and HL-23) are perhaps an indication that viral oncogenic studies in primates may lead to results with apparently equal if not greater application to the problem of isolation of a human oncogenic virus or viruses than similar studies in mice, although the latter cannot be discussed as irrelevant to the problem of human proliferative diseases.

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REFERENCES

Aaronson, S. A., and Stephenson, J. R. (1974). *Proc. Natl. Acad. Sci. U.S.A.* **71,** 1957–1961.

Ablashi, D. V., and Easton, J. M. (1976). *Cancer Res.* **36,** 701–703.

Abrell, J. W., and Gallo, R. C. (1973). *J. Virol.* **12,** 431–439.

Ahmed, M., Korol, W., Schidlovsky, G., Vidrine, J., and Mayyasi, S. (1973). *Proc. Am. Assoc. Cancer Res.* **14,** 34.

Ahmed, M., Schidlovsky, G., Korol, W., Vidrine, J. G., and Cicmanec, J. L. (1974). *Cancer Res.* **34,** 3504–3508.

Ahmed, M., Schidlovsky, G., Larson, D. L., Manousos, M., and Mayyasi, S. A. (1976). *J. Gen. Virol.* **30,** 11–20.

Aoki, T., and Sibal, L. R. (1976). *Cancer Res.* **36,** 591–597.
Aoki, T., and Todaro, G. J. (1973). *Proc. Natl. Acad. Sci. U.S.A.* **70,** 1598–1602.
August, J. T., Bolognesi, D. P., Fleissner, E., Gilden, R. V., and Nowinski, R. C. (1974). *Virology* **60,** 595–601.
Aurelian, L. (1972). *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **31,** 1651–1659.
Aurelian, L. (1973). *Cancer Res.* **33,** 1539–1547.
Aurelian, L. (1974a). *Cancer Res.* **34,** 1126–1135.
Aurelian, L. (1974b). *Am. J. Med. Technol.* **40,** 496–512.
Aurelian, L., and Roizman, B. (1965). *J. Mol. Biol.* **11,** 539–548.
Aurelian, L., and Strnad, B. C. (1976). *Cancer Res.* **36,** 810–820.
Aurelian, L., Strandberg, J. D., Melendez, L. V., and Johnson, L. A. (1971). *Science* **174,** 704–707.
Aurelian, L., Strandberg, J. D., and Davis, H. J. (1972). *Proc. Soc. Exp. Biol. Med.* **140,** 404–408.
Aurelian, L., Smith, M. F., and Cornish, D. J. (1976). *J. Natl. Cancer Inst.* **56,** 471–477.
Axel, R. C., Gulati, S. C., and Spiegelman, S. (1972a). *Proc. Natl. Acad. Sci. U.S.A.* **69,** 3133–3137.
Axel, R. C., Schlom, J., and Spiegelman, S. (1972b). *Nature (London)* **235,** 32–36.
Baltimore, D. (1970). *Nature (London)* **226,** 1209–1211.
Baltimore, D., and Smoler, D. F. (1972). *J. Biol. Chem.* **247,** 7282–7287.
Baltimore, D., Verma, I. M., Drost, S., and Mason, W. S. (1974). *Cancer* **34,** 1395–1397.
Baluda, M. A. (1972). *Proc. Natl. Acad. Sci. U.S.A.* **69,** 576–580.
Baringer, J. R. (1974). *N. Engl. J. Med.* **291,** 828–830.
Bates, H. A., Bankole, R. O., and Swaim, W. R. (1969). *Blood* **34,** 430–440.
Bauer, H. (1974). *Adv. Cancer Res.* **20,** 275–329.
Baumgartner, L. E., Olson, C., Miller, J. M., and Van der Maaten, M. J. (1975). *J. Am. Vet. Med. Assoc.* **166,** 249–251.
Baxt, W. G. (1974). *Proc. Natl. Acad. Sci. U.S.A.* **70,** 2853–2857.
Baxt, W., Hehlmann, R., and Spiegelman, S. (1972). *Nature (London), New Biol.* **240,** 72–75.
Baxt, W., Yates, J. W., Wallace, H. J., Holland, J. F., and Spiegelman, S. (1973). *Proc. Natl. Acad. Sci. U.S.A.* **70,** 2629–2632.
Becker, Y., Dym, H., and Sarov, I. (1968). *Virology* **36,** 185–192.
Bedigian, H. G., and Meier, H. (1975). *J. Natl. Cancer Inst.* **55,** 1007–1010.
Benedetti, E. L., Bernhard, W., and Oberling, C. (1956). *C. R. Hebd. Seances Acad. Sci.* **242,** 2891–2894.
Bendixen, H. J. (1965). Ph.D. Thesis, Virology Resources Branch, Natl. Cancer Inst., Washington, D.C.
Bentvelzen, P., Daams, J. H., Hageman, P., and Calafat, J. (1970). *Proc. Natl. Acad. Sci. U.S.A.* **67,** 377–384.
Benveniste, R. E., and Todaro, G. J. (1974). *Nature (London)* **252,** 456–459.
Benveniste, R. E., Lieber, M. D., Livingston, D. M., Sheer, C. J., Todaro, G. J., and Kalter, S. S. (1974). *Nature (London),* **248,** 17–20.
Bernhard, W. (1960). *Cancer Res.* **20,** 712–727.
Bernhard, W., and Guérin, M. (1958). *C. R. Hebd. Seances Acad. Sci.* **247,** 1802–1805.
Bernhard, W., Guérin, M., and Oberling, C. (1956). *Acta Unio Int. Cancrum* **12,** 544–557.
Bessis, M., and Thiery, J. P. (1962). *Nouv. Rev. Fr. Hematol.* **2,** 577–601.
Bierwolf, D., Rudolph, M., Niezabitowski, A., Jorde, A., Widmaier, R., and Graffi, A. (1975). *Arch. Geschwulstforsch.* **45,** 628–633.

Biggs, P. M., de Thé, G., and Payne, L. N., eds. (1972). "Oncogenesis and Herpesviruses." Int. Agency Res. Cancer, Lyon, France.

Birkmayer, G. D., Balda, B. R., and Miller, F. (1974). *Eur. J. Cancer* **10,** 419–424.

Bishop, D. H. L., Ruprecht, R., Simpson, R. W., and Spiegelman, S. (1971). *J. Virol.* **8,** 730–741.

Bishop, J. M., and Varmus, H. E. (1975). *In* "Cancer" (F. F. BEcker, ed.), Vol. 2, pp. 3–48, Plenum, New York.

Bishop, J. M., Faras, A. J., Garapin, A. C., Hansen, C., Jackson, N., Levinson, W. E., Taylor, J. M., and Varmus, H. E. (1973). *Collect. Pap., Annu. Symp. Fundam. Cancer Res.* **25,** 229–254.

Bogden, A. E., Cobb, W. R., Ahmed, M., Alex, S., and Mason, M. M. (1974). *J. Natl. Cancer Inst.* **53,** 1073–1077.

Bolognesi, D. P., Bauer, H., Gelderblom, H., and Huper, G. (1972a). *Virology* **47,** 551–566.

Bolognesi, D. P., Gelderblom, H., Bauer, H., Milling, K., and Huper, G. (1972b). *Virology* **47,** 567–578.

Bolognesi, D. P., Luftig, R., and Sharper, J. H. (1973). *Virology* **56,** 549–564.

Booth, K., Burkitt, D. P., Bassett, D. J., Cooke, R. A., and Biddulph, J. (1967). *Br. J. Cancer* **21,** 657–664.

Boothe, A. D., and Van der Maaten, M. J. (1974). *J. Virol.* **14,** 197–204.

Boothe, A. D., Van der Maaten, M. J., and Malmquist, W. A. (1970). *Arch. Gesamte Virusforsch.* **31,** 373–384.

Bouillant, A. M. P., Genest, P., and Greig, A. S. (1973). *In Vitro* **9,** 92–102.

Bouillant, A. M. P., Greig, A. S., Lieber, M. M., and Todaro, G. J. (1975). *J. Gen. Virol.* **27,** 173–180.

Bowen, J. M., East, J. L., Allen, P. T., Maruyama, K., Priori, E. S., Georgiades, J., Chan, J. C., Miller, M. F., Seman, G., and Dmochowski, L. (1974). *In* "Viruses, Evolution and Cancer" (E. Kurstak and K. Maramorosch, eds.), pp. 403–426. Academic Press, New York.

Bowen, J. M., Dmochowski, L., Seman, G., and Scanlon, M. (1976a). *Proc. Am. Assoc. Cancer Res.* **17,** 58 (Abstract No. 229).

Bowen, J. M., Dmochowski, L., Miller, M. F., Priori, E. S., Seman, G., Dodson, M. L., and Maruyama, K. (1976b). *Cancer Res.* **36,** 759–764.

Braunsteiner, H., Fellinger, K., and Pakesch, F. (1960). *Blood* **15,** 476–479.

Breese, S. S., Jr. (1970). *Arch. Gesamte Virusforsch.* **30,** 401–404.

Britten, R. J., and Kohne, D. E. (1968). *Science* **161,** 529–540.

Brodsky, J., and West, W. (1976). *Proc. Am. Assoc. Cancer Res.* p. 127 (Abstract No. 508).

Burger, C. L., Harris, W. W., Anderson, N. G., Bartlett, T. W., and Kinseley, R. M. (1964). *Proc. Soc. Exp. Biol. Med.* **115,** 151–156.

Burkitt, D. (1958). *Br. J. Surg.* **46,** 218–223.

Burkitt, D. P., and Wright, D. H. (1970). "Burkitt's Lymphoma," Vol. I. Livingstone, Edinburgh.

Bykovsky, A. F., Miller, G. G., Yershov, F. I., Ilyin, K. V., and Zhdanov, V. M. (1973). *Arch. Gesamte Virusforsch.* **42,** 21–35.

Calafat, J., Hageman, P. C., and Ressang, A. A. (1974). *J. Natl. Cancer Inst.* **52,** 1251–1257.

Calnek, B. W., Aldinger, H. K., and Kahn, D. E. (1970). *Avian Dis.* **14,** 219–233.

Cawley, J. C., and Karpas, A. (1974). *Eur. J. Cancer* **10,** 559–562.

Centifanto, J. M., Drylie, D. M., Deardourff, S. L., and Kaufman, H. E. (1972). *Science* **178,** 318–319.

Chan, E., Peters, W. P., Sweet, R. W., Ohno, T., Kufe, D. W., Spiegelman, S., Gallo, R. C., and Gallagher, R. E. (1976). *Nature (London)* **260,** 266–268.

Chandra, S., Liszczak, T., Korol, W., and Jensen, E. M. (1970). *Int. J. Cancer* **6,** 40–45.
Chervernick, P. A. (1974). *Dis.-Mon.* Dec. 1974, pp. 5–29.
Chopra, H. C. (1970). *Proc. Am. Assoc. Cancer Res.* **30,** 2081–2086.
Chopra, H. C., and Mason, M. M. (1970). *Cancer Res.* **30,** 2081–2086.
Chopra, H. C., Bogden, A. E., Zelljadt, I., and Jensen, E. M. (1970). *Eur. J. Cancer* **6,** 287–290.
Chopra, H. C., Hooks, J. J., Walling, M. J., and Gibbs, C. J., Jr. (1972). *J. Natl. Cancer Inst.* **48,** 451–463.
Church, R. B. (1973). *In* "Molecular Techniques and Approaches in Developmental Biology" (M. J. Chrispools, ed.), pp. 223–301. Wiley, New York.
Clarke, J. K., Attridge, J. T., and Gay, F. W. (1969). *J. Gen. Virol.* **4,** 183–188.
Coffin, J. M., and Temin, H. M. (1972). *J. Virol.* **9,** 766–775.
Cohen, M. H., Benneth, J. M., Berard, C. W., Ziegler, J. L., Vogel, C., Sheagren, L., and Carbone, P. P. (1969). *Cancer* **23,** 1259–1272.
Colcher, D., Spiegelman, S., and Schlom, J. (1974). *Proc. Natl. Acad. Sci. U.S.A.* **71,** 4975–4979.
Croshaw, J. E., Jr., Abt, D. A., Marshak, R. R., Hare, W. C. D., Switzer, J. W., Ipsen, J., and Dutcher, R. M. (1963). *Ann. N.Y. Acad. Sci.* **108,** 1193–1202.
Cuatico, W., Cho, J. R., and Spiegelman, S. (1973). *Proc. Natl. Acad. Sci. U.S.A.* **70,** 2789–2793.
Cuatico, W., Cho, J. R., and Spiegelman, S. (1974). *Proc. Natl. Acad. Sci. U.S.A.* **71,** 3304–3308.
Dalton, A. J. (1972a). *Cancer Res.* **32,** 1351–1353.
Dalton, A. J. (1972b). *J. Natl. Cancer Inst.* **48,** 1095–1099.
Dalton, A. J., and Haguenau, F. (eds.) (1973). "Ultrastructure of Animal Viruses and Bacteriophages: An Atlas." Academic Press, New York.
Dalton, A. J., Heine, U. I., and Melnick, J. L. (1975). *J. Natl. Cancer Inst.* **55,** 941–943.
Daniel, M. D., Melendez, L. V., Hunt, R. D., King, N. W., and Williamson, M. E. (1970). *Bacteriol. Proc.* p. 195.
Das, M. R., Sadasivan, E., Koshy, B., Vaidya, A. B., and Sirsat, S. M. (1972). *Nature (London), New Biol.* **239,** 92–95.
DeHarven, E. (1974). *Adv. Virus Res.* **19,** 221–264.
Deinhardt, F., Wolfe, L. G., Theilen, G. H., and Snyder, S. P. (1970). *Science* **167,** 881.
de Thé, G. (1972). *In* "Oncogenesis and Herpesviruses" (P. M. Biggs, G. de Thé, and L. N. Paynne, eds.), pp. 275–284. Int. Agency Res. Cancer, Lyon, France.
de Thé, G., and Geser, A. (1974). *Cancer Res.* **34,** 1194–1206.
Dickson, C., and Skehel, J. J. (1974). *Virology* **58,** 387–395.
Dion, A. S., Vaidya, A. B., Fout, G. S., and Moore, D. H. (1974). *J. Virol.* **14,** 40–46.
Dmochowski, L. (1954). *J. Natl. Cancer Inst.* **15,** 785–790.
Dmochowski, L. (1958). *Int. Symp. Mammary Cancer, Proc., 2nd, 1957* pp. 655–708.
Dmochowski, L. (1960a). *Prog. Med. Virol.* **3,** 363–494.
Dmochowski, L. (1960b). *Cancer Res.* **20,** 977–1015.
Dmochowski, L. (1963). *Prog. Exp. Tumor Res.* **3,** 35–147.
Dmochowski, L. (1965a). *Natl. Cancer Conf., Proc., 5th, 1964* pp. 57–96.
Dmochowski, L. (1965b). *In* "Disease of Poultry" (H. E. Biester and L. H. Schwarte, eds.), 5th ed., pp. 568–604. Iowa State Coll. Press, Ames.
Dmochowski, L. (1968). *In* "Perspectives in Leukemia" (D. W. Dameshek and R. M. Dutcher, eds.), pp. 34–63. Grune & Stratton, New York.
Dmochowski, L. (1971). *In* "Oncology 1970" (R. L. Clark, ed.), pp. 134–145. Yearbook Medical Publishing Co., Chicago.

Dmochowski, L. (1972). *Hosp. Pract.* **7,** 73–81.
Dmochowski, L. (1973). *Bibl. Haematol. (Basel)* **39,** 1194–1207.
Dmochowski, L., and Bowen, J. M. (1973). *Natl. Cancer Conf., Proc., 7th* pp. 697–710. 697–710.
Dmochowski, L., and Bowen, J. M. (1974). *Am. J. Clin. Pathol.* **62,** 167–172.
Dmochowski, L., and Grey, C. E. (1957). *Tex. Rep. Biol. Med.* **15,** 704–756.
Dmochowski, L., and Grey, C. E. (1958). *Blood* **13,** 1017–1042.
Dmochowski, L., and Passey, R. D. (1952). *Ann. N.Y. Acad. Sci.* **54,** 1035–1066.
Dmochowski, L., Gross, L., and Padgett, F. (1962). *Proc. Soc. Exp. Biol. Med.* **110,** 504–508.
Dmochowski, L., Grey, C. E., Langford, P. L., Williams, W. C., Sykes, J. A., Young, E. L., and Migliore, P. J. (1967a). *In* "Carcinogenesis, A Broad Critique," pp. 211–256. Williams & Wilkins, Baltimore, Maryland.
Dmochowski, L., Yumoto, T., Grey, C. E., Hales, R. L., Langford, P. L., Taylor, H. G., Freireich, E. J., Schullenberger, C. C., Shively, J. A., and Howe, C. D. (1967b). *Cancer* **20,** 760–777.
Dmochowski, L., Seman, G., Myers, B., and Gallager, H. S. (1968). *Med. Rec. Ann.* **61,** 384–388.
Dmochowski, L., Seman, G., and Gallager, H. S. (1969). *Cancer* **24,** 1241–1249.
Dmochowski, L., Priori, E. S., Williams, W. C., Myers, B., and Bowen, J. M. (1973). *Proc. Symp. Fundam. Res. Mammary Tumors, 1972* pp. 351–360.
Dmochowski, L., Hoshino, M., Shigematsu, T., Hiraki, S., and Priori, E. S. (1974). *In* "Viral Immunodiagnosis" (E. Kurstak and R. Morisset, eds.), pp. 183–213. Academic Press, New York.
Dmochowski, L., Maruyama, K., Ohtsuki, Y., Seman, G., Bowen, J. M., Newton, W. A., and Johnson, D. E. (1975). *Cancer Chemother. Rep.* **59,** 17–31.
Dmochowski, L., Bowen, J. M., Myers, B., Priori, E. S., Miller, M. F., Seman, G., Chan, J. C., Dodson, M. L., and Scanlon, M. (1976). *Bibl. Haematol. (Basel)* **43,** 417–424.
Duesberg, P. H. (1968). *Proc. Natl. Acad. Sci. U.S.A.* **60,** 1511–1558.
Duesberg, P. H., and Canaani, E. (1970). *Virology* **42,** 783–788.
Duesberg, P. H., and Vogt, P. K. (1970). *Proc. Natl. Acad. Sci. U.S.A.* **67,** 1673–1680.
Duesberg, P. H., and Vogt, P. K. (1973). *Virology* **54,** 207–219.
Duesberg, P. H., Martin, G. S., and Vogt, P. K. (1970). *Virology* **41,** 631–646.
Duesberg, P. H., von der Helm, K., and Canaani, E. (1971). *Proc. Natl. Acad. Sci. U.S.A.* **68,** 2505–2509.
Dutcher, R. M., Larkin, E. P., and Marshak, R. R. (1964). *J. Natl. Cancer Inst.* **33,** 1056–1064.
Dutta, S. K., Larson, V. L., Sorenson, D. K., Perman, V., Weber, A. F., Hammer, R. F., and Shope, R. E. (1970). *Bibl. Haematol. (Basel)* **36,** 548–554.
East, J. L., Allen, P. T., Knesek, J. E., Chan, J. C., Bowen, J. M., and Dmochowski, L. (1973). *J. Virol.* **11,** 709–720.
Eckner, R. J., Priori, E. S., Mirand, E. A., and Dmochowski, L. (1974). *Cancer Res.* **34,** 2521–2529.
Elliott, A. Y., Fraley, E. E., Castro, A. E., Cleveland, P., Hakala, T. R., and Stein, N. (1973a). *Surgery* **74,** 46–50.
Elliott, A. Y., Fraley, E. E., Cleveland, P., Castro, A. E., and Stein, N. (1973b). *Science* **179,** 393–395.
Elliott, A. Y., Cleveland, P., Cervenka, J., Castro, A. E., Stein, N., Hakala, T. R., and Fraley, E. E. (1974). *J. Natl. Cancer Inst.* **53,** 1341–1349.
Engle, G. C., Shirahama, S., and Dutcher, R. M. (1969). *Cancer Res.* **29,** 603–609.

Epstein, M. A. (1962). *J. Cell Biol.* **12,** 589–597.
Epstein, M. A., and Achong, B. G. (1970). *In* "Burkitt's Lymphoma" (D. P. Burkitt and D. H. Wright, eds.), pp. 231–248. Livingstone, Edinburgh.
Epstein, L. B., and Brecher, G. (1965). *Blood* **25,** 197–203.
Epstein, M. A., Achong, B. G., and Barr, Y. M. (1964). *Lancet* **1,** 702–703.
Epstein, M. A., Hunt, R. D., and Rabin, H. (1973a). *Int. J. Cancer* **12,** 309–318.
Epstein, M. A., Rabin, H., Ball, G., Rickinson, A. B., Jarvis, J., and Melendez, L. V. (1973b). *Int. J. Cancer* **12,** 319–332.
Epstein, M. A., Achong, B. G., and Ball, G. (1974). *J. Natl. Cancer Inst.* **53,** 681–688.
Erikson, E., and Erikson, R. L. (1971). *J. Virol.* **8,** 254–256.
Essex, M., Sliski, A., Hardy, W. D., Jr., and Cotter, S. M. (1976). *Cancer Res.* **36,** 640–645.
Evans, A. S., and Niederman, J. C. (1972). *In* "Oncogenesis and Herpesviruses" (P. M. Biggs, G. de Thé, and I. N. Payne, eds.), pp. 315–356. Int. Agency Res. Cancer, Lyon France.
Evans, D. L., Barnett, J. W., and Dmochowski, L. (1973). *Tex. Rep. Biol. Med.* **31,** 755–770.
Falk, L. A. (1974). *Lab. Anim. Sci.* **24,** 182–192.
Falk, L. A., Wolfe, L. G., Hoekstra, J., and Deinhardt, F. (1972). *J. Natl. Cancer Inst.* **48,** 523–530.
Falk, L. A., Wright, J., Deinhardt, F., Wolfe, L. G., Schaffer, P., and Benyesh-Melnick, M. (1976). *Cancer Res.* **6,** 707–710.
Fanshier, L., Garapin, A. C., McDonnell, J., Faras, A., Levinson, W. E., and Bishop, J. M. (1971). *J. Virol.* **7,** 77–86.
Faras, A. J., Fanshier, L., Garapin, A. C., Levinson, W. E., and Bishop, J. M. (1971). *J. Virol.* **7,** 539–548.
Faras, A. J., Taylor, J. M., McDonnell, J. P., Levinson, W. E., and Bishop, J. M. (1972). *Biochemistry* **11,** 2334–2342.
Faras, A. J., Garapin, A. C., Levinson, W. E., Bishop, J. M., and Goodman, H. M. (1973). *J. Virol.* **12,** 334–342.
Feldman, D. G., and Gross, L. (1966). *Cancer Res.* **26,** 412–426.
Feldman, D. G., and Gross, L. (1971). *Cancer Res.* **31,** 1261–1267.
Feldman, D. G., Ehrenreich, T., and Gross, L. (1974). *J. Natl. Cancer Inst.* **53,** 1843–1846.
Feldman, S. P., Schlom, J., and Spiegelman, S. (1973). *Proc. Natl. Acad. Sci. U.S.A.* **70,** 1976–1980.
Feller, W. F., and Chopra, H. C. (1968). *J. Natl. Cancer Inst.* **40,** 1359–1365.
Feller, W. F., and Chopra, H. C. (1969). *Cancer* **24,** 1250–1254.
Feller, W. F., Chopra, H. C., and Bepko, F. (1967). *Surgery* **62,** 750–755.
Ferrer, J. F., Abt, D. A., Bhatt, D. M., and Marshak, R. R. (1974). *Cancer Res.* **34,** 893–900.
Fine, D. L., Pienta, R. J., Malan, L. B., Kubicek, M. T., Bennett, D. G., Landon, J. C., Valerio, M. G., West, D. M., Fabrizio, D. A., and Chopra, H. C. (1974). *J. Natl. Cancer Inst.* **52,** 1135–1142.
Fink, M. A., Malmgren, R. A., Rauscher, F. J., Orr, H. C., and Karon, M. (1964). *J. Natl. Cancer Inst.* **33,** 581–588.
Fleissner, E. (1971). *J. Virol.* **8,** 778–785.
Foá, C., Foá, J., and Carcassonne, Y. (1976). *Cancer* **37,** 1718–1724.
Fogel, M., and Sachs, L. (1964). *Exp. Cell Res.* **34,** 448–462.
Fong, C. K. Y., and Hsiung, G. D. (1976). *Virology* **70,** 385–398.
Fraser, M. J., Robin, E. Z., and Allen, G. (1970). *Can. J. Biochem.* **48,** 501–509.

Frenkel, N., Roizman, B., Cassai, E., and Nahmias, A. (1972). *Proc. Natl. Acad. Sci. U.S.A.* **69,** 3784–3789.

Fucillo, D. A., Moder, F. L., Catalano, L. W., Vincent, M. M., and Sever, J. L. (1970). *Proc. Soc. Exp. Biol. Med.* **133,** 735–739.

Fujinaga, K., Parsons, J. T., Beard, J. W., Beard, D., and Green, M. (1970). *Proc. Natl. Acad. Sci. U.S.A.* **67,** 1432–1439.

Gall, J. G., and Pardue, M. L. (1971). *In* "Methods in Enzymology" (K. Moldave and L. Grossman, eds.), Vol. 21, Part D, pp. 470–480. Academic Press, New York.

Gallagher, R. E., and Gallo, R. C. (1975). *Science* **187,** 350–353.

Gallagher, R. E., Salahuddin, S. Z., Hall, W. T., McCredie, K. B., and Gallo, R. C. (1975). *Proc. Natl. Acad. Sci. U.S.A.* **72,** 4137–4141.

Gallbraith, P., Mitus, W. J., Gollerkeri, M., and Dameshek, W. (1963). *Blood* **22,** 630–638.

Gallo, R. C. (1972). *Blood* **39,** 117–137.

Gallo, R. C., Miller, N. R., Saxinger, W. C., and Gillespie, D. (1973). *Proc. Natl. Acad. Sci. U.S.A.* **70,** 3219–3224.

Gallo, R. C., Sarin, P. S., Sarngadharan, M. G., Reitz, M. S., Smith, R. G., and Bobrow, S. N. (1974). *Collect. Pap., Annu. Symp. Fundam. Cancer Res.* **25,** 477–494.

Gallo, R. C., Gallagher, R. E., Miller, N. R., Mondal, H., Saxinger, W. C., Mayer, R. J., Smith, R. G., and Gillespie, D. H. (1975). *Cold Spring Harbor Symp. Quant. Biol.* **39,** 933–961.

Garapin, A. C., Fanshier, L., Leong, J. A., Jackson, J., Levinson, W., and Bishop, J. M. (1973). *J. Virol.* **7,** 227–232.

Gardner, M. B., Henderson, B. E., Rongey, R. W., and Huebner, R. J. (1973a). *J. Natl. Cancer Inst.* **50,** 719–734.

Gardner, M. B., Henderson, B. E., Estes, J. D., Menck, H., Parker, J. C., and Huebner, R. J. (1973b). *J. Natl. Cancer Inst.* **50,** 1571–1579.

Gardner, M. B., Rongey, R. W., Johnson, E. Y., DeJournett, R., and Huebner, R. J. (1971). *J. Natl. Cancer Inst.* **47,** 561–565.

Gazzolo, L., de Thé, G., Vuillaume, M., and Ho, H. C. (1972). *J. Natl. Cancer Inst.* **48,** 73–86.

Geder, L., and Skinner, G. R. B. (1971). *J. Gen. Virol.* **12,** 179–182.

Geering, G., Aoki, T., and Old, L. J. (1970). *Nature (London)* **226,** 265–266.

Gelb, L., Aaronson, S. A., and Martin, M. (1971). *Science* **172,** 1353–1355.

Gelderblom, H., and Schwartz, H. (1976). *J. Natl. Cancer Inst.* **56,** 635–637.

Gelderblom, H., Bauer, H., Bolognesi, D. P., and Frank, H. (1972). *Zentralbl. Bakteriol., Parasitenkd., Infekionskr. Hyg., Abt. 1:* Orig., *Reihe A* **220,** 79–90.

Gelderblom, H., Bauer, H., Ogura, H., Wigand, R., and Fischer, A. B. (1974). *Int. J. Cancer* **13,** 246–253.

Gerard, G., Rottman, F., and Green, M. (1974). *Biochemistry* **13,** 1632–1641.

Gergely, L., Klein, G., and Ernberg, I. (1971). *Virology* **45,** 10–21.

Gerwin, I. B., Ebert, P., Chopra, H. C., Smith, S. G., Kvedar, J., Albert, S., and Brennan, M. (1973). *Science* **180,** 198–201.

Gilden, R. V., and Oroszlan, S. (1972). *Proc. Natl. Acad. Sci. U.S.A.* **69,** 1021–1025.

Gilden, R. V., Long, C. W., Hanson, M. Toni, R., Charman, H. P., Oroszlan, S., Miller, J. M., and Van der Maaten, M. J. (1975). *J. Gen. Virol.* **29,** 305–314.

Gillespie, D., and Spiegelman, S. (1965). *J. Mol. Biol.* **12,** 829–842.

Gillespie, D. S., Marshall, S., and Gallo, R. C. (1972). *Nature (London), New Biol.* **244,** 51–54.

Gold, P., and Freedman, S. O. (1965). *J. Exp. Med.* **121,** 439–462.

Goldberg, R. J., and Gravell, M. (1976). *Cancer Res.* **36,** 795–799.
Goldberg, R. J., Scolnick, E. M., Parks, W. P., Yakovleva, L. A., and Lapin, B. A. (1974). *Int. J. Cancer* **14,** 722–730.
Goldenberg, G. J., and Brandes, L. J. (1976). *Cancer Res.* **36,** 720–723.
Goodman, N. C., and Spiegelman, S. (1971). *Proc. Natl. Acad. Sci. U.S.A.* **68,** 2203–2206.
Goodman, N. C., Ruprecht, R. M., Sweet, R. W., Massey, R., Deinhardt, F., and Spiegelman, S. (1973). *Int. J. Cancer* **12,** 752–760.
Gotlieb-Stematsky, T., Vonsover, A., Ramot, B., Zaizov, R., Nordan, U., Aghai, E., Kende, G., and Modan, M. (1975). *Cancer* **36,** 1640–1645.
Granboulan, N., Huppert, J., and Lacour, F. (1966). *J. Mol. Biol.* **16,** 571–575.
Granoff, A. (1973). *Cancer Res.* **33,** 1431–1433.
Gravell, M. (1971). *Virology* **43,** 730–733.
Greaves, F. M., Brown, G., and Rickinson, A. B. (1975). *Clin. Immunol. Immunopathol.* **3,** 514–524.
Green, M., and Gerard, G. F. (1974). *Prog. Nucleic Acid Res. Mol. Biol.* **14,** 187–334.
Green, R. W., and Bolognesi, D. P. (1974). *Anal. Biochem.* **57,** 108–117.
Green, R. W., Bolognesi, D. P., Schafer, W., Pister, L., Hunsmann, G., and De Noronha, F. (1973). *Virology* **56,** 565–579.
Greenland, T. B., de Thé, G., and Day, W. E. (1972). *In* "Oncogenesis and Herpesviruses" (P. M. Biggs, G. de Thé, and L. N. Payne, eds.), pp. 302–307. Int. Agency Res. Cancer, Lyon, France.
Gregoriades, A., and Old, L. J. (1969). *Virology,* **37,** 189–202.
Griffin, E. R., Wright, D. H., Bell, T. M., and Ross, M. G. R. (1966). *Eur. J. Cancer* **2,** 353–358.
Gross, L. (1970). "Oncogenic Viruses." Pergamon, Oxford.
Gulati, S. C., Axel, R., and Spiegelman, S. (1972). *Proc. Natl. Acad. Sci. U.S.A.* **69,** 2020–2024.
Haguenau, F. (1959). *Bull. Assoc. Fr. Cancer* **46,** 177–211.
Hall, W. T., and Schidlovsky, G. (1976). *J. Natl. Cancer Inst.* **56,** 639–642.
Hehlmann, R. (1976). *Curr. Top. Microbiol. Immunol.* **73,** 141–215,
Hehlmann, R., Kufe, D., and Spiegelman, S. (1972a). *Proc. Natl. Acad. Sci. U.S.A.* **69,** 435–439.
Hehlmann, R. Kufe, D., and Spiegelman, S. (1972b). *Proc. Natl. Acad. Sci. U.S.A.* **69,** 1727–1731.
Hehlmann, R., Baxt, W., Kufe, D., and Spiegelman, S. (1973). *Am. J. Clin. Pathol.* **60,** 65–79.
Heine, J. W., Spear, P. G., and Roizman, B. (1972). *J. Virol.* **9,** 431–439.
Henderson, B. E., Gardner, M. B., Gilden, R. V., Estes, J. D., and Huebner, R. J. (1974). *J. Natl. Cancer Inst.* **53,** 1091–1092.
Henle, E., and Henle, G. (1970). *J. Infect. Dis.* **121,** 303–310.
Henle, E., and Henle, G. (1974). *Cancer* **34,** 1368–1374.
Henle, G., and Henle, W. (1966). *J. Bacteriol.* **91,** 1248–1256.
Henle, G., Henle, W., and Diehl, V. (1968). *Proc. Natl. Acad. Sci. U.S.A.* **59,** 94–101.
Henle, G., Henle, W., Klein, G., Gunven, P., Clifford, P., Morrow, R. H., and Ziegler, J. L. (1971a). *J. Natl. Cancer Inst.* **46,** 861–871.
Henle, G., Henle, W., and Klein, G. (1971b). *Int. J. Cancer* **8,** 272–282.
Henle, W., and Henle, G. (1972). *In* "Oncogenesis and Herpesviruses" (P. M. Biggs, G. de Thé and L. N. Payne, eds.), pp. 269–274. Int. Agency Res. Cancer, Lyon, France.
Henle, W., Henle, G., Ho, H. C., Burtin, P., Cachin, Y., Clifford, P., DeSchryver, A., de Thé, G., Diehl, V., and Klein, G. (1970). *J. Natl. Cancer Inst.* **44,** 225–231.

Herrera, I., Valenciano, L., Sanchez-Garrido, F., and Botella-Llusia, J. (1974). *Acta Cytol.* **18,** 45–50.

Hilgers, J., Williams, W. C., Myers, B., and Dmochowski, L. (1971). *Virology* **45,** 470–483.

Hinze, H. C., and Wegner, D. L. (1973). *Cancer Res.* **33,** 1434–1435.

Hirshaut, Y., Pei, D. T., Marcove, R. C., Mukherji, B., Spielvogel, A. R., and Essner, E. (1974a). *N. Engl. J. Med.* **291,** 1103–1107.

Hirshaut, Y., Reagan, R. L., Perry, S., DeVita, V., and Barile, M. F. (1974b). *Cancer* **34,** 1080–1089.

Ho, H. C. (1972). *Adv. Cancer Res.* **15,** 57–92.

Hollinshead, A. C., Lee, O. B., Chrétien, P. B., Tarpley, J. L., Rawls, W. E., and Adam, E. (1973). *Science* **182,** 713–715.

Hollinshead, A. C., Farro, G., Foster, W. A., Jr., Seigel, L. J., and Jaffurs, W. (1974). *Cancer Res.* **34,** 1122–1125.

Hollinshead, A. C., Chrétien, P. B., Lee, O. B., Tarpley, J. L., Kerney, S. E., Silverman, N. A., and Alexander, J. C. (1976). *Cancer Res.* **36,** 821–828.

Honess, R. W., Powell, K. L., Robinson, D. J., Sim, C., and Watson, D. H. (1974). *J. Gen. Virol.* **22,** 159–169.

Hooks, J., Gibbs, C. J., Chopra, H. C., Lewis, D., and Gajdusek, D. C. (1972). *Science* **176,** *1420*–1422.

Hoshino, M., and Dmochowski, L. (1973). *Cancer Res.* **33,** 2551–2561.

Hsiung, G. D. (1972). *J. Natl. Cancer Inst.* **49,** 567–570.

Hsiung, G. D., Kaplow, L. S., and Booss, J. (1971). *Am. J. Epidemiol.* **93,** 298–307.

Hsiung, G. D., Fong, C. K. Y., and Gross, P. A. (1973). *Cancer Res.* **33,** 1436–1442.

Huang, E. S., Chen, S. T., and Pagano, J. S. (1973). *J. Virol.* **12,** 1473–1481.

Huebner, R. J., and Todaro, G. J. (1969). *Proc. Natl. Acad. Sci. U.S.A.* **64,** 1087–1094.

Hull, R. N. (1973). *In* "The Herpesviruses" (A. S. Kaplan, ed.), pp. 389–426. Academic Press, New York.

Hunt, R. D., and Melendez, L. V. (1969). *Lab. Anim. Care* **19,** 221–234.

Hurwitz, J., and Leis, J. P. (1972). *J. Virol.* **9,** 116–129.

Ihle, J. N., Hanna, M. G., Schafer, W., Hunsmann, G., Bolognesi, D. P., and Huper, G. (1975). *Virology* **63,** 60–67.

Ilyin, K. V., Bykovsky, A. F., and Zhdanov, V. M. (1973). *Cancer* **32,** 89–96.

Jarrett, W. F. H., Martin, W. B., Crighton, J. W., Dalton, R. G., and Stewart, M. F. (1964). *Nature (London)* **202,** 566–567.

Jarvis, J. E., Ball, G., Rickinson, A. B., and Epstein, M. A. (1974). *Int. J. Cancer* **14,** 716–721.

Jensen, E. M., and Schidlovsky, G. (1964). *J. Natl. Cancer Inst.* **33,** 1029–1033.

Jensen, E. M., Zelljadt, I., Chopra, H. C., and Mason, M. M. (1970). *Cancer Res.* **30,** 2388–2393.

Jondal, M., and Klein, G. (1973). *J. Exp. Med.* **138,** 1365–1378.

Josey, W., Nahmias, A., and Naib, Z. (1972). *Obstet. & Gynecol. Surv.* **27,** 295–302.

Kacian, D. L., Watson, K. F., Burny, A., and Spiegelman, S. (1971). *Biochim. Biophys. Acta* **246,** 365–383.

Kalter, S. S., and Heberling, R. L. (1971). *Bacteriol. Rev.* **35,** 310–364.

Kalter, S. S., Helmke, R. J., Heberling, R. L., Panigel, M., Fowler, A. K., Strickland, J. E., and Hellman, A. (1973a). *J. Natl. Cancer Inst.* **50,** 1081–1084.

Kalter, S. S., Helmke, R. J., Panigel, M., Heberling, R. L., Felsburg, P. J., and Axelrod, L. R. (1973b). *Science* **179,** 1332–1333.

Kalter, S. S., Heberling, R. L., Helmke, R. J., Panigel, M., Smith, G. C., Kraemer, D. C., Hellman, A., Fowler, A. K., and Strickland, J. E. (1974). *In* "Comparative Leukemia

Research 1973" (Y. Ito and R. M. Dutcher, eds.), pp. 391–401. Univ. Tokyo Press, Tokyo.
Kalter, S. S., Heberling, R. L., Smith, G. C., Panigel, M., Kraemer, D. C., Helmke, R. J., and Hellman, A. (1975a). *J. Natl. Cancer Inst.* **54,** 1173–1176.
Kalter, S. S., Heberling, R. L., Smith, G. C., and Helmke, R. J. (1975b). *J. Natl. Cancer Inst.* **55,** 735–736.
Kaplan, A. S. (1973). *Cancer Res.* **33,** 1393–1398.
Kaufman, H. E., Ecker, S., and Vincent, M. M. (1970). *Science* **170,** 540–542.
Kaufman, R. H., Gardner, H. L., Rawls, W. E., Dixon, R. E., and Young, R. L. (1973). *Cancer Res.* **33,** 1446–1451.
Kawakami, T. E., Moore, A. L., Theilen, G. H., and Munn, R. J. (1970). *Bibl. Haematol.* **36,** 471–475.
Kawakami, T. G., Theilen, G. H., Dungworth, D. L., Munn, R. J., and Beall, S. G. (1967). *Science* **158,** 1049–1050.
Kawakami, T. G., Huff, S. D., Buckley, P. M., Dungworth, D. L., Snyder, S. P., and Gilden, R. V. (1972). *Nature (London)* **235,** 170–171.
Kawakami, T. G., Buckley, P. M., McDowell, T. S., and DePaoli, A. (1973). *Nature (London), New Biol.* **246,** 105–107.
Kessler, I. I. (1976). *Cancer Res.* **36,** 783–791.
Kessler, I. I., Kulcar, Z., Rawls, W. E., Smerdel, S., Strand, M., and Lilienfeld, A. M. (1974). *J. Natl. Cancer Inst.* **52,** 369–376.
Kieff, E. D., Bachenheimer, S. L., and Roizman, B. (1971). *J. Virol.* **8,** 125–132.
Kirkwood, J., Geering, G., and Old, L. J. (1972). *In* "Oncogenesis and Herpesviruses" (P. M. Biggs, G. de Thé and L. N. Payne, eds.), p. 479. Int. Agency Res. Cancer, Lyon, France.
Klein, G. (1969). *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **28,** 1739–1753.
Klein, G. (1971). *Adv. Immunol.* **14,** 187–250.
Klein, G. (1975). *N. Engl. J. Med.* **293,** 1353–1357.
Klein, G., Clifford, P., Klein, E., and Stjernsward, J. (1966). *Proc. Natl. Acad. Sci. U.S.A.* **55,** 1628–1635.
Klein, G., Pearson, G., Nadkarni, J. S., Nadkarni, J. J., Klein, E., Henle, G., Henle, W., and Clifford, P. (1968). *J. Exp. Med.* **128,** 1011–1020.
Klein, G., Geering, G., Old, L. J., Henle, G., Henle, W., and Clifford, P. (1970). *Int. J. Cancer* **5,** 185–194.
Klein, G., Pearson, G., Rabson, A., Ablashi, D., Falk, L. A., Wolfe, L., Deinhardt, F., and Rabin, H. (1973). *Int. J. Cancer* **12,** 270–289.
Klein, G., Giovanella, B. C., Lindahl, T., Fialkow, P. J., Singh, S., and Stehlin, J. S. (1974). *Proc. Natl. Acad. Sci. U.S.A.* **71,** 4737–4741.
Kotler, M., Weinberg, E., Haspel, O., Olshevsky, U., and Becker, Y. (1973). *Nature (London), New Biol.* **244,** 197–200.
Kovi, J., Tillman, R. L., and Lee, S. M. (1974). *Am. J. Clin. Pathol.* **61,** 702–710.
Kufe, D., Hehlmann, R., and Spiegelman, S. (1972). *Science* **175,** 182–185.
Kufe, D., McGrath, I. T., Ziegler, J. L., and Spiegelman, S. (1973a). *Proc. Natl. Acad. Sci. U.S.A.* **70,** 737–741.
Kufe, D., Peters, W. P., and Spiegelman, S. (1973b). *Proc. Natl. Acad. Sci. U.S.A.* **70,** 3810–3814.
Kurstak, E., and Kurstak, C. (1974). *In* "Viral Immunodiagnosis" (E. Kurstak and R. Morisset, eds.), pp. 3–30. Academic Press, New York.
Kurstak, E., and Morisset, R., eds. (1974). "Viral Immunodiagnosis." Academic Press, New York.

Kurstak, E., Tijssen, P., Kurstak, C., and Morisset, R. (1975). *Ann. N.Y. Acad. Sci.* **254,** 369–384.

Laird, H. M., Jarrett, O., Crighton, J. W., and Jarrett, W. F. H. (1968). *J. Natl. Cancer Inst.* **41,** 867–878.

Lange, J., Frank, H., Hansmann, G., Moenning, V., Wollmann, R., and Schafer, W. (1973). *Virology* **53,** 457–462.

Lapin, B. A. (1975). *In* "Comparative Leukemia Research 1973" (Y. I. Ito and R. M. *Dutcher, eds.), pp. 75–84.* Univ. of Tokyo.

Lapin, B. A., Yakovleva, L. A., Indhziia, L. V., Agrba, V. Z., Tsiripova, G. S., Voevodin, A. F., Ivanov, M. T., and Djatchenko, A. G. (1975). *Proc. R. Soc. Med.* **68,** 141–145.

Lasfargues, E. Y., Kramarsky, B., Lasfargues, J. C., and More, D. H. (1974). *J. Natl. Cancer Inst.* **53,** 1831–1833.

Lasfargues, E. Y., Lasfargues, J. C., and Moore, D. H. (1976). *Proc. Am. Assoc. Cancer Res.* **17,** 33 (Abstract No. 130).

Laufs, R., and Steinke, H. (1976). *Cancer Res.* **36,** 704–706.

Leis, J. P., and Hurwitz, J. (1972). *J. Virol.* **9,** 130–142.

Levine, P. H. (1972). *In* "Oncogenesis and Herpesviruses" (P. M. Biggs, G. de Thé, and L. N. Payne, eds.), pp. 384–389. Int. Agency Res. Cancer, Lyon, France.

Levine, P. H., and Cho, B. R. (1974). *Cancer Res.* **34,** 1219–1221.

Levine, P. H., Horoszewicz, J. S., Chai, J. T., Ellison, R. R., and Holland, J. F. (1967). *Cancer* **21,** 563–577.

Levy, J. A. (1973). *Science* **182,** 1151–1153.

Lewis, B. J., Abrell, J. W., Smith, R. G., and Gallo, R. C. (1974). *Science* **183,** 867–869.

Lieber, M. M., Benveniste, R. E., Livingston, D. M., and Todaro, G. J. (1973). *Science* **182,** 56–59.

Lieber, M. M., Sherr, C. J., Benveniste, R. E., and Todaro, G. J. (1975). *Virology* **66,** 616–619.

Long, C., Sachs, R., Norwell, J., Huebner, V., Hatanaka, M., and Gilden, R. (1973). *Nature (London) New Biol.* **241,** 147–149.

Louie, S., Curtis, J. E., and McCulloch, E. A. (1976). *Proc. Am. Assoc. Cancer Res.* **17,** 33 (Abstract No. 133).

Lowry, S. P., Bronson, D. L., and Rawls, W. E. (1971). *J. Gen. Virol.* **11,** 47–51.

Lowy, D. R., Chattopadhyay, S. K., Teich, N. M., Rowe, W. P., and Levine, A. S. (1974). *Proc. Natl. Acad. Sci. U.S.A.* **71,** 3555–3559.

Lucké, B. (1938). *J. Exp. Med.* **68,** 457–468.

Lunger, P. D., and Clark, H. F. (1974). *J. Natl. Cancer Inst.* **53,** 533–540.

Lunger, P. D., Lucas, J. C., and Shipley, F. H. (1964). *Cancer* **17,** 549–557.

Lunger, P. D., Hardy, W. D., and Clark, H. F. (1974). *J. Natl. Cancer Inst.* **52,** 1231–1235.

McAllister, R. M. (1973). *Prog. Med. Virol.* **16,** 48–85.

McAllister, R. M., Nicolson, M., Gardner, M. B., Rongey, R. W., Rasheed, S., Sarma, P. S., Huebner, R. J., Hatanaka, M., Oroszlan, S., Gilden, R. V., Kabigting, A., and Vernon, L. (1972). *Nature (London), New Biol.* **235,** 3–6.

McCarthy, B. J., and Church, R. B. (1970). *Annu. Rev. Biochem.* **39,** 131–150.

McCarthy, K., and Tosolini, F. A. (1975). *Proc. R. Soc. Med.* **68,** 145–150.

McCormick, J. J., Larson, L. J., and Rich, M. A. (1974). *Nature (London)* **251,** 737–740.

McDonough, S. K., Larsen, S., Brodey, R. S., Stock, N. D., and Hardy, W. D. (1971). *Cancer Res.* **31,** 953–956.

McDugald, L. V., Panem, S., and Kirsten, W. H. (1970). *Int. J. Cancer* **5,** 64–71.

McGrath, C. M., Grant, P. M., Soule, H. D., Glancy, T., and Rich, M. A. (1974). *Nature (London)* **252,** 247–250.

McKissick, G. E., and Lamont, P. H. (1970). *J. Virol.* **5,** 247–257.
Maisel, J., Klement, V., Lai, M., Ostertag, W., and Duesberg, P. H. (1973). *Proc. Natl. Acad. Sci. U.S.A.* **70,** 3536–3540.
Mak, T. W., Aye, M. T., Messner, H., Sheinin, R., Till, J. E., and McCulloch, E. A. (1974a). *Br. J. Cancer* **29,** 433–437.
Mak, T. W., Manaster, J., Howatson, A. F., Till, E. A., McCulloch, E. A., and Till, J. E. (1974b). *Proc. Natl. Acad. Sci. U.S.A.* **71,** 4336.
Malmquist, W. A., Van der Maaten, M. J., and Boothe, A. D. (1969). *Cancer Res.* **29,** 188–200.
Malonov, G., and Malonova, Y. (1972). *Nature (London)* **237,** 33–34.
Mangel, W. F., Delius, H., and Duesberg, P. H. (1974). *Proc. Natl. Acad. Sci. U.S.A.* **71,** 4541–4545.
Marek, J. (1907). *Dtsch. Tieraerztl. Wochenschr.* **15,** 417–421.
Marshak, R. R., Hare, W. C. D., Abt, D. A., Croshaw, J. E., Jr., Switzer, J. W., Ipsen, J., Dutcher, R. M., and Martin, J. E. (1963). *Ann. N.Y. Acad. Sci.* **108,** 1284–1301.
Maruyama, K., Dmochowski, L., Bowen, J. M., and Hales, R. (1968). *Tex. Rep. Biol. Med.* **26,** 545–565.
Mason, M. M., Bogden, A. E., Ilievski, V., Esber, H. J., Baker, J. R., and Chopra, H. C. (1972). *J. Natl. Cancer Inst.* **48,** 1323–1331.
Mathé, G., and Seman, G. (1962). *Rev. Prat.* **12,** 2687–2691.
Mayer, R. J., Smith, R. G., and Gallo, R. D. (1974). *Science* **185,** 864–867.
Melendez, L. V., Daniel, M. D., Hunt, R. D., and Garcia, F. G. (1968). *Lab. Anim. Care* **18,** 374–381.
Melendez, L. V., Hunt, R. D., Daniel, M. D., Fraser, C. E. O., Barahona, H. H., Garcia, F. G., and King, N. W. (1972a). *In* "Oncogenesis and Herpesviruses" (P. M. Biggs, G. de Thé, and L. N. Payne, eds.), pp. 451–461. Int. Agency Res. Cancer, Lyon, France.
Melendez, L. V., Hunt, R. D., King, N. W., Barahona, H. H., Daniel, M. D., Fraser, C. E. O., and Garcia, F. G. (1972b). *Nature (London), New Biol.* **235,** 182–184.
Melnick, J. L., and McCombs, R. M. (1966). *Prog. Med. Virol.* **8,** 400–409.
Melnick, J. L., Courtney, R. J., Powell, K. L., Schaffer, P. A., Benyesh-Melnick, M., Dreesman, G. R., Anzai, T., and Adam, E. (1976). *Cancer Res.* **36,** 845–856.
Michalides, R., Spiegelman, S., and Schlom, J. (1975). *Cancer Res.* **35,** 1003–1008.
Miller, G. G., Zhdanov, V. M., Lozinsky, T. F., Volkova, M. Y., Ilyin, K. V., Golubev, D. B., Irlin, I. S., and Bykovsky, A. F. (1974). *J. Natl. Cancer Inst.* **52,** 357–364.
Miller, G. J. (1974). *J. Infect. Dis.* **130,** 187–205.
Miller, J. M., and Olson, C. (1972). *J. Natl. Cancer Inst.* **49,** 1459–1462.
Miller, J. M., and Van der Maaten, M. J. (1974). *J. Natl. Cancer Inst.* **53,** 1694–1702.
Miller, J. M., Miller, L. D., Olson, G., and Gillette, K. G. (1969). *J. Natl. Cancer Inst.* **43,** 1297–1305.
Miller, L. D., Miller, J. M., and Olson, C. (1972). *J. Natl. Cancer Inst.* **48,** 423–427.
Miller, M. F. (1974). *Princ. Tech. Electron Microsc.* **4,** 89–128.
Miller, M. F., Allen, P. T., and Dmochowski, L. (1973). *J. Gen. Virol.* **21,** 57–68.
Moennig, V., Frank, H., Hunsmann, G., Ohms, P., Schwartz, H., Schafer, W., and Strandström, H. (1974). *Virology* **57,** 179–188.
Molling, K., Bolognesi, D. P., Bauer, H., Busen, W., Plassman, H. W., and Hausen, P. (1973). *Nature (London), New Biol.* **234,** 240–243.
Mondal, H., Gallagher, R. E., and Gallo, R. C. (1975). *Proc. Natl. Acad. Sci. U.S.A.* **72,** 1194–1198.
Moore, D. H. (1963). *Nature (London)* **198,** 429–433.

Moore, D. H., Pillsbury, N., and Pullinger, B. D. (1969). *J. Natl. Cancer Inst.* **43,** 1263–1273.

Moore, D. H., Charney, J., Kramarsky, B., Lasfargues, E. Y., Sarkar, N. H., Brennan, M. J., Burrows, J. H., Sirsat, S. M., Paymaster, J. C., and Vaidya, A. B. (1971). *Nature (London)* **229,** 611–629.

Morgan, C., Rose, H. M., Holden, M., and Jones, E. P. (1959). *J. Exp. Med.* **110,** 643–656.

Morgan, C., Rose, H. M., and Mednis, B. (1968). *J. Virol.* **2,** 507–516.

Moroni, C. (1972). *Virology* **47,** 1–7.

Morton, D. L., and Malmgren, R. A. (1968). *Science* **162,** 1279–1281.

Morton, D. L., Hall, W. T., and Malmgren, R. A. (1969). *Science* **165,** 813–815.

Müller, von M., and Grossman, H. (1972). *Nature (London), New Biol.* **237,** 116–117.

Müller, von M., Zotter, S., Wagner, H., and Grossman, H. (1971). *Arch. Geschwulstforsch.* **38,** 221–231

Müller, von M., Kemmer, C., Zotter, S., Grossman, H., and Micheel, B. (1973). *Arch. Geschwulstforsch.* **41,** 100–106.

Nadel, E., Banfield, W., Burstein, S., and Tousimis, A. J. (1967). *J. Natl. Cancer Inst.* **38,** 979–982.

Nahmias, A. J., and Roizman, B. (1973). *N. Engl. J. Med.* **289,** 667–674, 719–725, and 781–789.

Nahmias, A. J., Dowdle, W. R., Naib, Z. M., Josey, W. E., and Luce, C. F. (1969). *Br. J. Vener. Dis.* **45,** 294–298.

Nahmias, A. J., Del Buono, I., Schneweis, K. E., Gordon, D. S., and Thies, D. (1971). *Proc. Soc. Exp. Biol. Med.* **138,** 21–27.

Nahmias, A. J., Shore, S. L., Kohl, S., Starr, S. E., and Ashman, R. B. (1976). *Cancer Res.* **36,** 836–844.

Naib, Z. M., Nahmias, A. J., and Josey, W. E. (1966). *Cancer* **19,** 1026–1031.

Naib, Z. M., Nahmias, A. J., Josey, W. E., and Zaki, S. A. (1973). *Cancer Res.* **33,** 1452–1463.

Nayak, D. P. (1971). *J. Virol.* **8,** 579–588.

Nazerian, K. (1973). *Cancer Res.* **33,** 1427–1430.

Nelson-Rees, W. A., and Flandermeyer, R. R. (1975). *Science* **191,** 96–98.

Nermut, M. V., Frank, H., and Schafer, W. (1972). *Virology* **49,** 345–358.

Nilsson, K., Klein, G., Henle, W., and Henle, G. (1971). *Int. J. Cancer* **8,** 443–450.

Nishioka, K., Tachibana, Y., Hirayama, T., de Thé, G., Klein, G., Takada, M., and Kawamura, A. (1971). *In* "Recent Advances in Human Tumor Virology and Immunology" (W. Nakahara *et al.*, eds.), pp. 410–420. Univ. of Tokyo Press, Tokyo.

Nonoyama, M., and Pagano, J. S. (1971). *Nature (London) New Biol.* **233,** 103–106.

Nonoyama, M., Kawai, Y., Huang, C. H., Pagano, J. S., Hirshaut, Y., and Levine, P. H. (1974). *Cancer Res.* **34,** 1228–1231.

Nooter, K., Aarssen, A. M., DeGroot, F. G., and Van Pelt, F. G. (1975). *Nature (London)* **256,** 595–597.

Nowinski, R. C., Old, L. J., Sarkar, N. H., and Moore, D. H. (1970). *Virology,* **42,** 1152–1157.

Nowinski, R. C., Edynak, E., and Sarkar, N. H. (1971a). *Proc. Natl. Acad. Sci. U.S.A.* **68,** 1608–1612.

Nowinski, R. C., Sarkar, N. H., Old. L. J., Moore, D. H., Scheer, D. I., and Hilgers, J. (1971b). *Virology* **46,** 21–38.

Nowinski, R. C., Fleissner, E., Sarkar, N. H., and Aoki, T. (1972). *J. Virol.* **9,** 359–366.

Nowinski, R. D., and Kaehler, S. L. (1974). *Science* **185,** 869–871.

O'Conor, G. T., Rappaport, H., and Smith, E. B. (1965). *Cancer* **18,** 411–417.
Ohtsuki, Y., Seman, G., Maruyama, K., Johnson, D. E., and Dmochowski, L. (1976). *Cancer* **37,** 2295–2305.
Okabe, H., Gilden, R. V., Hatanaka, M., Stephenson, J. R., Gallo, R. C., Tronick, S. T., and Aaronson, S. A. (1976). *Nature (London)* **260,** 264–266.
Old, L. J., and Boyse, E. A. (1965). *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **64,** 1009–1017.
Old, L. J., Boyse, E. A., Geering, G., and Oettgen, H. F. (1968). *Cancer Res.* **28,** 1288–1299.
Olshevsky, U., and Becker, Y. (1970). *Virology* **40,** 948–960.
Olson, C., Hoss, H. E., Miller, J. M., and Baumgartner, L. E. (1973). *J. Am. Vet. Med. Assoc.* **163,** 355–357.
Opler, S. R. (1967). *J. Natl. Cancer Inst.* **38,** 797–800.
Oriel, J. D. (1971). *Br. J. Vener. Dis.* **47,** 1–12.
Oshiro, L. S., Taylor, D. O. N., Riggs, J. L., and Lennette, E. H. (1972). *J. Natl. Cancer Inst.* **48,** 1419–1424.
O'Sullivan, B., and McLaughlin, H. (1975). *J. Ir. Med. Assoc.* **68,** 247–254.
Pacsa, A. S., Kummerlander, L., Pejtsik, B., and Pali, K. (1975). *J. Natl. Cancer Inst.* **55,** 775–781.
Pagano, J. S., and Huang, E. S. (1974). *In* "Viral Immunodiagnosis" (E. Kurstak and R. Morisset, eds.), pp. 279–299. Academic Press, New York.
Panem, S., and Kirsten, W. H. (1975). *J. Gen. Virol.* **26,** 1–14.
Panem, S., Schwartz, S. A., and Kirsten, W. H. (1973). *Bibl. Haematol. (Basel)* **39,** 281–287.
Panem, S., Prochownik, E. W., Reale, F. R., and Kirsten, W. H. (1975). *Science* **189,** 297–298.
Parks, W. P., and Todaro, G. J. (1972). *Virology* **47,** 673–683.
Parks, W. P., Scolnick, E. M., Ross, J., Todaro, J. G., and Aaronson, S. A. (1972). *J. Virol.* **9,** 110–115.
Parks, W. P., Gilden, R. V., Bykovsky, A. F., Miller, G. G. Zhdanov, V. M., Soloviev, V. D., and Scolnick, E. M. (1973). *J. Virol.* **12,** 1540–1547.
Parson, P. G., Gross, P., and Pope, J. H. (1974). *Int. J. Cancer* **3,** 379–391.
Passey, R. D., Dmochowski, L., Astbury, W. T., Reed, R., and Johnson, P. (1950). *Nature (London)* **165,** 107.
Paul, J. R., and Bunnel, W. W. (1932). *Am. J. Med. Sci.* **183,** 90.
Payne, L. N. (1972). *In* "Oncogenesis and Herpesviruses" (P. M. Biggs, G. de Thé, and L. N. Payne, eds.), pp. 21–37. Int. Agency Res. Cancer, Lyon, France.
Pearson, G. R. (1974). *Cancer Res.* **34,** 1237–1240.
Pearson, G. R., Henle, G., and Henle, W. (1971). *J. Natl. Cancer Inst.* **46,** 1243–1250.
Plummer, G. (1967). *Prog. Med. Virol.* **9,** 302–340.
Plummer, G. (1973). *Cancer Res.* **33,** 1469–1476.
Plummer, G., Waner, J. L., Phuangsab, A., and Goodheart, C. R. (1970). *J. Virol.* **5,** 51–59.
Porter, G. H., Dalton, A. J., Moloney, J. B., and Mitchell, E. Z. (1964). *J. Natl. Cancer Inst.* **33,** 547–556.
Powell, K. L., Buchan, A., Sim, C., and Watson, D. H. (1974). *Nature (London)* **249,** 360–361.
Powell, L. C., Jr. (1972). *Clin. Obstet. Gynecol.* **15,** 948–955.
Prince, A. M., and Adams, W. R. (1966). *J. Natl. Cancer Inst.* **37,** 153–166.
Priori, E. S., and Dmochowski, L. (1974). *Prog. Exp. Tumor Res.* **19,** 182–193.
Priori, E. S., Dmochowski, L., Myers, B., and Wilbur, J. R. (1971a). *Nature (London), New Biol.* **232,** 61–62.

Priori, E. S., Wilbur, J. R., and Dmochowski, L. (1971b). *J. Natl. Cancer Inst.* **46,** 1299–1308.

Priori, E. S., Anderson, D. E., Williams, W. C., and Dmochowski, L. (1972). *J. Natl. Cancer Inst.* **48,** 1131–1135.

Prochownik, E. V., and Kirsten, W. H. (1976). *Nature (London)* **260,** 64–67.

Purchase, H. G. (1976). *Cancer Res.* **36,** 696–700.

Purchase, H. G., Okazaki, W., and Burmester, B. R. (1972). *Avian Dis.* **16,** 34–44.

Rabin, H., Pearson, G. R., Klein, G., Ablashi, D. V., Wallen, W., and Cicmanec, J. (1973). *Am. J. Phys. Anthropol.* **38,** 497–500.

Rabin, H., Neubauer, R. H., Pearson, G. R. Cicmanec, J. L., Wallen, W. C., Loeb, W. F., and Valerio, M. G. (1975). J. Natl. Cancer Inst. **54,** 499–502.

Rabson, A. S., O'Conor, G. T., Lorenz, D. E., Kirschtein, R. L., Legallais, F. Y., and Tralka, T. S. (1971). *J. Natl. Cancer Inst.* **46,** 1099–1109.

Rawls, W. E., Tompkins, W. A. F., and Melnick, J. L. (1969). *Am. J. Epidermiol.* **89,** 547–554.

Rawls, W. E., Gardner, H. L., Flanders, R. W., Lowry, S. P., Kaufman, R. H., and Melnick, J. L. (1971). *Am. J. Obstet. Gynecol.* **110,** 682–689.

Rawls, W. E., Adam, E., and Melnick, J. L. (1973). *Cancer Res.* **33,** 1477–1490.

Rawls, W. E., Garfield, C. H., Seth, P., and Adam, E. (1976). *Cancer Res.* **36,** 829–835.

Reedman, B. M., and Klein, G. (1973). *Int. J. Cancer* **11,** 499–520.

Rickard, C. G., Barr, L. M., De Noronha, F., Dougherty E., and Post J. E. (1967). *Cornell Vet.* **57,** 302–307.

Rickard, C. G., Post, J. E., De Noronha, F., Dougherty, E., and Barr, L. M. (1973). In "Biohazards in Biological Research." (A. Hellman, M. R. Oxman, and R. Pollack, eds.), pp. 166–178. Cold Spring Harbor Press, Cold Spring Harbor, New York.

Riggs, J. L., Oshiro, L. S., Taylor, D. O. N., and Lennette, E. H. (1969). *Nature (London)* **222,** 1190–1191.

Ritter, H. (1965). *Dtsch. Tieraertzl. Wochenschr.* **72,** 56–60.

Robinson, W. S., and Robinson, H. L. (1971). *Virology* **44,** 457–462.

Robinson, W. S., and Robinson, H. L. (1972). *In* "Membrane Research" (C. F. Fox, ed.), pp. 187–201. Academic Press, New York.

Roizman, B. (1965). *Perspect. Virol.* **4,** 283–304.

Roizman, B. (1972). *In* "Oncogenesis and Herpesviruses" (P. M. Biggs, G. de Thé, and L. N. Payne, eds.), pp. 1–17. Int. Agency Res. Cancer, Lyon, France.

Roizman, B., and Frenkel, N. (1973). *Cancer Res.* **33,** 1402–1416.

Roizman, B., and Spear, P. G. (1973). *In* "Ultrastructure of Animal Viruses and Bacteriophages: An Atlas" (A. J. Dalton and F. Haguenau, eds.), pp. 83–107. Academic Press, New York.

Rosenbaum, M. J., Phillips, I. A., Sullivan, E. J., Edwards, E. A., and Miller, L. F. (1963). *Proc. Soc. Exp. Biol. Med.* **113,** 224–229.

Roy-Burman, P. (1971). *Int. J. Cancer* **7,** 409–415.

Royston, I., and Aurelian, L. (1970a). *Am. J. Epidemiol.* **91,** 531–538.

Royston, I., and Aurelian, L. (1970b). *Proc. Natl. Acad. Sci. U.S.A.* **67,** 204–212.

Sanders, F. K. (1974). *In* "Human Tumor Cells in Vitro" (J. Fogh, ed.), pp. 487–515. Plenum, New York.

Sarin, P. S., and Gallo, R. C. (1973). *Biochem., Ser. One* **6,** 000–000.

Sarkar, N. H., and Dion, A. S. (1975). *Virology* **64,** 471–491.

Sarkar, N. H., and Moore, D. H. (1972). *Nature (London)* **236,** 103–106.

Sarkar, N. H., Nowinski, R. C., and Moore, D. H. (1971). *Virology* **46,** 1–20.

Sarkar, N. H., Manthey, W. J., and Sheffield, J. B. (1975). *Cancer Res.* **35,** 740–749.

Sarma, P. S., Ueberhorst, P. J., Zeve, V., Whang-Peng, J., and Huebner, R. (1970). *Bibl. Haematol. (Basel)* **36,** 574–577.
Sarngadharan, M. G., Sarin, P. S., Reitz, M. S., and Gallo, R. C. (1972). *Nature (London) New Biol.* **240,** 67–72.
Sarngadharan, M. G., Allaudeen, H. S., and Gallo, R. C. (1976). *Methods Cancer Res.* **12,** 3–42.
Savage, T., Roizman, B., and Heine, J. W. (1972). *J. Gen. Virol.* **17,** 31–48.
Schafer, W., Lange, J., Bolognesi, D. P., De Noronha, F., Post, J. E., and Rickard, C. G. (1971). *Virology* **44,** 73–82.
Schafer, W., Lange, J., Fischinger, P., Frank, H., Bolognesi, D. P., and Pister, L. (1972). *Virology* **47,** 210–228.
Schafer, W., Hunsmann, G., Moennig, V., De Noronha, F., Bolognesi, D. P., Green, R. W., and Huper, G. (1975). *Virology* **63,** 48–59.
Schidlovsky, G., and Ahmed, M. (1973). *J. Natl. Cancer Inst.* **51,** 225–233.
Schlom, J., and Spiegelman, S. (1971). *Science* **174,** 840–843.
Schlom, J. Spiegelman, S., and Moore, D. H. (1971). *Nature (London)* **231,** 97–100.
Schlom, J., Spiegelman, S., and Moore, D. H. (1972). *Science* **175,** 542–544.
Schneweis, K. E., and Nahmias, A. J. (1971). *Z. Immunitaetsforsch., Exp. Klin. Immunol.* **141,** 471–487.
Schumacher, H. R., Szekely, I. E., and Park, S. A. (1973). *Lancet* **1,** 601–602.
Schwartz, J., and Roizman, B. (1969). *J. Virol.* **4,** 879–889.
Scolnick, E. M., Parks, W. P., and Livingston, D. M. (1972a). *J. Immunol.* **109,** 570–577.
Scolnick, E. M., Parks, W. P., Todaro, G. J., and Aaronson, S. A. (1972b). *Nature (London), New Biol.* **235,** 35–40.
Scolnick, E. M., Parks, W. P., and Todaro,G. J. (1973). *Science* **177,** 1119–1121.
Sebring, E. D., and Salzman, N. P. (1964). *Anal. Biochem.* **8,** 126–129.
Seman, G. (1968). *Rev. Fr. Etud, Clin. Biol.* **13,** 763–772.
Seman, G. (1969). *Rev. Fr. Etud. Clin. Biol.* **14,** 482–486.
Seman, G. (1974). *Cancer Bull.* **26,** 74–79.
Seman, G., and Dmochowski, L. (1973a). *Cancer Res.* **33,** 1238–1242.
Seman, G., and Dmochowski, L. (1973b). *Cancer* **32,** 822–829.
Seman, G., and Dmochowski, L. (1976). *Methods Cancer Res.* **14,** 178–248.
Seman, G., and Seman, C. (1968). *Cancer* **22,** 1033–1045.
Seman, G., Myers, B., Williams, W. C., Gallager, H. S., and Dmochowski, L. (1969). *Tex. Rep. Biol. Med.* **27,** 839–866.
Seman, G., Gallager, H. S., Lukeman, J. M., and Dmochowski, L. (1971). *Cancer* **28,** 1431–1442.
Seman, G., Dmochowski, L., and Hunter, S. (1974). *Proc. Electron Microsc. Soc. Am.* **32,** 248–249.
Seman, G., Levy, B. M. Panigel, M., and Dmochowski, L. (1975). *J. Natl. Cancer Inst.* **54,** 251–252.
Sever, J. L. (1962). *J. Immunol.* **88,** 320–329.
Shanmugaratnam, K. (1971). *Int. Rev. Exp. Pathol.* **10,** 362–406.
Sheldon, P. J., Hemsted, E. H., Papamichail, M., and Holborow, E. J. (1973). *Lancet* **1,** 1153–1155.
Sherr, C. J., and Todaro, G. J. (1974). *Virology* **61,** 168–181.
Sherr, C. J. Lieber, M. M., Benveniste, R. E., and Todaro, G. J. (1974). *Virology* **58,** 492–503.
Shope, T. C., and Miller, G. (1974). *In* "Comparative Leukemia Research 1973" (Y. Ito and R. M. Dutcher, eds.), pp. 375–383. Univ. of Tokyo Press, Tokyo.

Shoyab, M., Baluda, M. A., and Evans, R. (1974). *J. Virol.* **13,** 331–339.
Simons, M. J., Day, N. E., Wee, G. B., Shanmugaratnam, K., Ho, H. C., Wong, S. H., Ti, T. K., Yong, N. K., Darmalingam, S., and de Thé, G. (1974). *Cancer Res.* **34,** 1192–1195.
Skinner, G. R. B., Thouless, M. E., and Jordan, J. A. (1971). *J. Obstet. Gynaecol. Br. Commonw.* **78,** 1031–1038.
Skinner, M. S., and Mizell, M. (1972). *Lab. Invest.* **26,** 671–681.
Smith, G. C., Kalter, S. S., Helmke, R. J., Heberling, R. L., Panigel, M., and Kraemer, D. C. (1975). *Proc. Soc. Exp. Biol. Med.* **148,** 1212–1216.
Smith, J. W., Lowry, S. P., Melnick, J. L., and Rawls, W. E. (1972). *Infect. Immun.* **5,** 305–310.
Snyder, S. P. (1971). *J. Natl. Cancer Inst.* **47,** 1079–1085.
Snyder, S. P., and Theilen, G. H., (1969). *Nature (London)* **221,** 1074–1075.
Spear, P. G., and Roizman, B. (1972). *J. Virol.* **9,** 143–159.
Spiegelman, S. (1975). *Prog. Hematol.* **9,** 305–330.
Spiegelman, S., Burny, A., Das, M. R., Keydar, J., Schlom, J., Travnicek, M., and Watson, K. (1970a). *Nature (London)* **227,** 563–567.
Spiegelman, S., Burny, A., Das, M. R., Keydar, J., Schlom, J., Travnicek, M., and Watson, K. (1970b). *Nature (London)* **228,** 430–432.
Srivastava, B. I. S. (1974). *Cancer Res.* **34,** 1015–1026.
Srivastava, B. I. S. (1976). *Cancer Res.* **36,** 1825–1830.
Stephenson, J. R., and Aaronson, S. A. (1972). *Proc. Natl. Acad. Sci. U.S.A.* **69,** 2798–2801.
Stephenson, J. R., Anderson, G. R., Troncik, S. R., and Aaronson, S. A. (1974). *Cell* **2,** 87–94.
Stern, E., and Longo, L. D. (1963). *Acta Cytol.* **7,** 295–299.
Stewart, S. E., Kasnick, G., Draycott, C., Feller, W. Golden, A., Mitchell, E., and Ben, T. (1972a). *J. Natl. Cancer Inst.* **48,** 273–277.
Stewart, S. E., Kasnick, G., Draycott, C., and Ben, T. (1972b). *Science* **175,** 198–199.
Stock, N. D., and Ferrer, J. F. (1972). *J. Natl. Cancer Inst.* **48, 985**–996.
Strand, M., and August, J. T. (1971). *Nature (London) New Biol.* **233,** 137–140.
Strand, M., and August, J. T. (1974). *J. Virol.* **13,** 171–180.
Strandström, H., Veijalainen, P., Moennig, V., Hunsmann, G., Schwartz, H., and Schafer, W. (1974). *Virology* **57,** 175–178.
Stromberg, K. (1972). *J. Virol.* **9,** 684–697.
Stromberg, K., Hurley, N. E., Davis, N. L., Reuckert, R. R., and Fleissner, E. (1974). *J. Virol.* **13,** 513–528.
Sun, C. N., Byrne, G. E., and Pinkerton, H. (1972). *Exp. Pathol.* **6,** 72–79.
Sutton, W. D. (1971). *Biochim. Biophys. Acta* **240,** 522–531.
Svoboda, D., Kirchner, F., and Shanmugaratnam, K. (1965). *Exp. Mol. Pathol.* **4,** 189–204.
Sweet, R. W., Goodman, N. C., Cho, J., Ruprecht, R. M., Redfield, R. R., and Spiegelman, S. (1974). *Proc. Natl. Acad. Sci. U.S.A.* **71,** 1705–1709.
Symposium: Herpesvirus and Cervical Cancer. Am. Cancer Soc., Key Biscayne, Fla., Dec. 8–10, 1972. (1973). *Cancer Res.* **33,** 1349–1557.
Symposium: International Symp. on Human Tumors Associatied with Herpesviruses. N. I. H., Bethesda, Maryland, March 26–28, 1976. (1974). *Cancer Res.* **34,** 1086–1241.
Symposium: Immunological Control of Virus-associated Tumors in Man: Prospects and Problems. N. I. H., Bethesda, Maryland, April 7–9, 1975. (1976). *Cancer Res.* **36,** Part 2, 563–867.
Szekely, I. E., Fischer, D. R., and Schumacher, H. R. (1976). *Cancer* **37.** 805–811.
Szybalski, W. (1968). *In* "Methods in Enzymology" (L. Grossman and K. Moldave, eds.), Vol. 12, Part B, pp. 330–360. Academic Press, New York.

Takemoto, K. K., Mattern, C. F. T., Stone, L. B., Coe, J. E., and Lavelle, G. (1971). *J. Virol.* **7,** 301–308.
Takemoto, K. K., Aoki, T., Garon, C., and Sturm, M. M. (1973). *J. Natl. Cancer Inst.* **50,** 543–547.
Teich, N. M., Weiss, R. A. Salahuddin, S. Z., Gallagher, R. E., Gillespie, D. H., and Gallo, R. C. (1975). *Nature* (*London*) **256,** 551–556.
Temin, H. M. (1964). *Proc. Natl. Acad. Sci. U.S.A.* **52,** 323–329.
Temin, H. M. (1971). *J. Natl. Cancer Inst.* **46** 3–7.
Temin, H. M., and Baltimore, D. (1972). *Adv. Virus Res.* **17,** 129–186.
Temin, H. M., and Mizutani, S. (1970). *Nature* (*London*) **226,** 1121–1123.
Teramoto, J. A., Puentes, M. J., Young, L. J. T., and Cardiff, R. D. (1974). *J. Virol.* **13,** 411–418.
Theilen, G. H., Gould D., Fowler, M., and Dungworth, D. L. (1969). *J. Natl. Cancer Inst.* **47,** 881–889.
Thormar, H. (1965). *Res. Vet. Sci.* **6,** 117–129.
Todaro, G. J., and Gallo, R. C. (1973). *Nature* (*London*) **244,** 206–209.
Todaro, G. J., and Huebner, R. J. (1972). *Proc. Natl. Acad, Sci. U.S.A.* **69,** 1009–1015.
Todaro, G. J., Arnstein, P., Parks, W. P., Lennette, E. H., and Huebner, R. J. (1973). *Proc. Natl. Acad. Sci. U.S.A.* **70,** 859–862.
Todaro, G. J., Benveniste, R. E., Lieber, M. M., and Sherr, C. J. (1974). *Virology* **58** 65–74.
Tooze, J. (1973). "The Molecular Biology of Tumour Viruses," Monogr. Cold Spring Harbor Lab., Cold Spring Harbor, New York.
Tronick, S. R., Stephenson, J. R., and Aaronson, S. A. (1974). *J. Virol.* **14,** 125–132.
Twardzik, D. R. Papas, T. S., and Portugal, F. H. (1974). *J. Virol.* **13,** 166–170.
Tweedill, K. S. (1967). *Cancer Res.* **27,** 2042–2052.
Vaidya, A. K., Black, M., Dion, A. S., and Moore, D. H. (1974). *Nature* (*London*), **249,** 565–566.
Van der Maaten, M. J. and Boothe, A. D. (1972). *Arch. Gesamete Virusforsch.* **37,** 85–96.
Van der Maaten, M. J., Boothe, A. D., and Seger, C. L. (1972) *J. Natl. Cancer Inst.* **49,** 1649–1657.
Van Furth, R.,Gorter, H., Nadkarni, J., S., Klein, E., and Clifford, P. (1972). *Immunology* **22,** 847–857.
Varmus, H. E., Levinson, W. E., and Bishop, J. M. (1971). *Nature* (*London*), *New Biol.* **233,** 19–21.
Varmus, H. E., Heasley, S., and Bishop, J. M. (1974). *J. Virol.* **14,** 895–903.
Verma, I. M., Meuth, N. L., Fan, H., and Baltimore, D. (1974). *J. Virol.* **13,** 1075–1082.
Vernon, M. L. (1974). *J. Natl. Cancer Inst.* **52,** 987–989.
Viola, M. V., Frazier, M., Wiernick, P. H., McCredie, K. B., and Spiegelman, S. (1976). *N. Engl. J. Med.* **294,** 75–80.
Vosika, G. J., Balazs, P., and Kennedy, B. J. (1976). *Proc. Am. Assoc. Cancer Res.* **17,** 177 (Abstract No. 705).
Walker, P. M. B., and McLaren, A. (1965). *Nature* (*London*) **208,** 1175–1179.
Waters, L. C., and Yang, W. K. (1974). *Cancer Res.* **34,** 2585–2593.
Weber, W. T. (1963). *Ann. N.Y. Acad. Sci.* **108,** 1270–1283.
Weinmann, B. J., Kluge, N., Dube, S. K., von Ehrenstein, G., Krieg, J. C., Kind, J., and Ostertag, W. (1975). *J. Natl. Cancer Inst.* **55,** 537–542.
Werner, J., Wolf, H., Apodaca, J., and Zur Hausen, H. (1975). *Int. J. Cancer* **15,** 1000–1008.
White, J. G. (1966). *Cancer* **19,** 877–884.

Witkin, S. S., Ohno, T., and Spiegelman, S. (1975). *Proc. Natl. Acad. Sci. U.S.A.* **72,** 4133–4136.

Witter, R. L., Nazerian, K., Purchase, H. G., and Burgoyne, J. H. (1970). *Am. J. Vet. Res.* **31,** 525—538.

Wolfe, L. G., Deinhardt, F., Theilen, G. H., Rabin, H., Kawakami, T., and Bustad, L. K. (1971a). *J. Natl. Cancer Inst.* **47,** 1115–1120.

Wolfe, L. G., Falk, L. A., and Deinhardt, F. (1971b). *J. Natl. Cancer Inst.* **47,** 1145–1162.

Woods, W. A., Papas, T. S., Hirumi, H., and Chirigos, M. A. (1973). *J. Virol.* **12,** 1184–1186.

Worlledge, S. M., and Dacie, J. V. (1969). *In* "Infectious Mononucleosis" (R. L. Carter and H. G. Penman, eds.), pp. 82–98. Blackwell, Oxford.

Wright, D. H. (1972). In "Oncogenesis and Herpesviruses" (P. M. Biggs, G. de Thè, and L. N. Payne, eds.), pp. 217–229. Int. Agency Res. Cancer, Lyon, France.

Yakovela, L. (1975). *Proc. Annu. J. Working Conf., Virus Cancer Program, 10th, 1975* p. 1.

Yaniv, A., Ohno, T., Kacian, D., Colcher, D., Witkin, S., Schlom, J., and Spiegelman, S. (1974). *Virology* **59,** 335–338.

Yeh, J., Ahmed, M., Lyles, J., Larson, D., and Mayyasi, S. A. (1975). *Int. J. Cancer* **15,** 632–639.

Yumoto, T., and Dmochowski, L. (1968). *Acta Pathol. Jpn.* **18,** 394–400.

Zech, L., Haglund, U., and Nilsson, K. (1976). In press.

Zeigel, R. F., and Clark, H. F. (1969). *J. Natl. Cancer Inst.* **43,** 1097–1102.

Zeigel, R. F., and Clark, H. F. (1971). *J. Natl. Cancer Inst.* **46,** 309–321.

Zhdanov, V. M., Soloviev, V. D., Bektemirov, T. A., Filatov, F. P., and Bykovsky, A. F. (1972). *Arch. Gesamte Virusforsch.* **39,** 309–316.

Zurcher, C., Brinkhof, J., Bentvelzen, P., and DeMan, J. C. H. (1975). *Nature (London)* **254,** 457–459.

Zur Hausen, H. (1976). *Cancer Res.* **36,** 794.

Zur Hausen, H., Schulte-Holthausen, H., Klein, G., Henle, W., Henle, G., Clifford, P., and Santesson, L. (1970). *Nature (London)* **228,** 1052–1058.

Zur Hausen, H., Schulte-Holthausen, H., Wolf, H., Dorries, K., and Egger, H. (1974). *Int. J. Cancer.* **13,** 657–664.

Chapter 5

Control of Viral Diseases by Vaccines

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AND G. CONTRERAS

I. INTRODUCTION

Vaccination is the best means presently available for the prevention of viral disease in man. This procedure has developed from the use of variolation by the Chinese, centuries before the published works of Jenner, to some of the more sophisticated approaches of today, such as the use of chemically induced temperature-sensitive influenza mutants as recombinants in the production of influenza vaccines. Highlights in the development of viral vaccines are summarized in Table I.

Vaccination and the use of vaccines in disease control dates from the work of Jenner (1798) on the use of cowpox to induce immunity to smallpox. Variolation, the inoculation of smallpox to produce active immunity, had been practiced for centuries by the Chinese. The procedure was introduced into England from Turkey in 1721 by Lady Montagu, but with a mortality rate of close to 2% this practice soon declined in England. The successful use of variolation in the United States in 1738 resulted in the reintroduction of the technique into England and, by 1750, the procedure had gained acceptance in several of the European countries. Even at Jenner's time this improved variolation technique produced a death rate between 1:200 and 1:500. The acceptance of such a tremendous risk (far beyond our current safety standards) gives us an indication of how much smallpox was dreaded at the time. By the second half of the eighteenth century many farmers in England had observed that persons who had contracted cowpox were protected from smallpox (Langer, 1976). However, it was Jenner who demonstrated conclusively in 1796 that the relationship between these two conditions could be used to protect from smallpox. Jenner's first experiment in humans consisted of transferring material from a cowpox lesion of a milkmaid to the arm of a boy. When inoculated with smallpox 6 weeks later the boy was immune to the disease.

The potential of vaccination in disease control is exemplified by smallpox. A program was introduced by the World Health Organization (WHO) in 1967 to eradicate the disease (Henderson, 1975). By June 1976 it remained endemic only in Ethiopia. The last reported cases in Asia were on October 16, 1975 in Bangladesh, and on May 24, 1975 in India. The cases detected in Ethiopia are of variola minor. Thus it appears that variola major, with a case fatality rate of 20 to 30% has already been eradicated.

Even with all the shrewdness and courage of Jenner's work, the nature of the agents and the processes involved remained a mystery. A step forward in the understanding of the role of microorganisms in disease was accomplished 90 years later when Pasteur demonstrated that rabies virus could be attenuated by repeated intracerebral passage in rabbits. This

TABLE I
Development of Viral Vaccines

Highlights	Vaccine	Date	Vaccine efficacy
Discovery of vaccination; use of naturally existing attenuated virus strains	Smallpox	1798	Smallpox has disappeared from all countries of the world, except one. It could be eradicated in 1977
Experimental attenuation *in vivo* of a naturally pathogenic virus	Rabies	1885	Human rabies has almost disappeared; in domestic animals it is greatly reduced in spite of wild life reservoirs
Attenuation of virus in cultures of minced tissues of mice and chick embryos *in vitro;* multiplication of viruses in chick embryo *in vivo*	Yellow fever, influenza	1937	Yellow fever is present in very isolated areas connected with wild life reservoirs
Attenuation of viruses in cell cultures often using genetic selection; multiplication of viruses in a variety of cell cultures	Poliomyelitis, measles, mumps, rubella, adenovirus	1949	Poliomyelitis has almost disappeared in many countries; its eradication has become possible. Measles has been greatly reduced

virus, when partially inactivated, was subsequently shown to protect a boy severely bitten by a rabid dog (Pasteur, 1895). Pasteur prepared his vaccine by drying rabbit spinal cords infected with fixed virus for various lengths of time. This resulted in a series of preparations ranging from zero to maximum infectivity as measured by intracerebral inoculation of rabbits. He used the term "fixed" virus for the virus that had been obtained experimentally after successive animal passage as opposed to the term "street" virus applied to a recent isolate. Pasteur's strain of fixed virus became the seed virus for most of the subsequent vaccine preparations.

New concepts and methods were required for the development of a vaccine against yellow fever. These techniques, developed at the Yellow Fever Laboratory in New York, included: (i) the use of large, homogeneous populations of small laboratory animals, including mice and chick embryos, and (ii) the *in vitro* cultivation of minced embryonic tissue of mice and chickens. By repeated passage of the Aisibi strain of yellow fever virus, first in minced mouse embryonic tissue, then in minced whole chick embryo, and, finally, in minced chick embryo from which the brain and spinal cord had been removed, Theiler and Smith developed what was to become the 17D strain of yellow fever vaccine. Following over 160 subcultures in the latter type of culture, they showed that the attenuated 17D strain had lost its virulence and neurotropic properties for monkeys (Theiler and Smith, 1937). In 1938 these authors reported that this vaccine was both safe and effective in humans (Theiler, 1951).

During the 1930's studies were also conducted on the feasibility of developing a vaccine for influenza. Influenza had been isolated by Smith, Andrewes, and Laidlaw in 1933 following intranasal inoculation of ferrets. While early attempts at immunization were largely unsuccessful, vaccination against influenza seemed to be feasible. During an extensive study in 1942–1943 by the Commission on Influenza in the United States (Francis, 1945), it became apparent that subcutaneous inoculation of an inactivated vaccine prepared from the allantoic fluid of chick embryos could prevent influenza. Since this vaccine has to be administered yearly to maintain immunity, vaccination against influenza has not been as widely accepted as vaccination against other viral diseases. Furthermore, because of the antigenic drift exhibited by influenza viruses, new vaccines have to be prepared and administered whenever a new antigenic variant appears in order to maintain protection against the disease.

After the successful development of yellow fever vaccine, the next important breakthrough came in 1949 when Enders, Weller, and Robbins showed that poliovirus could multiply in nonnervous tissue of human origin. The first attempts to immunize humans against poliomyelitis were

made in the 1930's by using infected monkey spinal cord suspensions inactivated with formalin or sodium ricinoleate. These attempts were a failure since several vaccinees developed poliomyelitis (Brodie and Park, 1936; Kolmer, 1936). However, the crucial observation by Enders and his group opened up new possibilities of research for poliomyelitis vaccines and for the study and possible development of vaccines against several other diseases. By 1953, Salk had introduced an inactivated poliomyelitis vaccine which had been prepared in monkey kidney cultures. This vaccine was shown to be effective in reducing the incidence of paralytic poliomyelitis in a large field trial directed by Francis in 1954 (Francis *et al.*, 1957). The use of inactivated vaccine was followed shortly by the development in primary monkey kidney cell cultures of attenuated poliovirus strains (Sabin, 1955; Koprowski *et al.*, 1956; Cox *et al.*, 1959). Following extensive testing of the Sabin strains for their lack of neuropathogenicity and for the stability of their attenuated character, live poliovirus vaccine was licensed and has been used successfully all over the world.

The use of the new cell culture techniques led to the isolation of a host of viruses. The development and use of vaccines for measles, mumps, and rubella followed within a few years and with the increasing number of diseases for which vaccines have become available, attention has been directed toward the use of combined vaccines. Rapid progress in viral technology will undoubtedly lead to the development of new vaccines for other viral diseases.

II. VACCINES IN CURRENT USE

The majority of viral vaccines in current use are prepared with live, attenuated virus strains; three inactivated vaccines are also widely employed (Table II). All these viruses except smallpox have a single-stranded RNA genome. The molecular weight of the nucleic acids varies between 2.8 to 160 million daltons. The number of proteins of each different virion is not certain at the present time and the numbers given in Table II are the ones most generally accepted by different investigators. Conversely, the number of serotypes is well known. With the exception of poliovirus vaccine which contains all three serotypes, and influenza which usually contains types A and B, all vaccines are monovalent. Monovalent live polio vaccines have been used successfully, however, for the specific purpose of controlling an epidemic (Yofe *et al.*, 1962; Zuñiga *et al.*, 1962). Monovalent influenza vaccines could be used when a pandemic due to a new influenza A virus variant is anticipated.

The necessity of using living cells as hosts for the propagation of

TABLE II
Viral Vaccines in Current Use[a]

Attenuated	Inactivated	Viral genome		Viral proteins (number)	Number of serotypes[d]
		Class[b]	MW[c]		
Influenza	Influenza	RNA S-S	4	7	2
Measles		RNA S-S	7	6–7	1
Mumps		RNA S-S	7	(6–7)	1
Poliomyelitis	Poliomyelitis	RNA S-S	2.8	4	3
Rabies	Rabies	RNA S-S	7	4	1
Smallpox		DNA D-S	160	>20	1
Yellow Fever		RNA S-S	4	3	1

[a] Other vaccines such as adenovirus vaccines and vaccines against some of the equine encephalitis are used only in restricted populations; therefore, they are omitted from this table.
[b] RNA or DNA; single-stranded (S-S) or double-stranded (D-S).
[c] MW: molecular weight (10^6 daltons).
[d] Number of serotypes usually present in the vaccine. Influenza type C is not included in the vaccine.

viruses for vaccine production increases the risk of contamination by extraneous viruses present in apparently normal cells. This type of contamination has been detected and poses a real danger in any vaccine preparation. For instance, the presence of simian virus 40 (SV40) in inactivated poliovirus vaccine raised concerns about the possible production of tumors in recipients (Sweet and Hilleman, 1960; Gerber, 1967). Subsequent studies have not detected any abnormal pathology in these human groups (Robbins, 1968). The availability of tissue culture techniques to detect SV40 has eliminated the danger of this agent as a contaminant. Nevertheless, in the control of vaccines one must always consider that other agents, for which techniques are not yet available, may be present.

The social and economic impacts of vaccination programs on a large scale are enormous. For instance, the reduction in the number of cases of one disease, measles, has resulted in substantial savings of both lives and money. Since its introduction in the United States "measles vaccine has saved an estimated 2400 lives (one in 10,000 cases), prevented 8000 cases of mental retardation (one in 3000 cases), and averted 140,000 hospitalizations. If one considers that this represents 78 million school days saved and 12 million doctor visits averted, the monetary equivalent of these savings totals $1.3 billion dollars" (Schreier, 1974).

The development of viral vaccines has involved many fields such as immunology, cell biology, and epidemiology. Frequently, increased knowledge in any one discipline has resulted in modification of vaccine testing

or vaccination programs. The information discussed below will reflect some of the more recent knowledge concerning viral vaccines and will cover a number of relevant facts in this field.

A. Poliomyelitis

The tragic lifelong crippling of children and adults due to poliomyelitis was a powerful stimulus for the development of an effective vaccine against this disease. Efforts began soon after the demonstration by Landsteiner and Popper in 1909 that the paralytic disease could be transmitted to monkeys. As mentioned in Section I, the first attempts at vaccination using infected monkey spinal cord suspensions were unsuccessful because several vaccinees contracted the disease. In order to prepare an effective vaccine it was necessary to have a nonnervous but susceptible tissue as a source of large amounts of virus having minimal contamination by host components. Such a source became available in 1949 so that production of inactivated and live, attenuated poliovirus vaccines became possible.

1. Development and Characteristics of Vaccines

a. Inactivated Vaccine. Shortly after the demonstration by Enders and his group (Enders *et al.*, 1949) that poliovirus multiplied in nonnervous tissue, monkey kidney cell cultures were found suitable for the production of vaccine. Within 3 years, Salk and his group developed a formalin-inactivated vaccine (Salk, 1953). A large field trial of this vaccine showed that it was safe and effective in reducing the incidence of paralytic poliomyelitis (Francis *et al.*, 1957). The vaccine was licensed in 1955 in the United States and by 1956 several other countries had become involved in large vaccination programs. Serious troubles were encountered with the initial inactivation of crude viral preparations. As a result some lots of commercial vaccines containing residual infectious virus were used and caused paralytic poliomyelitis in vaccine recipients (Nathanson and Langmuir, 1963). Fortunately, it was soon recognized that a safe vaccine could be produced when filtered virus was used.

Vaccination with inactivated virus results in seroconversion of 90% of those immunized with four or more doses. Antibody levels for all three types fall to approximately 20% of their maximum within 2 years and more slowly thereafter. Therefore, booster injections every 3–5 years are recommended. Immunization provided by this vaccine does not prevent infection of the alimentary tract although infection of the oropharyngeal mucosa and tonsils is generally prevented. Although live, attenuated vaccine has almost completely replaced inactivated vaccine in the United

States, other countries including Sweden, the Netherlands, and some provinces in Canada, continue to use the inactivated vaccine.

b. Live, Attenuated Oral Vaccine. During the development of the inactivated poliovirus vaccine, attempts were also made by several investigators to attenuate wild poliovirus strains for use in a live-virus vaccine. It was hoped that such vaccine would give better and longer lasting protection by simulating natural infection. It would immunize the gastrointestinal tract and would be cheaper and easier to administer. Attenuated viruses developed in tissue cultures independently by Sabin (1955), Koprowski *et al.* (1956), and Cox *et al.* (1959) for the preparation of vaccines have all been used successfully in large field trials. Of the candidate strains, those of Sabin were found most stable following attenuation and showed the least neurovirulence in the monkeys. Sabin (1955) selected strains for each of the poliovirus types and studied them by different procedures. A detailed description of the work which led to the preparation of the Sabin Original (SO) strains was published years later (Sabin and Boulger, 1973). The seed virus now used worldwide for the preparation of live poliovirus vaccines represents only three or four further tissue culture passages from the SO virus. After extensive testing of the vaccine strains for their lack of neuropathogenicity and the stability of their attenuated character in humans, live, attenuated poliovirus vaccine was licensed in the United States in 1962.

The possible, causal relationship of attenuated vaccine with paralytic poliomyelitis is of worldwide concern. The figures calculated from all cases typical of paralytic poliomyeltis occurring in persons within 5 to 30 days after vaccine was fed are probably an overestimate of the risk of vaccination since association in some cases must be purely coincidental. In the United States from 1962 to 1964, when approximately 270 million doses of monovalent vaccines were distributed, the average rate of paralytic cases possibly associated with vaccination was one per five million recipients (Henderson *et al.*, 1964). With the use of trivalent vaccines in the United States from 1965 to 1973, when approximately 207 million doses were distributed, this rate was even lower, one case per 16 million recipients (Center for Disease Control, 1975a). The rate of paralytic poliomyelitis cases in the contacts of vaccine recipients is more difficult to determine; however, the estimated number of cases in the United States from 1965 to 1973 was one per five million doses distributed (Center for Disease Control, 1975a).

Contamination with simian viruses was also detected in the experimental lots of live, attenuated vaccine. Special precautions in manufacture and in testing were taken so that commercial live, oral vaccines are

free of SV40 (Murray, 1968). Contamination of monkey kidney cells with simian viruses prompted the search for a more suitable host tissue. Live, oral vaccines have been successfully prepared in human diploid cell lines, such as WI-38, and are now used in several countries.

In most individuals an oral dose of one virus type produces gastrointestinal infection with the excretion of a high concentration of virus for 4 to 6 weeks and the development of serum antibodies in 3 to 4 weeks. Seroconversion occurs in about 95% of those previously seronegative. Antibody levels after the administration of live, oral vaccine are similar to those following natural infection.

c. Comparison of Inactivated and Live, Attenuated Vaccines. Inactivated vaccine is now of high potency and adequate purity, and has the advantage of being safe and effective provided it is properly used. Such is the case in Sweden and some provinces in Canada where poliomyelitis has been practically eliminated by the exclusive use of this vaccine. Its disadvantages are the following: (i) it does not result in lifelong immunity, therefore booster immunizations are required every 3–5 years; (ii) it fails to prevent intestinal infection and fecal excretion of the virus; (iii) its administration by injection is time-consuming and expensive; (iv) the vaccine is more costly since a greater antigenic mass is required per dose; and (v) additional doses are needed to ensure proper immunity.

Live, attenuated vaccine has the following advantages: (i) it appears to simulate natural infection producing long lasting immunity; (ii) it induces synthesis and excretion of IgA antibody in the intestinal tract producing resistance to reinfection; (iii) it confers herd immunity by allowing spread of vaccine virus in the community; (iv) its effectiveness approaches 100% when properly used; and (v) it is easily administered and is relatively less expensive than inactivated vaccine. The disadvantages are (i) even though the vaccine is considered safe, there exists a very small risk of paralysis in vaccine recipients and their contacts; and (ii) the possibility that contaminant viruses, which are very difficult to eliminate completely, may be present in the vaccine. However, the probability of this is currently very low.

2. Control of Poliomyelitis by Vaccination

After 1955 when inactivated vaccine was introduced, and especially after 1962 when live, attenuated vaccine became available, several countries of the world experienced a reduction in the incidence of poliomyelitis of an order seldom achieved in the control of disease by vaccination. In 1955 a total of over 76,000 cases of poliomyelitis was reported in several countries of the world. In these same countries in 1967 only 1013 cases

were recorded, a reduction of 98.7% in 12 years (World Health Organization, 1969). Massive application of live, oral vaccine can also be used to contain poliomyelitis outbreaks.

On the other hand, there are large areas of Africa, Asia, and Latin America where the incidence of poliomyelitis has been rising (Cockburn and Drozdov, 1970). The analysis of the epidemiological and virological data gathered through extensive and worldwide use of poliomyelitis vaccines suggests that a high level of immunity can be achieved also in the population of warm climate countries. This required regular vaccination of new susceptibles with adequate doses of potent, live vaccines and revaccination at regular intervals (Cockburn and Drozdov, 1970). Since there seem to be no natural reservoirs for polioviruses, this secular disease, along with smallpox, is amenable to eradication. Such a goal, which has nearly been achieved with smallpox, has not been formulated yet for poliomyelitis, although the conditions necessary for success may be near.

B. Measles

Measles is an acute, highly contagious viral disease that is followed by complete recovery in most cases. Serious complications involving the respiratory tract and the central nervous system occur in a minority of cases in developed countries. In other parts of the world, however, the high morbidity and mortality associated with measles present serious problems. For example, in Chile in 1964 before mass vaccination was implemented, measles by itself accounted for 3.5% of all deaths registered in the country (Borgoño and Greiber, 1971). In the United States, the mortality rate from measles has declined from 10 deaths per 100,000 during the early 1900's, but even in the period 1958–1963, 2523 deaths from measles were recorded (Barkin, 1975). Moreover, measles virus has been implicated also as the etiologic agent of subacute sclerosing panencephalitis. Development and licensing of the vaccine in the early 1960's was met with considerable optimism that measles eradication was imminent, but even now, 13 years later, measles is still an epidemiological problem.

1. Development and Characteristics of Vaccines

Development of the first measles vaccine followed the growth in tissue culture of measles virus isolated by Enders and Peebles (1954). The original Edmonston vaccine (Milovanović *et al.*, 1957; Katz and Enders, 1959), derived by attenuation of the isolate in chick embryo cell culture, produced many adverse reactions in recipients and further attenuation was required. The strains now represent further passage of the original

Edmonston strain in cell cultures of chick embryo. The vaccines have proved to be safe and effective although some adverse reactions such as fever and rash are seen in about 15% of persons 5–12 days following vaccination.

An inactivated vaccine for measles was introduced in 1962 in several countries. It soon became apparent that the antibody levels induced with this vaccine were low and the immunity was of short duration. In addition, it was found that serious reactions, similar to the Arthus phenomenon, developed in vaccinees who were exposed in later years to wild measles virus or live attenuated vaccine (Rauh and Schmidt, 1965; Fulginiti *et al.*, 1967; Buser, 1967). As a result the vaccine was withdrawn from the market in the United States and Canada, and only live, attenuated vaccines are presently licensed. Following the administration of live, attenuated vaccine, antibodies develop in over 95% of vaccinees. The level of antibodies is lower than following natural infection (Krugman, 1971); however, protection rates of close to 100% are obtained (Katz *et al.*, 1962; Krugman, 1971). Krugman (1973) reports that the persistance of antibody levels over a 12-year period following vaccination has been similar in vaccinated and naturally infected children, although the antibody levels are lower in vaccinees.

Numerous reports of measles infections in immunized children have appeared in the literature (Rawls *et al.*, 1975) and the waning of antibody levels has resulted in concern and considerable debate on the incidence of reinfection with wild virus in vaccinated children.

Several factors have been implicated in the failure of measles vaccination (Conrad *et al.*, 1971; Krugman, 1971; Plotkin, 1973). The most important is the practice of vaccinating children under 1 year of age. This results in vaccine failure due to neutralization of vaccine virus by maternal antibodies. Consequently, if vaccination of a child under 1 year of age is advised for medical reasons, revaccination must be carried out for lasting immunity to be attained. A similar failure exists when γ-globulin has been given with the vaccine. Another cause of failure is improper handling of the vaccine either through inadequate refrigeration or by exposure to light (Borkow, 1975). If proper precautions are observed there is every indication that measles vaccination will be effective. The question whether vaccine induced immunity will last as long as immunity following natural infection remains to be answered.

2. *Control of Measles by Vaccination*

Following the introduction of measles vaccination in the United States in 1963, there was a marked drop in the incidence of measles from over 480,000 cases in 1962 to a low of 22,231 cases in 1968 (Center for Disease

Control, 1970, 1974). This decline was reversed when the United States Federal Government halted funds for measles vaccination in favor of a rubella vaccination program. The dramatic resurgence of measles in 1971, when 75,290 cases were reported, indicates the need for an ongoing program if measles is to be controlled. Reallotment of funds to the measles program in 1971, in addition to licensing of combined measles–rubella and measles–mumps–rubella vaccines, again resulted in a drop in the incidence of measles, which has remained about 25,000 cases per year since 1972.

The incidence of measles in Canada is high with 12,400 cases reported in 1975 (Health and Welfare Canada, 1975). Accurate immunization rates are not available for all of Canada but estimates for Ontario in 1974 indicate that only 67% of children entering school have been immunized (Ontario Ministry of Health, 1974). This is comparable to the 1974 rate in the United States where only 64.7% of the population from 1 to 4 years and only 53.7% of the 1 year age group had been vaccinated (Center for Disease Control, 1975b).

C. Rubella

Rubella is usually a mild illness with few serious complications. However, an infection during pregnancy, especially in the first trimester, may result in a malformed infant with congenital rubella syndrome (CRS). This association of rubella infection during pregnancy with subsequent development of congenital malformations was first described by Gregg in 1941. Rubella vaccine thus became the only virus vaccine which was designed not to protect the individual vaccinated but to protect the unborn human fetus.

1. Development and Characteristics of Vaccines

It was 21 years from the time of Gregg's description of CRS until growth of rubella virus in tissue culture was achieved (Weller and Neva, 1962; Parkman *et al.*, 1962). Within 4 years, Meyer *et al.* (1966) reported on the development of the HPV-77 vaccine in which rubella virus had been successfully attenuated by 77 tissue culture passages in primary monkey kidney cells. The HPV-77 virus was further passaged in duck embryo cell cultures and the vaccine (HPV-77, DE-5) was licensed for use in the United States and in Canada. Clinical trials in the United States indicated that the vaccine was immunogenic and safe. In contrast, use of a second vaccine prepared by passage of the HPV-77 strain in dog kidney cells (HPV-77, DK-12) has been discontinued because of an increase in the severity and incidence of joint manifestations and neuropathy. The

other vaccine presently licensed for use in Canada and the United States is the Cendehill strain, isolated in green monkey kidney cells and attenuated by 51 passages in rabbit kidney cells (Huygelen *et al.*, 1969). A fourth vaccine, the RA27/3 strain (Plotkin *et al.*, 1969) isolated and passaged 25 times in human diploid (WI-38) cells, is licensed in England and several European countries. Immunity induced by this vaccine is similar to that produced by natural infection, and artificial challenge studies indicate that there is a lower incidence of reinfection than with other vaccines (Plotkin *et al.*, 1973).

Rubella vaccine is administered as a single subcutaneous injection. In order to eliminate the major source of rubella transmission to pregnant women, Canada and the United States have adopted a policy of vaccination of all children from the ages of 1 to 12. Vaccine is also offered to adolescent girls and adult women who are found to be seronegative and in whom pregnancy can be prevented for at least 2 months after vaccination. In Britain, a different approach was taken with vaccination recommended only for girls aged 11–14 years.

The vaccine stimulates production of antibodies to rubella virus in approximately 95% of susceptible individuals. Antibody titers following vaccination are lower than those following natural infection; however, long-term protection is expected, although the duration is not yet known. Studies by Meyer and Parkman on a small group of children given the HPV-77, DE-5 vaccine indicate that immunity in vaccinated persons persists for at least 7 years (Modlin *et al.*, 1975). Horstmann (1975) reports, however, that those vaccinees who have a low antibody response, approximately 26%, will probably be seronegative after 5 years.

The risk of reinfection with wild virus to fetuses of immunized women is of primary concern in assessing the efficacy of any rubella vaccine. Reinfection with wild virus occurs in naturally immune persons (Horstmann *et al.*, 1970; Davis *et al.*, 1971), however, the frequency in immunized persons is greater (Chang *et al.*, 1970; Horstmann *et al.*, 1970). Krugman (1973) reports that viremia is not associated with reinfection and, therefore, there is probably no danger to the fetuses of vaccinated women. Several seemingly conflicting reports on rubella reinfection during pregnancy have appeared in the literature (Boué *et al.*, 1971; Northrop *et al.*, 1972; Haukenes and Haram, 1972; Biano *et al.*, 1975). In one case, virus was isolated from the products of conception (Northrop *et al.*, 1972), but a subsequent report (Robbins, 1972) questions whether this was not in fact due to a primary infection. In two other cases, serological tests in either the mother's or infant's serum had resulted in false positive diagnoses of rubella infections during pregnancy (Haukenes *et al.*, 1973; Biano *et al.*, 1975). In three other women with serological evidence

of reinfection, all had normal full-term infants (Boué *et al.*, 1971). Thus the evidence to date would appear to support Krugman's contention that reinfection poses little danger to the fetus. However, additional studies are still required.

Since the vaccine contains live virus there has also been considerable concern about the risk of fetal infection with vaccine virus. In view of this concern, the Center for Disease Control (CDC) in Atlanta, Georgia has initiated an ongoing program to assess the risk of vaccination during pregnancy (Modlin *et al.*, 1976). Several reports indicate that infection of a fetus with rubella vaccine virus can occur during the first 3 months of development (Vaheri *et al.*, 1972; Modlin *et al.*, 1976). Conversely, data from 172 women whose pregnancies went to term following vaccination indicate that there was no serological or clinical evidence of congenital infection. Included in this group were 38 known susceptible and 12 suspected susceptible women (Modlin *et al.*, 1976). From the data, these authors calculate that the maximum risk of fetal infection following rubella vaccination during pregnancy is between 5 and 10% and suggest that "the true risk is probably less." Regardless of the risk, rubella vaccination is contraindicated 2 months prior to and during pregnancy.

2. *Control of Rubella by Vaccination*

Since the introduction of rubella vaccine in 1969, the incidence of rubella in the United States has declined from over 56,500 total cases in 1970 to a low of just over 11,900 cases in 1974 (Center for Disease Control, 1974), although in 1975 there was an increase with over 16,000 cases reported (Center for Disease Control, 1976). While immunization rates are not available for Canada, data for Ontario in 1974 indicate that only 42% of children entering school have been immunized (Ontario Ministry of Health, 1974). Furthermore, data from the United States indicate that only 49% of 1 year olds have been immunized as compared to 70% at 5 years of age (Center for Disease Control, 1975b). Although a predicted outbreak of rubella in the early 1970's based on previous rubella cycles did not occur, the recent trend in present immunization practices must be improved if any impact on CRS is to be attained. In fact, a better criterion for evaluation of a rubella immunization program is probably the incidence of CRS in the population. Since 1969, when the National Registry for CRS was established in the United States, there has been a drop from 77 cases of CRS in 1970 to 28 cases in 1975 (Center for Disease Control, 1974, 1976). The short period of time the registry has been in existence makes it difficult to assess the significance of these data. Schoenbaum *et al.* (1976) point out that the overall incidence of CRS has not changed in

the last 5 years, although the total number of rubella cases has decreased fivefold. They also suggest that other factors such as the decline in birth rate and an increase in the number of abortions may have contributed to the lower incidence of CRS. It is probably still too early, however, to obtain an accurate assessment of the rubella vaccination program on CRS. The question at this time appears to be whether the duration of immunity in girls vaccinated early in life will be sufficient to prevent infection of the fetus during their child-bearing years.

D. Mumps

Mumps infection is a relatively mild disease characterized by enlargement of one or both of the parotid glands. Complications following infection are rare. Nevertheless, mumps accounts for approximately 15% of all cases of encephalitis in the United States, the incidence being three times more common in males than females. Orchitis, which is not a reportable complication in the United States, occurs in approximately 20% of cases but is rarely bilateral and concerns with subsequent sterility are unfounded.

1. Development and Characteristics of Vaccines

Mumps virus was first isolated in the amniotic cavity of chick embryos in 1945 (Habel, 1945; Levens and Enders, 1945). The first vaccines available were inactivated, and while they were found to protect man (Habel, 1951a,b; Henle *et al.*, 1951) the immunity they induced was transitory and repeated booster injections were required (Henle *et al.*, 1951). Early attempts to produce a live, attenuated vaccine were unsuccessful since the virus would change from fully virulent to overattenuated in only a few passages. Mumps virus was successfully attenuated in chick embryo tissue culture first by Smorodintsev and Klyatchko (1958) and then by Buynak and Hilleman (1966). The attenuated vaccine, the Jeryl Lynn strain, developed by the latter authors is produced in chick embryo cell cultures and is licensed for use in Canada and the United States.

Vaccination with the Jeryl Lynn strain results in seroconversion in over 95% of susceptible persons. However, as with other live, attenuated vaccines, antibody levels are lower than those following natural infection. Weibel *et al.* (1975) have found that while the mean antibody titers of naturally infected persons fell, those of vaccinated individuals increased, suggesting that reinfection may have occurred more frequently in the latter group. Nonetheless, children vaccinated for $6\frac{1}{2}$ or 8 years were protected when exposed to wild mumps (Weibel *et al.*, 1975).

2. *Control of Mumps by Vaccination*

In the past, mumps has shown a wide fluctuation in incidence from year to year and this has made the evaluation of the mumps immunization program difficult. Since the introduction of vaccination in the United States there has been an indication of a decrease in incidence of mumps from approximately 150,000 cases in 1968 to 59,000 in 1975 (Center for Disease Control, 1974, 1976). This decrease has not been as dramatic as has occurred with polio or measles, due in part to the fact that mumps vaccination has not been as actively pursued. For example, in the United States, only 29% of 1 year olds and 45% of 5 year olds have been vaccinated against mumps (Center for Disease Control, 1975b). In Massachusetts, where an extensive immunization program has been undertaken, the number of reported cases has declined from over 9000 in 1968 to just over 1000 in 1974, an 88% reduction (Center for Disease Control, 1975c). With the introduction of combined vaccines it is hoped that a similar effect will be seen nationwide.

E. Influenza

Influenza is generally an uncomplicated disease resulting in relatively mild discomfort to the infected individual. Nonetheless, in elderly and in persons with chronic debilitating conditions, a significant degree of mortality is associated with influenza infection each year. The most striking incidence of mortality from influenza infection occurred during the great pandemic of 1918 in which 500,000 people died in the United States and 20 million died worldwide. While many of the deaths in 1918 were due to secondary bacterial infections, the 1957 and 1968 pandemics which resulted in approximately 98,000 excess deaths in the United States indicate that influenza is still a serious problem today. Even in the interpandemic years, influenza can be the cause of a significant degree of mortality. Dowdle *et al.* (1974) report that in the period from 1959 to 1972, excluding 1968, there were 160,000 excess deaths in the United States which were attributable to influenza infection.

The three influenza viruses, types A, B, and C, are grouped according to the antigenic character of the internal ribonucleoprotein. Influenza types A and B are unique in that the two surface antigens hemagglutinin (HA) and neuraminidase (NA) undergo continual change. It is because of these changes that man has been unable to adequately prevent or control influenza. Major antigenic changes, termed "shift," occur in the HA and/or NA of only influenza A viruses at approximately 10-year intervals. Such shifts in influenza A were responsible for pandemics in 1947, 1957,

and 1968. Minor changes, termed "drift," in the HA or NA of both influenza A and B are responsible for minor epidemics which occur between major shifts. Influenza C, which is associated with endemic upper respiratory infections, undergoes only minor changes in antigenicity. Since epidemics are not usually associated with this type it is not included in any of the present vaccines.

1. Development and Characteristics of Inactivated Vaccines

Results from large-scale field trials conducted in the United States and the U.K. in the 1940's and 1950's indicated that the efficacy of influenza vaccines ranged from 40 to 90% (Francis, 1945; Medical Research Council Influenza Committee, 1953, 1957, 1958). It was realized that the greater the antigenic mass, the better the protection (Salk, 1948). However, these early highly concentrated preparations induced serious local and systemic reactions, especially in the young and the elderly.

The introduction by Reimer *et al.* in 1967 of zonal ultracentrifugation for purification of influenza vaccine has led to a product which is free of most of the impurities of the older vaccines. This has made possible the use of higher dose levels and studies have shown that, even with an increase in antigenicity, there is less reactogenicity (Peck, 1968; Mostow *et al.*, 1969). Two types of vaccines are available, whole virion or split. With whole virus vaccines the majority of the particles are complete while the split vaccines contain the HA and NA of the virion separated from the nucleoprotein. The latter vaccine has the advantage that febrile response and reactogenicity are reduced. Both vaccines produce a good immune response in vaccinees although the split vaccines appear to be less efficacious than the whole virus vaccines.

Although not available for the general population, adjuvant (mineral oil) vaccines have been used in the armed forces of both the United States and the United Kingdom and have produced adequate protection even at reduced dosages (Hobson, 1975). The use of adjuvants has been tempered by the suggestion that such preparations may be oncogenic in humans. In addition, it is questionable whether such vaccines could be produced and used on a large scale since not only are uniform adjuvant vaccines difficult to produce but increased care in administration must be used to avoid an increase in adverse reactions.

2. Development and Characteristics of Live Attenuated Vaccines

During the past few years there has been a resurgence of interest and effort in the development of techniques for the attenuation of influenza viruses, with the expectation that the most promising strains might be

useful for the prevention of influenzal disease. Several approaches have been used for the attenuation of influenza viruses. Live, attenuated vaccines studied and used by Smorodintsev (1969) for many years in the USSR were attenuated by serial passage of the virus in eggs. Other techniques include (i) recombination of the pathogenic variant with a distinct laboratory-adapted strain known to be nonpathogenic for man, for example influenza A/PR8 (Beare and Hall, 1971); (ii) selection of cold-adapted mutant strains derived by serial passage at low temperature (Smorodintsev *et al.*, 1965; Maassab, 1969); (iii) use of a chemical mutagen to induce temperature-sensitive (ts) mutants (Murphy *et al.*, 1972, 1973); and (iv) selection of inhibitor-resistant strains by serial passage of virus in the presence of horse serum (Beare and Bynoe, 1969; Huygelen *et al.*, 1973). Attenuated vaccines prepared by the latter technique have recently been licensed in some countries.

Clinical results obtained with these live, attenuated influenza vaccines are encouraging. However, further evidence is required that such vaccines can be produced quickly, administered by a simple and acceptable method in the field, and still induce a satisfactory immune response without causing serious adverse reactions, particularly in children.

3. Control of Influenza by Vaccination

The efficacy of inactivated influenza vaccines is often debated. On the one hand, it is estimated that the present zonally purified vaccines protect 75% of vaccinees and, in addition, the severity and duration of illness is reduced in those who contract the disease (Hobson, 1975). On the other hand, several factors have contributed to the general conception that influenza vaccines were inadequate. First, accurate estimates of vaccine efficacy in clinical trials have been complicated by the appearance of acute respiratory illness of noninfluenza origin in vaccinees. Second, new influenza vaccines have frequently become available too late to arrest an outbreak. However, the use of recombinant viruses should enable manufacturers to produce vaccines containing new antigenic determinants more quickly. Such recombinants, used effectively in the past few years, may finally provide, in 1976, a vaccine in time to prevent an epidemic as a result of a major antigenic shift.

F. Rabies

Although human rabies is rare in the United States and Canada, thousands of persons receive rabies prophylaxis each year. The vaccines available for this purpose have caused adverse reactions, including permanent disability and even death. Fortunately, there has been a continu-

ous improvement of rabies vaccines which will be reviewed briefly. The management of persons possibly exposed to rabies infection is of paramount importance but is beyond the scope of this chapter and will not be dealt with here.

1. Development and Characteristics of Vaccines

The first advancement after Pasteur's original vaccine was achieved when Semple developed a phenol-inactivated vaccine which contained a 5% brain suspension from rabbits infected with fixed virus (Semple, 1911). Such phenolized vaccines are still in use in several western countries. The incidence of encephalomyelitis during or following immunization with the adult nervous tissue vaccines has been found to differ in various countries and even between different studies in the same country. In one extensive analysis of 156,596 vaccinated persons, there were 96 neuroparalytic accidents (a rate of 1:1630) with 14 deaths (Abdussalam and Bögel, 1971). In order to reduce the demyelinating complications of immunization with the Semple vaccine, other inactivated vaccines have been prepared in nervous tissues of immature animals such as suckling mouse brain in Latin America (Fuenzalida *et al.*, 1964) and suckling rat brain in Russia (Svet-Moldavski *et al.*, 1965). Rabies virus propagated in embryonated duck eggs and inactivated by β-propiolactone has been widely used in the United States and other countries since 1957 (Peck *et al.*, 1956). This vaccine is almost devoid of encephalitogenic factors. The incidence of neurological complications during or after treatment of 172,000 persons was one in 25,000 with only one death possibly related to the vaccine administration (Abdussalam and Bögel, 1971).

Recently, rabies virus has been successfully propagated in tissue cultures of animal (hamster kidney) and human (WI-38) origin. These vaccines have shown greater purity than those obtained from animal tissues. Virus grown in hamster kidney cultures (Fenje, 1960) is already being used as a preexposure vaccine in Canada. Inactivated, purified, and concentrated experimental vaccines prepared in WI-38 cell cultures have been subjected to several successful human trials (Wiktor, 1975; Plotkin *et al.*, 1976).

2. Control of Rabies by Vaccination

Rabies is a worldwide disease of warm-blooded animals. In the Western Hemisphere, a major reservoir of rabies virus exists in bats, which sometimes carry the virus without signs of illness. All other mammals, such as foxes and wolves, usually die after natural rabies infection. This means its elimination will be difficult; however, vaccination programs have proved successful in eliminating the virus from domestic animals in many urban

areas of Canada, the United States, and Europe. This has resulted in a shift in rabies incidence in nature. For instance, in the United States, the incidence of rabies in dogs fell from 6000 cases in 1953 to 185 cases in 1970, while in the same period the incidence in skunks increased from 319 to 1235 cases (Fischman, 1973).

Although no controlled studies are available to evaluate the efficacy of human rabies vaccines derived from nervous tissue, one can estimate vaccine efficacy by epidemiological means. In one study of 809 persons, who had been bitten by animals proved to be rabid, only 8% of 581 who completed treatment died of rabies, whereas among 153 who refused the vaccine, 50% succumbed to rabies (Davis *et al.*, 1973).

Once vaccines eliciting fewer adverse reactions became available, it was possible to use them in preexposure vaccination of human beings. Preexposure vaccination is employed to protect persons involved in the handling of animals. In addition, preexposure protection is recommended for individuals, especially children, living in areas of the world where rabies is a constant threat.

In postexposure vaccination, the vaccine is used frequently in combination with rabies immune serum, for treatment of the exposed person. For a detailed description of the immunization procedures the reader should refer to publications by the WHO Rabies Expert Committee and the Recommendations of the U.S. Public Health Advisory Committee (World Health Organization, 1973; Center for Disease Control, 1972a).

G. Smallpox

The last case of smallpox may occur in 1976 due to the success of the eradication program. This program may serve as an example for other health programs concerning measles and poliomyelitis where eradication may also be possible (Henderson, 1975).

1. Animal Reservoirs

Eradication of an infectious viral disease can be effective only if there is no animal reservoir for the etiologic agent. Because of this, the WHO has conducted an intensive program to detect any such reservoir for smallpox. The appearance in 1970 of monkeypox infection in humans raised concern that monkeys could act as a reservoir for variola virus (Foster *et al.*, 1972). Although the disease is clinically indistinguishable from smallpox, the virus recovered from the patients was identified as monkeypox (Foster *et al.*, 1972; Rondle and Sayeed, 1972; Marennikova *et al.*, 1972). All cases have occurred in unvaccinated persons, mostly children, in smallpox-free areas and transmission among humans, even if

unvaccinated, does not occur. Therefore, the data available seem to rule out monkeys as a possible reservoir for smallpox (Henderson and Arita, 1973; Kaplan, 1975).

Another finding which has puzzled scientists has been the isolation of two poxviruses from kidney tissue of healthy monkeys. These viruses were different from monkeypox virus and resembled variola virus (Gispen and Brand-Saathof, 1972). They have been provisionally named "white poxviruses" since they produce white lesions in the chorioallantoic membrane of chick embryos in contrast to the hemorrhagic pocks produced by monkeypox viruses (Gispen and Brand-Saathof, 1972). White poxviruses cannot be distinguished from variola in the laboratory and do not seem to have evolved from monkeypox (Gispen, 1975). Although epidemiological data are consistent with the hypothesis that these isolates from healthy monkeys have no relation with human disease, the significance of white poxviruses is at present obscure. It is important to note that in countries with large simian populations, such as Malaysia and the Philipines, smallpox has been eliminated, recurring only when imported. Although the existence of an animal reservoir seems remote it is essential to continue ecologic surveys on mammals, not only nonhuman primates, and to pursue further research on the biology and ecology of variolalike diseases in man (Henderson and Arita, 1973).

2. Development and Characteristics of Vaccines

Vaccinia virus is the term used to designate the agent which is propagated in laboratories and used for vaccine preparation. The history and origin of strains used for vaccination are obscure; they could be derived either from cowpox virus or an attenuated smallpox virus. Whatever the origin, vaccinia has properties different from both cowpox and smallpox viruses.

For the purpose of vaccine production, vaccinia virus has been propagated on the skin of several animal species or on the chorioallantoic membrane of chick embryos. Current smallpox vaccine is a suspension of vaccinia virus propagated on calf skin. Once the suspension is obtained it can be either mixed with glycerine or dried from the frozen state. Mixing the virus suspension with an equal volume of glycerine has proved adequate for use where refrigeration facilities are available. Freeze-drying the virus suspension prior to glycerine addition has yielded a product with an increased thermostability, adequate for use in warmer climates.

3. Control of Smallpox by Vaccination

The intensified global program of smallpox eradication began in January, 1967. This program, currently close to its successful completion,

developed into an outstanding human and technical experience (Henderson, 1975). Its success has been a result of the application of the enormous ingenuity, technical ability, and organizational capacity of the human race. It became quite clear after the initiation of the program that vaccination alone would be insufficient and a different strategy had to be devised if smallpox was to be eradicated. Since the detection and containment of smallpox outbreaks interrupted the transmission of the disease, even in poorly vaccinated populations, the emphasis of the campaign was shifted from mass vaccination to an excellent reporting system and the containment of local outbreaks (Henderson, 1975). The ensuing success proved that this strategic concept was correct.

4. Current Recommendations for Smallpox Prophylaxis

Smallpox vaccination has several complications which have prompted many countries to change their recommendations for its use. For instance, in 1971 the U.S. Public Health Service changed its recommendations from routine smallpox vaccination of children to selective vaccination of persons at special risk. Complications following vaccination can be prevented or treated with immune globulin prepared from adults recently immunized with vaccinia virus. Prophylactically, vaccinia immune globulin should be used along with vaccination in the following situations: pregnancy, eczema (or history of it), other chronic skin ailments, immunodeficiency conditions, and malignancy. It can also be used to treat eczema vaccinatum, vaccinia necrosum, and ocular vaccinia. It is recommended also in cases of generalized vaccinia, but it is of no benefit in the treatment of postvaccination encephalitis. The prophylactic dose of vaccinia immune globulin is 0.3 ml/kg given intramuscularly. The therapeutic dose is 0.6 ml/kg given intramuscularly.

H. Yellow Fever

Yellow fever was a mysterious and dreaded disease for the first European traders in Africa until its transmission by *A. aegypti* was recognized at the beginning of this century. This provided the first step in controlling yellow fever, and was followed about 30 years later by the development of the attenuated 17D vaccine. At present, cases of yellow fever are reported from only Africa and South America. Two forms of yellow fever—urban and jungle—are distinguishable epidemiologically, although clinically and etiologically, they are identical. Urban yellow fever is an epidemic disease of man transmitted by the *Aedes aegypti* mosquito. The jungle form is

enzootic in nonhuman hosts and is transmitted by a variety of mosquito vectors, although human cases occur infrequently.

Two live vaccines are available. One is an attenuated virus preparation made from the 17D strain grown in chick embryos. (see Section I). The other contains the Dakar virus attenuated by serial passage in mouse brain. The Dakar vaccine elicits higher antibody response and is more thermostable, but because it has been associated with a significant incidence (0.5%) of meningoencephalitic reactions, its use has been limited. The 17D vaccine has proved to be very effective and has caused no significant complications. In the infected areas of the world, it is used in mass vaccinations of the populations at risk. In the yellow fever-free areas its use is limited to travelers going to infected areas.

The yellow fever problem has changed little in the last two decades, and the following statement made in 1955 is still fully applicable: "Yellow fever is not a disease which has been conquered. It is not a disease which has been eliminated from consideration as a permanent threat. Too many health authorities get alarmed about yellow fever only when it becomes an urban disease, overlooking entirely the fact that for the jungle populations and for rural workers who have to go into the forest, yellow fever carries the same threat that it previously had for the people in the cities" (Soper, 1955).

I. Other Vaccines

1. Adenovirus

Both inactivated and live vaccines have been experimentally successful in humans. The use of adenovirus vaccine appears to be primarily indicated to protect closed populations, such as military recruits, since the incidence of adenovirus disease is usually low in the general population. Adenovirus infections in military trainees has been a serious problem and an effective vaccine program using live adenovirus vaccines has been conducted for several years in the United States Army (Top, 1975). The vaccine given as an enteric-coated capsule, contains types 4 and 7 and produces an asymptomatic infection of the gastrointestinal tract resulting in the development of serum-neutralizing antibodies. However, since some common pathogenic adenoviruses such as type 7, which is included in the vaccine, are oncogenic for laboratory animals, the use of these vaccines in humans is debatable. One way of avoiding this potential danger of oncogenesis has been to use adenovirus preparations free from their nucleic acids. Purified and crystallized hexon and fiber components of

adenovirus type 5 have been used successfully in human volunteers (Couch *et al.*, 1973).

2. *Arboviruses (Togaviruses)*

There are at least fifteen arbovirus serotypes that affect man, some of which cause severe disease with high mortality (Casals, 1971). Questionable protection has been afforded by formalin-inactivated vaccines grown in the developing chick embryo. More recently, some promising vaccines have been prepared with viruses grown in tissue cultures, either from chick embryos or from human cell lines, such as St. Louis (Darwish and Hammon, 1966) and Japanese encephalitis (Darwish *et al.*, 1967). Inactivated subunit vaccines are also being developed, but so far they are less potent than whole virus vaccines (Mussgay and Weiland, 1973). A number of attenuated arboviruses are being used in experimental vaccines, such as western (Binn *et al.*, 1966) and Venezuelan encephalitis (Alevizatos *et al.*, 1967). Although still in an experimental stage, the vaccine against Venezuelan encephalitis has been given successfully to several thousand persons, including many laboratory workers (Johnson and Ellis, 1974). The magnitude of the ecological problems generated by these viruses, the resulting injury, and constant threat for human populations in so many areas requires that these promising vaccines should soon be developed.

III. COMBINED AND SIMULTANEOUS ADMINISTRATION OF VIRAL VACCINES

With the increasing number of vaccines for viral infections considerable attention has been directed toward the effective use of combined vaccines. The advantages of combining different vaccines have been recognized for years. The three types of inactivated polioviruses were combined in the 1950's with diphtheria and tetanus toxoids and/or pertussis vaccine and were shown to be as efficacious as vaccines given separately.

Combined viral vaccines now in use include trivalent poliovirus vaccine, measles–smallpox, measles–smallpox–yellow fever, measles–mumps–rubella (MMR), measles–mumps, measles–rubella, and mumps–rubella. Data on combined vaccines for MMR, mumps–rubella, measles–rubella, and measles–mumps indicate that the immune response and persistance of antibodies are similar to those following use of monovalent vaccine (Weibel *et al.*, 1975; Schwarz *et al.*, 1975). Data indicate that antibodies persist for at least 5 years following immunization with MMR or mumps–rubella vaccine (Weibel *et al.*, 1975). Adverse reactions to the

various combined vaccines are within acceptable limits and comparable to those found with individual vaccines. The Center for Disease Control (1972b) has reported that the simultaneous administration of MMR with a booster dose of trivalent poliomyelitis vaccine has no effect on the immunological response to each of these antigens. The United States Public Health Service Advisory Committee on Immunization Practices and the American Academy of Pediatrics (Steigman, 1974) state that, where desirable, these vaccines may be administered simultaneously. However, the combination of one manufacturer's vaccine with other manufacturer's vaccines have not been tested sufficiently to recommend their simultaneous administration at this time (Center for Disease Control, 1972a). Although it is preferable to give yellow fever and smallpox vaccines at least 1 month apart, they may be given on the same day at different sites without any loss of efficacy (Center for Disease Control, 1972a). If two live virus vaccines are to be administered, an interval of 2 days to 2 weeks between vaccinations should be avoided since interference between vaccines is most likely to occur during this period (Center for Disease Control, 1972a).

IV. IMMUNIZATION PRACTICES AGAINST VIRAL DISEASES IN THE UNITED STATES AND CANADA

The following immunization schedules and recommendations are taken, in part, from the advisory Committee on Immunization Practices (ACIP) of the U.S. Public Health Service (Center for Disease Control, 1972a), the American Academy of Pediatrics (AAP), (Steigman, 1974), and the National Advisory Committee on Immunizing Agents (NACIA) of Canada (Health and Welfare Canada, 1973). For a detailed description of these recommendations, the reader should refer to the reports of these committees. Recommended schedules for active immunization of infants and children are summarized in Tables III and IV. In Canada, these recommendations differ only in that either killed, or killed-live, poliomyelitis vaccines are used in some provincial immunization programs. Vaccines against influenza, rabies, smallpox, and yellow fever are not routinely used, and, hence their administration is not described here.

A. Poliomyelitis

The first viral vaccine to be given to an infant is trivalent oral poliomyelitis vaccine (TOPV) (Table III) starting with a three-dose immunization series at 6 to 12 weeks of age (Center for Disease Control, 1972a), com-

TABLE III
Recommended Schedule for Active Immunization of Normal Infants in the United States and Canada

Age	Vaccines[a]
2 months	DTP, TOPV
4 months	DTP, TOPV
6 months	DTP, TOPV[b,c]
12 months	Measles, rubella, mumps[d]
18 months	DTP, TOPV
4–6 years	DTP, TOPV
14–16 years	Td (continue every 10 years), TOPV[c]

[a] DTP, diphtheria and tetanus toxoids combined with pertussis vaccine; Td, combined tetanus and diphtheria toxoid (adult type); TOPV, trivalent oral poliovaccine.

[b] Recommended by the American Academy of Pediatrics (Steigman, 1974).

[c] Recommended by the Canadian Advisory Committee on Immunizing Agents (Health and Welfare Canada, 1973).

[d] These vaccines can be administered in monovalent or combined form.

TABLE IV
Recommended Schedule for Active Immunization of Children in the United States Who Did Not Receive Vaccines During First Year of Life

	Age[a]	
Time interval	1–5 years	6 years and older
First visit	DTP, TOPV	Td, TOPV
1 month later	Measles, rubella, mumps[b]	Measles, rubella, mumps[b]
2 months later	DTP, TOPV	Td, TOPV
4 months later	DTP, TOPV[c]	—
6–12 months later	DTP, TOPV	Td, TOPV
At age 14–16 years	Td (continue every 10 years)	Td (continue every 10 years)

[a] Abbreviations are identified in Table III.

[b] These vaccines can be administered in monovalent or combined forms.

[c] Recommended by the American Academy of Pediatrics (Steigman, 1974).

monly with the first dose of diphtheria, tetanus toxoid, and pertussis vaccine (DTP). The second and third doses should be given at intervals of not less than 6 and preferably 8 weeks. The fourth dose is an integral part of primary immunization and should be administered 8–12 months after the third dose. On entering kindergarten or first grade, all children who have completed the primary series of TOPV should be given a single dose of TOPV.

In Canada, for those provinces using a killed-live vaccine for immunization against poliomyelitis, NACIA suggests that killed polio vaccine (preferably combined with other antigens) be administered at 2 to 3 months and 4–5 months of age, followed by the first dose of live vaccine at 6 months of age. Additional reinforcing doses of live vaccine should be administered as outlined in Table III.

Where killed vaccine alone is used to achieve and maintain immunity, the vaccine (preferably combined with other antigens) should be given at 2–3, 4–5 and 5–6 months, followed by reinforcing doses at $1\frac{1}{2}$ years, school entry, midschool, and school exit.

For unimmunized children and adolescents through high school age, the primary series is four and three doses, respectively (Table IV). Routine poliomyelitis immunization for adults residing in the continental United States is not necessary because of the extreme unlikelihood of exposure. However, an unimmunized adult at increased risk through contact with a known case or travel to areas where polio is epidemic or occurs regularly should receive TOPV as indicated for children and adolescents. Persons employed in hospitals, medical laboratories, and sanitation facilities might also be at increased risk.

Pregnancy is not an indication for vaccine administration, or is it a contraindication when protection is required.

Contraindications

Altered Immune States. Infection with live, attenuated viruses (polio, measles, mumps, and rubella) might be potentiated by severe underlying diseases, such as leukemia, lymphoma, or generalized malignancy, or by lowered resistance, such as results from therapy with steroids, alkylating drugs, antimetabolites, or radiation; vaccination should not be conducted where these conditions exist.

B. Measles

For maximum efficacy, measles vaccine should be administered when children are at least 12 months old (Table III). Children who have not re-

ceived vaccine during infancy may be immunized at any age. In areas where the incidence of natural measles occurring before 1 year of age is great, live vaccines may be given with fairly reliable results as early as 6–9 months of age. However, if vaccine is given before 12 months, a second dose should be given at 12 to 16 months of age because the rate of successful seroconversion prior to age 12 months is variable.

Vaccination of adults at the present time is rarely necessary because nearly all persons in the United States and Canada over age 15 are immune. Limited data indicate that adverse reactions to vaccine are no more common in adults than in children.

High Risk Groups. Immunization against measles is particularly important for children with chronic illnesses such as heart disease, cystic fibrosis, and tuberculosis, and for those who are malnourished or are institutionalized. These children are more prone to severe disease and complications.

1. Prior Immunization with Inactivated Measles Virus Vaccine

Atypical measles, sometimes severe, has occasionally followed exposure to natural measles in children previously inoculated with inactivated measles virus vaccines. Untoward local reactions, such as induration and edema, have at times been observed when live measles virus vaccine was administered to persons who had previously received inactivated vaccine.

Despite the risk of local reaction, children who have previously been given only inactivated vaccine should be revaccinated with live vaccine to avoid the severe atypical form of natural measles and to provide full and lasting protection.

2. Contraindications

a. Severe Febrile Illness. Vaccination should be postponed until the patient has recovered.

b. Tuberculosis. Exacerbation of tuberculosis known with natural measles infection might, by analogy, be associated with the live, attenuated measles virus. Therefore, an individual with known active tuberculosis should be under treatment when vaccinated.

Although tuberculin skin testing is desirable as part of ideal health care, it need not be a routine prerequisite in community measles immunization programs. The value of protection against natural measles far outweighs the theoretical hazard of possible exacerbation of unsuspected tuberculosis.

c. Recent Immune Serum Globulin (ISG) Administration. After administration of ISG, vaccination should be deferred for 3 months. Persistence

of measles antibody from the globulin might interfere with optimal response to the vaccine.

d. Hypersensitivity to Vaccine Components. Theoretically, measles, mumps, and rubella vaccine should not be given to individuals clearly hypersensitive to egg protein (mumps and measles are produced in chick embryo cell culture) or the animals from which cells are derived for use in vaccine production, or to other components of the vaccine. To date, there have been no documented reports of serious reactions to measles, mumps, or rubella vaccine clearly attributable to hypersensitivity.

e. Altered Immune States. See poliomyelitis vaccine.

f. Pregnancy. On theoretical grounds, it is prudent to avoid vaccinating pregnant women with live measles or mumps vaccine. Pregnant women should not, under any circumstances, be given rubella vaccine.

C. Mumps

Live mumps vaccine may be used at any age after 12 months (Table III). It should not be administered to children less than 1 year old because of possible persistence of interfering maternal antibody. The vaccine is of particular value in children approaching puberty, adolescents, and adults, especially males who have not had mumps or who have no serological evidence of mumps immunity. The mumps skin test with currently available antigens is not a reliable indicator of immunity.

Contraindications

a. Severe Febrile Illness. See measles vaccine.

b. Marked Hypersensitivity to Vaccine Components. See measles vaccine.

c. Altered Immune States. See poliomyelitis vaccine.

d. Pregnancy. See measles vaccine.

D. Rubella

Live rubella virus vaccine is recommended for all children between the age of 1 year and puberty (Tables III and IV). It should not be administered to infants less than 1 year old due to possible failure to respond to vaccination.

Priority for immunization should be given to children in kindergarten and elementary school because they are the major sources of virus dissemination in the community. For optimum program effectiveness, it is essential that immunization activities be developed to ensure ongoing,

routine immunization of preschool children as well. A history of rubella is not reliable; all children should receive vaccine.

It is desirable that programs of rubella vaccine use in adolescent girls and adult women be extended. Because of the precautions which must apply, potential vaccinees in these groups should be considered individually. They should receive vaccine only if they are shown to be susceptible by serological testing and if they agree to prevent pregnancy for 2 months after immunization.

Contraindications

a. Severe Febrile Illness. See measles vaccine.

b. Marked Hypersensitivity to Vaccine Components. See measles vaccine.

c. Altered Immune States. See poliomyelitis vaccine.

d. Pregnancy. Pregnant women should not under any circumstances be given rubella vaccine.

V. IMMUNIZATION RECOMMENDATIONS FOR TRAVELERS

An international certificate of vaccination or revaccination against smallpox, yellow fever, and cholera may be required for travel to and from some countries. A pamphlet outlining the requirements and possible exemptions from vaccination for travelers is published by the WHO. In general, the only exemptions from vaccinations are for infants under 1 year of age and for those persons with a signed statement from a physician stating that vaccination is not recommended on medical grounds. Amendments to these regulations are published weekly and the latest requirements for travelers to specific areas may be obtained from local health departments. Although not legally binding, it is also advisable when traveling to some areas where poliomyelitis is still prevalent that travelers be vaccinated against this disease.

A. Yellow Fever

An international certificate of vaccination or revaccination against yellow fever is valid for 10 years starting 10 days after vaccination or on the day of revaccination. Vaccination against yellow fever must be done at a center designated by the WHO and only with vaccine approved by the WHO. The vaccine is generally not given to infants under 1 year of age due to the risk of encephalitis. When yellow fever and smallpox vaccinations are both required it is preferable that a 3-week period be left

between vaccinations although both vaccines may be given at the same time at different inoculation sites.

B. Smallpox

An international certificate of vaccination or revaccination against smallpox is valid for 3 years, starting 8 days after a successful primary vaccination or on the day of revaccination. Since some countries do not allow a minimum age limit of 1 year, infants should be vaccinated unless a medical certificate stating otherwise is obtained.

VI. PROSPECTS FOR NEW VIRAL VACCINES

Since vaccination is the most effective means of preventing viral infection it is not surprising that extensive research is being conducted on the development of vaccines for many viral diseases.

A. Hepatitis Viruses

Research on hepatitis over the last several years has shown that the hepatitis B antigen exists in three forms, 20-nm spherical and filamentous particles and a spherical 42-nm Dane particle, which is probably the complete hepatitis B virion. The 20-nm particles carry the same antigenic specificity as the coat or surface of the 42-nm paticle (hepatitis B surface antigen, HBsAg). Vaccines now under study are prepared by separating the 20-nm particles from plasma of chronic HBsAg carriers. These preparations are treated with formalin to inactivate any residual infectious agents. Four hepatitis subtypes designated *ayw, ayr, adw,* and *adr* have been identified (Krugman, 1975). Recent studies in chimpanzees indicate that these purified HBsAg preparations are immunogenic and safe (Purcell and Gerin, 1975; Buynak *et al.*, 1976). Purcell and Gerin (1975) immunized animals with HBsAg subtypes *ayw* or *adr* and showed that they were protected when challenged 6 months later with infectious plasma containing hepatitis B subtype *ayw*. Hilleman *et al.* (1975) have demonstrated an immune response in chimpanzees immunized with a vaccine comprised of two subtypes. Test results on the efficacy and safety of this vaccine in chimpanzees are encouraging (Buynak *et al.*, 1976). Clinical trials in humans, to determine the safety, antigenicity, and efficacy of these vaccines, are still required. Further studies will also be needed to determine if a vaccine prepared from one of the four subtypes will provide protection against infection with the other subtypes. However, the ultimate control

of hepatitis type B is hindered by the fact that the agent has not yet been cultivated in tissue culture.

The identification of the hepatitis type A particle by immune electron microscopy (Feinstone *et al.*, 1973) and the subsequent demonstration that similar particles are associated with acute cases of hepatitis A infection (Maynard, 1975) raise the possibility that a vaccine for hepatitis A virus may be feasible. This development is dependent on the propagation of the virus in cell culture but this has not yet been achieved.

B. Herpesviruses

Development of vaccines for members of the herpesvirus group have been accompanied by considerable controversy on the possible oncogenicity and latency of these viruses. Tests conducted with a vaccine prepared from killed whole herpes simplex virus types 1 and 2 indicate that the vaccine is immunogenic in animals but the immune response in humans is poor (Hilleman, 1976). Inactivated vaccine for *Herpesvirus saimiri* have been shown in one study to induce lymphomas in monkeys following inoculation (Ablashi *et al.*, 1973). Other investigators have found that an inactivated vaccine against this virus protects animals on subsequent challenge (Laufs and Steinke, 1976). These studies underline the complexities involved in producing a vaccine against the herpesviruses. Once a safe and effective vaccine is developed, the question remains of whom to vaccinate. For instance, it is doubtful that a vaccine would have any effect in preventing recurrent herpes since most persons who experience recurrent infections do so in the presence of relatively high levels of neutralizing antibody.

Limited trials have also been conducted with experimental vaccines for two other members of the herpesvirus group, cytomegalovirus (CMV) and varicella-zoster (VZ). Both CMV and VZ usually produce relatively mild infections, especially in children, and there has not been as great an impetus for a vaccine as with other viral infections. However, CMV infection can also be serious and may involve the liver and lung or result in the development of a mononucleosis-like syndrome. Infection in persons receiving multiple transfusions, or in immunosuppressed patients, is generally more severe. Of greater concern, however, is the problem of prenatal infection with CMV. Stern *et al.* (1969) report that not only is prenatal CMV infection the most prevalent congenital infection today but that it is also probably the most important cause of congenital mental retardation. Evidence indicates that 1% of all newborn children have been infected *in utero* and of these, 10 to 33% show some brain damage. Thus CMV vacci-

nation, as with rubella, would be intended primarily for the prevention of fetal infection. Data from two laboratories indicate that experimental live, attenuated vaccines stimulate an immune response in volunteers (Elek and Stern, 1974; Plotkin *et al.*, 1975). Similarly, a live, attenuated vaccine for varicella recently developed by Japanese investigators, has been shown to stimulate antibodies in seronegative children (Takahashi *et al.*, 1974). As with herpesvirus types 1 and 2, the advisability of administering a vaccine for CMV or VZ has been tempered by observations that these agents may produce a latent infection or may be oncogenic. Whether these vaccines can prevent activation of a latent infection or whether the vaccine virus may itself become latent remains to be answered. The demonstration that irradiated human CMV can transform hamster embryo fibroblasts has increased the concern expressed by many scientists although there is no evidence yet to suggest that CMV or VZ are oncogenic in man.

C. Rhinoviruses

Vaccination against rhinovirus infection has been encumbered by the large number of serotypes which may exceed 100. Inactivated rhinovirus vaccines have been shown to induce both an immune response and resistance to subsequent challenge with homotypic virus (Perkins *et al.*, 1969; Douglas and Couch, 1972). However, the large number of serotypes has limited the feasibility of preparing an effective vaccine against rhinovirus infections. The suggestion that infection with one rhinovirus serotype may produce antibody which cross-reacts with a heterotypic serotype (Fleet *et al.*, 1968; Mogabgab *et al.*, 1975) raises the question of whether a vaccine containing a few selected antigenic types could be used in an effective vaccine. Recent studies using inactivated vaccines with several different serotypes indicated that such an approach may be feasible (Hamory *et al.*, 1975; Mogabgab *et al.*, 1975).

D. Respiratory Syncytial Virus

Respiratory syncytial virus (RSV) causes a significant degree of morbidity in children and is responsible for a large number of deaths in infants. Attempts to produce an effective, inactivated vaccine have been largely unsuccessful because immunity to RSV is probably correlated with secretory antibody and early inactivated vaccines induced high levels of circulating, but no secretory, antibody (Fulginiti *et al.*, 1969; Chanock, 1970). In addition, 80% of immunized infants in one study

developed a severe illness when they were infected later with wild virus (Kim *et al.*, 1969). Recent trials with live experimental vaccines by McIntosh *et al.* (1974) and Parrott *et al.* (1975) have shown some promise in providing protection against subsequent infection. In both studies, increase in serum and local antibody was detected following administration of the vaccine. Both groups found, however, that the vaccine strains had some residual pathogenicity for infants and also showed some genetic instability. Nonetheless, these data do indicate that the preparation of an effective vaccine for RSV may be possible once the problems of genetic instability and pathogenicity of the vaccine virus have been resolved.

E. Parainfluenza Virus

Efforts to produce a vaccine for parainfluenza viruses have encountered problems similar to those for RSV. Following immunization with an inactivated parainfluenza type 1 vaccine, Smith *et al.* (1966) found that significant titers of circulating antibody to parainfluenza 1 failed to prevent infection of volunteers challenged with virus. In contrast, experimentally infected volunteers who possessed secretory antibody were protected. Inactivated vaccines have also failed to protect children against natural parainfluenza infection, although the vaccine had stimulated serum hemagglutination inhibition antibodies in a large proportion of the seronegative children (Chin *et al.*, 1969; Fulginiti *et al.*, 1969). As found with RSV, a more promising approach would be the development of a live, attenuated vaccine which would be capable of inducing secretory antibodies in the nasal tract.

F. Chronic Viral Diseases

The developments described so far will provide vaccines for most of the acute viral diseases. There is increasing evidence that viruses play an important role in chronic diseases resulting either from degenerative processes or from neoplastic growth. However, several virological as well as immunological questions regarding the pathogenesis of chronic diseases will have to be answered before suitable vaccines can be developed.

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REFERENCES

Abdussalam, M., and Bögel, K. (1971). *Sci. Publ., Pan. Am. Health Organ.* **226,** 54–59.

Ablashi, D. V., Loeb, W. F., Pearson, G., Valerio, M. G., Armstrong, G. R., Kingsbury, E. W., and Heine, V. (1973). *Nature (London), New Biol.* **242,** 28–30.

Alevizatos, A. C., McKinney, R. W., and Feigin, R. D. (1967). *Am. J. Trop. Med. Hyg.* **16,** 762–768.

Barkin, R. M. (1975). *Am. J. Epidemiol.* **102,** 341–349.

Beare, A. S., and Bynoe, M. L. (1969). *Br. Med. J.* **4,** 198–201.

Beare, A. S., and Hall, T. S. (1971). *Lancet* **2,** 1271–1273.

Biano, S., Cochran, W., Herrmann, K., Hall, A. D., and Chang, T. W. (1975). *Am. J. Dis. Child.* **129,** 1353–1356.

Binn, L. N., Sponseller, M. L., Wooding, W. L., McConnell, S. J., Spertzel, R. O., and Yager, R. H. (1966). *Am. J. Vet. Res.* **27,** 1599–1604.

Borgoño, J. M., and Greiber, R. (1971). *Sci. Publ., Pan. Am. Hlth. Organ.* **226,** 203–206.

Borkow, R. B. (1975). *N.Y. State J. Med.* **75,** 424–425.

Boué, A., Nicolas, A., and Montagnon, B. (1971). *Lancet* **1,** 1251–1253.

Brodie, M., and Park, W. H. (1936). *Am. J. Pub. Health* **26,** 119–125.

Buser, F. (1967). *N. Engl. J. Med.* **277,** 250–251.

Buynak, E. B., and Hilleman, M. R. (1966). *Proc. Soc. Exp. Biol. Med.* **123,** 768–775.

Buynak, E. B., Roehm, R. R., Tytell, A. A., Bertland, A. U., Lampson, G. P., and Hilleman, M. R. (1976). *Proc. Soc. Exp. Biol. Med.* **151,** 694–700.

Casals, J. (1971). *Sci. Publ., Pan Am. Health Organ.* **226,** 47–48.

Center for Disease Control. (1970). *Morbidity, Mortality Week. Rep.* **19,** Annu. Suppl., Summ. p. 4.

Center for Disease Control. (1972a). *Morbidity, Mortality Week. Rep. Suppl.* **21,** 1–34.

Center for Disease Control. (1972b). *Morbidity, Mortality Week. Rep.* **21,** 402.

Center for Disease Control. (1974). *Morbidity, Mortality Weekly Rep.* **23,** Annu. Suppl. No. 53, p. 2.

Center for Disease Control. (1975a). *Poliomyelitis Surveillance, Annu. Summ., 1973* pp. 1–13.

Center for Disease Control. (1975b). *U.S. Immunization Surv., 1974* pp. 1–56.

Center for Disease Control. (1975c). *Morbidity, Mortality Week, Rep.* **24,** 214.

Center for Disease Control. (1976). *Morbidity, Mortality Week. Rep.* **24,** 445.

Chang, T. W., DesRosiers, S., and Weinstein, L. (1970). *N. Engl. J. Med.* **283,** 246–248.

Chanock, R. M. (1970). *Science* **169,** 248–256.

Chin, J., Magoffin, R. L., Shearer, L. A., Schieble, J. H., and Lennette, E. H. (1969). *Am. J. Epidemiol.* **89,** 449–463.

Cockburn, W. C., and Drozdov, S. G. (1970). *Bull. W. H. O.* **42,** 405–417.

Conrad, J. L., Wallace, R., and Witte, J. J. (1971). *Am. J. Public Health* **61,** 2304–2310.

Couch, R. B., Kasel, J. A., Pereira, H. G., Haase, A. J., and Knight, V. (1973). *Proc. Soc. Exp. Biol. Med.* **143,** 905–910.

Cox, H. R., Cabasso, V. J., Markham, F. S., Moses, M. J., Moyer, A. W., Roca-Garcia, M., and Ruegsegger, J. M. (1959). *Br. Med. J.* **2,** 591–597.

Darwish, M. A., and Hammon, W. McD. (1966). *Proc. Soc. Exp. Biol. Med.* **123,** 242–246.

Darwish, M. A., Hammon, W. McD., and Sather, G. E. (1967). *Am. J. Trop. Med. Hyg.* **16,** 361–370.

Davis, B. D., Dulbecco, R., Eisen, H. R., Ginsberg, H. S., and Wood, B. W. (1973). *In* "Microbiology," 2nd ed., pp. 1368–1376. Harper, New York.

Davis, W. J., Larson, H. E., Simsarian, J. P., Parkman, P. D., and Meyer, H. M., Jr. (1971). *J. Am. Med. Assoc.* **215,** 600–608.
Douglas, R. G., and Couch, R. B. (1972). *Proc. Soc. Exp. Biol. Med.* **139,** 899–902.
Dowdle, W. R., Coleman, M. T., and Gregg, M. B. (1974). *Prog. Med. Virol.* **17,** 92–135.
Elek, S. D., and Stern, H. (1974). *Lancet* **1,** 1–5.
Enders, J. F., and Peebles, T. C. (1954). *Proc. Soc. Exp. Biol. Med.* **86,** 277–286.
Enders, J. F., Weller, T. H., and Robbins, F. C. (1949). *Science* **109,** 85–87.
Feinstone, S. M., Kapikian, A. Z., and Purcell, R. H. (1973). *Science* **182,** 1026–1028.
Fenje, P. (1960). *Can. J. Microbiol.* **6,** 605–609.
Fischman, H. R. (1973). *In* "Preventive Medicine and Public Health" (P. E. Sartwell, ed.), 10th ed., pp. 343–361. Appleton, New York.
Fleet, W. F., Douglas, R. G., Jr., Cate, T. R., and Couch, R. B. (1968). *Proc. Soc. Exp. Biol. Med.* **127,** 503–509.
Foster, S. O., Brink, E. W., Hutchins, D. L. *et al.* (1972). *Bull. W. H. O.* **46,** 569–576.
Francis, T., Jr. (1945). *Am. J. Hyg.* **42,** 1–11.
Francis, T., Jr., Napier, J. A., Voight, R. B. *et al.* (1957). *Mich., Univ., Poliomyelitis Vaccine Evaluation Cent.* pp. i–xiv and 1–63.
Fuenzalida, E., Palacios, R., and Borgoño, J. M. (1964). *Bull. W. H. O.* **30,** 431–436.
Fulginiti, V. A., Eller, J. J., Downie, A. W., and Kempe, C. H. (1967). *J. Am. Med. Assoc.* **202,** 1075–1080.
Fulginiti, V. A., Eller, J. J., Sieber, O. F., Jayner, J. W., Minamitani, M., and Meiklejohn, G. (1969). *Am. J. Epidemiol.* **89,** 435–448.
Gerber, P. (1967). *Proc. Soc. Exp. Biol. Med.* **125,** 1284–1287.
Gispen, R. (1975). *Trans. R. Soc. Trop. Med. Hyg.* **69,** 299–302.
Gispen, R., and Brand-Saathof, B. (1972). *Bull. W. H. O.* **46,** 585–592.
Gregg, N., McA. (1941). *Trans. Ophthalmol. Soc. Aust.* **3,** 35–46.
Habel, K. (1945). *Pub. Health Rep.* **60,** 201–212.
Habel, K. (1951a). *Am. J. Hyg.* **54,** 295–311.
Habel, K. (1951b). *Am. J. Hyg.* **54,** 312–318.
Hamory, B. H., Hamparian, V. V., Conant, R. M., and Gwaltney, J. M., Jr. (1975). *J. Infect. Dis.* **132,** 623–629.
Haukenes, G., and Haram, K. O. (1972). *N. Engl. J. Med.* **287,** 1204.
Haukenes, G., Haram, K., and Solberg, C. O. (1973). *N. Engl. J. Med.* **289,** 429.
Health and Welfare Canada. (1973). *Epidemiol. Bull.* **17,** 157.
Health and Welfare Canada. (1975). *Can. Dis. Week. Rep.* **1,** 134.
Henderson, D. A. (1975). *J. Clin. Pathol.* **28,** 843–849.
Henderson, D. A., and Arita, I. (1973). *WHO Chron.* **27,** 145–148.
Henderson, D. A., White, J. J., Morris, L., and Langmuir, A. D. (1964). *J. Am. Med. Assoc.* **190,** 31–48.
Henle, G., Bashe, W. J., Jr., Burgoon, J. S., Burgoon, C. F., Hunt, G. R., and Henle, W. (1951). *J. Immunol.* **66,** 561–577.
Henle, W., Crawford, N. M., Henle, G., Tabio, H. F., Deinhardt, F., Chabau, A. G., and Olshin, I. G. (1959). *J. Immunol.* **83,** 17–28.
Hilleman, M. R. (1976). *Cancer Res.* **36,** 857–858.
Hilleman, M. R., Buynak, E. B., Roehm, R. R., Tytell, A. A., Bertland, A. U., and Lampson, G. P. (1975). *Am. J. Med. Sci.* **270,** 401–404.
Hobson, D. (1975). *Proc. Symp. Int. Assoc. Biol. Stand., 47th, 1974* Vol. 28, pp. 285–293.
Horstmann, D. M. (1975). *Ann. Intern. Med.* **83,** 412–417.
Horstmann, D. M., Liebhaber, H., LeBouvier, G. L., Rosenberg, D. A., and Halstead, S. B. (1970). *N. Engl. J. Med.* **283,** 771–778.

Huygelen, C., Peetermans, J., and Prinzie, A. (1969). *Prog. Med. Virol.* **11,** 107–125.
Huygelen, C., Peetermans, J., Vascoboinic, E., Berge, E., and Colinet, G. (1973). *Proc. Int. Symp. Influenza Vaccines Man Horses, 1972* Vol. 1, pp. 52–57.
Jenner, E. (1798). Pamphlet Vol. 4232. Army Med. Libr., Washington, D.C. (reprinted by Cassell, London, 1896).
Johnson, R. H., and Ellis, R. J. (1974). *Ann. Intern. Med.* **81,** 61–67.
Kaplan, C. (1975). *Trans. R. Soc. Trop. Med. Hyg.* **69,** 293–298.
Katz, S. L., and Enders, J. F. (1959). *Am. J. Dis. Child.* **98,** 605.
Katz, S. L., Enders, J. F., and Holloway, A. (1962). *Am. J. Dis. Child.* **103,** 340–344.
Kim, H. W., Canchola, J. G., Brandt, C. D., Pyles, G., Chanock, R. M., Jensen, K., and Parrott, R. H. (1969). *Am. J. Epidemiol.* **89,** 422–434.
Kolmer, J. A. (1936). *Am. J. Public Health* **26,** 126–135.
Koprowski, H., Norton, T. W., Jervis, G. A. *et al.* (1956). *J. Am. Med. Assoc.* **160,** 954–966.
Krugman, S. (1971). *J. Pediatr.* **78,** 1–16.
Krugman, S. (1973). *Proc. Symp. Int. Assoc. Biol. Stand., 45th, 1973* Vol. 22, pp. 55–63.
Krugman, S. (1975). *J. Pediatr.* **87,** 1067–1077.
Langer, W. L. (1976). *Sci. Am.* **234,** 112–117.
Laufs, R., and Steinke, H. (1976). *Cancer Res.* **36,** 704–706.
Levens, J. H., and Enders, J. F. (1945). *Science* **102,** 117–120.
Maassab, H. F. (1969). *J. Immunol.* **102,** 728–732.
McIntosh, K., Arbeter, A. M., Stahl, M. K., Orr, I. A., Hodes, D. S., and Ellis, E. F. (1974). *Pediatr. Res.* **8,** 689–696.
Marennikova, S. A., Sěluhina, E. M., Mal'ceva, N. N., Cimiskjan, K. L., Macevič, G. R. *et al.* (1972). *Bull. W. H. O.* **46,** 599–611.
Maynard, J. E. (1975). *Am. J. Pathol.* **81,** 683–693.
Medical Research Council Influenza Committee. (1953). *Br. Med. J.* **2,** 1173–1177.
Medical Research Council Influenza Committee. (1957). *Br. Med. J.* **2,** 1–7.
Medical Research Council Influenza Committee. (1958). *Br. Med. J.* **1,** 415–418.
Meyer, H. M., Parkman, P. D., and Panos, T. C. (1966). *N. Engl. J. Med.* **275,** 575–580.
Milovanović, M. V., Enders, J. F., and Mitus, A. (1957). *Proc. Soc. Exp. Biol. Med.* **95,** 120–127.
Modlin, J. F., Brandling-Bennett, A. D., Witte, J. J., Campbell, C. C., and Meyers, J. D. (1975). *Pediatrics* **55,** 20–29.
Modlin, J. F., Herrmann, K., Brandling-Bennett, A. D., Eddins, D. L., and Hayden, G. F. (1976). *N. Engl. J. Med.* **294,** 972–974.
Mogabgab, W. J., Holmes, B. J., and Pollock, B. (1975). *Proc. Symp. Int. Assoc. Biol. Stand., 47th, 1974* Vol. 28, pp. 400–411.
Mostow, S. R., Schoenbaum, S. C., Dowdle, W. R., Coleman, M. R., and Kaye, H. S. (1969). *Bull. W. H. O.* **41,** 525–530.
Murphy, B. R., Chalhub, E. G., Nusinoff, S. R., and Chanock, R. M. (1972). *J. Infect. Dis.* **126,** 170–178.
Murphy, B. R., Chalhub, E. G., Nusinoff, S. R., Kasel, J., and Chanock, R. M. (1973). *J. Infect. Dis.* **128,** 479–487.
Murray, R. (1968). *Natl. Cancer Inst., Monogr.* **29,** 460.
Mussgay, M., and Weiland, E. (1973). *Intervirology* **1,** 259–268.
Nathanson, N., and Langmuir, A. D. (1963). *Am. J. Hyg.* **78,** 16–28.
Northrop, R. L., Gardner, W. M., and Geittmann, W. F. (1972). *Obstet. Gynecol.* **39,** 524–526.

Ontario Ministry of Health. (1974). "Computer Assisted School Health (CASH) Programme" Min. Health. Ontario.
Parkman, P. D., Buescher, E. L., and Artenstein, M. S. (1962). *Proc. Soc. Exp. Biol. Med.* **111,** 225–230.
Parrott, R. H., Kim, H. W., Brandt, C. D., and Chanock, R. M. (1975). *Proc. Symp. Int. Assoc. Biol. Stand., 47th, 1974* Vol. 28, pp. 389–398.
Pasteur, L. (1895). *C.R. Hebd. Seances Acad. Sci.* **101,** 765–772.
Peck, F. B. (1968). *J. Am. Med. Assoc.* **206,** 2277–2282.
Peck, F. B., Powell, H. M., and Culbertson, C. G. (1956). *J. Am. Med. Assoc.* **162,** 1373–1376.
Perkins, J. C., Tucker, D. N., Knofp, H. L. S., Wenzel, R. P., Kapikian, A. Z., and Chanock, R. M. (1969). *Am. J. Epidemiol.* **90,** 519–526.
Plotkin, S. A. (1973). *J. Pediatr.* **82,** 908–911.
Plotkin, S. A., Farquhar, J. D., Katz, M., and Buser, F. (1969). *Am. J. Dis. Child.* **118,** 178–185.
Plotkin, S. A., Farquhar, J. D., and Ogra, P. L. (1973). *J. Am. Med. Assoc.* **225,** 585–590.
Plotkin, S. A., Furukawa, T., Zygraich, N., and Huygelen, C. (1975). *Infect. Immun.* **12,** 521–527.
Plotkin, S. A., Wiktor, T. J., Koprowski, H., Rosanoff, E. I., and Tint, H. (1976). *Am. J. Epidemiol.* **103,** 75–80.
Purcell, R. H., and Gerin, J. L. (1975). *Am. J. Med. Sci.* **270,** 395–399.
Rauh, L. W., and Schmidt, R. (1965). *Am. J. Dis. Child.* **109,** 232–237.
Rawls, W. E., Rawls, M. L., and Chernesky, M. A. (1975). *Can. Med. Assoc. J.* **113,** 941–944.
Reimer, C. B., Baker, R. S., van Frank, R. M., Newlin, T. E., Cline, G. B., and Anderson, N. G. (1967). *J. Virol.* **1,** 1207–1216.
Robbins, F. C. (1968). *Natl. Cancer Inst., Monogr.* **29,** 457–461.
Robbins, F. C. (1972). *N. Engl. J. Med.* **287,** 615.
Rondle, C. J. M., and Sayeed, K. A. R. (1972). *Bull. W. H. O.* **46,** 577–583.
Sabin, A. B. (1955). *Ann. N.Y. Acad. Sci.* **61,** 924–938.
Sabin, A. B., and Boulger, R. L. (1973). *J. Biol. Stand.* **1,** 115–118.
Salk, J. E. (1948). *J. Immunol.* **58,** 369–395.
Salk, J. E. (1953). *J. Am. Med. Assoc.* **151,** 1081–1098.
Schoenbaum, S. C., Biano, S., and Mack, T. (1976). *J. Am. Med. Assoc.* **235,** 248.
Schreier, H. A. (1974). *N. Engl. J. Med.* **290,** 803–804.
Schwarz, A. J. F., Jackson, J. E., Ehrenkranz, N. J., Ventura, A., Schiff, G. M., and Walters, V. W. (1975). *Am. J. Dis. Child.* **129,** 1408–1412.
Semple, D. (1911). *Sci. Mem. Med. Sanit. Dep. India* No. 44.
Smith, C. B., Purcell, R. H., Bellanti, J. A., and Chanock, R. M. (1966). *N. Engl. J. Med.* **275,** 1145–1152.
Smith, W., Andrewes, C. H., and Laidlaw, P. P. (1933). *Lancet* **2,** 66–68.
Smorodintsev, A. A. (1969). *Bull. W. H. O.* **41,** 585–588.
Smorodintsev, A. A., and Klyatchko, N. S. (1958). *Acta Virol. (Engl. Ed.)* **2,** 137–151.
Smorodintsev, A. A., Alexandrova, G. A., Chalkina, O. M., and Selivanov, A. A. (1965). *Proc. Annu. Symp. Appl. Resp. Infect. 1st, 1964* pp. 142–165.
Soper, F. L. (1955). *Am. J. Trop. Med. Hyg.* **4,** 573–582.
Steigman, A. J., ed. (1974). "Report of The Committee on Infectious Diseases," 17th ed., Am. Acad. Pediatr., Evanston, Illinois.
Stern, H., Elek, S. D., Booth, J. C., and Fleck, D. G. (1969). *Lancet* **2,** 443–448.

Svet-Moldavskij, G. J., Andjaparidze, O. G., Unanov, S. S., *et al.* (1965). *Bull. W. H. O.* **32,** 47–58.

Sweet, B. H., and Hilleman, M. R. (1960). *Proc. Soc. Exp. Biol. Med.* **105,** 420–427.

Takahashi, M., Otsuka, T., Okuno, Y., Yazaky, A. Y., and Isomuras, T. (1974). *Lancet* **2,** 1288–1290.

Theiler, M. (1951). *In* "Yellow Fever" (G. K. Strode, ed.), pp. 39–136. McGraw-Hill, New York.

Theiler, M., and Smith, H. H. (1937). *J. Exp. Med.* **65,** 787–800.

Top, F. H., Jr. (1975). *Yale J. Biol. Med.* **48,** 185–195.

Vaheri, A., Vesckari, T., Oker-Blom, N., Seppala, M., Parkman, P. D., Veronelli, J., and Robbins, F. C. (1972). *N. Engl. J. Med.* **286,** 1071–1074.

Weibel, R. E., Buynak, E. B., McLean, A. A., and Hilleman, M. R. (1975). *Pediatrics* **56,** 380–387.

Weller, T. H., and Neva, F. A. (1962). *Proc. Soc. Exp. Biol. Med.* **111,** 215–225.

Wiktor, T. J. (1975). *Wistar Inst. Res. Rep.* pp. 1–63.

World Health Organization, (1969). *Bull. W. H. O.* **40,** 925–945.

World Health Organization. (1973). *W.H.O., Tech. Rep. Ser.* **523.**

Yofe, J., Goldblum, N., Eylan, E., and Melnick, J. L. (1962). *Am. J. Hyg.* **76,** 225–238.

Zuñiga, M., Kerschbaum de Pizze, A., Speulvéda, A., *et al.* (1962). *Rev. Chil. Pediatr.* **33,** 89–98.

Chapter 6

Chemotherapy of Viral Diseases: Present Status and Future Prospects

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I. INTRODUCTION

Therapy of established viral diseases is limited almost exclusively to supportive measures designed to augment the patients' natural defenses against infection. Although the patient may be provided symptomatic relief, there is little that can be done directly to influence the course of the virus infection itself. The only widely used measures in defense against viral diseases are prophylactic, i.e., the vaccines where available, and γ- or immune serum globulins. There is a wide range of viral infections where immune prophylaxis by either method is impractical or unavailable and for which there is a need to develop other means of control. Two such means are through the use of antiviral substances and immunotropic agents, both of which may be utilized as chemotherapeutic or chemoprophylactic agents in viral diseases.

Progress in the development of clinically acceptable antiviral substances has been slow because our knowledge of the biosynthetic pathways utilized by both the host cells and the virus is still evolving and acceptable antiviral efficacy depends upon agents which selectively inhibit or block viral biosynthesis but have minimal or no adverse effect on host cell biosyntheses or function.

We are now entering a period where several agents currently under investigation appear to hold promise as effective antivirals with acceptable therapeutic indexes. Although this chapter cannot be all-inclusive in its coverage, it does attempt to describe those agents which have been considered promising in the past as well as those which continue to hold promise for the future.

II. GENERAL CONSIDERATIONS

Antiviral substances with recognized clinical potential have been available for investigational human use for more than a decade. Agents such as methisazone, amantadine, and topical idoxuridine have received extensive clinical trials and are now recognized as having a legitimate place among the antimicrobials available for the prophylaxis or therapy of infectious diseases. As detailed later in this chapter, others, such as cytosine arabinoside and systemically administered idoxuridine, which appeared to have promise as antiviral agents, have fallen into disuse because of their inherent toxicity.

Among the antimetabolites which have been studied over the past 25 years, the nucleoside analogs have received special attention as potential

antivirals since many of the biosynthetic pathways they block or inhibit are utilized by both the dividing host cell and the replicating virus. A problem with many of these agents is that they tend to be nonselective in their actions, their antiviral efficacy and their anticell activity parallel each other in relation to dose. The result is, of course, a therapeutic dose range close or equal to the toxic dose range. In general, the toxicity of the nucleoside analogs is first manifested in rapidly dividing cells, such as bone marrow, the epithelium of the gastrointestinal tract, and oral mucosa, with changes also seen in skin, hair, and nails. Early symptoms of toxicity due to this class of drugs are usually anorexia and nausea, followed by diarrhea.

As is true for the antineoplastic effects of the nucleoside analogs, their antiviral activity appears to be dependent upon the intracellular phosphorylation of the inactive nucleoside base to the nucleotide level. As the nucleotide analog, they act as competitive inhibitors of their natural nucleotide counterparts with enzymes involved in nucleotide or nucleic acid synthesis. In the case of the purine nucleotides, their actions might also produce alterations in intracellular cyclic nucleotide levels modifying the physiologic activity of multiple organ systems. For example, ara-ATP has been reported to competitively inhibit adenyl cyclase, the enzyme which catalyzes the production of cyclic 3′,5′-AMP (Ortiz, 1972). Both the therapeutic and the adverse effects of the purine nucleoside analogs might thus be more complex than those of pyrimidine nucleoside analogs, whose principle site of action is clearly nucleic acid synthesis.

The dual roles of the cyclic nucleotides, cyclic adenosine 3′,5′-monophosphate (cAMP) and cyclic guanosine 3′,5′-monophosphate (cGMP), in viral replication have not yet been clearly determined. Obviously, as messengers or mediators of cellular metabolic and biosynthetic activities, their potential role is important. A fuller explanation for both the antiviral and toxic effects of many antiviral compounds, including interferons, may come with the demonstration of their effects on cyclic nucleotide levels.

An exception to the role of "lethal" phosphorylation could be adenine arabinoside (ara-A), an analog of deoxyadenosine. There is still some doubt whether this compound is phosphorylated intracellularly, although experimental evidence exists that it is (Brink and Le Page, 1964a,b; Cohen, 1966; Bennett *et al.*, 1975). Ara-A poses a special case in another way, however, since much of its antiviral activity appears to take place following deamination to hypoxanthine arabinoside (ara-Hx), a nucleoside analog of deoxyguanosine. Whether ara-Hx is phosphorylated intracellularly to the nucleotide level is also open to question (Le Page, 1970).

If significant amounts of the monophosphate are formed, however, the relatively lower toxicity of ara-A could in part be due to a more complex inhibitory effect divided between the two purine nucleotide forms.

In conrast to the mechanisms of action of the nucleoside analogs, the interferons act mainly to inhibit the translation of viral or virally induced mRNA into protein. Cellular DNA, RNA, and protein syntheses are affected much less by the antiviral action of the interferons. Although the high cost of production of exogenous interferons and the lack of potent, nontoxic inducers of endogenous interferon have slowed the development of this broad-spectrum antiviral, new inducers and improved methods of stimulation through superinduction offer hope that the practical application of interferon in antiviral therapy may soon be feasible.

It will become obvious in reading this chapter that the era of antiviral agents has really just begun. A great deal of *in vitro* and *in vivo* work needs to be carried out testing the antiviral potency and toxicity of these substances per se, in select combination with each other, and in combination with immunotropic agents. With this and with the development of still newer agents, we should be able to broaden the spectrum of activity and open the gap between efficacy and toxicity ever wider.

III. NUCLEOSIDE ANALOGS

The antiviral activity of nucleoside analogs has been recognized since the discovery in 1949 that several 5-substituted uracils inhibited the replication of vaccinia virus in chick embryo cell cultures (Thompson *et al.*, 1949). Since that time, the antiviral activity of a very large number of both purine and pyrimidine analogs have been tested *in vitro* and *in vivo*. For a detailed account of this work the reader is referred to the comprehensive review by Schabel and Montgomery (1972). Only those analogs which currently appear to have potential for clinical use will be discussed here.

A. Pyrimidines

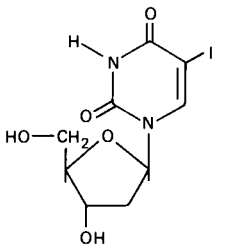
1. Idoxuridine (IDU)

a. History. During the search for pyrimidine analogs which might have potential for antiviral as well as antineoplastic therapy, a number of potent antimetabolites were tested. Since thymine is involved principally in DNA synthesis and fluorides, like fluoroacetate, are powerful metabolic poisons, some of the earliest compounds of this type developed were fluoride and other halogenated analogs of thymidine. One of these, idox-

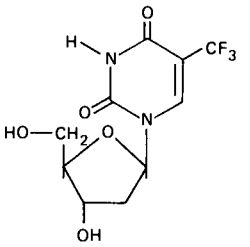
uridine (IDU) (Stoxil and Smith, Kline and French), was found to have interesting antiviral properties as well as antineoplastic activity. The discovery by Herrmann that idoxuridine had a potent suppressive effect on the replication of certain DNA viruses led to the pioneering work of Kaufman in its therapeutic application to *Herpesvirus hominis* (HVH) keratitis (Herrmann, 1961; Kaufman, 1962).

b. Antiviral Activity and Mechanism of Action. The formula of IDU is shown in Fig. 1. The iodine atom at the 5-position on the pyrimidine ring makes this compound an analog of thymidine, a nucleoside involved almost exclusively with DNA synthesis.

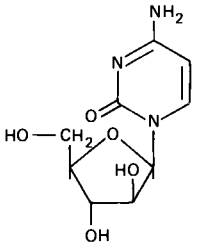
IDU is active *in vitro* against several DNA viruses including HVH



Idoxuridine (IDU)



Trifluorothymidine (F_3T)



Cytosine Arabinoside

Fig. 1. Pyrimidine nucleosides.

types I and II, cytomegalovirus, vaccinia, and pseudorabies (Goz and Prusoff, 1970; Sidwell *et al.*, 1970; Loddo *et al.*, 1963; Kaplan and Ben Porat, 1966). As might be expected, it lacks activity against RNA viruses except those which, like Rous sarcoma virus, form DNA intermediates (Force and Stewart, 1964).

Both the antimetabolite and antiviral effects of the drug itself appear to be due to the incorporation of the fraudulent nucleotide into DNA during synthesis (McCrea and Lipman, 1967). The compound, like other purine and pyrimidine analogs, is probably inactive until it is converted enzymatically into the nucleotide. The nucleotides of IDU then act as competitive inhibitors to the enzymatic utilization of the analogous nucleotides of thymidine during the biosynthesis of DNA (Delamore and Prusoff, 1962). These actions may occur both during viral and cellular DNA synthesis.

c. Absorption, Fate, and Excretion. IDU is rapidly degraded within cells to the base, 5-iodouracil, which is, in turn, converted to uracil and iodide (Delamore and Prusoff, 1962). Following intravenous administration, IDU has a serum half-life of about 30 minutes with only 20% of it excreted unchanged in the urine (Calabresi *et al.*, 1961, 1963).

d. Toxicity. The compound is mutagenic in *E. coli* and possibly teratogenic (Lorkiewicz, 1963; Welch and Prusoff, 1960). Toxicity is related to dose and reflects the inhibitory effect of IDU on DNA synthesis. Thus rapidly dividing cells, such as those of the gastrointestinal tract, bone marrow, and skin, are affected most with toxicity principally manifested by myelosuppression, alopecia, and loss of nails (Calabresi, 1963).

IDU has a low therapeutic index. Any selectivity of the compound for viral DNA as compared to host appears to be due to the more rapid, sustained synthesis of viral DNA during replication (Payrau and Dohlman, 1964). Its use is currently limited to topical application.

e. Therapeutic Uses. At present, IDU is licensed for topical use in herpes simplex virus keratitis. Its efficacy in acute HVH keratitis has been established in several well designed double-blind placebo-controlled trials where healing occurred in almost 75% of the treated cases and in less than 25% of the placebo controls (Laibson and Leopold, 1964; Leopold, 1965). The drug is most efficacious in superficial, epithelial infections and markedly less so in stromal infections.

In the past, IDU also has been employed in the treatment of systemic herpesvirus infections and HVH encephalitis. Even though its toxicity was recognized, some of the anecdotal reports indicated that the drug favorably influenced the clinical course and outcome of these more serious infections (Nolan *et al.*, 1970; Silk and Roome, 1970).

A more recent report, however, cited the experience of two groups in placebo-controlled double-blind efficacy studies of IDU in HVH encepha-

litis. In both groups, the study was stopped because of unacceptable toxicity and a lack of efficacy (Boston Interhospital Virus Study Group and NIAID Sponsored Cooperative Antiviral Study Report, 1975). The toxicity was manifested by the appearance of severe leukopenia, thrombocytopenia, and a deteriorating clinical course, all at a uniform time after institution of IDU therapy. Six of eight biopsy-proved cases on IDU therapy died and of the two survivors, one had a severe neurologic deficit and the other a seizure disorder. Four of the six deceased whose HVH encephalitis was biopsy-proved before treatment, had histologic and virologic evidence of continuing active necrotizing herpes encephalitis at autopsy. None of the deaths was directly attributed to the drug's toxicity.

f. Preparations and Untoward Effects. Idoxuridine (Stoxil) is available as a 0.1% ophthalmic solution in 15-ml dropper bottles and as a 0.5% oplthalmic ointment in 4-gm tubes. Adverse effects include irritation and inflammation with itching or pain. Toxicity may also be manifested by delayed wound healing resulting from the effect of the drug on the conjunctiva. Viral resistance to IDU can also develop during therapy and naturally resistant forms may explain some of the therapeutic failures.

2. Trifluorothymidine

a. Chemistry and Mechanism of Action. Trifluorothymidine (F_3T) is a halogenated pyrimidine analog whose structural formula is shown in Fig. 1. Like IDU, the compound is recognized as thymidine by enzymes involved in thymidine nucleotide biosynthesis and, following phosphorylation to the nucleotide level, it serves as a competitive antagonist to thymidine uptake in DNA synthesis (Heidelberger, 1967). It has further been reported to show a selective inhibitory effect on vaccinia viral DNA polymerase activity in comparison to the host cell (HeLa) DNA polymerases (Heidelberger, 1975).

b. Antiviral Activity. F_3T has an *in vitro* antiviral spectrum similar to IDU. *In vivo* studies in experimental *Herpesvirus hominis* infections have shown it to be superior to IDU, both in ocular herpes in rabbits and in herpes encephalitis in mice (Sugar *et al.*, 1973; Allen and Sidwell, 1972). In both types of animal model, it appeared comparable to ara-A in efficacy, but in the mouse encephalitis model F_3T had to be used in high doses with a narrow margin of safety to achieve the same effect as ara-A. Part of the superiority of F_3T to IDU is because of its much greater solubility. This, however, raises questions concerning its potential toxicity during prolonged topical application since, like IDU and ara-C, F_3T is a potent antimetabolite (Heidelberger, 1967).

c. Therapeutic Uses. In a large double-blind study comparing the efficacy of F_3T and IDU in ocular herpes, F_3T displayed significant superior-

TABLE I
Comparison of Clinical Effects of IDU, Ara-A, and F_3T in Topical Ocular Antiviral Therapy[a]

Effect	IDU	F_3T	Ara-A
Toxicity	+ +	+	±
Antiviral action	+ +	+ + +	+ + +

[a] ±, Minimal; +, mild; + +, moderate; + + +, potent.

ity both in the rate of complete healing and in healing time (Wellings *et al.*, 1972). No cross-toxicity was seen between the two drugs and toxicity was seen less frequently with F_3T even though the concentration of F_3T (1.0% eye drops) was ten times greater than that of IDU (0.1% drops). In another well designed double-blind efficacy study, F_3T, topically applied, was compared to topical ara-A in 102 ocular HVH infections (Coster *et al.*, 1976). No significant difference was seen between the two drugs in overall efficacy but F_3T showed a suggestive although not statistically significant superiority over ara-A in the treatment of the larger and more serious ameboidal ulcers. A comparison of clinical effect modified from Jones (Jones *et al.*, 1976) is shown in Table I.

F_3T would appear to have good clinical potential, at least for ocular herpes. At present, the high cost of manufacture and the need for further studies on toxicity combine to delay its more widespread use.

3. *Cytosine Arabinoside (ara-C)*

a. History. Cytosine arabinoside (cytarabine, ara-C) was reported by Evans to have a potent antitumor effect (Evans *et al.*, 1961). It has since proved to produce clinical remissions in acute leukemia and is currently an important agent in the chemotherapy of both acute myelogenous and acute lymphoblastic leukemias. In addition to its antineoplastic activity, it possesses immunosuppressive and antiviral activities (Underwood *et al.*, 1965; Mitchell *et al.*, 1969).

b. Chemistry and Mechanism of Action. Ara-C is a pyrimidine nucleoside analog whose structure is shown in Fig. 1. The 2′-carbon on the pentose ring lacks a *cis*-hydroxyl group making ara-C an analog of 2′-deoxycytidine. This translocation of the hydroxyl group appears to interfere with rotation of the pyrimidine base leading to abnormal stacking of the nucleotide bases during nucleic acid synthesis and thus, ara-C is poorly incorporated into DNA (Chu and Fischer, 1965; Nagyvary *et al.*, 1968). Following phosphorylation, the nucleotides of ara-C compete with similar nucleotides of deoxycytidine for enzymes, such as nucleoside

diphosphate reductase and the DN polymerases (Evans *et al.*, 1964; Furth and Cohen, 1967). The compound also serves as a substrate for the enzyme cytidine deaminase and, when administered intravenously to humans in the therapy of neoplasia, ara-C is rapidly degraded by this enzyme to uracil arabinoside which lacks both antineoplastic and antiviral activity (Creasey *et al.*, 1966).

c. Antiviral Activity. Ara-C has demonstrated antiviral activity *in vitro* against herpesviruses including HVH types I and II, varicella-zoster, and cytomegalovirus and poxviruses and rabies virus (Campbell *et al.*, 1968). The compound has also demonstrated topical efficacy in animals with HVH and vaccinia virus keratitis (Underwood, 1962; Sidwell *et al.*, 1968).

d. Therapeutic Uses and Untoward Effects. Ara-C has been used for the treatment of a variety of human herpesvirus infections, including varicella-zoster, cytomegalovirus, and *Herpesvirus hominis*, with mixed results. Anecdotal reports of efficacy have frequently been favorable (Chow *et al.*, 1970; Juel-Jensen, 1970). Others, however, have reported a lack of efficacy (Douglas *et al.*, 1969; Seligman and Rosner, 1970). In a recent double-blind placebo-controlled study of the efficacy of parenterally administered ara-C against herpes zoster, however, the results suggested that although this agent has an *in vitro* antiviral effect, it has the opposite effect in man (Stevens *et al.*, 1973). A total of 31 patients were enrolled in the study. In those receiving placebo, dissemination ceased within 5 days. Dissemination continued for 6 days or longer in 25% of the treatment (ara-C) group. Immune suppression, demonstrated by depressed antibody titers and leukocyte counts and delay in interferon response, was associated significantly with ara-C.

In view of these adverse reactions and its lack of efficacy, ara-C does not appear suitable for systemic use in humans as an antiviral substance.

B. Purines

1. Adenine Arabinoside (Ara-A) (Vidarabine)

a. History. Arabinosyl nucleosides were first isolated in 1950 from a Caribbean sponge, *Cryptotethia crypta* (Bergmann and Feeney, 1950). Ara-A was studied as a potential anticancer drug in the early 1960's and somewhat later was reported to have a good antiviral activity *in vitro* against HVH and vaccinia viruses (Privat de Garilhe and De Rudder, 1964). It was not until it was recognized that large-scale production of ara-A at reasonable cost was feasible through a fermentation process that extensive studies of the compound were undertaken.

In spite of its rather limited spectrum of antiviral activity, ara-A and its

congeners are demonstrating impressive therapeutic efficacy with a wide margin of safety both topically and parenterally in humans.

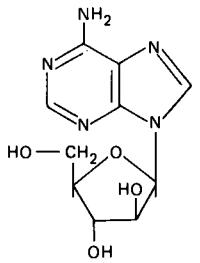
b. Chemistry and Mechanism of Action. The structure of ara-A is shown in Fig. 2. The 2'-carbon on the arabinose lacks a *cis*-hydroxyl group and thus ara-A acts as an analog of the deoxyribonucleoside of adenine. In *in vivo* studies, ara-A is rapidly deaminated and converted to hypoxanthine arabinoside (ara-Hx) (Fig. 2) (Sweetman *et al.*, 1975). Ara-Hx has been shown *in vitro* to possess about one-tenth the antiviral activity of ara-A, although *in vivo* their antiviral efficacy is similar (Hyndiuk *et al.*, 1975). There is also experimental evidence that ara-Hx is reaminated *in vivo* to form ara-A (Le Page, 1970). The reaction may be in balance intracellularly resulting in the presence of small amounts of ara-A along with the larger amounts of ara-Hx over prolonged periods.

The precise mechanism(s) of antiviral action of ara-A is still uncertain. The best evidence is that the portion of it which is not converted to adenine ribonucleotides or deaminated to ara-Hx is converted to ara-AMP and to ara-ATP (Schwartz *et al.*, 1974). This, in turn, causes inhibition of viral DNA polymerase, inhibition of viral ribonucleotide reductase, or inhibition of other virus-specific enzymes involved with viral DNA synthesis (Bennett *et al.*, 1975). The phosphorylation of ara-A is probably mediated by deoxyadenosine kinase, although this has not yet been demonstrated (Cohen, 1966; Ilan *et al.*, 1970).

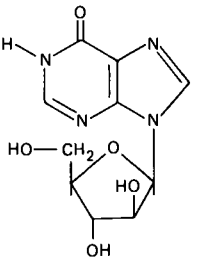
The mechanism of action of the principal antiviral metabolite of ara-A, ara-Hx, is unclear but it could be very different from ara-A. If some of the nucleoside is phosphorylated, the triphosphate could act as a competitive inhibitor of deoxyguanosine nucleotides in DNA synthesis. Unfortunately, studies of the inhibitory effect of the triphosphate of ara-Hx on DNA polymerase or ribonucleotide reductase have not been reported.

Ara-A neither directly inactivates virus nor prevents its attachment and cell penetration. At low dose (375 μmoles) it does not appear to inhibit significantly DNA, RNA, or protein synthesis in normal, uninfected cells *in vitro*, but does not inhibit viral DNA synthesis in infected cells (Bennett, *et al.*, 1975). There is good evidence that the activity of ara-A which inhibits HVH multiplication occurs early in the virus replicative cycle, between 2 and 4 hours postinfection, a time corresponding to the onset of viral DNA synthesis (Shannon, 1975).

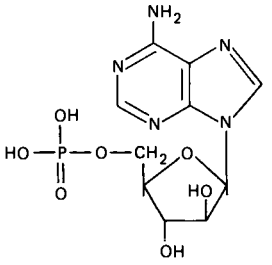
It thus appears that at least one of the virus-induced enzymes involved in DNA synthesis is inhibited by ara-A or its nucleotides much more than the corresponding host cell enzyme(s). The viral enzymes which might be involved are thymidine kinase, ribonucleotide reductase, deoxyribonuclease, and DNA polymerase. All of these differ from their host cell counterparts physically, chemically, and antigenically (Shannon, 1975).



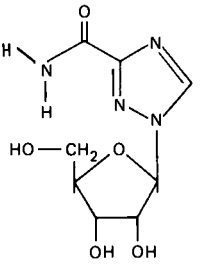
Adenine Arabinoside (ara-A)



Hypoxanthine Arabinoside (ara-Hx)



Adenine Arabinoside Monophosphate (ara-A-MP)



Ribavirin

Fig. 2. Purine nucleosides.

c. Antiviral Activity. Ara-A has been found to be active against a number of DNA viruses *in vitro* (Shannon, 1975). Included are the herpesvirus group: HVH types I and II, varicella-zoster, cytomegalovirus, pseudorabies, and other animal types of herpesvirus and the poxviruses. The compound has a more limited effect against the adenoviruses and the papovaviruses. It lacks activity against RNA viruses, in general, with the exception of the rhabdoviruses and the oncornaviruses (Shannon, 1975).

Ara-A has been studied in a variety of animal systems with consistent antiviral activity and has demonstrated effective antiviral action regardless of the route of administration. The drug's antiviral spectrum is approximately the same *in vivo* as it is *in vitro*. It has proved especially effective against experimental vaccinial and herpesvirus keratitis, encephalitis, and skin lesions (Shannon, 1975).

d. Toxicity. Because of its low solubility in water (0.7 mg/ml), ara-A was found to be moderately to severely irritating in animals when injected intramuscularly. The severity of the irritation varied directly with dose administered. Rats tolerated a dose of 150 mg/kg/day administered intramuscularly as a 20% suspension for 28 days without clinical, laboratory, or pathological evidence of toxicity (Kurtz, 1975). Dogs tolerate 50 mg/kg/day for 28 days with a similar lack of toxicity. Rhesus monkeys tolerate a dilute ara-A solution, i.e., 15 mg/kg/day, intravenously for 28 days with no evidence of toxicity. When the dose level was increased to 25 mg/kg/day or higher, they develop signs of neurotoxicity with the lag time before onset dose-related. New world monkeys, on the other hand, tolerate much higher doses without evidence of toxicity (Kurtz, 1975).

When rodents were given high doses of ara-A either as a single dose at the LD_{50} level or as equivalent moderate doses, they developed hepatomegalocytosis following a latent period of several weeks. The effect disappears without residual pathology 8 months to 1 year afterward (Kurtz, 1975).

Ara-A is teratogenic in both rabbits and rats but the effect is most pronounced in rabbits where the threshold dose is 3–5 mg/kg. No teratogenesis was observed in rhesus monkeys, however. In the standard dominant lethal and host-mediated assays using known mutagens as controls, ara-A was not mutagenic. Current evidence indicates that the compound is not oncogenic (Kurtz, 1975).

e. Absorption, Distribution, and Excretion. The amount of information available on the absorption and fate of ara-A in man is limited. A recent study of the compound in seven persons who were treated with ara-A for 5 days showed only low or barely detectable levels of ara-A in the plasma, even during a period of continuous intravenous drip (Kinkel and Buchanan, 1975). Plasma levels of ara-Hx, however, increased throughout

the period of infusion and decreased rapidly thereafter. Urinary excretion of ara-Hx accounted for the major portion of the dose administered. The cumulative amount excreted in the urine rose during the first 3 days and then leveled off at a steady rate. Interestingly, the urinary excretion rate of ara-Hx was highest during the 12-hour period following completion of the infusion, indicating that the compound was emerging from a cellular or fluid compartment other than plasma. The plasma half-life of ara-Hx was estimated at 3.5 hours. Levels of ara-Hx in erythrocytes paralleled those in the plasma and CSF levels were about one-third of the plasma level, indicating that the parent drug has potential for the treatment of CNS infections. Following the development of steady-state excretion on day 3, approximately 50% of the daily dose was excreted in the urine. One patient with severely imparied renal function following transplant exhibited tenfold higher levels of ara-Hx in his plasma than the others by day 4 but then leveled off and declined slowly through the postinfusion period. This seems to indicate the existence of alternative mechanisms to urinary excretion for elimination of the compound (Kinkel and Buchanan, 1975).

f. Therapeutic Uses. Ara-A is currently available for investigational use only, although its licensure as a 3% ophthalmic ointment for use in herpes keratitis is anticipated. A growing number of clinical trials are now confirming the drug's efficacy or its lack of efficacy in a number of DNA viral infections. A partial listing is presented in Table II.

In the uncontrolled, open protocol studies it was not possible to demonstrate efficacy by the very nature of the study, so all results are either equivocal (promising) or negative. In each of these early studies, ara-A showed promise and the investigators felt that controlled, double-blind studies should be carried out. In ocular herpes, placebo-controlled studies could not be conducted since a drug of proved efficacy, IDU, was already licensed and in wide use. In these studies, ara-A had a therapeutic efficacy at least equal to IDU and lacked the latter drug's tendency for toxicity and reactogenicity (Laibson and Krachmer, 1975; Pavan-Langston, 1975; O'Day *et al.*, 1975).

The relative lack of toxicity of ara-A in comparison to IDU during systemic administration might make it the drug of choice in the treatment of HVH uveitis where topical administration of an antiviral agent is inappropriate (Abel *et al.*, 1975).

The lack of efficacy of topical ara-A in the treatment of HVH-2 herpes progenitalis is difficult to explain in view of the supposed mechanism of action of ara-A and the close similarity in the replicative cycles of HVH-1 and HVH-2 (Goodman *et al.*, 1975; Adams *et al.*, 1976). It is possible that topically applied ara-A did not penetrate the deeper layers of epithelium in adequate antiviral concentrations because of its poor solubility. An

TABLE II
Therapeutic Trials with Ara-A

Disease	Route of administration	Study design[a]	Result[b]	Reference
Severe mucocutaneous HVH	i.v.	a	±	Ch'ien *et al.* (1975)
Neonatal herpes	i.v.	a	±	Whitley *et al.* (1975)
Varicella-zoster	i.v.	a	±	Luby *et al.* (1975)
Cytomegalovirus	i.v.	a	±	Baublis *et al.* (1975)
HVH keratitis	Topical	c	+	Laibson and Krachmer (1975)
HVH keratitis	Topical	c	+	Pavan-Langston (1975)
HVH keratitis	Topical	c	+	O'Day *et al.* (1975)
HVH keratouveitis	i.v.	b	+	Abel *et al.* (1975)
KVH keratitis	Topical	c	+	McKinnon *et al.* (1975)
Varicella-zoster	i.v.	d	±	Whitley *et al.* (1976)
Varicella-zoster	i.v.	d	+	Ch'ien *et al.* (1976)
Herpes progenitalis	Topical	b	−	Goodman *et al.* (1975)
Herpes progenitalis	Topical	b	−	Adams *et al.* (1976)
Herpes encephalitis	i.v.	a	±	Taber *et al.* (1975)
Smallpox	i.v.	b	−	Koplan *et al.* (1975)

[a] Study design: a, uncontrolled; b, double-blind, placebo-controlled; c, double-blind, other drug controlled; d, double-blind crossover, placebo-controlled.
[b] Result: +, has efficacy; ±, equivocal; −, lacks efficacy.

alternative possibility is that in comparison to cutaneous HVH-1, HVH-2, with its shorter period of lesion production, i.e., from onset of symptoms to crusting, has a brief period where symptoms are due to viral tissue invasion and replication and a relatively longer period where the signs and symptoms are due to the host immune response. Whatever the cause, topical ara-A as tested appears ineffective in the treatment of mucocutaneous herpes progenitalis.

Although a double-blind placebo-controlled study of ara-A in varicella-zoster infections of immune compromised hosts is currently underway, the results from the earlier double-blind crossover study clearly demonstrate therapeutic efficacy (Ch'ien *et al.*, 1976). Highly significant reductions in new vesicle formation, vesicle virus titers, and resolution of cutaneous disease were seen in patients first treated with intravenous ara-A in comparison to those first treated with the placebo. The crossover design did not permit an assessment of the comparative effect of ara-A in preventing cutaneous dissemination, visceral disease, late drug toxicity, or sequelae since after 5 days on initial therapy all patients were changed over to alternate therapy. That is, after 5 days' therapy, patients on ara-A were placed on 5 days of placebo therapy and vice versa. The

current true placebo-controlled study should answer these questions (Whitley *et al.*, 1976).

A study is currently in progress to determine the efficacy of ara-A in biopsy-proved herpes encephalitis (C. A. Alford, personal communication). In view of its proved efficacy in ocular herpes and herpes zoster and its ability to penetrate the blood–brain barrier, it is hoped that the drug will prove effective against this highly fatal form of herpesvirus infection.

g. Preparations and Dosage. For topical use in ocular herpes infections, ara-A is available for investigational use as a 3% ointment. For systemic administration, because of its low solubility, ara-A is diluted in standard intravenous solutions at concentrations no greater than 0.7 mg/ml. On the basis of toxicity and human tolerance studies cited below, the optimum dose level appears to be 10 mg/kg/day administered over a period of 12 hours. A 70-kg patient would thus receive 700 mg of ara-A administered in 1 liter or more of intravenous solution.

h. Untoward Effects. A recent report of adverse reactions to ara-A describes the following results (Ross *et al.*, 1976). During a 2-year period, 42 patients with severe herpesvirus infections were treated with one of the following regimens: placebo (6 patients), ara-A at 10 mg/kg/day (10 patients), 15 mg/kg/day (3 patients), 20 mg/kg/day (22 patients), and 30 mg/kg/day (1 patient). The incidence of nausea and vomiting was 76% in the 20 mg/kg group versus 20% in the 10 mg/kg group. Weakness was seen in 40% of the patients on the 20-mg dose versus 30% at the 10-mg dose. Tremors were seen in four patients, two on the 20-mg and two on the 15-mg dosage.

A subsequent report using lower doses describes 143 cases who were on daily doses of ara-A ranging from 2.5 to 29 mg/kg/day with a mean of 9 mg/kg/day (Keeney, 1975). The duration of therapy ranged from 1 day to 1 month. All received the medication through slow intravenous drip with the exception of two cases who received the first few doses intramuscularly. All patients were monitored clinically and in the laboratory for evidence of adverse reactions. Based on serum glutamic-oxaloacetic transaminase (SGOT) and serum bilirubin levels, there was no evidence of hepatotoxicity due to ara-A. Similarly, studies of blood urea nitrogen (BUN) levels and serum creatinine levels indicated no evidence of nephrotoxicity at the dosage levels utilized. The urinalyses remained unchanged throughout the study. Hematological measurements monitored included hemoglobin, hematocrit, and white blood cell and platelet counts. There was no evidence of erythrocyte toxicity as evidenced by hemolysis nor was there a reduction of hematocrit or hemoglobin levels. The data also failed to show depression of leukocyte or platelet counts during or following the

medication. A total of 30 patients exhibited adverse reactions possibly attributable to the drug, all but four of which were mild to moderate gastrointestinal symptoms, principally nausea and/or vomiting. Two patients developed mental confusion during therapy and two had pain at the injection site following intramuscular administration.

i. Congeners of Ara-A. In an attempt to achieve a more potent antiviral effect with toxicity equal to or less than that of ara-A, a number of related purine arabinosides have been studied. Of these, the most promising in *in vitro* and *in vivo* studies have been the 5′-monophosphate of adenine arabinoside (ara-AMP)(Fig. 2) and the 5′-monophosphate of hypoxanthine arabinoside (ara-HxMP)(Revankar *et al.*, 1975; Sidwell *et al.*, 1975; Sloan, 1975). Both compounds compare favorably to ara-A in these studies and have the added advantage of much greater solubility and, thus, penetrability. This, however, is probably their principal advantage. Most of the ara-AMP is dephosphorylated at the cell surface prior to transport across the membrane as ara-A (R. A. Buchanan, personal communication). Intracellularly it is promptly deaminated to ara-Hx. It is likely that ara-HxMP is similarly degraded to ara-Hx and if there is a balance achieved in the ara-A ⇆ ara-Hx reaction intracellularly, both compounds may achieve much the same *in vivo* antiviral effect as ara-A at the cellular level (Le Page, 1970). Nevertheless, solubility is exceedingly important in the problem of distribution and it appears likely that one or both of these compounds will replace the parent, ara-A, in future antiviral regimens.

j. Deaminase Inhibitors. The rapid conversion of ara-A intracellularly to the hypoxanthine through deamination has led to a search for inhibitors of the effector enzyme, adenosine deaminse.

Two such inhibitors have been under study *in vitro* and *in vivo* as potentiators of the therapeutic effect of ara-A. Both are structurally similar to ara-A and deoxyadenosine but have a novel seven-membered ring in place of the pyrimidine ring of the purines. The first of these compounds tested was coformycin and the second, more potent compound, isolated from culture filtrates of a strain of *Streptomyces antibioticus,* has been called Covidaribine (Parke-Davis) or "DI" for deaminase inhibitor (Woo *et al.*, 1974).

In vitro studies in HEp-2 cells showed an enhancement of the antiviral activity of ara-A by DI of from five- to tenfold against HVH and ten- to twentyfold against vaccinia virus (Shannon, 1975). *In vivo* studies in mice have yielded similar results. Against HVH, ara-A with DI achieved 50% survival in mice at one-tenth the dose level required of ara-A alone (Sloan, 1975). Against experimental herpes keratitis, a fivefold potentia-

tion of the antiviral activity of ara-A has been seen with deaminase inhibitor (Sloan, 1975).

Other investigators have found even greater *in vitro* enhancement of ara-A activity utilizing DI (Williams and Lerner, 1975). The amounts of DI administered *in vivo* were small, ranging from 0.2 to 3.0 mg/kg/day.

Although the deaminase inhibitors offer the prospect of additional uses of ara-A, as well as potentiation of current uses, it will be necessary to repeat all of the *in vitro* and *in vivo* studies of metabolic disposition, toxicity, teratogenicity, mutagenicity, and oncogenicity of the inhibitor and ara-A. It is quite possible that ara-A plus a deaminase inhibitor, while exhibiting a more potent antiviral activity, will also be more toxic than ara-A alone.

Ara-A and its congeners have good clinical potential for use against an important group of viruses, the herpesviruses. The teratogenic potential of the active drug must be considered, however, whenever it is used as therapy in women capable of childbearing. While this is not a problem with its use against the serious forms of infection, it may restrict the drug's use, at least systemically, against less serious, more common herpesvirus infections.

2. *Ribavirin*

a. History. Ribavirin (Virazole, ICN) is a synthetic nucleoside analog of guanosine, structurally similar to the purine precursor 5-aminoimidazole 4-carboxamide ribonucleotide (AICR). The synthesis of the compound, whose formula is shown in Fig. 2, was first reported in 1972 (Witkowski *et al.*, 1972).

b. Antiviral Activity and Mechanism of Action. Unlike most of the other antivirals, ribavirin has been shown to be active against a wide spectrum of both DNA and RNA viruses (Huffman *et al.*, 1973). It is most active against influenza, parainfluenza, and murine hepatitis viruses. In *in vitro* studies it proved to be equally as effective as ara-A, ara-C, and IDU against DNA viruses and appeared to be superior to amantadine *in vivo* as well as *in vitro* against experimental influenza A, A_2, and B and parainfluenza virus of animals.

The mechanism of action of ribavirin as a nucleoside analog has been rather well described (Streeter *et al.*, 1973). It has been shown that ribavirin interferes with steps leading to biosynthesis of guanylic acid nucleotides. Following *in vivo* administration, ribavirin is phosphorylated in the liver and other tissues to the nucleotide, ribavirin 5′-monophosphate (Streeter *et al.*, 1973). The nucleotide has been shown to be a potent inhibitor of inosine monophosphate (IMP) dehydrogenase, apparently rec-

ognized by the enzyme as GMP (Streeter *et al.*, 1973). In support of this, X-ray crystallographic studies have shown ribavirin to have a crystalline structure very similar to guanosine (Prusiner and Sundaralingarm, 1973). Although ribavirin or the 5′-monophosphate may also inhibit GMP kinase, and to some extent, GMP synthetase, its principle site of action appears to be its inhibition of IMP dehydrogenase, blocking a critical step in the synthesis of guanosine nucleotides between IMP and xanthylic acid (Sidwell *et al.*, 1974).

Ribavirin does not prevent viral attachment, penetration, or uncoating nor does it directly inactivate viruses or induce interferon. Its antiviral action appears to be due to its effect on guanine nucleotide synthesis through the inhibition of biosynthesis of viral DNA, RNA, and protein.

c. Absorption and Fate. Following oral administration in rats, ribavirin is concentrated in the liver, lung, and spleen especially and then rapidly appears in the urine. The major portion of the metabolites of the compound (more than 80%) is found in the urine within 24 hours (R. W. Sidwell, personal communication.) Ribavirin is disseminated through most of the tissues and metabolized intracellularly by phosphorylation, deribosylation, and hydrolysis (R. W. Sidwell, personal communication).

d. Toxicity. The following information from the toxicity studies of ribavirin has not been published. It has been made available through personal communication from Drs. R. W. Sidwell and L. N. Simon of ICN Pharmaceuticals, for the further information of the reader.

Ribavirin was seen to exhibit some toxicity at higher dose levels. Acute toxicity studies showed an LD_{50} dose in rhesus monkeys of greater than 10 g/kg. Subacute and chronic studies have been carried out as follows. A two-year study in rats showed that animals receiving more than 60 mg/kg/day for 2 years exhibited no increased incidence of tumors in the treated animals versus the control animals. At 120 and 200 mg/kg/day for 30 days, depression of hemoglobin and red blood cell counts were seen. Rhesus monkeys receiving 200 mg/kg/day for 30 days exhibited similar findings but in both the monkeys and rats reversal of symtomology was seen upon withdrawal of the drug. Monkeys administered 120 mg/kg/day for 6 months showed no gross or histopathological signs of toxicity, although a decrease in red blood cell and hemoglobin levels occurred in these animals.

The drug was found to be teratogenic to rats when administered during the second trimester of pregnancy (days 6–13) but to have no effect on teratogenicity, fertility, or reproduction when given at mating, after birth, or during the first or third trimester. While the drug was embryotoxic to rabbits at doses as low as 10 mg/kg, no teratogenicity was observed in those animals who were born alive.

Ribavirin was shown to be nonmutagenic in nine different tests of mutagenicity, including the Ames *in vitro* test. When ribavirin was evaluated for immunosuppression potential, it was found that no suppression of the humoral immune system was observed in animals receiving doses as high as 100 mg/kg. Suppression of cellular immunity was seen as a weak response and only at doses approaching toxic levels (100–200 mg/kg/day).

Finally, rats treated with 60 mg/kg/day for 18 months were found to have normal humoral immune systems as judged by the SRBC test and to have a normal cellular immune response system as judged by delayed hypersensitivity and adjuvant-induced arthritis test.

In humans no side effects with the exception of mild frontal headaches, abdominal cramps, and fatigue was seen in normal volunteers given 600 mg/day of ribavirin for 28 days. Dose levels of 1200 mg/day for 13 days caused a depression of hemoglobin, hematocrit, and red blood cell count but this depression was reversed after withdrawal of the drug. No other drug related side effects were observed (R. W. Sidwell and L. N. Simon, personal communication).

e. Therapeutic Uses. Ribavirin is available for investigational use only. The drug has undergone extensive therapeutic trials in humans against several viral infections in both uncontrolled and placebo-controlled studies. A partial listing of these trials is shown in Table III.

The results of these trials are mixed. In the first two studies of ribavirin in hepatitis (Table III), a more rapid reduction in serum transaminase levels was reported in the treated group but no statistical analysis was performed on the data. In the studies reported by Vilela, a reduction in the amount of circulating hepatitis surface antigen in hepatitis B cases was reported and possibly a shortened duration of antigenemia, but statistically significant differences were not claimed. Trials of ribavirin in influenza A infections yielded conflicting results. In a double-blind study of 48

TABLE III
Therapeutic Trials with Ribavirin

Country	Disease	Route of administration	Study design[a]	Reference
Brazil	"Viral" hepatitis	Oral	b	Zuñiga *et al.* (1974)
Brazil	"Viral" hepatitis	Oral	b	Galvao and Castro (1975)
Brazil	"Viral" hepatitis	Oral	a	Vilela *et al.* (1974)
Mexico	Influenza A_2	Oral	a	Salida-Rengall *et al.* (1977)
United States	Influenza B	Oral	b	Togo and McCracken (1976)
United States	Influenza A	Oral	b	Cohen *et al.* (1976)

[a] a, Uncontrolled; b, placebo-controlled double-blind.

patients with influenza A infections, Salida-Rengall reported statistically significant improvement in clinical response and viral isolations between the treated and placebo groups (Salida-Rengall *et al.*, 1977). Cohen, on the other hand, in a double-blind study of ribavirin, amantadine, and placebo in 30 volunteers challenged with influenza A virus, found ribavirin to lack both prophylactic and significant therapeutic effect (Cohen *et al.*, 1976). Togo, in a study of the prophylactic efficacy of ribavirin against influenza B infections, noted marginal symptomatic benefit in the treated group but no difference in virus titers or antibody response between the treated and placebo groups (Togo and McCracken, 1976).

The dosages of ribavirin employed in the hepatitis studies were 100 mg four times daily for 8 or 10 days. In the influenza studies, the dosages were 100 mg, three times daily, for 5 days in Salida-Rengall's study and 200 mg, three times daily, for 10 days in the Cohen and Togo studies. No adverse effects other than drowsiness were seen in any of these studies.

At present, the therapeutic efficacy of ribavirin as an antiviral substance remains to be determined. Its potential for clinical use will also depend heavily on further studies of its teratogenicity. If this poses a real hazard, its systemic use, at least in women of child-bearing potential, would probably be limited to those more serious types of infections where its efficacy has been demonstrated.

IV. THE INTERFERONS

A. General Background

a. History. Interferons are relatively low molecular weight protein macromolecules whose antiviral activity was first reported by Isaacs and Lindenmann in 1957. It was noted subsequently that, following infection of cells with one virus, the induced interferon inhibited the multiplication of a broad spectrum of other viruses, both related and unrelated. Interferon, as it was named by its discoverers, is produced by many species of animals, including man, and appears to form a major natural defense mechanism against viral infection (Wheelock and Sibley, 1964). For a comprehensive survey of the current literature on interferon, the reader is referred to the recent review by Ho and Armstrong (1975).

b. Chemistry and Mechanism of Action. The precise chemical structure of interferon(s) is unknown. Studies of rabbit and human interferon have indicated that they are glycoproteins (Cesario and Tilles, 1977; Dorner *et al.*, 1973). The molecular weights of interferons vary with the

animal species, the type of cell, and the type of inducer, i.e., virus or polymer (Hallum *et al.*, 1965; Cesario *et al.*, 1977; Smith and Wagner, 1967). The molecular weights are found to range from less than 20,000 to about 90,000 daltons (Hallum *et al.*, 1965). There are reports that human interferons may have "monomer" units of 15,000 and 21,000 daltons (Vilcek and Havell, 1977). The suggestion is made that the different molecular species are multimeric combinations of smaller monomers or at times artifacts due to binding with other proteins (Ke and Ho, 1967; Carter, 1970). While the mechanism of production of interferon is largely understood, the mechanisms of its induction are less clear. Studies employing actinomycin D, puromycin, and other specific metabolic inhibitors suggest that interferon is produced by postinduction transcription of mRNA and its ribosomal translation (Tan *et al.*, 1970, 1971). It is then actively secreted from the synthesizing cell, reacts with the membranes on neighboring cells, and activates an antiviral state within those cells (Chany, 1977).

A current hypothesis attributes the interferon-induced antiviral state to the derepression of a host cell genome resulting in the production of a second intracellular protein which inhibits viral reproduction (Taylor, 1964). This inhibitory protein apparently interferes with the translation of viral mRNA at the ribosome, inhibiting the synthesis of virus-specific proteins (Falcoff *et al.*, 1973). A recent report suggests that the inhibitory factor is a polypeptide with a molecular weight of 48,000 daltons (Samuel and Joklik, 1974). Whatever the mechanism of action of interferon, it does not prevent adsorption or cell penetration by the virus but blocks viral replication at some point before assembly (Finter, 1973).

Cell systems exposed to low doses of interferon may be primed and produce larger than usual amounts of interferon when subsequently exposed to an inducer. This occurs especially when a virus or other polynucleotide serves as the inducer (Tovell and Cantell, 1971). Following exposure to high doses of interferon, however, "blocking" occurs and on subsequent exposure to an inducer interferon production is inhibited (Stewart *et al.*, 1971). Interferon also may be strongly inhibitory to antibody production (Johnson and Baron, 1975).

c. Absorption, Distribution, and Excretion. Following induction by a synthetic polymer, interferon has an estimated serum half-life of under 12 hours (Tilles, 1974). Following initial intravenous inoculation of exogenous human interferon in the rabbit, its half-life in the serum has been measured at from less than 20 minutes to more than an hour (Ho and Postic, 1967; Tilles, 1974). From that point, the levels fall more slowly until no interferon can be detected 24 hours after the injection. When the administration of interferon is extended over longer periods, the serum

half-life increases up to 24–48 hours, indicating that interferon is initially held in some nonvascular fluid compartment (Jordan *et al.*, 1974).

Studies of decay curves of serum interferon in mice in general confirm this (Spahn *et al.*, 1977). The curve seen following intravenous administration of 2×10^5 units of interferon was divided into three phases. The initial phase was a rapid decline in serum levels with a half-life of 2 minutes. During the second phase from 16 minutes to 3 hours after administration, the half-life increased to 21 minutes. In the third phase through 9 hours, the half-life reached 90 minutes. The curves seem to indicate rapid diffusion of interferon out of the serum initially, into other fluid compartments, with slow back-diffusion occurring during the prolonged third phase of decay.

Intramuscular injection results in lower initial serum levels, but with a half-life of more than an hour they persist for a longer period of time (Ho and Postic, 1967). The importance of sustained blood levels is problematical, however, since once the cells are exposed to interferon they retain their antiviral state over a considerable period of time (Finter, 1967).

As a macromolecule, interferon is impeded in its passage through certain cell barriers such as respiratory tract epithelium, the blood-brain barrier, and placenta (Finter, 1968; Cathala and Baron, 1970; Overall and Glasgow, 1970). Following oral administration it is almost completely destroyed in the gastrointestinal tract; less than 1% is recoverable in the bloodstream of mice following oral administration of high doses (Schafer *et al.*, 1972). A number of body fluids inactivate interferon slowly *in vitro*, including serum, spinal fluid, saliva, and urine, and it is probable that almost all human interferon is destroyed through such inactivation and degradation *in vivo* with only a small amount excreted in the urine and feces (Cesario and Tilles, 1973; Cesario *et al.*, 1977).

d. Therapeutic Forms. Although the interferons would appear to be ideal antiviral substances, a number of problems remain to be resolved before they can be considered practical therapeutic agents. Probably the most important is the fact that they are relatively species-specific and exogenous interferons for human use must be synthesized from high-yield human cell cultures, such as leukocyte or fibroblast. Until human interferons can be produced more efficiently, the difficult and expensive methods of production in current use will severely restrict their application. An alternative, of course, is the utilization of an inducer, such as a synthetic polynucleotide. Following the initial response, this has the disadvantage of producing a hyporeactive response to further induction, which lasts several days. There is evidence, however, that the antiviral state persists during this hyporeactive period (Ho *et al.*, 1965). At

present, both exogenous interferons and inducers are utilized on an investigational basis.

B. Exogenous Interferon

Exogenous interferon is prepared from induced cultures of human leukocytes or fibroblasts, principally the former. Following a 24-hour incubation of the cell cultures and inducer, the supernatant fluid containing from 5,000 to 20,000 units of interferon per mililiter is precipitated and concentrated by centrifugation. This results in a highly purified interferon with a potency ranging up to 2×10^6 units/mg of protein (Strander *et al.*, 1973). For storage and shipment, the interferon is lyophilized and held at 4°C. Under these conditions it retains its potency for periods of at least a year (T. C. Merigan, personal communication).

1. Therapeutic Uses

Interferon is most effective as a prophylactic agent and is therapeutically most efficacious when pathogenesis is dependent on slow and continued replication of virus. Therefore, it is most potent when administered early in the disease where it can be more effective in limiting the ultimate severity of infection. This view has been amply supported experimentally (Neumann-Haefelin *et al.*, 1976; Merigan *et al.*, 1975). It has also been shown that increasingly high doses of interferon are required to control infection as the time elapsed from initial infections increases (Kishida, 1975).

In early therapeutic trials, exogenous interferon lacked efficacy against HVH viral infections such as keratitis (Tommila, 1963). In a large field trial of the prophylactic efficacy of intranasal interferon during an epidemic of influenza A_2 in the Soviet Union from 1967 to 1969, however, significant efficacy was seen. More than 6000 persons were included in the treatment and control groups and a highly significant difference was seen in the attack rates between the interferon-treated group (7.7%) and placebo group (17.6%)(Soloviev, 1968, 1969). These results have not been achieved in similar trials elsewhere, however, and questions have arisen concerning the dosage used and the possibility that the inducer agent, Newcastle disease virus (NDV), was not completely inactivated in the product used (Finter, 1973). Nevertheless, it has been demonstrated that the interferon available to the general public in the Soviet Union is free of inducer and contains the specified potency (1000 units/ml)(G. J. Galasso, unpublished data). The Soviet experience contrasts sharply with that of Merigan and his Colleagues, where 8×10^5 units of interferon intranasally

TABLE IV
Therapeutic Trials with Human Interferon

Country	Route administered	Disease treated	Reference
Soviet Union	Nasal spray	Influenza A	Soloviev (1968) Soloviev (1969)
France	Intravenous	Cytomegalovirus; generalized herpes	Falcoff *et al.* (1966)
Great Britain	Intranasal	Rhinovirus	Scientific Committee (1970)
United States	Nasal spray	Influenza B	Merigan *et al.* (1973)
United States	Nasal spray	Rhinovirus 4	Merigan *et al.* (1973)
United States	Intravenous	Varicella-zoster	Merigan *et al.* (1975)
United States	Topical	HVH keratitis	Kaufman *et al.* (1976)
West Germany	Topical	HVH keratitis	Sundmacher *et al.* (1976)
Great Britain	Topical	HVH keratitis	Jones *et al.* (1976)
United States	Intravenous	Neonatal cytomegalovirus	Arvin *et al.* (1976)
United States	Intravenous	Hepatitis B antigen carriers	Greenberg *et al.* (1976)

per patient were inadequate to prevent influenza B infection (Merigan *et al.*, 1973). They were, however, able to prove statistically significant therapeutic efficacy against rhinovirus with very large doses of interferon in a small study of 32 subjects (Merigan *et al.*, 1973). In this study, 16 subjects received 14×10^6 units of interferon in divided doses before and for 3 days after challenge with rhinovirus type 4 while the 16 control subjects were given a placebo.

A partial summary of the clinical trials of human interferon as an antiviral substance is shown in Table IV.

The study by Merigan and his colleagues (1975) on the efficacy of human leukocyte interferon in varicella infections appears to be particularly promising. Although the study is still in progress, a recent report on the results of the preliminary trials with interferon in a randomized double-blind study of 85 varicella-zoster patients is most encouraging. In over 55 of those patients who entered the study early in the course of their infection, a fivefold lesser frequency of visceral complications was observed in the interferon-treated group. This group also showed a reduction in pain in the primary dermatome. Other studies currently underway involve clinical trials of interferon in ocular herpes, cytomegalovirus, and hepatitis B virus infections, but results are not yet available.

In addition to therapeutic trials of interferon in viral infection, the substance has undergone extensive investigation as an antineoplastic agent. Trials against Hodgkin's disease, malignant melanoma, leukemia, and re-

ticulum cell sarcoma have been either negative or indecisive (Kishida, 1975). Interferon has been reported tentatively, however, to have produced remissions in 16 cases of osteogenic sarcoma treated with 9×10^6 units of interferon per week for periods up to 18 months (Strander *et al.*, 1976).

2. *Adverse Effects*

The untoward effects of human interferon are minimal. Following intravenous administration, a low-grade fever of from 38° to 39°C has commonly been seen, presumably due to contaminants in the material (Strander *et al.*, 1973). This effect is not being seen with the more highly purified interferons currently in use (T. C. Merigan, personal communication).

In neither man nor experimental animals have signs of toxicity such as weight loss or specific pathological changes in organ systems been seen (Kishida, 1975), or has tolerance to interferon or antibody formation against it been observed. Although humans have not been studied for the adverse effects of massive doses of interferon, mice have tolerated doses in terms of human equivalents of over 6×10^8 units without adverse effects (Kishida, 1975).

C. Interferon Inducers

Interferons are induced by two general types of inducers. The first includes viruses, both RNA and DNA, double-stranded and sometimes single-stranded RNA. The second type of inducers are substances which are mitogenic for T and B lymphocytes. Miscellaneous inducers include macrophage-phagocytosed bacteria and intracellular organisms. A partial listing of interferon inducers modified from Ho and Armstrong (1975) is shown in the tabulation on p. 274.

1. *Therapeutic Use*

Of the synthetic inducers of interferon currently available, the double-stranded polyribonucleotides are by far the most active (Torrence *et al.*, 1975). The inducer of this class most widely used is polyriboinosinic polyribocytidylic acid [poly(I)·poly(C)]. Poly(I)·poly(C) is well tolerated systemically at doses up to 40 mg/kg daily but serum interferon levels are low (Field *et al.*, 1972). This is probably due to hydrolysis of the inducer by normal serum hydrolytic enzymes.

A derivative, poly(I)·poly(C)-poly-L-lysine-CMC, has been found to be more resistant to enzymatic hydrolysis and appears to be a more potent inducer of interferon *in vivo* (Levy *et al.*, 1975). It currently is undergoing testing in humans as a topical antiviral in herpes keratitis and is also being

Type IA Inducers (polynucleotide induced)
1. RNA and DNA animal viruses
2. Plant and insect viruses
3. Mycophages and bacteriophages
4. Cellular double-stranded RNA
5. Synthetic double-stranded RNA
6. DNA and single-stranded RNA

Type IB Other Antigens
1. Macrophage phagocytosed bacteria and intracellular microorganisms (chlamydia, toxoplasma, mycoplasma, etc.)
2. Bacterial products (polysaccharides, lipopolysaccharides, etc.)
3. Polymers (pyran, polyacrylic acid, polysulfates, etc.)
4. Low molecular weight substances (tilorone, basic dyes, propanediamine, aminothiols, etc.)

Type II Lymphocyte Mitogens
1. Phytohemagglutinin
2. Concanavalin A
3. Pokeweed mitogen

administered investigationally in cases of St. Louis and herpes encephalitis (Kaufman, 1975; H. Levy, personal communication).

2. Adverse Effects

Two major problems are posed by the use of interferon inducers systemically. One is the toxicity of most of them at dosage levels required for a useful antiviral effect and the other is the development of tolerance or hyporeactivity (Ho and Armstrong, 1975). Both problems occur with either high or low molecular weight inducers. Toxicity is manifested most frequently by inhibition of DNA, RNA, or protein synthesis and fever. Tolerance is exhibited by a diminished response to subsequent administration of the inducer following a satisfactory initial response. This appears to be the result of a "repressor" protein which is produced by the induced cell at or about the same time as interferon. Presumably the repressor is synthesized following transcription and early translation of interferon mRNA and acts at the translational level blocking further interferon synthesis (Borden *et al.*, 1975). This view is supported by *in vitro* studies of induced cells which are treated with cycloheximide or actinomycin D. In both instances, prolonged synthesis of interferon or "superinduction" occurs, apparently due to inhibition of the repressor protein synthesis or its mRNA transcription (Sehgal *et al.*, 1976).

Toxicity can be circumvented partially by topical application of the inducer but amounts of interferon achieved by this route are generally too low to show a therapeutic effect (Finter, 1973).

D. Superinduction

In an effort to achieve more prolonged synthesis of interferon following administration of an inducer, substances which act as reversible inhibitors of nuclear heterogeneous RNA synthesis are being actively investigated. One which appears particularly promising at the present time is a halobenzimidazole riboside called DRB. It has a structure similar to a purine nucleoside but with a bichlorinated benzene ring in place of the six member ring of the purine base. DRB inhibits the transcription of the interferon "repressor" mRNA and prolonged production of interferon or "superinduction" results when cells *in vitro* are treated with the drug immediately after exposure to an inducer (Sehgal *et al.*, 1976). Although further production of interferon mRNA is also inhibited, the already transcribed interferon mRNA continues to be translated at the ribosome free of the blocking effect of the "repressor" protein. In this manner, *in vitro* yields of interferon have been increased fivefold (Sehgal *et al.*, 1976). The effect is reversible and, following removal of DRB, nuclear RNA transcription proceeds as before.

E. Summary

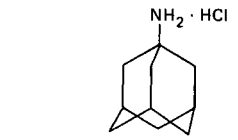
The applicability of this system to man remains to be seen. However, if practical, "superinducers" such as DRB along with more potent inducers such as poly(I)·poly(C)-poly-L-lysine might be first steps in the application of interferon in medical therpeutics. This, together with further progress in the development of more economical production methods for exogenous interferon, holds promise for the increased use of interferons in clinical medicine. The practical application of interferon in medical practice as a broad-spectrum antiviral will probably have to await its synthetic production at a reasonable cost.

V. MISCELLANEOUS ANTIVIRALS

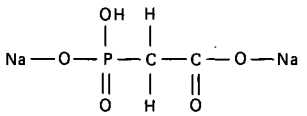
A. Amantadine

1. History

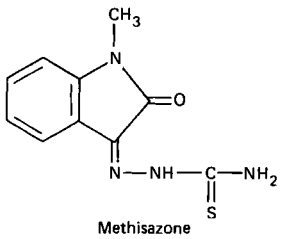
Amantadine (Symmetrel, Endo) is a synthetic compound whose antiviral activity was first reported by Davies *et al.* (1964). The drug, used clinically as the hydrochloride, is a primary amine of an uniquely symmetrical structure. Its formula is shown in Fig. 3.



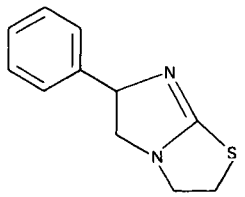
Amantadine Hydrochloride



Disodium Phosphonoacetate



Methisazone



Levamisole

Fig. 3. Miscellaneous antivirals.

2. *Antiviral Activity*

Amantadine is currently licensed for clinical use as an antiviral substance against strains of influenza A_2 virus and its antigenic variants. It has been shown to have *in vitro* activity against other strains of influenza A, parainfluenza, and rubella viruses (Hoffmann *et al.*, 1965; Maassab and Cochran, 1964; Oxford and Schild, 1967; Neumayer *et al.*, 1965). It lacks antiviral activity against influenza type B and measles viruses as well as other RNA and RNA viruses (Dickinson *et al.*, 1966).

3. *Mechanism of Action*

The mechanism of antiviral action of amantadine is not clear. It does not affect viral replication, assembly, or release. It is neither directly virucidal nor does it prevent adsorption of the virus to the cell membrane. Instead, it appears to inhibit transport of the virus into the cell, preventing replication and leaving the virus vulnerable to the hosts' immune defenses (Hoffmann *et al.*, 1965).

Amantadine has a dopaminergic effect in the central nervous system which makes it a useful pharmacological agent in the treatment of Parkinson's disease and syndrome (Clark *et al.*, 1976).

4. *Absorption, Fate, and Toxicity*

Amantadine is well absorbed following oral administration with maximum blood levels reached within 4 hours. About 90% of the drug is excreted unchanged in the urine following oral administration (Bleidner *et al.*, 1965).

The drug exhibits no specific toxicity in animals at dose levels comparable to those used in man. At higher doses, about twelve times the level of the recommended dose for humans (50 mg/kg/day), the drug is embryotoxic and teratogenic in rats. This effect was not seen in rabbits at doses up to 25 times the recommended human dose (Physicians's Desk Reference, 1976). Although the drug does not show anticholinergic action in animals at ordinary dosage, it does produce an increase in dopamine in the animal brain.

5. *Therapeutic Uses and Untoward Effects*

In double-blind studies, amantadine has demonstrated prophylactic efficacy in preventing attacks of influenza A infections whether naturally acquired or through intranasal challenge (Galbraith *et al.*, 1969; Finklea *et al.*, 1967; Quilligan *et al.*, 1966; Wendel *et al.*, 1966). At least 50% protection was observed in these studies.

The drug has also been studied relative to its efficacy in the treatment of acute influenza A virus infections (Wingfield *et al.*, 1969; Togo *et al.*, 1970; Galbraith *et al.*, 1971). Since influenza is normally a self-limited disease of short duration, measurements of clinical efficacy are difficult to obtain. Nevertheless, in the above double-blind, placebo-controlled studies, which were carried out during outbreaks of both the Asian and Hong Kong strains of influenza A_2, significant reduction in the duration of fever was seen in the amantadine-treated groups. A more recent study incorporating measurements of pulmonary physiology found that in naturally acquired influenza A infections, amantadine reduced the duration of

time of increased peripheral airways resistance seen following the acute phase of the illness (Douglas *et al.*, 1977). Such an effect could be responsible for some of the symptomatic improvement seen in patients with influenza treated with amantadine. Since this resembles a catecholaminergic response to pulmonary airways constriction, it raises an interesting question of whether a more basic cellular mechanism is influenced by amantadine, which mediates both the central nervous system and the pulmonary airways catecholaminergic actions.

Amantadine hydrochloride, N. F. (Symmetrel), is available in gelatin capsules containing 100 mg of the drug and as a syrup containing 10 mg/ml. The adult daily dosage for prophylaxis of influenza A infections is 200 mg given either as a single oral dose or in two doses of 100 mg each.

Untoward effects of the drug are primarily related to the central nervous system. These include depression, psychosis, hallucinations, anxiety, ataxia, and dizziness. Other adverse effects include anorexia, nausea, and constipation. Serious toxicity at the recommended dosage levels is extremely rare.

Influenza is unusual in that the virus undergoes periodic antigenic drift and shift, resulting in pandemics in approximately 11-year cycles. There obviously may be a lag time between isolation of the new virus and the availability of the specific vaccine. For this reason, it is most important to have an effective antiviral available for high-risk populations. Amantadine may very well fit this need.

B. Methisazone

1. History

Methisazone (Marboran), a thiosemicarbazone, is a synthetic antiviral substance whose structural formula is shown in Fig. 3. The antiviral activity of the thiosemicarbazones was first noted in 1950 when benzaldehyde thiosemicarbazone was observed to inhibit the growth of vaccinia virus in mice (Hamre *et al.*, 1950).

2. Mechanism of Action and Antiviral Activity

The precise mechanism of antiviral action of methisazone, as well as other thiosemicarbazones, is unknown. The antiviral activity is probably dependent upon the presence of the benzene ring and the side chain containing sulfur since it has been demonstrated with other thiosemicarbazones that substitution of either of these results in a loss of activity (Hamre *et al.*, 1951). The compound appears to inhibit translation of late viral mRNA, effecting a sharp reduction in the synthesis of "late" virus

protein (Woodson and Joklik, 1965). Infective virus is not produced, but infected, treated cells continue to produce viral DNA, viral antigen, and incomplete viral particles (Bauer and Sadler, 1960). Methisazone is active *in vitro* against the poxviruses and several adenoviruses. Its parent compound, isatin 3-thiosemicarbazone, possesses *in vitro* activity against several human RNA viruses as well (Bauer and Apostolov, 1966; Bauer *et al.*, 1970).

3. Therapeutic Uses

Methisazone has undergone a number of field trials as a prophylaxis against smallpox following an early suggestion (Bauer, 1955) that the parent compound, isatin 3-thiosemicarbazone, might be effective in protecting individuals exposed to smallpox. A large-scale study of methisazone's prophylactic efficacy was carried out during an outbreak of smallpox in Madras, India in 1963 (Bauer *et al.*, 1969). Although the study was unblinded and no placebo was used, the results were impressive. Of 2610 contacts of smallpox cases who were either partially or fully treated with methisazone, only 18 (0.7%) developed the disease and four died. Of 2710 contacts who served as untreated controls, 113 (4.2%) developed smallpox and 21 died. The two groups were equal in their vaccination status and degree of exposure to the index case. Two subsequent studies (Rao *et al.*, 1969; Heiner *et al.*, 1971), both of which were double-blinded, failed to demonstrate a significant protective effect for methisazone in contacts of smallpox cases. In the larger of the two studies, 106 contacts were in the treated group and 103 in the control. In the treated group, four contacts developed smallpox and, in the untreated group, five contacts developed smallpox. None of the contacts had had previous immunization (Heiner *et al.*, 1971).

A controlled study of the compound's efficacy in the treatment of clinical smallpox failed to demonstrate a significant effect, although the mortality rate in the treatment group (3.6%) was somewhat lower than that of the placebo group (6.5%) (Rao *et al.*, 1969). The drug was found to have prophylactic efficacy, however, in reducing the incidence of alastrim (variola minor) among contacts of the disease (do Valle *et al.*, 1965).

It is unfortunate that the prophylactic efficacy of methisazone in variola major remains in doubt. The declining incidence of smallpox in all parts of the world may make further therapeutic trials of the drug, to resolve this question, difficult. In the face of declining immunization levels and until smallpox is truly eradicated, it would be useful to have an efficacious chemoprophylactic agent available for this disease.

A number of impressive anecdotal reports testify to the effectiveness of methisazone as a therapeutic agent in the serious complication of vaccinia

immunization (Bauer, 1965, 1972; Kempe *et al.*, 1968). One recent report cites the effectiveness of the drug in eight cases of eczema vaccinatum, all of whose lesions yielded vaccinia virus on culture (McLean, 1977). In seven of the cases, prompt symptomatic relief was noted and drying of the lesions occurred within 1 to 2 days after initiation of therapy. The remaining case involved vaccinia necrosum in a 69-year-old patient with chronic myeloid leukemia. Healing began 4 days after initiation of therapy and was complete after 4 weeks.

4. Administration and Untoward Effects

Methisazone is quite insoluble and since it is absorbed well from the gastrointestinal tract, it is administered orally as a suspension in syrup. Little data are available on its distribution, metabolism, and excretion following absorption. Peak blood levels are reached within 6 hours after oral administration with a serum half-life of about 3 hours (Kempe *et al.*, 1965).

The only recognized untoward effect is vomiting. This is noted frequently and may be severe, requiring the use of antiemetic drugs, such as the phenothiazines. Vomiting usually begins in from 4 to 6 hours following administration of the drug and may be in part responsible for the differences noted in past efficacy studies, since an unknown amount of the drug may be lost in the process (do Valle *et al.*, 1965; Bauer *et al.*, 1969; Heiner *et al.*, 1971).

Its current use in the United States is as an investigational drug for the therapy of eczema vaccinatum and vaccinia gangrenosa. It is given orally with an initial dose of 200 mg/kg followed by 500 mg/kg in divided doses over a 48-hour period (Bauer, 1965).

C. Phosphonoacetic Acid (PAA)

1. History

Phosphonoacetic acid (PAA) has been known since 1924 but only during the past few years has its antiviral potential been recognized. Shipkowitz *et al.* (1973) reported its suppressive action against HVH infections, both *in vitro* and *in vivo*.

2. Chemistry

Because of its irritative properties as an acid, PAA is used as the disodium salt in *in vivo* studies. The formula for disodium phosphonoacetate is shown in Fig. 3. In several important aspects the configuration of PAA is similar to pyrophosphoric acid, but whether this similarity is related to its effectiveness as an antiviral substance is unknown.

3. *Antiviral Activity*

Phosphonoacetic acid has a relatively narrow spectrum of antiviral activity. It appears to be limited to DNA viruses such as HVH types I and II, equine abortion virus, murine and human cytomegalovirus, vaccinia virus and, to some extent, adenovirus-2 (Shipkowitz *et al.*, 1973; Overby *et al.*, 1975; Huang, 1975; Bolden *et al.*, 1975).

The *in vivo* studies in animals show this compound to have promising clinical potential particularly with HVH type II and cytomegalovirus (Kern *et al.*, 1975; Overall *et al.*, 1976. One study compared the effectiveness of 5% PAA, 3% ara-A, and 3% ara-AMP as an intravaginal topical treatment in mice inoculated with HVH type II (Kern *et al.*, 1975). Treatment was initiated at various intervals postinoculation to determine what the therapeutic potential would be. When treatment was initiated at 3 hours, viral replication was completely inhibited locally with no subsequent mortality. Treatment initiated at 24 to 72 hours resulted in sharp reduction in HVH type II titers in the vagina but the mice eventually died of encephalitis. Ara-A and ara-AMP had no effect on HVH type II replication in the genital tract in any of the treatment groups in this experiment nor did they protect the mice from subsequent encephalitis.

A second study tested the efficacy of PAA *in vivo* against murine cytomegalovirus (MCMV) infections in mice and *in vitro* against human cytomegalovirus (HCMV) in human cells (Overall *et al.*, 1976). In the mouse MCMV model, the untreated control mice had a final mortality of 53% while those treated with PAA had mortalities of 7% when therapy was begun 2 hours postviral challenge, 7% at 24 hours, and 33% at 48 hours. HCMV exhibited a similar level of sensitivity in human cells *in vitro*. Previous experiments with the same models demonstrated negligible protective effects with IDU, ara-C, or ara-A.

Another *in vivo* study tested the efficacy of topical 5% PAA in localized skin lesions of rabbits produced by the intradermal inoculation of Shope fibroma virus (SFV) and vaccinia virus (VV). Again, therapeutic efficacy was observed. When treatment was begun 24 hours after inoculation with VV, complete suppression of lesions was seen. Significant efficacy against SFV was seen when treatment was begun either 24 or 72 hours postinoculation (Friedman-Kien *et al.*, 1976).

4. *Mechanism of Action*

The mechanism of action of PAA is unclear; it does not appear to block adsorption or penetration of the virus nor does it have a specific virucidal action.

Against HVH it has no effect on the release of virus from host cells and,

when the drug is removed from infected cells, the typical viral cytopathic effects reappear within a few days. It thus appears that the antiviral action of PAA is effected through inhibition of viral replication (Overby *et al.*, 1974). Although it does not inhibit synthesis of HVH viral RNA or protein in infected cells, the drug sharply inhibits viral DNA synthesis. It is thought to bind to the DNA polymerase of susceptible viruses, interfering with viral DNA synthesis (Overby *et al.*, 1974). PAA also inhibits some of the host cell DNA polymerases but its inhibition of virally induced DNA polymerase, *in vitro*, is more potent (Bolden *et al.*, 1975; Huang *et al.*, 1975). Studies which utilized intact mammalian cell nuclei in culture, both infected and uninfected with virus, have demonstrated the differential effect of PAA on the cell and viral DNA polymerases (Bolden *et al.*, 1975). In these studies, utilizing HeLa cell nuclei, the cell α-DNA polymerase was inhibited by PAA to about the same extent as HVH and vaccinia virus-induced polymerases. The cell γ-DNA polymerase was inhibited to a somewhat less extent, while the cell β-DNA polymerase was minimally inhibited and then only at the highest concentrations of PAA (Bolden *et al.*, 1975).

5. Metabolism and Toxicity

Information is lacking in the published literature concerning the metabolism and toxicity of PAA.

6. Therapeutic Use

At this writing, PAA has not been used in the therapy of human viral infections. If *in vivo* studies of toxicity, reactogenicity, teratogenicity, and mutagenicity are favorable, this compound should be a candidate for efficacy studies against HVH infections, especially type II herpes progenitalis.

D. Photodynamic Inactivation

1. History

The antiviral activity of light plus photoreactive dyes, such as neutral red, acridine orange, and proflavine, has been studied extensively during the past decade (Yamamoto, 1958; Hiatt *et al.*, 1960; Wallis and Melnick, 1965; Felber *et al.*, 1973).

2. Mechanism of Action

The dye acts by associating with viral (or cellular) DNA and by absorbing light causing a photochemical oxidation of the DNA. Each of the dyes

absorbs a maximum amount of energy from a specific range of wavelength characteristic for the dye. The dye can penetrate into the virion or passes through the cell membrane and becomes associated with viral nucleic acid. On exposure to light of the appropriate wavelength, the virus is inactivated (Hiatt *et al.*, 1960). The method appears to be most practical when used against superficial lesions of the large DNA viruses such as the herpesviruses (Wallis and Melnick, 1965).

3. Therapeutic Use

Therapeutic use of photodynamic inactivation is limited to superficial HVH lesions. The method of treatment is quite simple. The herpetic vesicles are unroofed with a needle and painted with sufficient dye to permit it to soak thoroughly into the tissue. After this, the treated lesions are exposed for 10 to 15 minutes to a source emitting light of the proper intensity and wavelength at a distance of 6 to 8 inches (Wallis *et al.*, 1972). The method has been anecdotally reported to be especially useful for the photodynamic inactivation of HVH infections of the lip, skin, or genital area (Moore *et al.*, 1972; Schneider and Geary, 1971).

Two accounts have been published during the past few years concerning larger efficacy studies of this method as a treatment for cutaneous HVH infections. The first gave an account of a double-blind placebo-controlled study of 32 patients presenting with recurrent facial or genital herpes (Wallis *et al.*, 1972). A 0.1% solution of neutral red was used as the vital dye and a nonphotosensitizing red dye, phenolsulfonphthalein, was used as a control. There was a 50% reduction in both healing time and recurrence rate in the treated group as compared to the control. However, perhaps because of the degree of variation in the characteristics of oral and genital herpes and the small size of the sample, the differences were not statistically significant.

A second account described an uncontrolled study of 0.1% proflavine in a series of 49 cases of herpes progenitalis in women (Kaufman *et al.*, 1973). Only seven of the women failed to respond favorably to the treatment and almost half experienced immediate symptomatic relief upon completion of the period of light exposure.

In contrast to these and a number of encouraging anecdotal reports, a large double-blinded placebo-controlled study of light with neutral red or phenolsulfonphthalein in cutaneous HVH infections failed to demonstrate efficacy for photodynamic inactivation (Myers *et al.*, 1975). The study included 96 patients with 170 episodes occurring over more than a year. In terms of crusting, healing, and time intervals between recurrences, the neutral red group fared worse than the placebo group, although the differences were not statistically significant. Two other similar studies, one

using proflavine and the other neutral red, also have failed to demonstrate efficacy (Taylor and Doherty, 1975; Roome *et al.*, 1975).

4. Untoward Effects

Concern has been expressed by Rapp that photodynamic inactivation may be hazardous (Rapp, 1973). He provides data showing that the noninfective and probably defective virus resulting from the treatment are capable of transforming normal mammalian cells in a manner associated with a malignant potential. The importance of the results cited by Rapp in terms of human disease has been challenged (Friedrich, 1974; Melnick and Rawls, 1973).

Questions have also been raised concerning the appropriateness of the wavelength of the light source used in the largest of the efficacy studies reported (J. L. Melnick, personal communication). In spite of these questions, however, until the problem of safety is resolved and there is a clear cut demonstration of efficacy based on further double-blinded, placebo-controlled trials, photodynamic inactivation cannot be considered an appropriate method of antiviral therapy at this time.

VI. IMMUNE POTENTIATORS

A number of substances are known which augment the response of one or more components of the immune system to an infection or other immune stimulant. The inducers of interferon are examples of such substances and the effects of many of them are, of course, not limited to interferon induction. Other substances appear to have a more general immune stimulant effect without interferon induction through augmentation of the lymphoproliferative response to infection. Two of these, representing different cellular mechanisms of action, levamisole and isoprinosine, are discussed in this section.

A. Levamisole

1. History, Chemistry, and Mechanism of Action

Levamisole, a phenylimidazothiazole, has been demonstrated to be effective in anthelminthic therapy (Lionel *et al.*, 1969). Its structural formula is shown in Fig. 3. About 4 years ago it was discovered that levamisole stimulated the immune system of laboratory animals and since that time the drug has been extensively studied as a potential anticancer agent (Renoux and Renoux, 1971, 1972; Hirshaut *et al.*, 1973). While it is not an

antiviral agent per se, the precise mechanism of action of levamisole is not clear. It boosts the cell-mediated immune (CMI) response but only when cell-mediated immunity is depressed (Symoens and Brugmans, 1977). Levamisole has also been noted to cause significant increases in serum IgA levels in the elderly (Renoux and Renoux, 1972). Its action appears to be mediated by alterations in lymphocyte cyclic nucleotide levels following mitogen stimulation (Lima *et al.*, 1974; Hadden *et al.*, 1977).

2. Therapeutic Use and Untoward Effects

In a recently reported study, 21 patients with recurring HVH infections were treated with levamisole for periods ranging up to 5 months (Glogau *et al.*, 1975). Although the results are inconclusive, they suggested that the drug might have efficacy in reducing the number of recurrences.

Another study measured the effect of levamisole on the frequency of recurrence and the immune response in recurrent herpes progenitalis. The study was an unblinded open trial but reduction in frequency of recurrence was seen in almost two-thirds of the cases. The clinical response in these patients appeared to be associated with an enhanced lymphoproliferative response to HVH (O'Reilly and Lopez, 1977). The dosage of levamisole used in both studies above was 150 mg orally daily for 2 days each week for periods up to 5 months.

A double-blind placebo-controlled study of the efficacy of levamisole in reducing the incidence of respiratory infections among 70 children with chronically relapsing upper respiratory tract infections has recently been reported. Levamisole significantly reduced the number, the duration, and the severity of the infections. The effect was most striking in children receiving the highest dose, >2.5 mg/kg/day. No adverse effects were noted (Van Eygen *et al.*, 1976).

Common side effects encountered with levamisole include anorexia, nausea, diarrhea, skin rashes, irritability, and fatigue. However, these reactions are rare at routine dosage levels.

B. Isoprinosine

1. Mechanism of Action and Antiviral Activity

Isoprinosine, a derivative of the purine inosine, has been reported to have both antiviral and immune potentiating effects (Glasky *et al.*, 1975; Gordon and Brown, 1972; Gordon *et al.*, 1974). These studies have indicated that at least part of the *in vivo* antiviral effects of the compound are the result of an augmentation of both cellular and humoral immune responses. Unlike levamisole, isoprinosine does not appear to alter cyclic

nucleotide levels in lymphocytes but augments their proliferation in some other manner (Hadden *et al.*, 1976). The compound possibly enhances polyribosome synthesis after proliferation has begun.

Isoprinosine was observed to reduce the morbidity and mortality in animals infected with influenza type A and HVH (Chang and Weinstein, 1973). This finding was not confirmed in a coordinated study carried out in six laboratories where the antiviral activity of isoprinosine was tested in five animal species using eleven viruses (Glasgow and Galasso, 1972). No therapeutic efficacy was demonstrated by isoprinosine in these model systems except for a favorable suppression of fibroma virus lesions in rabbits.

2. Therapeutic Use and Untoward Effects

At least three separate placebo-controlled, double-blind challenge studies using isoprinosine as an antiviral prophylactic agent have been reported during the past several years (Longley *et al.*, 1973; Pachuta *et al.*, 1974; Waldman and Ganguly, 1977). The first (Longley *et al.*, 1973) challenged 30 volunteers with influenza A (H3N2) virus. No difference was seen in the two groups except a slight but significant reduction in virus shedding in the isoprinosine-treated group.

In the second study, a total of 37 volunteers were challenged with either rhinovirus 44 (17 subjects) or rhinovirus 32. In both trials the number and severity of colds were greater in the placebo-controlled groups, but the difference was not statistically significant.

In a third study (Waldman and Ganguly, 1977), 39 volunteers were challenged with rhinovirus type 21. Five of 19 volunteers in the isoprinosine group became ill in contrast to 14 of 20 in the placebo group who became ill. This difference was satistically significant ($p < 0.02$). A reduction in the duration of virus shedding was also noted.

The only untoward effect observed in the studies was a rise in serum uric acid levels occurring in the isoprinosine groups after the first few days of therapy. No other adverse effects were noted during any of the trials.

Since isoprinosine has a different mode of action than levamisole for its immune potentiating effect, the suggestion has been made that the two agents be combined in future therapeutic trials (Hadden *et al.*, 1976).

VII. ANTIVIRAL PERSPECTIVES

The development and therapeutic use of antiviral substances has proceeded fitfully during the past 25 years since the recognition of the anti-

viral properties of the thiosemicarbazones. The problems inherent in the development of these agents have, at times, seemed overwhelming. Since viruses are obligate intracellular parasites which take genetic control of the host cell's metabolism to achieve their replication, it appeared unlikely to many that a substance could be found which would selectively inhibit viral replication without serious damage to the host cell.

Indeed, it has seemed at times to the investigators that this point of view was correct. The apparent failure of methisazone to live up to its early hopes and the controversy that continues over the usefulness of amantadine are illustrative of the frustrations which have beset this field. The discovery that the apparent effectiveness of ara-C and IDU as therapeutic agents against serious herpesvirus infections was illusory and that instead they may have had a negative effect are other illustrations of these frustrations.

The practicing medical profession too has been pessimistic over the likelihood of finding clinically useful antiviral chemotherapeutic agents. The course of many viral infections is acute and self-limiting leaving little time to undertake specific chemotherapy even if such existed. In many cases the multiplication of the virus is at a maximum and the tissue damage irreversible by the time the illness becomes clinically apparent. The main use of antivirals in these cases would be prophylactic, i.e., to protect individuals known to be exposed to the infection. Rabies represents a classic example of the need for this type of antiviral chemoprophylaxis.

There are, however, viral diseases with a more protracted clinical course for which antivirals would be most useful, such as measles, varicella, and the adenovirus infections. Others which fit this category frequently involve an external locus, such as herpes progenitalis, ocular herpes, or herpes zoster, and are apparent from the outset. Some, like hepatitis B infections or the newly recognized disorders that are associated with persistent viruses, such as subacute sclerosing panencephalitis, can be chronic with a duration of active infection extending over years. The opportunity for successful intervention with appropriate chemotherapy in all of these instances is obvious.

The early disappointments in the achievement of effective antiviral chemotherapy have not been fruitless in that they have produced much to help the investigator in the development and testing of newer agents. Knowledge of the details of purine and pyrimidine metabolism has increased to the point that one can identify virally induced enzymes in their biosynthesis which differ from the corresponding host enzymes in their response to antimetabolites. The brilliant work which has gone into the investigations of the molecular mechanisms of action of the newer antivirals are examples of this. While not all of these agents may prove to be

ideal chemotherapeutic agents, their development is illustrative of the manner in which knowledge gained from past failure can be utilized in the search for new agents.

The studies of interferon and its inducers as antivirals must be viewed from two perspectives. The first and obvious one is that the structural formula of interferon itself will probably be determined and that it will be feasible to synthesize at least the active portion of the molecule for use as a broad-spectrum antiviral agent. The second, and perhaps more important perspective, is the recognition that we are clearly in the process of uncovering basic cellular mechanisms which are themselves antiviral. The reported ribosomal protein which specifically inhibits viral mRNA translation (Samuel and Joklik, 1974) represents an exciting step in this process.

The demonstration that the immune potentiators, such as levamisole, have efficacy in the prophylaxis of certain respiratory infections calls attention to the overriding importance of the immune responses in determining the severity and outcome of viral infections. While immunotropic agents probably cannot be used in patients who must be immunosuppressed therapeutically, they otherwise may represent an adjuvant to therapy with other antiviral agents. Following appropriate *in vivo* safety studies in animals of the immunotropic agent plus antivirals, it may be possible to increase substantially both the safety and the antiviral efficacy of our currently available drugs.

There is also a need to study carefully other combinations of antivirals both *in vitro* and *in vivo* in animals. A recent report described a markedly enhanced *in vitro* antiviral effect without increase in toxicity, produced by combinations of nucleoside analogs (Babiuk *et al.*, 1975). While in many cases such combinations might increase the toxicity of the drugs, it is entirely possible that the combination of agents with different sites of action might, in other cases, enhance therapeutic efficacy without adverse effects. The resulting reduction in dosage levels would minimize the potential for toxicity.

Expeditious progress in the development of antivirals will rely heavily on the availability of resources for testing candidate antiviral agents and combinations of agents in suitable systems of animal models of human viral diseases. The failure to use such systems in the past undoubtedly contributed to some of the earlier disappointments with antiviral therapy. Only after the clear demonstration of their safety and efficacy in these systems should candidate agents be considered for human trials.

The ultimate measure of therapeutic efficacy should rest on the results of double-blinded, randomized, controlled studies in humans. The recent experiences with both ara-C and IDU in serious herpesvirus infections serve as examples of the need for such studies for each candidate agent.

Only in the case of diseases with a very high case-fatality rate, such as clinically recognizable rabies, should an exception be made to this rule. Even here the demonstration of efficacy in animal models should be convincing to avoid adding to the burden of an already critically ill patient.

To summarize, it is hoped that the reader will agree that the era of antiviral therapy is at hand. Agents with demonstrated efficacy are available for the prophylaxis or treatment of a variety of viral disorders and the number of attractive candidate antiviral agents is growing. While no dramatic breakthroughs akin to those achieved by penicillin and the broad-spectrum antibiotics in the management of bacterial infections are foreseen, it is possible to predict with confidence the logical development of a growing armamentarium of effective antiviral chemotherapeutic agents during the coming decade.

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REFERENCES

Abel, R., Jr., Kaufman, H. E., and Sugar, J. (1975). *In* "Adenine Arabinoside: An Antiviral Agent" (D. Pavan-Langston, R. Buchanan, and C. Alford, Jr., eds.), pp. 393–400. Raven, New York.

Adams, H. G., Benson, E. A., Alexander, E. R., Vonter, L. A., Remington, M. A., and Holmes, K. K. (1976). *J. Infect. Dis., Suppl.* **133,** A151–A159.

Allen, L. B., and Sidwell, R. W. (1972). *Antimicrob. Agents & Chemother.* **2,** 229–233.

Arvin, A. M., Yeager, A. S., Merigan, T. C. (1976). *J. Infect. Dis. Suppl.* **133,** A205–A210.

Babiuk, L. A., Meldrum, B., Gupta, V. S., and Rouse, B. T. (1975). *Antimicrob. Agents & Chemother.* **8,** 643–650.

Baublis, J. V., Whitley, R. J., Ch'ien, L. T., and Alford, C. A., Jr. (1975). *In* "Adenine Arabinoside: An Antiviral Agent" (D. Pavan-Langston, R. Buchanan, and C. Alford, Jr., eds.), pp. 247–260. Raven, New York.

Bauer, D. J. (1955). *Br. J. Exp. Pathol.* **36,** 105–114.

Bauer, D. J. (1965). *Ann. N. Y. Acad. Sci.* **130,** 110–117.

Bauer, D. J. (1972). *Int. Encycl. Pharmacol. Ther.* **1,** Sect. 61.

Bauer, D. J., and Apostolov, K. (1966). *Science* **154,** 796–797.

Bauer, D. J., and Sadler, P. W. (1960). *Br. J. Pharmacol. Chemother.* **15,** 101–110.

Bauer, D. J., St. Vincent, L., Kempe, C. H., Young, P. A., and Downie, A. W. (1969). *Am. J. Epidemiol.* **90,** 130–145.

Bauer, D. J., Apostolov, K., and Selway, J. W. T. (1970). *Ann. N. Y. Acad. Sci.* **173,** 314–319.

Bennett, L. L., Jr., Shannon, W. M., Allan, P. W., and Arnett, G. (1975). *Ann. N. Y. Acad. Sci.* **255,** 342–358.

Bergmann, W., and Feeney, R. J. (1950). *J. Am. Chem. Soc.* **72,** 2809–2810.

Bleidner, W. E., Harmon, J. B., Hewes, W. E., Lynes, T. E., and Herrmann, E. C. (1965). *J. Pharmacol. Exp. Ther.* **150,** 484.

Bolden, A., Aucker, J., and Weissbach, A. (1975). *J. Virol.* **16,** 1584–1592.

Borden, E. C., Prochownik, E. V., and Carter, W. A. (1975). *J. Immunol.* **114,** 752–756.

Boston Interhospital Virus Study Group and the NIAID Sponsored Cooperative Antiviral Clinical Study Report (1975). *N. Engl. J. Med.* **292,** 599–603.

Brink, J. J., and Le Page, G. A. (1964a). *Cancer Res.* **24,** 312–318.

Brink, J. J., and Le Page, G. A. (1964b). *Cancer Res.* **24,** 1042–1049.

Calabresi, P. (1963). *Cancer Res.* **23,** 1260–1267.

Calabresi, P. Cardoso, S. S., Finch, S. C., Kligerman, M. M., von Essen, C. F., Chu, M. Y., and Welch, A. D. (1961). *Cancer Res.* **21,** 550–559.

Calabresi, P., Creasey, W. A., Prusoff, W. H., and Welch, A. D. (1963). *Cancer Res.* **23,** 583–592.

Campbell, J. B., Maes, R. R., Wilston, T. J., and Koprowski, H. (1968). *Virology* **34,** 701–708.

Carter, W. A. (1970). *Proc. Natl. Acad. Sci. U.S.A.* **67,** 620–628.

Cathala, F., and Baron, S. (1970). *J. Immunol.* **104,** 1355–1358.

Cesario, T. C., and Tilles, J. G. (1973). *J. Infect. Dis.* **127,** 311–314.

Cesario, T. C., Schryer, P. and Tilles, J. G. (1977). *Antimicrob. Agents & Chemother.* **11,** 291–298.

Chang, T. W., and Weinstein, L. (1973). *Am. J. Med. Sci.* **265,** 143–146.

Chany C. (1977). *Proc. Natl. Acad. Sci. U.S.A.* (in press).

Ch'ien, L. T., Whitley, R. J., Charomella, L. J., Buchanan, R. A., Cannon, N. J., Dismuckes, W. E., and Alford, C. A., Jr. (1975). *In* "Adenine Arabinoside: An Antiviral Agent" (D. Pavan-Langston, R. Buchanan, and C. Alford, Jr., eds.), pp. 205–224. Raven, New York.

Ch'ien, L. T., Whitley, R. J., Alford, C. A., Jr., Galasso, G. J., and Dolin, R. (1976). *J. Infect. Dis., Suppl.* **133,** A184–A191.

Chow, A. W. C., Foerster, J., and Hryniuk, W. (1970). *J. Am. Med. Assoc.* **214,** 1635.

Chu, M. Y., and Fischer, G. A. (1965). *Biochem. Pharmacol.* **14,** 333–341.

Clark, R., Smith, D. H., and Vernier, V. G. (1976). *Proc. Soc. Exp. Biol. Med.* **151,** 434–436.

Cohen, A., Togo, Y., Khakoo, R., Waldman, R. and Sigel, M. (1976). *J. Infect. Dis., Suppl.* **133,** A114–A120.

Cohen, S. S. (1966). *Prog. Nucleic Acid Res. Mol. Biol.* **5,** 1–88.

Coster, D. J., McKinnon, J. A., McGill, J., Jones, B. R., and Kraunfelder, F. T. (1976). *J. Infect. Dis., Suppl.* **133,** A173–A177.

Creasy, W. A., Papoc, R. J., Markiev, M. E., Calabresi, P., and Welch, A. D. (1966). *Biochem. Pharmacol.* **15,** 1417–1428.

Davies, W. L., Grunert, R. R., Haff, R. F., McGrahen, J. W., Neumayer, E. M., Paulshock, M., Watts, J. C., Wood, T. R., Herrmann, E. C., and Hoffman, C. E. (1964). *Science* **144,** 862–863.

Delamore, I. W., and Prusoff, W. H. (1962). *Biochem. Pharmacol.* **11,** 101–112.

Dickinson, P. C. T., Chang, T. W., and Weinstein, L. (1966). *Proc. 6th Interscience Conf. Antimicrob. Agents & Chemother.* pp. 521–526.

Dorner, F., Scriba, M., and Weil, R. (1973). *Proc. Natl. Acad. Sci. U.S.A.* **70,** 1981–1985.

Douglas, R. G., Jr., Anderson, S., Weg, J. G., Williams, T., Jenkins, D. E., Knight, V., and Beall, A. C., Jr. (1969). *J. Am. Med. Assoc.* **210,** 902–904.

Douglas, R. G., Jr., Hall, W. J., Roth F. K., and Speers, D. (1977). *Ann. N.Y. Acad. Sci.* (in press).

do Valle, L. A. R., De Melo, P. R., De Gomes, L. F., and Proenca, L. M. (1965). *Lancet* **2,** 976–978.

Evans, J. S., Musser, E. A., Mengel, G. D., Forsblad, K. R., and Hunter, J. H. (1961). *Proc. Soc. Exp. Biol. Med.* **106,** 350–353.

Evans, J. S., Musser, E. A., Bostwick, L., and Mengel, G. D. (1964). *Cancer Res.* **24,** 1285–1293.

Falcoff, E., Falcoff, R., Fournier, F., Chany, C., and Galliot, B. (1966). *Ann. Inst. Pasteur, Paris* **111,** 562–584.

Falcoff, E., Falcoff, R., Lebleu, B., and Revel, M. (1973). *J. Virol.* **12,** 421–430.

Felber, T. D., Smith, E. B., Knox, J. M., Wallis, C., and Melnick, J. L. (1973). *J. Am. Med. Assoc.* **223,** 289.

Field, A. K., Tytell, A. A., Piperno, E., Lampson, G., Nemes, M., and Hilleman, M. R. (1972). *Medicine (Baltimore)* **51,** 169–174.

Finklea, J. F., Hennessy, A. V., and Davenport, F. M. (1967). *Am. J. Epidemiol.* **85,** 402–412.

Finter, N. B. (1967). *J. Gen. Virol.* **1,** 395–397.

Finter, N. B. (1968). *Interferon, Ciba Found. Symp.*, pp. 204–215.

Finter, N. B. (1973). "Interferon and Interferon Inducers." Am. Elsevier, New York.

Force, E. E., and Stewart, R. C. (1964). *Proc. Soc. Exp. Biol. Med.* **116,** 803–806.

Friedman-Kein, A. E., Fondak, A. A., and Klein, R. J. (1976). *J. Invest. Dermatol.* **66,** 99–102.

Friedrich, E. G., Jr. (1974). *Obstet. Gynecol.* **43,** 304–305.

Furth, J. J., and Cohen, S. S. (1967). *Cancer Res.* **27,** 1528–1533.

Galbraith, A. W., Oxford, J. S., Schild, G. C., and Watson, G. I. (1969). *Lancet* **2,** 1026–1028.

Galbraith, A. W., Oxford, J. S., Schild, G. C., Potter, C. W., and Watson, G. I. (1971). *Lancet* **2,** 113–115.

Galvao, P. A. A., and Castro, I. O. (1975). *Rev. Bras. Clin. Ter.* **3,** 221.

Glasgow, L. A., and Galasso, G. J. (1972). *J. Infect. Dis.* **126,** 162–169.

Glasky, A., Pfadenhauser, E. H., Settineri, R., and Ginsberg, T. (1975). *In* "'Combined Immunodeficiency Disease and Adenosine Deaminase Deficiency: A Molecular Defect." H. J. Meuwissen *et al.*, eds., pp. 162–169. Academic Press, New York.

Glogau, R. G., Spittsv, L. E., Hanna, L., and Ostler, H. B. (1975). *Abst. Antivirals with Clinical Potential Symp., Stanford.* Session C2.

Goodman, E. L., Luby, J. P., and Johnson, M. T. (1975). *Antimicrob. Agent & Chemother.* **8,** 693–697.

Gordon, P., and Brown, E. R. (1972). *Can. J. Microbiol.* **18,** 1463–1470.

Gordon, P., Ronsen, B., and Brown, E. R. (1974). *Antimicrob. Agents & Chemother.* **5,** 153–160.

Goz, B., and Prusoff, W. H. (1970). *Annu. Rev. Pharmacol.* **10,** 143–170.

Greenberg, H., Pollard, R., Lutwick, L., Gregory, P., Robinson, W., and Merigan, T. C. (1976). *N. Engl. J. Med.* **295,** 517–522.

Hadden, J. W., Lopez, C., O'Reilly, R., and Hadden E. M. (1977). Ann. N.Y. Acad. Sci. (in press).

Hadden, J. W., Hadden, E. M., and Coffey, R. G. (1976). *Infect. Immunol.* **13,** 382–387.

Hallum, J. V., Youngner, J. S., and Stinebring, W. R. (1965). *Virology* **27,** 429–431.
Hamre, D., Bernstein, J., and Donovick, R. (1950). *Proc. Soc. Exp. Biol. Med.* **73,** 275–278.
Hamre, D., Borwnlee, K. A., and Donovick, R. (1951). *J. Immunol.* **67,** 304–313.
Heidelberger, C. (1967). *Annu. Rev. Pharmacol.* **7,** 101–124.
Heidelberger, C. (1975). *Ann. N. Y. Acad. Sci.* **255,** 317–325.
Heiner, G. G., Fatima, N., Russell, P. K., Haase, A. T., Ahmad, N., Mohammed, N., Thomas, D. B., Mack, T. M., Khan, M. M., Knatterud, G. L., Anthony, R. L., and McCrumb, F. R., Jr. (1971). *Am. J. Epidemiol.* **94,** 435–449.
Herrmann, E. C., Jr. (1961). *Proc. Soc. Exp. Biol. Med.* **107,** 142–145.
Hiatt, C. W., Kaufman, E., Helprin, J. J., and Baron, S. (1960). *J. Immunol.* **84,** 480–484.
Hirshaut, U., Pinsky, C., Marquardt, H., and Oettgen, H. F. (1973). *Proc. Am. Assoc. Cancer Res.* **14,** 109.
Ho, M., and Armstrong, J. A. (1975). *Annu. Rev. Microbiol.* **29,** 131–161.
Ho, M., and Postic, B. (1967). *In* "First International Conference on Vaccines Against Viral and Rickettsial Diseases of Man," Publ. No. 147, PAHO WHO pp. 632–649.
Ho, M., Kono, Y., and Breinig, M. K. (1965). *Proc. Soc. Exp. Biol. Med.* **119,** 1227–1232.
Hoffmann, C. E., Neumayer, E. M., Haff, R. F., and Goldsky, R. A. (1965). *J. Bacteriol.* **90,** 623–628.
Huang, E.-S. (1975). *J. Virol.* **16,** 1560–1564.
Huang, E.-S., Huang, C.-H., Huong, S.-M., Selgrade, M. J., and Shaw, J. (1975). *J. Virol.* **16,** 1560.
Huffman, J. H., Sidwell, R. W., Khave, G. P., Witkowski, J. T., Allen, L. B., and Robins, R. K. (1973). *Antimicrob. Agents & Chemother.* **3,** 235–241.
Hyndiuk, R. A., Schultz, R. O., and Hull, D. S. (1975). *In* "Adenine Arabinoside: An Antiviral Agent" (D. Pavan-Langston, R. Buchanan, and C. Alford, Jr., eds.), pp. 331–344. Raven, New York.
Ilan, J., Tokuyasu, K., and Ilan, J. (1970). *Nature (London)* **228,** 1300–1301.
Isaacs, A., and Lindenmann, J. (1957). *Proc. R. Soc. London, Ser. B* **147,** 258–267.
Johnson, H. M., and Baron, S. (1975). *J. Immunol.* **114,** 403.
Jones, B. R., McGill, J. I., McKinnon, J. R., Holt-Wilson, A. D., and Williams, H. P. (1975). *In* "Adenine Arabinoside: An Antiviral Agent" (D. Pavan-Langston, R. Buchanan, and C. Alford, Jr., eds.), pp. 411–416. Raven, New York.
Jones, B. R., Coster, D. J., Falcon, M. G., and Cantell, K. (1976). *J. Infect. Dis., Suppl.* **133,** A169–A172.
Jordan, G. W., Freid, R. P., and Merigan, T. C. (1974). *J. Infect. Dis.* **130,** 56–62.
Juel-Jensen, B. E. (1970). *Br. Med. J.* **2,** 154–155.
Kaplan, A. S., and Ben-Porat, T. (1966). *J. Mol. Biol.* **19,** 320–332.
Kaufman, H. E. (1962). *Proc. Soc. Exp. Biol. Med.* **109,** 251–252.
Kaufman, H. E. (1975). *Abst. Antivirals with Clinical Potential Symp. Stanford.* Session VI.
Kaufman, H. E., Meyer, R., Laibson, P., Waltman, S., Nesburn, A., and Shuster, J. (1976). *J. Infect. Dis., Suppl.* **133,** A165–A168.
Kaufman, R. H., Gardner, H. L., Brown, D., Wallis, C., Rawls, W. E., and Melnick, J. L. (1973). *Am. J. Obstet. Gynecol.* **117,** 1144–1146.
Ke, Y. H., and Ho, M. (1967). *J. Virol.* **1,** 883–890.
Keeney, R. E. (1975). *In* "Adenine Arabinoside: An Antiviral Agent" (D. Pavan-Langston, R. Buchanan, and C. Alford, Jr., eds.), pp. 265–273. Raven, New York.
Kempe, C. H., Rodgerson, D., and Sieber, O. F., Jr. (1965). *Lancet* **1,** 824–825.
Kempe, C. H., Fulginiti, V., and Sieber, O. F., Jr. (1968). *Proc. Int. Congr. Chemother., 5th, 1967* Abstract, Port 2, p. 1234.
Kern, E. R., Overall, J. C., Jr., and Glasgow, L. A. (1975). *Antimicrob. Agents & Chemother.* **7,** 587–595.

Kinkel, A. W., and Buchanan, R. A. (1975). *In* "Adenine Arabinoside: An Antiviral Agent" (D. Pavan-Langston, R. Buchanan, and C. Alford, Jr., eds.), pp. 197–204. Raven, New York.

Kishida, T. (1975). *Asian Med. J.* **18,** 161–177.

Koplan, J. P., Monsur, K. A., Foster, S. O., Huq, F., Rahaman, M. M., Huq, S., Buchanan, R. A., and Ward, N. A. (1975). *J. Infect. Dis.* **131,** 34–39.

Kurtz, S. M. (1975). *In* "Adenine Arabinoside: An Antiviral Agent" (D. Pavan-Langston, R. Buchanan, and C. Alford, Jr., eds.), pp. 145–157. Raven, New York.

Laibson, P. R., and Krachmer, J. H. (1975). *In* "Adenine Arabinoside: An Antiviral Agent" (D. Pavan-Langston, R. Buchanan, and C. Alford, Jr., eds.), pp. 323–335. Raven, New York.

Laibson, P. R., and Leopold, I. H. (1964). *Trans. Am. Acad. Ophthalmol. Otolaryngol.* **68,** 22–34.

Leopold, I. H. (1965). *Ann. N. Y. Acad. Sci.* **130,** 181–191.

Le Page, G. A. (1970). *Can. J. Biochem.* **48,** 75–78.

Levy, H. B., Baer, G., Baron, S., Buckler, C. E., Gibbs, C., Ladarola, M., London, W., Rice, J., Stephen, E., and Kaufman, H. E. (1975). *J. Infect. Dis.*, **132,** 434–439.

Lima, A. O., Javierre, M. Q., Da Silva, W. D., and Camara, D. S. (1974). *Experientia* **30,** 945–946.

Lionel, N. D., Mirando, E. H., Nanayakkara, J. C., and Soysa, P. E. (1969). *Br. Med. J.* **4,** 340–341.

Loddo, B., Muntoni, S., and Ferrari, W. (1963). *Nature (London)* **198,** 510.

Longley, S., Dunning, R. L., and Waldman, R. H. (1973). *Antimicrob. Agents & Chemother.* **3,** 506–509.

Lorkiewicz, Z. (1963). *Nature (London)* **197,** 314.

Luby, J. P., Johnson, M. T., Buchanan, R. A. Ch'ien, L. T., Whitley, R. J., and Alford, C. A., Jr. (1975). *In* "Adenine Arabinoside: An Antiviral Agent" (D. Pavan-Langston, R. Buchanan, and C. Alford, Jr., eds.), pp. 237–245. Raven New York.

Maassab, H. F., and Cochran, K. W. (1964). *Science* **145,** 1443–1444.

McCrea, J. F., and Lipman, M. B. (1967). *J. Virol.* **1,** 1037–1044.

McKinnon, J. R., McGill, J. I., and Jones, B. R. (1975). *In* "Adenine Arabinoside: An Antiviral Agent" (D. Pavan-Langston, R. Buchanan, and C. Alford, Jr. eds.), pp. 401–410. Raven, New York.

McLean, D. M. (1977). *Ann N. Y. Acad. Sci.* (in press).

Melnick, J. L., and Rawls, W. W. (1973). *J. Am. Med. Assoc.* **226,** 79–80.

Merigan, T. C., Reed, S. E., Hall, T. S., and Tyrell, D. A. J. (1973). *Lancet* **1,** 563–567.

Merigan, T. C., Rand, K. H., Abdallah, P. S., Jordan, G. W., Feldman, S., and Fried, R. P. (1975). *Abst. Antivirals with Clinical Potential Symp. Stanford.* Session VII.

Mitchell, M. S., Wade, M. E. DeConti, R. C., Bertino, J. R., and Calabresi, P. (1969). *Ann. Int. Med.* **70,** 535–547.

Moore, C., Wallis, C., Melnick, J. L., and Kuns, M. D. (1972). *Infect. Immun.* **5,** 169–171.

Myers, M. G., Oxman, M. N., Clark, J. E., and Arndt, K. A. (1975). *N. Engl. J. Med.* **293,** 945–949.

Nagyvary, J., Provenzole, R., and Clark, J. M. (1968). *Biochem. Biophys. Res. Commun.* **31,** 508–514.

Neumann-Haefelin, D., Shrethsa, B., and Monthey, K. F. (1976). *J. Infect. Dis., Suppl.* **133,** A211–A216.

Neumayer, E. M., Haff, R. F., and Hoffmann, C. E. (1965). *Proc. Soc. Exp. Biol. Med.* **119,** 393–396.

Nolan, D. C., Corruthers, M. M., and Lerner, A. M. (1970). *N. Engl. J. Med.* **282,** 10–13.

O'Day, D. M., Poiner, R. H., and Elliot, J. H. (1975). *In* "Adenine Arabinoside: An Anti-

viral Agent" (D. Pavan-Langston, R. Buchanan, and C. Alford, Jr., eds.), pp. 357–369. Raven, New York.
O'Reilly, R. J., and Lopez C. (1977). *Ann. N. Y. Acad. Sci.* (in press).
Ortiz, P. J. (1972). *Biochem. Biophys. Res. Commun.* **46,** 1728–1733.
Overall, J. C., Jr., and Glasgow, L. A. (1970). *Science* **167,** 1139–1141.
Overall, J. C., Jr., Kern, E. R., and Glasgow, L. A. (1976). *J. Infect. Dis., Suppl.* **133,** A237–A244.
Overby, L. R., Robishaw, E. E., Schleicher, J. B., Reuter, A., Shipkowitz, N. L., and Mao, J. C. (1974). *Antimicrob. Agents & Chemother.* **6,** 360–365.
Overby, L. R., Duff, R. G., Mao, J. C., Shipkowitz, N. L., Geiszler, A., and von Esch, A. (1975). *Abst. Antivirals with Clinical Potential Sympo. Stanford.*
Oxford, J. S., and Schild, G. C. (1967). *Brit. J. Exp. Pathol.* **48,** 235–243.
Pachuta, O. M., Yashuhi, T., Hornick, R. B., Schwartz, A. R., and Tominaga, S. (1974). *Antimicrob. Agents & Chemother.* **5,** 403–408.
Pavan-Langston, D. (1975). *In* "Adenine Arabinoside: An Antiviral Agent" (D. Pavan-Langston, R. Buchanan, and C. Alford, Jr., eds.), pp. 345–356. Raven, New York.
Payrau, P., and Dohlman, C. H. (1964). *Am. J. Opthalmol.* **57,** 999–1002.
Physician's Desk Reference. (1976). Medical Economics Company, Oradell, New Jersey.
Privat de Garilhe, M., and De Rudder, J. (1964). *C. R. Hebd. Seances Acad. Sci.* **259,** 2725–2728.
Prusiner, P., and Sundaralingarm, M. A. (1973). *Nature (London), New Biol.* **244,** 116–117.
Quilligan, J. J., Jr., Hirayama, M., and Baernstein, H. D., Jr. (1966). *J. Pediatr.* **69,** 572–575.
Rao, A. R., Jacobs, E. S., Kamalakski, S., Bradbury, S., and Swamy, A. (1969). *Indian J. Med. Res.* **57,** 477–483.
Rapp, F. (1973). *J. Am. Med. Assoc.* **255,** 459–460.
Renoux, G., and Renoux, M. (1971). *C. R. Held. Seances Acad. Sci.* **272,** 349.
Renoux, G., and Renoux, M. (1972). *Nature (London) New Biol.* **240,** 217.
Revankar, G. R., Huffman, J. H., Allen, L. B., Sidwell, R. W., Robins, R. K., and Tolman, R. L. (1975). *J. Med. Chem.* **18,** 721–726.
Roome, A. P., Tinkler, A. E., and Hilton, A. L. (1975). *Br. J. Vener. Dis.* **51,** 130–133.
Ross, A. H., Julia, A., and Babkrishnor, C. (1976). *J. Infect. Dis., Suppl.* **133,** A192–A198.
Salida-Rengall, F., Nasser-Quinones, H., and Briseno-Garcia, B. (1977). *Ann. N. Y. Acad. Sci.* (in press).
Samuel, C. E., and Joklik, W. K. (1974). *Virology* **58,** 476–491.
Schabel, D. D., and Montgomery, D. D. (1972). *In* "Chemotherapy of Virus Diseases" (D. Bauer, ed.), pp. 231–363. Pergamon, Oxford.
Schafer, T. W., Lieberman, M., Cohen, M., and Came, P. E. (1972). *Science* **176,** 1326–1327.
Schneider, G. T., and Geary, W. L. (1971). *Clin. Obstet. Gynecol.* **14,** 1057.
Schwartz, P. M., Shipman, C., Jr., and Drach, J. C. (1974). *Proc. Intersci. Conf. Antimicrob. Agents Chemother. 14th,* Session 6, Paper 33.
Scientific Committee on Interferon (1970). *Ann. N. Y. Acad. Sci.* **173,** 770–781.
Sehgal, P. B., Tam., I., and Vilcek, J. (1976). *Virology* **70,** 542–545.
Seligman, B. R., and Rosner, F. (1970). *Lancet* **1,** 307–308.
Shannon, W. M. (1975). *In* "Adenine Arabinoside: An Antiviral Agent" (D. Pavan-Langston, R. Buchanan, and C. Alford, Jr., eds.), pp. 1–43. Raven, New York.
Shipkowitz, N. L., Bower, R. R., Appell, R. N., Nordeen, C. W., Overby, L. R., Roderick, W. R., Schleicher, J. B., and von Esch, A. M. (1973). *Appl. Microbiol.* **26,** 264–267.

Sidwell, R. W., Dixon, G. J., Sellers, S. M., and Schabel, R. M. (1968). *Appl. Microbiol.* **16,** 370–392.

Sidwell, R. W., Arnett, G., and Brockman, R. W. (1970). *Ann. N. Y. Acad. Sci.* **173,** 592–602.

Sidwell, R. W., Simon, L. N., Witkowski, J. T., and Robins, R. K. (1974). *Progr. Chemother., Proc. Int. Congr. Chemother. 8th* **2,** 889–903.

Sidwell, R. W., Allen, L. B., Huffman, J. H., Revankar, G. R., Simon, L. N., and Robins, R. K. (1975). *Abstr. Antivirals with Clinical Potential Sympo. Stanford.* Session B5.

Silk, B. R., and Roome, A. P. (1970). *Lancet* **1,** 411–412.

Sloan, B. J. (1975). *In* "Adenine Arabinoside: An Antiviral Agent" (D. Pavan-Langston, R. Buchanan, and C. Alford, Jr., eds.), pp. 45–94. Raven, New York.

Smith, T. J., and Wagner, R. R. (1967). *J. Exp. Med.* **125,** 579–593.

Soloviev, V. D. (1968). *In* "The Interferons" (G. Rita, ed.), pp. 233–243. Academic Press, New York.

Soloviev, V. D. (1969). *Bull. W. H.O.* **41,** 683–688.

Spahn, G. J., Du Buy, H. G., Gazdar, A. F., and Baron S. (1977). *Proc. Soc. Exp. Biol. Med.* (in press).

Stevens, D. A., Jordan, G. W., Waddell, T. F., and Merigan, T. C. (1973). *N. Engl. J. Med.* **289,** 873–878.

Strander, H., Cantell, K., Carström, G., and Jakobsson, P. A. (1973) *J. Natl. Cancer Inst.* **51,** 733–742.

Strander, H., Cantell, K., Ingimarsson, S., Jakobsson, P. A., Nikonne, U., and Södenberg, G. (1976). *J. Infect. Dis., Suppl.* **133,** A245–A248.

Stewart, W. E., II, Gosser, L. B., and Lockart, R. Z. (1971). *J. Gen. Virol.* **13,** 35–50.

Streeter, D. G., Witkowski, J. T., Khane, G. P., Sidwell, R. W., Bauer, R. J., Robins, R. K., and Simon, L. N. (1973). *Proc. Natl. Acad. Sci. U.S.A.* **70,** 1174–1178.

Sugar, J., Varnell, E., Centifanti, Y., and Kaufman, H. E. (1973). *Invest. Ophthalmol.* **12,** 532–533.

Sundmacher, R., Neumann-Haefelin, D., Manthey, K. F., and Müller, O. (1976). *J. Infect. Dis. Suppl.* **133,** A160–A164.

Sweetman, Z. Connor, J. D., Seshanani, R., Stuckey, M. A. Carey, S., Buchanan, R. (1975). *In* "Adenine Arabinoside: An Antiviral Agent" (D. Pavan-Langston, R. Buchanan, and C. Alford, Jr., eds.), pp. 135–144. Raven, New York.

Symoens, J., and Brugmans, J. (1977). *Fogarty Int. Cent. Proc.* No. 28 (in press).

Taber, L., Greenberg, S. B., Knight, V., and Couch, R. B. (1975). *Abst. Antivirals with Clinical Potential Sympo., Stanford.* Session C6.

Tan, Y. H., Armstrong, J. A., Ke, Y. H., and Ho, M. (1970). *Proc. Natl. Acad. Sci. U.S.A.* **67,** 464–471.

Tan, Y. H., Armstrong, J. A., and Ho, M. (1971). *Virology* **44,** 503–509.

Taylor, J. (1964). *Biochem. Biophys. Res. Commun.* **14,** 447.

Taylor, P. K., and Doherty, N. R. (1975). *Br. J. Vener. Dis.* **51,** 125–129.

Thompson, R. L., Wilkin, M. L., Hitchings, G. H., Elion, G. B., Falco, E. A., and Russell, P. B. (1949). *Science* **110,** 454.

Tilles, J. G. (1974). *Annu. Rev. Pharmacol.* **14,** 469–489.

Togo, Y., and McCracken, E. A. (1976). *J. Infect. Dis., Suppl.* **133,** A109–A113.

Togo, Y., Hornick, R. B., Felitti, V. J., Kaufman, M. L., Dawkins, A. T., Kilpe, V. E., and Claghorn, J. L. (1970). *J. Am. Med. Assoc.* **211,** 1149–1156.

Tommila, V. (1963). *Acta Opthalmol.* **41,** 478–489.

Torrence, P. F., De Clercq, E., and Witkop, B. (1975). *Proc. Natl. Aca. Sci. U.S.A.* **72,** 4564–4568.

Tovell, D., and Cantell, K. (1971). *J. Gen. Virol.* **13,** 485–489.
Underwood, G. E. (1962). *Proc. Soc. Exp. Biol. Med.* **11,** 660–664.
Underwood, G. E., Elliot, G. A., and Buthala, D. A. (1965). *Ann. N.Y. Acad. Sci.* **130,** 151–167.
Van Eygen, M., Zramensky, P. Y., Heck, E., and Raymackers, I. (1976). *Lancet* **1,** 382–385.
Viceck, J., and Havell, E. A. (1977). *Ann. N. Y. Acad. Sci.* (in press).
Vilela, M. P., Siqueira, W. A., Domingues, S. H. S., Rodrigues, L. D., and Rodrigues, F. S. (1974). *Rev. Bras. Clin. Ter.* **3,** 27–28.
Waldman, R. H., and Ganguly, R. (1977). *Ann. N. Y. Acad. Sci.* (in press).
Wallis, C., and Melnick, J. L. (1965). *Photochem. Photobiol.* **4,** 159.
Wallis, C., Melnick. J. L., and Kaufman, R. H. (1972). *Clin. Obstet. Gynecol.* **15,** 939–947.
Welch, A. D., and Prusoff, W. H. (1960). *Cancer Chemother. Rep.* **6,** 29–36.
Wellings, P. C., Awdry, P. N., Bors, F. H., Jones, B. R., Brown, D. C., and Kaufman, H. E. (1972). *Am. J. Opthalmol.* **73,** 932–942.
Wendel, H. A., Snyder, M. T., and Pell, S. (1966). *Clin. Pharmacol. Ther.* **7,** 38–43.
Wheelock, E. F., and Sibley, W. A. (1964). *Lancet* **2,** 382–385.
Whitley, R. J., Ch'ien, L. T., Nahmias, A. J., Buchanan, R. A., and Alford, C. A., Jr. (1975). *In* "Adenine Arabinoside: An Antiviral Agent" (D. Pavan-Langston, R. Buchanan, and C. Alford, Jr., eds.), pp. 225–235. Raven, New York.
Whitey, R. J., Dolin, R., Ch'ien, L. T., Galasso, G. J., and Alford, C. A. Jr. (1976). *N. Engl. J. Med.* **294,** 1193–1199.
Williams, B. B., and Lerner, A. M. (1975). *J. Infect. Dis.* **131,** 673–677.
Wingfield, W. L., Pollack, D., and Grunert, R. R. (1969). *N. Engl. J. Md.* **281,** 579–584.
Witkowski, J. T., Robins, R. K., and Sidwell, R. W. (1972). *Abstract. Med. Chem. Sect., MEDI 19,* 163rd Meet. Am. Chem. Soc., Boston.
Woo, P. W. K., Dion, H. W., Lange, S. M., Dahl, L. F., and Durham, L. J. (1974). *J. Heteroycl. Chem.* **11,** 641–643.
Woodson, B., and Joklik, W. K. (1965). *Proc. Natl. Acad. Sci. U.S.A.* **54,** 946–953.
Yamamoto, N. (1958). *J. Bacteriol.* **74,** 443–448.
Zuñiga, C. B., Almeida, c., Iervolino, A. C. L., Castro, I. O., and Galvao, P. A. A. (1974). *Rev. Assoc. Med. Bras.* **20,** 386–390.

Chapter 7

The World Health Organization Virus Information System

FAKHRY ASSAAD AND PAUL BRÈS

I. INTRODUCTION

Many countries have developed their own system of reporting communicable diseases, and some of these have been in operation for quite a long time. The purpose of reporting systems is essentially to monitor the prevalence of cases and to detect any increase in their incidence, thereby warning of threatened epidemics. Reporting systems are also often used for planning and evaluating programs for control or prevention of communicable diseases. The development of an efficient reporting system is not as easy as it may appear at first sight, particularly for viral diseases. In this field, several factors have to be considered: reporting based only on clinical diagnosis could be inaccurate; the geographic distribution of laboratories may be uneven; technical capabilities may differ widely; and coordination between laboratories, even in the same country, is often lacking. Laboratory confirmation of most viral diseases was not carried out until the 1960's when the use of tissue culture brought the diagnosis of viral diseases within the reach of many laboratories. It was at this time that the desirability of an international coordination of reporting systems for these diseases was felt. For these reasons, in 1961, WHO was asked to undertake an international program of collection, analysis, and dissemination of information on viral diseases. The development of the WHO reporting system on virus diseases, its operation, results achieved so far, and the problems encountered will be described below.

II. DEVELOPMENT OF THE REPORTING SYSTEM

The Scientific Group on Virus Diseases, which met in 1961, recommended WHO for developing a reporting system for viral diseases. It was felt that this system should be based on direct communication with laboratories and should not depend on statutory notifications.* In addition, it appeared that the problem differed according to the category of viral infections. One category concerned infections of the respiratory tract and central nervous system, the second, arbovirus infections, and the third, infections caused by rickettsiae, chlamydiae, and mycoplasmas. Although the latter three agents are not viruses, they are frequently dealt with in virus laboratories. For practical reasons, efforts were directed first toward the group of respiratory and enteric infections and those of the central nervous system, which represent the majority of clinical infections.

* Statutory notifications from all Member States of WHO are collated and published in the World Health Statistics Report and the World Health Statistics Annual.

When WHO was developing its own reporting system, the Public Health Laboratory Services (PHLS) in Great Britain seemed at the time to be an appropriate example (Bradstreet *et al.*, 1964, 1973). Each week, virus diagnostic laboratories in the PHLS, and many hospital laboratories outside the Service, reported infections confirmed by virus isolation and/or serologic tests. This information, collated in a weekly report, was circulated, confidentially, to the contributing laboratories, Area Medical Officers of Health, and the Department of Health and Social Security. This scheme was developed just before World War II and proved to be increasingly useful in the years that followed. Very few other countries had developed at this time a comparable, centrally coordinated reporting system. WHO then had to deal with the central service, where it existed, or to seek the direct collaboration of laboratories in countries where a central channel of communication did not exist. Furthermore, participation of laboratories in the scheme was only on a voluntary basis. The return they expected was to obtain as quickly as possible confidential information on the epidemiological situation in other countries. Each virologist could then anticipate the viruses likely to be isolated from the samples he received.

The WHO Virus Reporting System started in 1963 with a small number of participating laboratories. As mentioned above these laboratories had already established recording and reporting systems of their own. In designing the WHO system and, in particular, in deciding on the input, the experience already acquired by the participating laboratories was used. At that time there was little difference in the technical capabilities and the working practices of the small number of laboratories engaged in virological work. The number of reports received by WHO each year was about 8000 and the data were tabulated by hand. In the following years the number of laboratories joining the scheme increased and by 1966 the number of reports received was close to 18,000. In 1967, it became necessary to use electronic data processing to handle the increasing number of reports and to render feasible the storage and retrieval of information. However, in adopting this process, unless existing data were transferred to magnetic tape following the same layout as had been designed for the new system, retrieval of information which had accumulated over the preceding few years was impossible. Considering the technical difficulties at that time, and the expense, it was not considered advisable to transfer the data for the years 1963–1966 to magnetic tape.

As the number of participating laboratories continued to increase over the years, the differences among the laboratories in facilities, technical set up, interest in different groups of viruses, and adoption of different diagnostic methods and procedures, etc., increased even more. Therefore, in

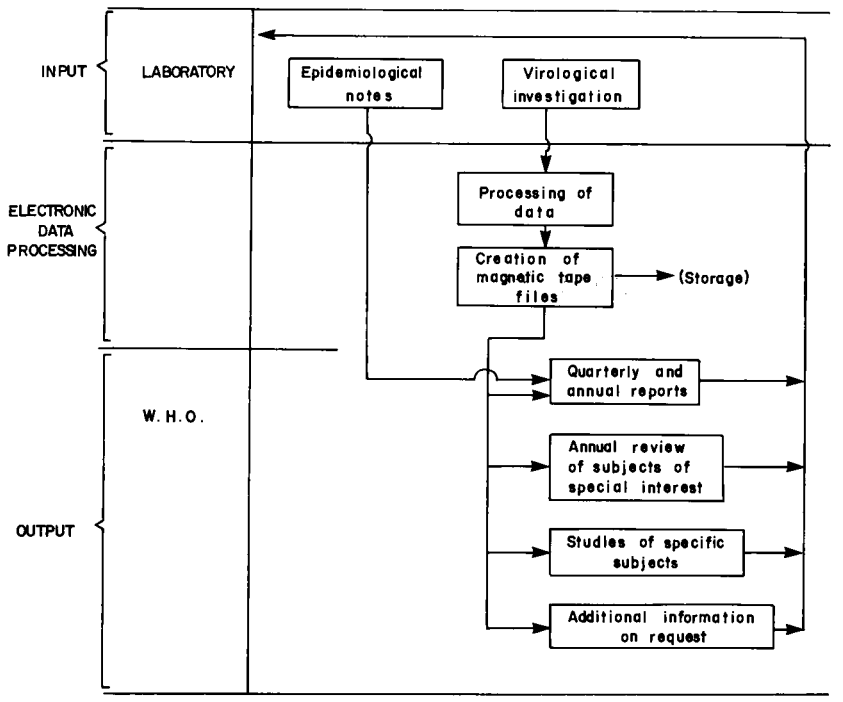


Fig. 1. WHO Virus Reporting System.

1973, the Virus Reporting System was reviewed and now functions as shown in Fig. 1 (Grist and Miller, 1973).

By the end of 1975, the number of laboratories participating in the reporting system from each of the WHO Regions was as follows: Africa, 7; America, 17; eastern Mediterranean, 3; Europe, 84; southeast Asia, 2; western Pacific, 6. These figures reveal the uneven coverage found in the different regions, a difficulty which cannot easily be overcome. This is inevitably the result of the uneven development of virus laboratories in the world. WHO is constantly trying to encourage the development of competent virus laboratories in countries where they do not exist and to bring them into the scheme, thus giving them an incentive to improve their standards.

III. OBJECTIVES

The two objectives of the reporting system are as follows: to serve as an epidemiologic information service and to promote public health laboratory services in countries where these are needed.

The first objective includes the following:

1. Surveillance of viral diseases: to obtain regular, systematic information on the distribution of particular virus infections in different parts of the world; to promptly recognize changes in their distribution; to obtain broad indications of the main clinical manifestations and age groups affected.

2. Assistance in disease control: to alert local, national, and regional health authorities to changes in disease distribution (particularly epidemics) that have more than local significance and require coordinated investigation and containment operations; to provide these authorities with data on the distribution of virus infections in their own and neighboring areas that may assist them in planning control programs; to coordinate vaccine surveillance (safety and efficacy).

3. Study of patterns of virus behavior: to recognize differences in the pattern of virus infection in communities with various social, economic, and geographical conditions and to follow the effects of changing environmental conditions which may assist understanding of their ecological behavior; to further investigate data obtained from reports of routine diagnostic virology by promoting planned studies based on defined populations (e.g., patterns of immunity and frequency of inapparent infection).

In addition, the information system plays a role, considered to be very important from the point of view of WHO, in stimulating the development of virus laboratories (its second objective). The following may be considered educational aims:

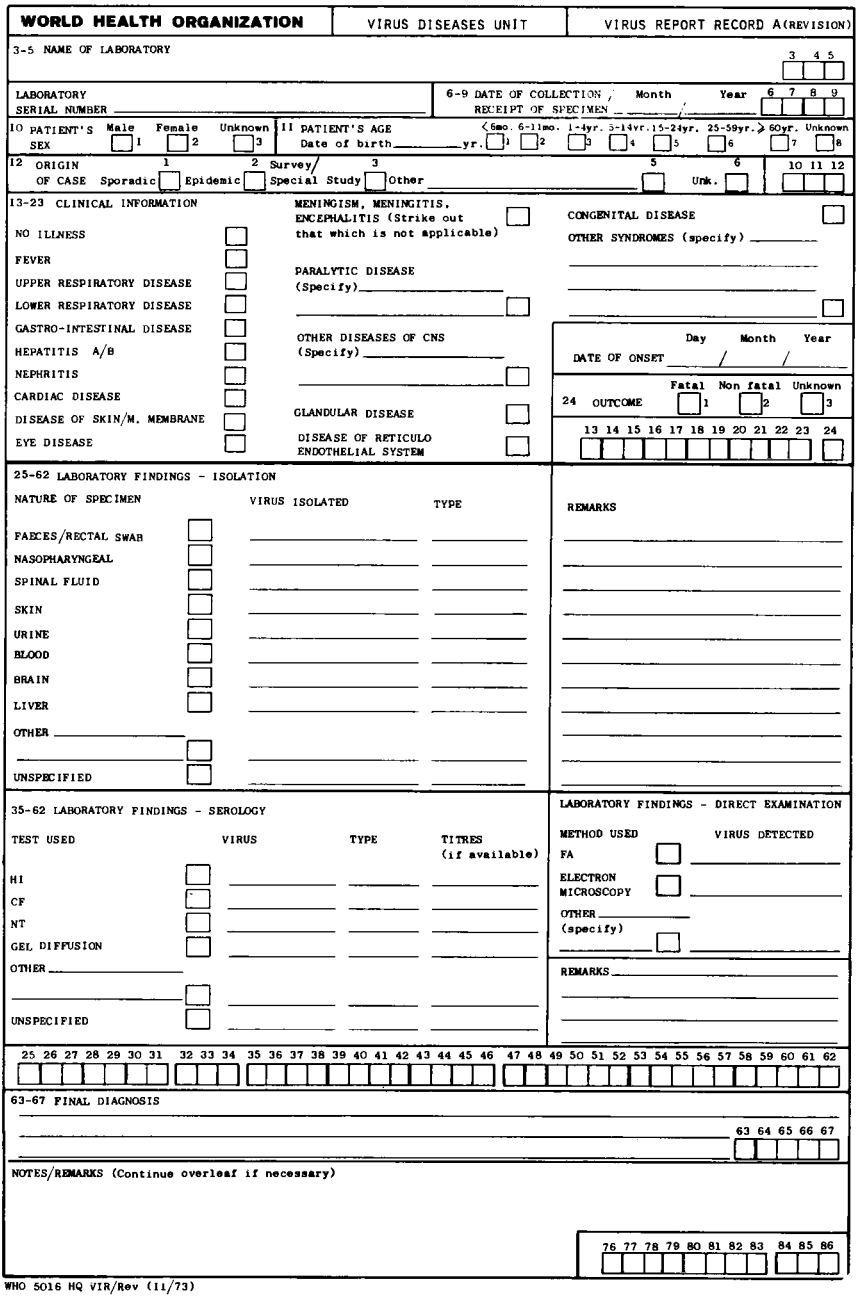
1. Exchange information: to offer a forum for exchange of information on local experiences in the investigation and control of virus infections and the use of new laboratory or other investigative techniques.

2. Data collection: to encourage the systematic collection and reporting of accurate statistics locally and at a national level; to show the value of consolidated reports to local laboratories, physicians, and health administrators in interpreting isolated cases and in understanding the behavior and directing the control of diseases in their areas.

3. Laboratory methods: to promote the development and trial of new techniques; to encourage the critical evaluation of laboratory performance.

IV. DATA INPUT FROM LABORATORIES

The following is the input which was adopted in 1974 and is entered on the form shown in Fig. 2.



WORLD HEALTH ORGANIZATION | VIRUS DISEASES UNIT | VIRUS REPORT RECORD A(REVISION)

3-5 NAME OF LABORATORY

LABORATORY SERIAL NUMBER

6-9 DATE OF COLLECTION / RECEIPT OF SPECIMEN — Month Year

10 PATIENT'S SEX: Male 1 Female 2 Unknown 3

11 PATIENT'S AGE Date of birth ____ yr. <6mo. 1 | 6-11mo. 2 | 1-4yr. 3 | 5-14yr. 4 | 15-24yr. 5 | 25-59yr. 6 | ≥60yr. 7 | Unknown 8

12 ORIGIN OF CASE: 1 Sporadic 2 Epidemic 3 Survey/Special Study 5 Other 6 Unk.

10 11 12

13-23 CLINICAL INFORMATION

NO ILLNESS
FEVER
UPPER RESPIRATORY DISEASE
LOWER RESPIRATORY DISEASE
GASTRO-INTESTINAL DISEASE
HEPATITIS A/B
NEPHRITIS
CARDIAC DISEASE
DISEASE OF SKIN/M. MEMBRANE
EYE DISEASE

MENINGISM, MENINGITIS, ENCEPHALITIS (Strike out that which is not applicable)
PARALYTIC DISEASE (Specify)
OTHER DISEASES OF CNS (Specify)
GLANDULAR DISEASE
DISEASE OF RETICULO ENDOTHELIAL SYSTEM

CONGENITAL DISEASE
OTHER SYNDROMES (specify)

DATE OF ONSET Day / Month / Year

24 OUTCOME: Fatal 1 Non fatal 2 Unknown 3

13 14 15 16 17 18 19 20 21 22 23 24

25-62 LABORATORY FINDINGS - ISOLATION

NATURE OF SPECIMEN	VIRUS ISOLATED	TYPE
FAECES/RECTAL SWAB		
NASOPHARYNGEAL		
SPINAL FLUID		
SKIN		
URINE		
BLOOD		
BRAIN		
LIVER		
OTHER		
UNSPECIFIED		

REMARKS

35-62 LABORATORY FINDINGS - SEROLOGY

TEST USED	VIRUS	TYPE	TITRES (if available)
HI			
CF			
NT			
GEL DIFFUSION			
OTHER			
UNSPECIFIED			

LABORATORY FINDINGS - DIRECT EXAMINATION

METHOD USED — VIRUS DETECTED
FA
ELECTRON MICROSCOPY
OTHER (specify)

REMARKS

25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60 61 62

63-67 FINAL DIAGNOSIS

63 64 65 66 67

NOTES/REMARKS (Continue overleaf if necessary)

76 77 78 79 80 81 82 83 84 85 86

WHO 5016 HQ VIR/Rev (11/73)

Fig. 2. WHO form for virus report record (facsimile).

A. Identification of Specimen

Every laboratory is given a code number. Up to 1973, the laboratory serial number of the specimen was included in the input. This was meant to be used as a cross reference between two reports sent at different times by the same laboratory and referring to the same case. However, this was very rare and arose only when (a) a report of an untyped virus was first sent to WHO and was then followed at some later date by the result of the typing; (b) more than one investigation was carried out on the same case and took different times to complete, e.g., isolation and serology; (c) more than one virus was identified and the time taken for typing them differed, etc. These conditions were not only rare, but very few laboratories ever referred to an earlier report. Therefore, starting in 1974, the participating laboratories were requested to send their reports only when the final diagnosis of the viral infection had been completed.

B. Date of Collection or Receipt of Specimen

No difference is made between the date of collection or date of receipt. It is understood that in most cases the date given is that of the receipt of the specimen which is readily available to the laboratory. The date requested is by month and year only. For some infections, e.g., influenza A, reporting by week might, at first sight, appear to be desirable. However, considering the need for a unified system—for ease of data processing and for comparison among laboratories—reporting by month has proved quite adequate.

C. Indication of Sex

This was introduced in 1974. However, its value remains to be assessed.

D. Age

Preset age groups are used, but for a number of laboratories which give the date of birth instead of age, space is provided for this purpose and the computer programme attributes the corresponding age group.

E. The Origin of the Case

The origin of the case, whether sporadic, epidemic, or a result of a special study, etc., is included. However, it is not always clear to the labora-

tory whether the case is epidemic or sporadic. Furthermore, a number of cases at the beginning of an epidemic could be thought to be sporadic. In certain studies it is important that cases forming part of special investigations, surveys, etc., be differentiated from those occurring at large.

F. Clinical Information

Clinical information is given according to anatomical systems whenever possible, but space is also left for nonlocalizing fever, congenital disease, etc. The coding allows for entry of up to five syndromes. The date of onset is included in the form but is not coded because it is not frequently supplied by reporting laboratories. When available it helps to assess the validity of the results, especially in serology.

G. Outcome

Whether the outcome is fatal or nonfatal is included. Only a small number of reports include this information.

H. Laboratory Findings

1. Isolation

The nature of the specimen is included. This is primarily used to assess the significance of a given isolation. Information on the system used for isolation of viruses is not requested. Because it is difficult, except as a planned experiment in one laboratory, to evaluate different test procedures, types of tissue culture, etc., it is impractical to collect such information on an international basis in a form suitable for useful analysis.

2. Serology

The serological test used is indicated and a space is provided for giving the titers when available. Knowledge of the type of test helps in assessing the value of a serologically positive finding. The titers confirm that there has been a fourfold or more rise between the acute and convalescent sera. Almost all laboratories supply information on the test used and many indicate the titers. In some instances only one titer is given, in which case the judgment of the laboratory is accepted. Nonetheless, the date of onset of the disease, the date of collection or receipt of serum, and the titer level are scrutinized. If there is any doubt a query is sent to the laboratory.

3. Direct Methods

Only a small number of laboratories report cases diagnosed on evidence of direct detection by fluorescent antibody technique or by electron microscopy. Recently, the number of cases in which the reovirus-like agent of infantile gastroenteritis is being reported has increased.

4. Considerations for Inclusion of Laboratory Findings

Except for enteroviruses, where isolation is required, diagnosis of other viruses can be made on serological findings alone or by direct methods, e.g., immunofluorescence, immunoperoxidase or electron microscopy. For the sake of simplicity in data processing, information on arboviruses, and nonviral agents, such as chlamydiae, rickettsiae, and *Mycoplasma pneumoniae* are also included in the input, although no use of them is made at present when analyzing the information, with the exception of *M. pneumoniae*. Up to three specimens and three viruses can be coded on the form. Beyond this, it has to be determined whether to drop the excessive entries. So far such cases have been extremely rare. The following untyped agents are rejected: influenza viruses, enteroviruses, chlamydiae, and rickettsiae.

I. Final Diagnosis

This is required and when given—which is not very frequently—is coded in accordance with the International Classification of Diseases.

The form on the whole has been well accepted by participating laboratories. In fact, many laboratories indicate the method of isolation, the serological test used, the dates of withdrawing blood, and the rising titer in the convalescent serum. However, a number of items are still poorly reported.

V. LIMITATIONS TO THE INPUT

The principal limitations are due to the following facts:

1. Capacity (facilities of a laboratory): number of specimens that can be handled, range of tests available
2. Skill and experience of laboratory workers: choice of tests, conduct of tests, reading of results, interpretations of observations. These may vary according to the special interest of workers in different viruses.

3. Quality of material
4. Reproducibility and accuracy (sensitivity and specificity) of test systems
5. Clinical criteria for investigating patients (including hospital admission policy), availability of medical care, costs, physicians' attitude to tests, etc.
6. Quality of specimens, range of illness, methods of collection, transport facilities, etc.

These inherent variations in the input of an international routine diagnostic reporting system are unavoidable and should be considered when evaluating the output.

VI. DATA PROCESSING

Reports received from laboratories can be divided into two groups: (a) reports made by completing the WHO form "Virus Report Record A" (Fig. 2), or those made on locally produced forms or lists, and (b) reports already coded and punched on IBM cards or on magnetic tapes. The flow charts in Fig. 3 show these alternatives.

The WHO Virus Report Record Form is easy to complete and requires no codification on the part of the laboratory. Once received in WHO it undergoes a series of three operations:

1. The entries are checked to determine that all pertinent data have been entered; that the final diagnosis agrees with the data given; that there are no "illegal" entries, e.g., entries into two age groups, virus type numbers which do not exist; whether there is a cross reference to an earlier report. If there are any queries, the report is returned to the laboratory for clarification.
2. The report is coded, using the boxes provided on the form. This process is time-consuming.
3. The coded data are punched on tape.

All data, i.e., those coded both at WHO and outside, punched on tapes or cards are processed by the International Computing Centre (ICC) on the IBM 3701175 VS computer. All programs have been prepared by the Data Processing Unit, WHO in PL/I language. The incoming data are treated as follows:

1. Data generated outside WHO are converted into WHO coding specifications.

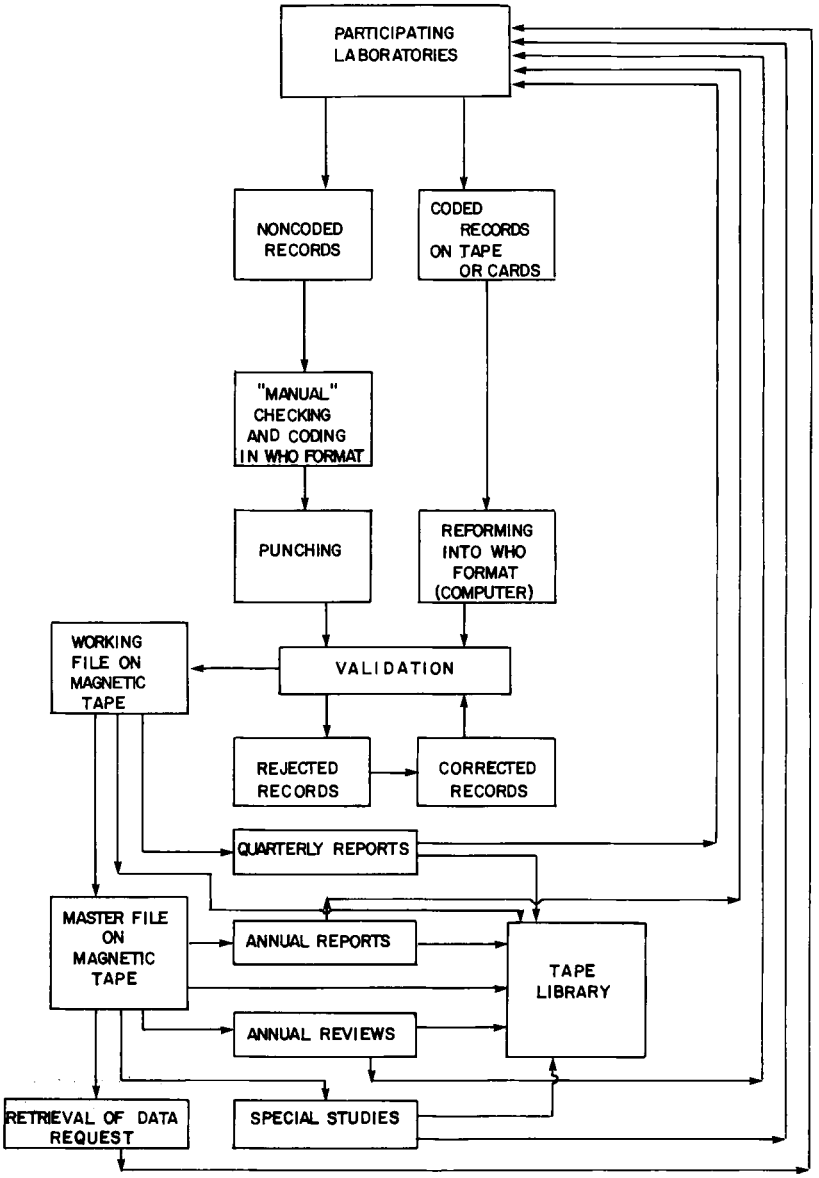


Fig. 3. Data processing.

2. All data are validated, i.e., they are checked by the computer to ensure that all pertinent items have been completed.

3. Correct records are put onto a working tape, while those with errors or missing information are rejected, and a list of these records with an indication of the errors is produced.

4. The rejected records which were coded at WHO are corrected, repunched, and rechecked. Mistakes in reports from the laboratories sending coded data are corrected if the mistake is obvious and, if it is not, a query is sent to the relevant laboratory and the corrected record is included in the next quarter.

5. Once all records for the quarter are corrected they are put on a working tape and the data for the quarterly report are tabulated.

6. During the tabulation a final validation is made by the computer including codes of viruses, their types, clinical conditions, age groups, etc. A list of rejected records is produced. The errors are then corrected and put on the file of the next quarter. The list also contains records of agents that are purposely stored in the file but are not tabulated in the reports, e.g., chlamydiae and rickettsiae.

7. As soon as the report for the fourth quarter has been tabulated, the data for the four quarters of the year are transferred, in the chronological order of their receipt, for processing onto a master tape, and the working tapes can be reused.

8. Data stored on the master tape serve to produce annual tabulations, reviews of subjects of special interest, specific studies, and *ad hoc* retrievals. The master tape has a backup copy which is kept in a fireproof safe. All tabulations are also copied on tape and are kept in the tape library where they are available whenever necessary.

VII. OUTPUT

The data are processed to produce quarterly and annual reports, annual reviews of subjects of special interest, studies of specific subjects, and *ad hoc* retrieval requested by laboratories.

A. Quarterly and Annual Reports

The computer has been programmed to produce a set of tables according to a preset format. These are designed to account for the limitations of the input already mentioned in Section IV as well as the use made of the quarterly and annual reports by the recipients. Tables have the same layout in both the quarterly and annual reports and are introduced

by a short text highlighting pertinent observations on the information gathered during the quarter or the year.

Table I of the report (Table I) gives the number of virus infections by country. Up to 1971, the findings were tabulated by laboratory. In some countries one laboratory acted as the reporting channel for the entire country, while in others, laboratories reported independently. In addition, as additional laboratories joined the scheme, the tabulations by laboratory became more and more cumbersome. With the approval of the reporting laboratories, therefore, the decision was made in 1972 to tabulate the reported viral infections by country rather than by laboratory. However, the data file contains the information by reporting laboratory.

In Table II of the report (Table II) viral infections are supplied according to the principal clinical conditions to which they were associated and in Table III by age (Table III). Nearly half of the reports relate to diseases of the respiratory tract and central nervous systems. Because of their importance as causes of morbidity, they are tabulated separately. Table IV gives viral infections of the central nervous system by age and Table V supplies viral infections of the respiratory tract by age. Reporting countries are grouped as follows (Tables VI and VII) in an attempt to determine the differences among the industrialized and the less developed countries in the frequency of viral infections associated with diseases of the central nervous system and the respiratory tract:

1. Africa, excluding North Africa
2. The Americas, excluding Canada and the United States
3. Asia, excluding Japan
4. North Africa and the Eastern Mediterranean, including Turkey and excluding Israel
5. Europe plus Israel
6. Australia, Canada, New Zealand, Japan and the United States

B. Annual Reviews of Subjects of Special Interest

Since 1971, reports on enteroviruses other than poliovirus have been reviewed annually. Annual reviews of respiratory virus infections started in 1975. Two publications (Assaad and Cockburn, 1972, 1974) and one report (Brès and Assaad, 1975) summarize the findings which have been made in the previous reporting years. It is recognized that there are many difficulties in comparing data from different laboratories, such as, variations in the level of technical competence or laboratory facilities, interest limited to certain groups of viruses, delays in reporting and in completion of tests (mainly the final typing of viruses), and the emphasis placed by

TABLE I
Virus Infections by Country (All Year 1974) (Facsimile)

	TOTAL	ARG	AUL	AUS	BEL	BRA	BUL	CAN	CAR	CZE	DEN	ECU	EGY	FIN	FRA	FRG
ADENOVIRUS																
TOTAL	5223	-	146	4	266	-	-	418	-	148	61	-	-	184	437	349
1	626	-	25	-	14	-	-	73	-	6	-	-	-	18	24	21
2	861	-	37	-	25	-	-	112	-	8	1	-	-	6	39	27
3	320	-	29	-	2	-	-	42	-	13	4	-	-	6	14	17
4	95	-	6	-	-	-	-	5	-	-	-	-	-	-	4	1
5	305	-	15	-	7	-	-	34	-	4	-	-	-	5	16	14
6	65	-	3	-	-	-	-	8	-	-	-	-	-	-	4	-
7	1045	-	22	-	3	-	-	27	-	14	10	-	-	1	65	62
8	41	-	-	-	-	-	-	-	-	2	-	-	-	-	4	1
9	4	-	-	-	1	-	-	-	-	-	-	-	-	-	2	1
10	20	-	-	-	-	-	-	-	-	-	-	-	-	-	1	1
11	7	-	-	-	1	-	-	-	-	-	-	-	-	-	-	2
12	4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
13	15	-	-	-	15	-	-	-	-	-	-	-	-	-	-	-
14	22	-	-	-	3	-	-	1	-	-	-	-	-	-	-	1
15	3	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-
17	2	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-
18	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
19	52	-	-	-	-	-	-	9	-	-	-	-	-	-	1	1
21	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
22	1	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-
23	1	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-
29	1	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-
30	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
31	8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
INFLUENZA VIRUS																
TYPE A	3688	-	302	9	16	-	6	279	1	-	191	-	-	150	200	345
TYPE B	2592	6	10	3	16	-	42	189	-	1	23	-	-	173	38	294
TYPE C	46	-	-	-	-	-	-	-	-	3	-	-	-	-	-	-
PARAINFLUENZA																
TOTAL	2248	-	140	2	75	-	3	210	-	61	-	-	-	28	159	125
1	613	-	8	-	13	-	2	53	-	3	-	-	-	28	16	34
2	389	-	47	-	16	-	1	39	-	20	-	-	-	-	20	7
3	1173	-	83	2	41	-	-	118	-	33	-	-	-	-	123	84
4	27	-	-	-	-	-	-	-	-	5	-	-	-	-	-	-

RS VIRUS	2850	-	128	-	77	-	-	122	-	46	5	-	-	87	90	110
RHINOVIRUS	513	-	141	-	-	-	-	-	-	11	-	-	-	-	-	4
COXSACKIE A																
TOTAL	741	-	40	-	1	-	-	8	1	1	18	-	-	32	29	25
1	26	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2	39	-	-	-	-	-	-	-	-	-	2	-	-	-	-	-
3	3	-	-	-	-	-	-	-	-	-	3	-	-	-	-	-
4	81	-	-	-	-	-	-	1	-	-	1	-	-	-	-	-
5	48	-	-	-	-	-	-	-	-	-	3	-	-	-	-	1
6	34	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-
7	16	-	-	-	-	-	-	-	-	-	1	-	-	-	1	-
8	20	-	-	-	-	-	-	-	-	-	3	-	-	-	-	-
9	245	-	35	-	-	-	-	6	-	1	1	-	-	5	19	14
10	43	-	-	-	-	-	-	-	-	-	2	-	-	-	-	-
11	5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
13	7	-	-	-	-	-	-	-	-	-	-	-	-	-	2	-
15	1	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-
16	129	-	2	-	-	-	-	1	-	-	1	-	-	27	-	5
17	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
19	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
20	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
21	7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
22	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
24	4	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-
COXSACKIE B																
TOTAL	2422	-	101	-	127	4	47	18	10	6	27	-	-	122	106	227
1	82	-	1	-	13	-	25	-	-	-	-	-	-	-	1	10
2	562	-	62	-	22	-	10	42	1	-	4	-	-	-	33	73
3	640	-	35	-	18	-	-	118	2	-	2	-	-	27	17	63
4	297	-	1	-	11	-	12	8	-	-	4	-	-	54	8	14
5	756	-	1	-	63	1	-	11	7	6	10	-	-	41	39	65
6	27	-	-	-	-	3	-	4	-	-	1	-	-	-	1	2

TABLE II
Virus Infections by Clinical Conditions (All Year 1974) (Facsimile)

	TOTAL	RESP. TOTAL	RESP. UPPER	RESP. LOWER	CNS TOTAL	MENG/ ENCPH	PARA- LYTIC	CARD- VASC
ADENOVIRUS								
TOTAL	5223	2545	1285	1178	363	263	27	60
1	626	291	150	126	40	27	4	6
2	861	426	219	184	56	32	6	6
3	320	158	96	59	14	8	-	3
4	95	34	14	14	2	2	-	-
5	305	138	76	58	24	14	2	2
6	65	30	23	7	6	4	1	1
7	1045	508	302	191	74	67	2	7
8	41	2	2	-	2	2	-	-
9	4	2	1	1	-	-	-	-
10	20	1	-	1	3	3	-	-
11	7	-	-	-	1	-	-	-
12	4	1	1	-	-	-	-	-
13	15	13	9	4	1	-	-	-
14	22	17	10	7	-	-	-	1
15	3	1	1	-	-	-	-	-
17	2	-	-	-	-	-	-	-
18	2	2	1	1	-	-	-	-
19	52	4	3	1	-	-	-	-
21	2	-	-	-	-	-	-	-
22	1	-	-	-	-	-	-	-
23	1	1	-	1	-	-	-	-
29	1	-	-	-	-	-	-	-
30	1	-	-	-	-	-	-	-
31	8	-	-	-	1	1	-	-
INFLUENZA VIRUS								
TYPE A	3688	2260	920	1296	93	70	2	40
TYPE B	2592	1620	964	602	70	48	1	16
TYPE C	46	33	13	20	3	3	-	2
PARAINFLUENZA								
TOTAL	2248	1677	615	1023	104	70	2	23
1	613	452	166	269	28	22	-	4
2	389	311	92	216	14	12	-	5
3	1173	854	338	499	59	33	2	13
4	27	23	10	13	-	-	-	-

RS VIRUS	2850	2540	580	1844	47	27	2	15
RHINOVIRUS	513	422	204	187	7	5	-	3
COXSACKIE A								
TOTAL	741	86	55	29	219	149	52	3
1	26	-	-	-	18	3	15	-
2	39	4	1	3	10	5	3	-
3	3	-	-	-	-	-	-	-
4	81	6	5	1	15	7	6	-
5	48	12	12	-	17	14	2	-
6	34	4	3	1	9	1	7	-
7	16	2	2	-	1	-	1	-
8	20	5	4	1	2	-	2	-
9	245	31	14	17	110	98	3	-
10	43	1	1	-	5	3	2	-
11	5	-	-	-	4	2	2	-
13	7	1	-	1	5	2	3	-
15	1	-	-	-	1	1	-	-
16	129	14	10	2	6	6	-	2
17	3	-	-	-	1	-	1	-
19	3	-	-	-	2	2	-	-
20	2	-	-	-	1	-	1	-
21	7	2	1	1	1	1	-	-
22	1	-	-	-	1	-	1	-
24	4	1	1	-	1	1	-	-
COXSACKIE B								
TOTAL	2422	529	301	219	761	702	24	79
1	82	15	9	6	17	13	3	3
2	562	143	74	68	187	174	4	13
3	640	134	74	57	203	184	6	28
4	297	59	36	21	68	60	6	12
5	756	161	98	60	259	245	4	18
6	27	7	6	1	3	3	-	1

TABLE III
Virus Infections by Age (All Year 1974) (Facsimile)

	TOTAL	0-5 MONTHS	6-11 MONTHS	1-4 YEARS	5-14 YEARS	CHILD. 0 - 14	15-24 YEARS	25-59 YEARS	60+ YEARS	ADULTS 15 & +
ADENOVIRUS										
TOTAL	5223	440	607	1912	1002	3961	410	532	86	1028
1	626	110	127	286	59	582	6	12	3	21
2	861	133	177	396	65	771	13	19	14	46
3	320	18	32	117	90	257	11	40	2	53
4	95	5	3	18	14	40	16	34	1	51
5	305	37	60	155	28	280	6	8	2	16
6	65	10	17	30	6	63	-	1	-	1
7	1045	58	71	348	328	805	97	98	7	202
8	41	1	-	-	4	5	3	26	6	35
9	4	-	-	-	-	-	1	2	-	3
10	20	2	-	2	2	6	2	8	1	11
11	7	-	1	2	3	6	-	1	-	1
12	4	1	1	2	-	4	-	-	-	-
13	15	2	3	6	1	12	-	-	-	-
14	22	-	1	7	9	17	1	2	-	3
15	3	1	-	1	-	2	1	-	-	1
17	2	1	-	1	-	2	-	-	-	-
18	2	1	-	-	-	1	-	-	-	-
19	52	5	-	5	2	12	12	19	4	35
21	2	-	-	-	-	-	-	2	-	2
22	1	-	-	-	-	-	-	1	-	1
23	1	-	1	-	-	1	-	-	-	-
29	1	-	-	1	-	1	-	-	-	-
30	1	-	-	-	1	1	-	-	-	-
31	8	1	1	6	-	8	-	-	-	-
INFLUENZA VIRUS										
TYPE A	3688	76	109	544	525	1254	529	1015	623	2167
TYPE B	2592	34	43	281	750	1108	578	536	232	1346
TYPE C	46	-	2	10	7	19	3	10	9	22
PARAINFLUENZA										
TOTAL	2248	239	277	1052	331	1899	73	139	59	271
1	613	40	74	292	101	507	17	47	19	83
2	389	28	34	195	72	329	18	26	7	51
3	1173	164	158	542	141	1005	35	61	28	124
4	27	3	7	6	9	25	-	1	-	1

RS VIRUS	2850	930	523	919	202	2574	34	90	94	218
RHINOVIRUS	513	65	40	141	84	330	58	86	15	159
COXSACKIE A										
TOTAL	741	44	57	379	129	609	29	71	2	102
1	26	-	4	16	1	21	-	-	-	-
2	39	2	2	28	6	38	-	1	-	1
3	3	-	-	2	1	3	-	-	-	-
4	81	2	12	55	7	76	-	3	-	3
5	48	4	3	31	5	43	2	2	-	4
6	34	-	4	22	6	32	-	-	-	-
7	16	2	-	5	4	11	-	3	-	3
8	20	2	7	9	-	18	-	-	-	-
9	245	19	13	92	59	183	18	35	1	54
10	43	2	7	24	6	39	-	4	-	4
11	5	-	1	4	-	5	-	-	-	-
13	7	-	1	4	-	5	-	1	-	1
15	1	-	-	-	-	-	-	-	-	-
16	129	3	2	71	28	104	6	16	-	22
17	3	1	-	2	-	3	-	-	-	-
19	3	2	-	1	-	3	-	-	-	-
20	2	-	-	2	-	2	-	-	-	-
21	7	-	-	2	-	2	3	1	1	5
22	1	-	-	1	-	1	-	-	-	-
24	4	-	-	2	-	2	-	2	-	2
COXSACKIE B										
TOTAL	2422	309	160	868	496	1833	142	314	17	473
1	82	6	6	34	12	58	2	13	-	15
2	562	68	39	212	114	433	24	83	1	108
3	640	101	45	207	148	501	35	61	5	101
4	297	34	22	132	53	241	17	30	1	48
5	756	97	45	264	154	560	52	102	10	164
6	27	2	1	12	3	18	2	1	-	3

TABLE IV
Virus Infections of the Central Nervous System by Age (All Year 1974) (Facsimile)

	TOTAL	0-5 MONTHS	6-11 MONTHS	1-4 YEARS	5-14 YEARS	CHILD. 0 - 14	15-24 YEARS	25-59 YEARS	60+ YEARS	ADULTS 15 & +
ADENOVIRUS										
TOTAL	363	24	23	123	127	297	25	23	3	51
1	40	6	3	13	13	35	1	1	-	2
2	56	5	5	23	14	47	2	2	1	5
3	14	1	1	9	3	14	-	-	-	-
4	2	1	-	-	1	2	-	-	-	-
5	24	2	2	10	5	19	1	2	-	3
6	6	3	-	1	2	6	-	-	-	-
7	74	2	6	20	36	64	4	4	-	8
8	2	-	-	-	1	1	-	1	-	1
10	3	-	-	-	2	2	1	-	-	1
11	1	-	-	1	-	1	-	-	-	-
13	1	-	-	-	-	-	-	-	-	-
31	1	-	-	1	-	1	-	-	-	-
INFLUENZA VIRUS										
TYPE A	93	-	5	36	23	64	8	15	2	25
TYPE B	70	-	-	11	34	45	9	12	2	23
TYPE C	3	-	-	1	-	1	1	-	1	2
PARAINFLUENZA										
TOTAL	104	4	9	53	27	93	2	6	-	8
1	28	-	2	11	13	26	-	2	-	2
2	14	-	1	8	3	12	-	2	-	2
3	59	4	6	32	11	53	2	1	-	3
RS VIRUS	47	5	7	21	8	41	2	2	1	5
RHINOVIRUS	7	1	1	4	-	6	-	1	-	1

COXSACKIE A										
TOTAL	219	12	11	82	57	162	13	26	1	40
1	18	-	3	10	1	14	-	-	-	-
2	10	1	-	4	5	10	-	-	-	-
4	15	-	1	10	3	14	-	-	-	-
5	17	2	1	9	3	15	1	-	-	1
6	9	-	-	6	1	7	-	-	-	-
7	1	-	-	1	-	1	-	-	-	-
8	2	-	-	-	-	-	-	-	-	-
9	110	4	3	26	39	72	12	22	-	34
10	5	-	1	2	1	4	-	1	-	1
11	4	-	1	3	-	4	-	-	-	-
13	5	-	1	2	-	3	-	1	-	1
15	1	-	-	-	-	-	-	-	-	-
16	6	1	-	2	2	5	-	1	-	1
17	1	-	-	1	-	1	-	-	-	-
19	2	2	-	-	-	2	-	-	-	-
20	1	-	-	1	-	1	-	-	-	-
21	1	-	-	-	-	-	-	-	1	1
22	1	-	-	1	-	1	-	-	-	-
24	1	-	-	1	-	1	-	-	-	-
COXSACKIE B										
TOTAL	761	121	25	161	218	525	67	130	9	206
1	17	1	-	7	5	13	-	3	-	3
2	187	27	5	43	55	130	12	39	1	52
3	203	44	7	46	56	153	15	23	2	40
4	68	11	1	17	16	45	9	11	-	20
5	259	38	11	46	79	174	24	45	6	75
6	3	-	-	-	1	1	1	-	-	1

TABLE V
Virus Infections of the Respiratory Tract by Age (All Year 1974) (Facsimile)

	TOTAL	0-5 MONTHS	6-11 MONTHS	1-4 YEARS	5-14 YEARS	CHILD. 0 - 14	15-24 YEARS	25-59 YEARS	60+ YEARS	ADULTS 15 & +
ADENOVIRUS										
TOTAL	2545	204	346	1067	465	2082	192	166	30	388
1	291	42	67	144	23	276	-	4	1	5
2	426	63	90	214	26	393	7	7	4	18
3	158	8	21	60	60	149	4	4	1	9
4	34	4	2	11	6	23	5	3	-	8
5	138	20	32	71	10	133	2	2	-	4
6	30	2	11	14	2	29	-	1	-	1
7	508	33	44	214	145	436	40	18	2	60
8	2	-	-	-	1	1	-	1	-	1
9	2	-	-	-	-	-	-	1	-	1
10	1	1	-	-	-	1	-	-	-	-
12	1	-	-	1	-	1	-	-	-	-
13	13	2	3	5	1	11	-	-	-	-
14	17	-	-	5	7	12	1	2	-	3
15	1	-	-	1	-	1	-	-	-	-
18	2	1	-	-	-	1	-	-	-	-
19	4	2	-	1	-	3	1	-	-	1
23	1	-	1	-	-	1	-	-	-	-
INFLUENZA VIRUS										
TYPE A	2260	57	80	357	277	771	282	593	480	1355
TYPE B	1620	28	31	165	428	652	380	336	177	893
TYPE C	33	-	2	8	7	17	2	6	5	13
PARAINFLUENZA										
TOTAL	1677	193	223	829	217	1462	41	86	41	168
1	452	33	60	234	62	389	8	28	13	49
2	311	21	28	168	55	272	12	15	5	32
3	854	133	124	409	85	751	20	39	19	78
4	23	2	7	5	7	21	-	1	-	1
RS VIRUS	2540	896	486	800	155	2337	25	60	79	164
RHINOVIRUS	422	56	34	112	74	276	54	69	9	132

COXSACKIE A										
TOTAL	86	4	5	51	12	72	2	11	-	13
2	4	-	1	1	1	3	-	1	-	1
4	6	-	-	5	-	5	-	1	-	1
5	12	-	-	11	1	12	-	-	-	-
6	4	-	-	3	1	4	-	-	-	-
7	2	-	-	-	1	1	-	1	-	1
8	5	-	2	3	-	5	-	-	-	-
9	31	3	1	13	6	23	2	5	-	7
10	1	-	-	1	-	1	-	-	-	-
13	1	-	-	1	-	1	-	-	-	-
16	14	-	-	11	1	12	-	2	-	2
21	2	-	-	1	-	1	-	1	-	1
24	1	-	-	1	-	1	-	-	-	-
COXSACKIE B										
TOTAL	529	53	52	222	117	444	21	42	2	65
1	15	-	2	7	3	12	-	2	-	2
2	143	11	10	74	32	127	4	9	-	13
3	134	14	17	50	34	115	7	4	-	11
4	59	7	8	28	8	51	1	5	-	6
5	161	21	15	58	35	129	9	16	2	27
6	7	-	-	5	1	6	-	1	-	1

TABLE VI
Virus Infections of the Central Nervous System by Region[a] (All Year 1974) (Facsimile)

	TOTAL	I	II	III	IV	V	VI
ADENOVIRUS							
TOTAL	363	1	-	-	-	319	43
1	40	-	-	-	-	33	7
2	56	1	-	-	-	47	8
3	14	-	-	-	-	8	6
4	2	-	-	-	-	2	-
5	24	-	-	-	-	21	3
6	6	-	-	-	-	4	2
7	74	-	-	-	-	67	7
8	2	-	-	-	-	2	-
10	3	-	-	-	-	2	1
11	1	-	-	-	-	1	-
13	1	-	-	-	-	1	-
31	1	-	-	-	-	-	1
INFLUENZA VIRUS							
TYPE A	93	-	1	2	-	75	15
TYPE B	70	-	-	-	-	50	20
TYPE C	3	-	-	-	-	3	-
PARAINFLUENZA							
TOTAL	104	-	-	2	-	73	29
1	28	-	-	2	-	22	4
2	14	-	-	-	-	6	8
3	59	-	-	-	-	43	16
RS VIRUS	47	-	-	-	-	36	11
RHINOVIRUS	7	-	-	-	-	4	3
COXSACKIE A							
TOTAL	219	57	-	1	-	110	51
1	18	18	-	-	-	-	-
2	10	3	-	-	-	7	-
4	15	6	-	-	-	8	1
5	17	5	-	-	-	7	5
6	9	8	-	-	-	1	-
7	1	1	-	-	-	-	-
8	2	2	-	-	-	-	-
9	110	3	-	1	-	63	43
10	5	1	-	-	-	3	1
11	4	4	-	-	-	-	-
13	5	3	-	-	-	2	-
15	1	-	-	-	-	1	-
16	6	-	-	-	-	5	1
17	1	1	-	-	-	-	-
19	2	-	-	-	-	2	-
20	1	1	-	-	-	-	-
21	1	-	-	-	-	1	-
22	1	1	-	-	-	-	-
24	1	-	-	-	-	1	-
COXSACKIE B							
TOTAL	761	27	6	2	-	591	135
1	17	1	-	-	-	15	1
2	187	2	-	-	-	124	61
3	203	14	-	-	-	136	53
4	68	7	-	2	-	51	8
5	259	2	6	-	-	239	12
6	3	-	-	-	-	3	-

[a]REGION I AFRICA excluding NORTH AFRICA
REGION II THE AMERICAS excluding CANADA and the USA
REGION III ASIA excluding JAPAN
REGION IV NORTH AFRICA and the EASTERN MEDITERRANEAN including TURKEY and excluding ISRAEL
REGION V EUROPE plus ISRAEL
REGION VI AUSTRALIA, CANADA, NEW ZEALAND, JAPAN, the USA

TABLE VII
Virus Infections of the Respiratory Tract by Region[a]
(All Year 1974) (Facsimile)

	TOTAL	I	II	III	IV	V	VI
ADENOVIRUS							
TOTAL	2545	15	9	5	1	2124	391
1	291	5	3	-	-	230	53
2	426	2	4	-	-	316	104
3	158	-	-	3	-	93	62
4	34	-	-	-	-	28	6
5	138	1	-	-	-	101	36
6	30	-	-	-	-	21	9
7	508	4	1	2	-	465	36
8	2	-	-	-	-	2	-
9	2	-	-	-	-	2	-
10	1	-	-	-	-	1	-
12	1	-	1	-	-	-	-
13	13	-	-	-	-	13	-
14	17	3	-	-	-	13	1
15	1	-	-	-	-	-	1
18	2	-	-	-	-	2	-
19	4	-	-	-	-	2	2
23	1	-	-	-	-	1	-
INFLUENZA VIRUS							
TYPE A	2260	46	14	42	7	1689	462
TYPE B	1620	13	12	-	5	1324	266
TYPE C	33	5	-	-	-	28	-
PARAINFLUENZA							
TOTAL	1677	15	3	32	-	1281	346
1	452	5	-	8	-	381	58
2	311	3	1	1	-	227	79
3	854	7	2	23	-	622	200
4	23	-	-	-	-	15	8
RS VIRUS	2540	12	-	-	-	2248	280
RHINOVIRUS	422	-	-	2	-	294	126
COXSACKIE A							
TOTAL	86	1	-	-	-	48	37
2	4	-	-	-	-	4	-
4	6	-	-	-	-	3	3
5	12	-	-	-	-	1	11
6	4	1	-	-	-	-	3
7	2	-	-	-	-	2	-
8	5	-	-	-	-	3	2
9	31	-	-	-	-	23	8
10	1	-	-	-	-	-	1
13	1	-	-	-	-	1	-
16	14	-	-	-	-	9	5
21	2	-	-	-	-	2	-
24	1	-	-	-	-	-	1
COXSACKIE B							
TOTAL	529	7	9	3	-	373	137
1	15	-	-	-	-	15	-
2	143	1	-	2	-	108	32
3	134	1	-	-	-	67	66
4	59	-	8	1	-	46	4
5	161	5	-	-	-	125	31
6	7	-	1	-	-	4	2

[a]REGION I AFRICA excluding NORTH AFRICA
REGION II THE AMERICAS excluding CANADA and the USA
REGION III ASIA excluding JAPAN
REGION IV NORTH AFRICA and the EASTERN MEDITERRANEAN including TURKEY and excluding ISRAEL
REGION V EUROPE plus ISRAEL
REGION VI AUSTRALIA, CANADA, NEW ZEALAND, JAPAN, the USA

public health authorities on the importance of certain virus diseases in comparison to others. Despite these limitations, comparison of data from different laboratories can be useful, and the results may give a valid indication of world trends in viral infections, although a complete picture may not emerge.

1. Enteroviruses

A "four-year study of WHO virus reports on enteroviruses other than poliovirus" (Assaad and Cockburn, 1972) covering the period 1967–1970 has shown that among the coxsackieviruses A type 9 was reported most frequently. The most frequently reported coxsackievirus B was type 3. Among the echoviruses, types 9, 6, and 30 were more common than other types. The highest incidence for each of the three groups of viruses, coxsackie A, B, and echoviruses, was June–October in the Northern Hemisphere and November–February in the Southern Hemisphere. Most of the infections were in children and the clinical manifestations usually included aseptic meningitis, respiratory disease, skin eruptions, undifferentiated febrile illnesses, and gastroenteritis. The relative frequency of an association of a virus with a classic syndrome differed not only between the three groups of viruses but in a number of instances between the types within a group. As is well known, there were a number of instances in which a specific clinical syndrome was linked to certain specific viruses, e.g., hand, foot, and mouth disease to certain types of coxsackievirus A, and myalgia (Bornholm disease) and cardiac conditions to coxsackievirus B. There was also an apparent relationship between age and symptoms, e.g., those due to coxsackievirus B associated with Bornholm disease in persons over 15 years of age.

The 1974 review clearly revealed annual variations in the incidence of enteroviruses in different countries. Irrespective of an increasing number of reporting laboratories, the total number of coxsackievirus A reported was the smallest since the initiation of the study in 1967 and type A9 was still the dominant type. Coxsackievirus B showed the same annual pattern with minor variations; the types most frequently reported were B3, B5, and B2, respectively. In contrast to the coxsackieviruses, the number of reports in 1974 on echoviruses was the highest since 1967. Echovirus type 30 was the most frequently reported—about 400 reports were received from the Federal Republic of Germany. In the United Kingdom echovirus type 19 caused a number of outbreaks—550 reports were received by WHO. Other echoviruses were reported by a number of countries, but no particular virus showed any particular predominance in any one country.

2. *Respiratory Viruses*

In 1974 a review was also made of the respiratory viruses (Assaad and Cockburn, 1974). The study covered the period from 1967 to 1973. In the Northern Hemisphere, from which over 95% of the reports were received, a clear pattern of seasonal incidence of different respiratory tract infections emerged from the study. Over 70% of the total number of reported adenovirus infections, over 80% of the parainfluenza virus infections, and over 90% of the respiratory syncytial virus infections occured in children. Nonviral infections by *M. pneumoniae* were most frequently reported in adults. Influenza A virus infections were predominant in the adult population, with a high proportion in those aged 60 years and over. Influenza B infections were reported both in adults and in children, but over one-third were in children of school age. The proportion of lower respiratory tract infections to total respiratory tract infections varied from one virus to another, and ranged from less than one-half for adenovirus infections to over four-fifths for mycoplasma infections. Nonlocalizing fever was usually the second principal clinical condition reported in association with respiratory viruses.

3. *Viruses of the Central Nervous System*

Annual reviews may indicate areas for further investigation rather than provide answers to certain questions. Virus reports on diseases of the central nervous system over the period 1967–1971 were analyzed. An unexpected finding of this analysis was the implication of myxoviruses (other than mumps virus) in a relatively large number of cases of diseases of the central nervous system, including paralysis. A detailed study of the clinical and laboratory aspects could not be performed because of incomplete information routinely obtained from the laboratories participating in the WHO Virus Reporting System. A more detailed inquiry involving a small number of laboratories, eight in number, has therefore been organized by WHO.

C. Studies of Specific Subjects

Information on poliovirus isolation in the countries participating in the WHO Virus Reporting System has been used by Cockburn and Drozdov (1970) for an international study of poliomyelitis. It was recognized that there were many more laboratories in North America and Europe than in other parts of the world and that there were definite limitations to the conclusions which could be drawn from the incomplete data available. The most interesting finding was that in areas without adequate vaccination

programs, type 1 poliovirus was responsible for much of the disease of the central nervous system and spinal paralysis. On the other hand, in areas with good vaccination programs, approximately equal numbers of the three types were isolated from patients with disease of the central nervous system and from patients with other diseases or no illness.

The outcome of the viral infection is often not reported. Nonetheless, over the period 1967–1974, 2035 deaths associated with viral infections were reported to WHO. A study of these deaths was conducted and the findings were interesting. Over one-third of the cases were associated with influenza virus A infections and about one-sixth with herpesvirus. However, this pattern reflects the picture in the industrially developed world (which provides the majority of reports on viral infections). In the developing countries, enteroviruses, in particular, polioviruses, are high on the list.

Marked variations were noted in the percentage of fatal cases associated with influenza virus A infections from one year to another and the lowest percentage was seen in 1974 (approximately 14%). The highest percentage of reports on fatal influenza virus B infections were received in 1974 (over 8%), an observation which agrees well with the epidemiological pattern of influenza B in the Northern Hemisphere that year. Herpesvirus infections showed much less fluctuation from one year to the other. In the Northern Hemisphere, deaths associated with influenza A infections were reported most frequently during December, January, and February; those associated with influenza B infections occurred during February and March. Fatal herpes viral infections were reported throughout the year.

As expected, the highest proportion of influenza virus infections (over 80% of influenza A and over 65% of influenza B) were associated with diseases of the respiratory system. However, almost one-fifth of influenza B fatal infections were associated with a disease of the nervous system. Sixty-five percent of herpesvirus infections involved the central nervous system.

Over 85% of fatal influenza virus A infections occurred in adults while fata influenza virus B infections occurred both in children and adults. Forty percent of fatal herpesvirus infections were reported in children and 60% in adults. In the developing world almost 90% of deaths from enteroviruses, including polioviruses, occur in the young.

D. *Ad Hoc* Retrieval of Information

Since 1970, WHO has provided each laboratory with tabulations of the information which it had reported during the preceding year, thus facili-

tating the preparation of the laboratory's annual review. The individual annual tabulation provided by WHO has been very well received by many laboratories.

Ad hoc retrieval of information may be requested by any participating laboratory. It may be restricted to information provided by the laboratory or may extend to the entire information available in the system. An example of the former was that of one laboratory requesting detailed analysis of enteroviral infections by age. The analysis requested answers to a specific question—whether it was worth sampling the adult population for the various types of enteroviruses. In that particular case, the yield of positive results decreased with increasing age and it was decided that specimens would no longer be taken from those of middle age or older. Another example is that of one laboratory asking for the list of laboratories which undertook typing of herpes simplex virus at a time when herpes was not frequently reported by type. The purpose of the inquiry was to contact other laboratories with a similar interest to exchange detailed information.

VIII. LIMITATIONS OF THE OUTPUT

In spite of the simplifications that have been introduced in the input there are still a number of shortcomings in the output. Positive findings concerning the identification of a viral agent are reported by laboratories irrespective of either their significance as pathogens to humans or their frequency. Although some agents may be of interest to a few workers engaged primarily in research, they may be of little significance to laboratories working primarily in routine diagnostic work, to whom the reporting system is primarily aimed, or they may simply be rarely encountered.

Viruses which have hardly ever or never been reported by laboratories and, therefore, have not been included in the input, may suddenly become of worldwide interest. The following are two pertinent examples. Hepatitis antigen was first reported to WHO by four reports from one laboratory during the fourth quarter of 1969. During the first quarter of 1970 there was only one report. The number of reports increased and in the fourth quarter of the same year was up to 51; these are progressively increasing. Furthermore, the agent was reported under a number of different names. In 1972, HB Ag and HB Ab came to be generally used. In 1975, the new nomenclature HBsAg and HBcAg and the corresponding antibodies came into use.

Infantile gastroenteritis attracted the attention of laboratories in 1975.

Three hundred and seventy-seven reports were received in the first quarter of that year. Again, the causative virus was reported under several names. In the WHO reporting system the term "reovirus-like agent" was chosen. The stage at which these viral agents should be brought into the quarterly report depends partly on the number reported and partly on the anticipated interest of laboratories. For example, although there were only four reports of the "Australia antigen" in the fourth quarter of 1969 and only one in the first quarter of 1970, these were published in the reports.

The implication of a viral agent may be based on one or more laboratory tests. As mentioned above, except for the enteroviruses for which isolation is required, records of serology and/or direct examination (e.g., electron microscopy) without isolation are equally accepted for other viruses. During the period 1967–1973, laboratories were not asked to supply any information on the method used for isolation or the technique and titers for serological diagnosis. Since 1974, more detailed information has been requested on techniques used in laboratories. Not all of them provide the information, although an appreciable number do. Because of the lack of uniformity in reporting techniques, it was decided to use this information only for detailed studies rather than to include it in the routine quarterly and annual reports.

A number of reports mention untyped viruses. Experience has been the best guide on deciding which reports to include in the output and which to exclude. The majority of adenoviruses are reported as "untyped" and are shown as such in the quarterly tabulations. On the other hand, because of the different significance of influenza A, B, and C viruses, an untyped influenza virus is not accepted. The epidemiological and clinical differences among parainfluenza virus infections are far less marked than among the influenza viruses and untyped parainfluenza virus is accepted for tabulation. Rhinoviruses are an extreme case. There are a large number of types and only a few laboratories can do the typing. All the rhinoviruses reported to WHO are therefore tabulated under one entry without any attempt to indicate the various types. An untyped enterovirus is excluded from the input. Coxsackievirus A or B or an echovirus are included, even if not further typed. Recently, enteroviruses 68–71 have been included in the reporting system, but before 1974 when these enteroviruses were not recognized and designated and were reported as "untypable" or "untyped," they were excluded. Herpes simplex viruses were included in the report without further typing until 1972. However, as a distinction was made in additional reports between types 1 and 2, these two types were included in the tabulations. Since then, many more laboratories have been reporting herpes simplex virus by types.

One of the problems faced in designing the output tabulations was the so-called "multiple infections." Whereas for clinical decisions the basic unit is the patient, for epidemiological analysis of data in the present system the basic unit is the infection, i.e., the existence of a particular virus infection in a person. In other words, one person infected with two viruses constitutes two infections. Therefore, since 1973, clinical information has been given in the tabulations opposite each of the identified viruses, and no attempt has been made to decide which one is the more significant infection in relation to the clinical syndrome. The frequency of a particular association of two viruses with a given clinical feature could only be assessed in a separate study.

Clinical information is influenced by the particular terminology used in different countries. Frequently, information is limited to the clinical picture at admittance. Few laboratories have facilities to follow up the clinical condition of a patient and to obtain from the clinician the final diagnosis at discharge. The attempt to obtain uniform clinical information by requesting laboratories to indicate final diagnosis following the International Classification of Diseases has not yielded the desired response. Laboratories encounter difficulties in trying to obtain the final diagnosis. Clinical information frequently comprises more than one clinical syndrome or more than one clinical manifestation. Considering the length of tabulations it was decided to limit the clinical data to the most pertinent clinical condition. It was felt that a comparison of different clinical manifestations associated with a given viral infection could only be assessed in a separate study.

It is recognized that specimens can vary greatly according to the population from which they have been received. Nonetheless, an attempt was made to establish the origin of cases whether, epidemic, sporadic, special study, or clinical trial. The reliability of the information concerning the sporadic or epidemic nature of a case has been seriously questioned. The laboratory does not usually have adequate facilities or sufficient liaison with the public health service to determine whether sporadic cases would, by their number, have to be considered as an epidemic. Reports of cases forming part of surveys are excluded from special studies and from the annual reviews, but not from the quarterly tabulations (see below). The outcome is indicated only in a small number of cases. However, useful information can be obtained over a sufficiently long period of time and special study of fatal cases, over a period of 7 years, has yielded valuable data, as already mentioned. Ninety percent of the reports mention age. Tabulation by age, therefore, carries little bias. Comparison of data tabulated from the six different groups of countries would give more of an indication of the differences in laboratory facilities than in differences in the frequency

of the various viral infections. However, more and more laboratories in the developing world are joining the WHO scheme and, at the same time, examinations in these laboratories are becoming more elaborate and covering progressively a wider range of viral infections. Even with these severe limitations, pertinent observations can be made, e.g., the paramount role of polioviruses in diseases of the central nervous system in the developing world and the rarity of reports on these viruses in the developed world.

Information is given in the quarterly reports by date of reporting and by date of collection/receipt of specimen. The annual report summarizes the quarterly reports. In annual reviews and in special studies, use is made of the date of collection/receipt of specimen, which normally is closely related to the date of onset of the disease.

IX. NONCOMPUTERIZED INFORMATION

In addition to the information on individual cases of viral infection, laboratories are asked to supply notes on epidemiological or laboratory investigations which are of interest to other workers. Furthermore, extracts from unpublished reports outside the system and from published literature, especially that of restricted circulation, are also included in the quarterly and annual reports.

X. INFORMATION SYSTEM FOR INFLUENZA SURVEILLANCE

The information system for influenza surveillance was started in 1947 by Sir Christopher Andrewes and its further development was, in fact, one of the first tasks assigned to WHO. Since that time, the usefulness of the system has constantly been proved in enabling the rapid follow-up of frequent changes in the antigenic composition of influenza viruses A and B and the dissemination of information concerning influenza epidemics.

The system is based on a network of 97 National Influenza Centres in 69 countries. National Influenza Centres are officially designated by agreement between the relevant Government and WHO. According to the terms of agreement, the Centres can communicate directly with WHO. The laboratories which are designated are selected for their technical capabilities and also for their geographical location in order to ensure the

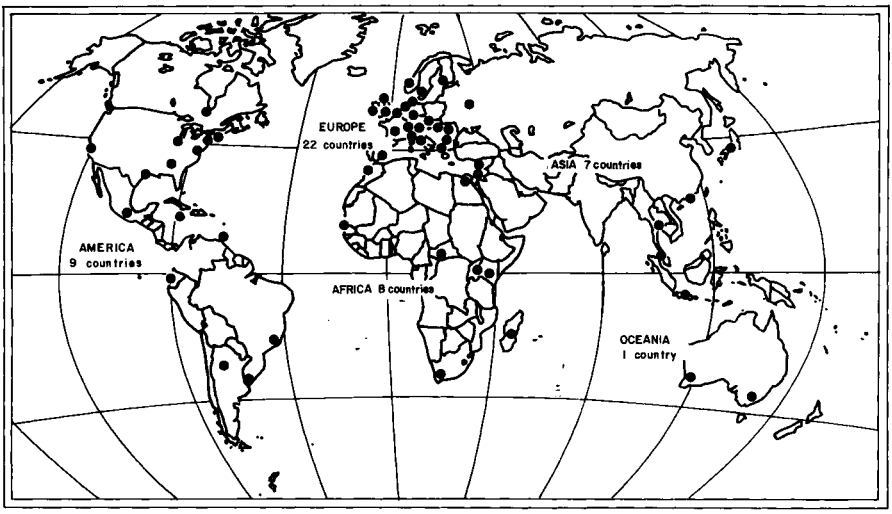


Fig. 4. WHO geographical distribution of Centres.

best possible epidemiological coverage all over the world. At present the geographical distribution of National Influenza Centres for each WHO Region is as follows (Fig. 4): Africa, 7; America, 18; Eastern Mediterranean, 4; Europe, 45; Southeast Asia, 6; Western Pacific, 15. As can be seen, the geographical coverage is again uneven, and the reasons for this are the same as those given previously for other viruses. Two WHO Collaborating Centres for Reference and Research on Influenza are the focal points of the system, one in London* (formerly the World Influenza Centre) and the other in Atlanta.†

The National Influenza Centres collect epidemiological information on influenza-like diseases and report direct to WHO on a weekly basis during the influenza season. They isolate current influenza strains and carry out preliminary identification with the aid of a kit of antigens and antisera provided annually by WHO. Information on outbreaks of influenza A and B and characteristics of isolates are analyzed in the Virus Diseases Unit of WHO in Geneva and published in the WHO Weekly Epidemiological Record. Whenever necessary, use is made of press releases, cables to governments, or the WHO automatic telex reply service. When a new variant is found by a National Influenza Centre it is sent without delay to

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† Center for Disease Control, Atlanta, Georgia (United States).

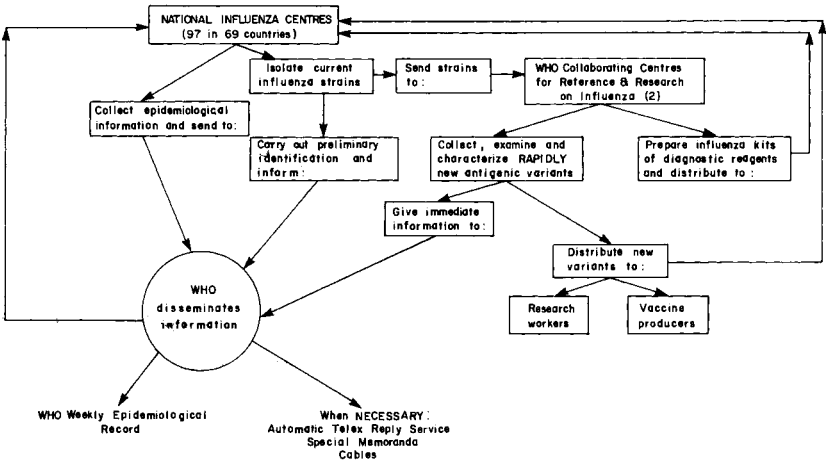

Fig. 5. WHO influenza program.

one of the two WHO Collaborating Centres for Reference and Research on Influenza.

The WHO Collaborating Centres for Reference and Research on Influenza in London and Atlanta carry out a detailed antigenic characterization of the new variants. Results of comparison of a new strain with the former prevalent variants are rapidly published in the WHO Weekly Epidemiological Record. If the strain is sufficiently different to be considered for introduction into the vaccine, a more detailed study is carried out. This includes its hemagglutinin and neuraminidase characteristics, its spread in different countries, the prevalence of natural antibodies against it in the population and, when feasible, the level of antibodies obtained in volunteers administered the new vaccine. A recommendation is then published in the Weekly Epidemiological Record in time for manufacturers to prepare stocks of vaccines for the next influenza season, i.e., early in the year for the Northern Hemisphere. The new strain is made available to research laboratories and vaccine producers as well as a high yielding recombinant with the new surface antigens. An endeavor is made to follow up as quickly as possible changes in the antigenic pattern of current strains. However, when an important change occurs, there is inevitably a delay of a minimum of 15 weeks between its detection and the availability of the vaccine. The influenza surveillance scheme is the best example of a system where the exchange of information and reagents between laboratories is carried out very rapidly. Figure 5 shows how this scheme is organized.

XI. USE OF EXCESS MORTALITY FROM RESPIRATORY DISEASES IN THE STUDY OF INFLUENZA

In contrast to the precise information about viruses obtained for the reporting system, the quantity and quality of the epidemiological information on influenza vary. Influenza is not a notifiable disease in most countries, and even in highly developed countries morbidity returns from general practitioners and hospitals are not usually published except during epidemics. Data on industrial and school absenteeism provide useful information during epidemic periods, but these data are not available in many countries.

In the United States and a few other countries with temperate climates, the "excess mortality" from all acute respiratory diseases (i.e., the number of deaths actually recorded in excess of the number expected on the basis of past seasonal experience) has proved to be a useful method for assessing the severity of influenza epidemics (Langmuir and Housworth, 1969). These mortality statistics are readily available in many countries during and between epidemics.

Since 1970, WHO has been conducting a collaborative study on the use of "excess mortality" from respiratory diseases in order to assess in thirteen different countries the severity of influenza epidemics (Assaad *et al.*, 1973). The use of computer-produced seasonal, expected and actual curves permits quick visual assessment of influenza activity in any one country, as well as comparisons between different countries (Fig. 6). So far, no definite pattern of occurrence of epidemics of influenza has emerged. The study will be countinued for a number of years and, apart from its value to individual countries, should provide useful comparisons between countries with different climates.

XII. ARBOVIRUSES, RICKETTSIAE, AND CHLAMYDIAE

These represent quite a number of agents of endemic or epidemic diseases. However, the Scientific Groups advising WHO considered that the conditions were not, at present, suitable to allow these viruses to be recorded in a system comparable to that for enteric and respiratory groups of viruses already described. The reason for this is that these agents are identified by specialized laboratories, the distribution of which is still more uneven than those dealing with other viruses. Any data on the geographical distribution of these agents would in fact represent the radius of activity of specialized laboratories. Arboviruses, first discovered in

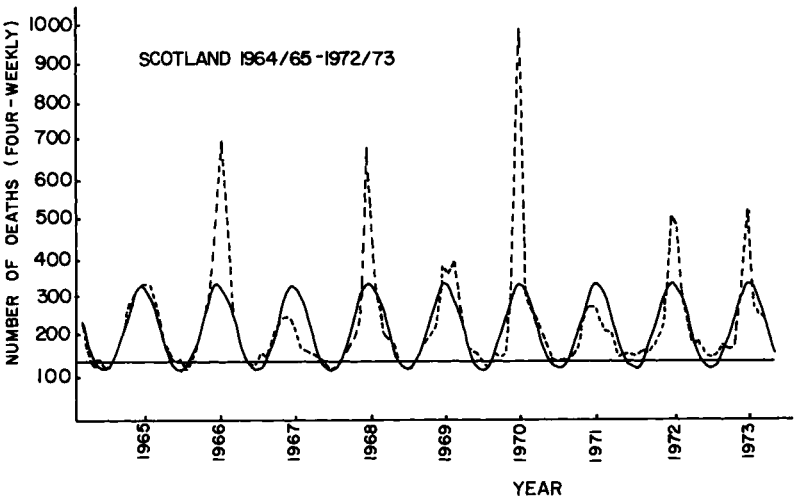

Fig. 6. Use of excess mortality from respiratory diseases in the study of influenza. Scotland 1964/1965–1972/1973.

Africa, may in the future be isolated on another continent. A catalog of arboviruses exists, published by the American Committee on Arthropod Borne Viruses, as well as an Arthropod Borne Virus Information Exchange Newsletter, to which the WHO Collaborating Centres for Arbovirus Reference and Research contribute. For nonviral agents, such as, rickettsiae and chlamydiae the situation is still less satisfactory because of the scarcity of laboratories presently actively engaged in this field. However, the WHO Collaborating Centres for Rickettsial Reference and Research* and the WHO Collaborating Centes for Reference and Research on trachoma and other chlamydial Infections† maintain close links with national laboratories working in this field.

XIII. FUTURE OF THE WHO VIRUS REPORTING SYSTEM

Over the past years, WHO has been able to develop a data collection and processing system that satisfies the needs of a very wide and diverse

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clientele. The WHO Reporting System has been developed over the past 13 years from a small network of virus laboratories providing data, which were manually processed in WHO, to an extensive network of laboratories. These laboratories differ in their technical capacity and areas of interest and provide data of varying quality. Thanks to computer facilities in WHO these can be processed to feed back information to the participating laboratories. Except for two countries which already provide their data on tapes, reports from other countries still have to be manually coded. With more laboratories joining the scheme and many of these extending their sphere of action, the number of reports that need coding has risen to over 50,000 per year, a load which can hardly be coped with.

WHO will make its experience on the processing of information available to participating countries, and it is expected that countries with a central point for collection of information will undertake to process their own information. Processed data will then be sent on tape to WHO for storage and retrieval. In this way WHO will still be able to manage a very important function of maintaining a record of virus infections on a global basis and still be able to act as a retrieval point for all participating laboratories.

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REFERENCES

Assaad, F., and Cockburn, W. C. (1972). *Bull. W. H. O.* **46,** 329–336.

Assaad, F., and Cockburn, W. C. (1974). *Bull. W. H. O.* **51,** 437–445.

Assaad, F., Cockburn, W. C., and Sundaresan, T. K. (1973). *Bull. W. H. O.* **49,** 219–233.

Bradstreet, C. M. P., Pereira, M. S., and Andrews, B. E. (1964). *Prog. Med. Virol.* **6,** 149–174.

Bradstreet, C. M. P., Pereira, M. S., and Pollock, T. M. (1973). *Prog. Med. Virol.* **16,** 241–268.

Brès, P., and Assaad, F. (1975). *International Virology 3, Abstracts 3rd International Congress for Virology, 1975* page 68.

Cockburn, W. C., and Drozdov, S. G. (1970). *Bull. W. H. O.* **42,** 405–417.

Grist, N. R., and Miller, D. L. (1973). WHO unpublished offset document VIR/RC/73.2.

Langmuir, A. D., and Housworth, J. (1969). *Bull. W. H. O.* **41,** 393–398.

Chapter 8

Viral Diagnostic Reagents

JOHN R. POLLEY

I. INTRODUCTION

Over the last 10–15 years there has been a major increase in the requests and identified needs for the laboratory diagnosis of viral infections in humans. In some cases the laboratory service is required to aid in a differential diagnosis and in other cases to identify a virus as the possible causative agent of an infection not readily diagnosed on clinical grounds alone. In many instances, the virus laboratory has expanded in a few years from a one-man operation to a separate department. Physicians have been relying increasingly on laboratory services to assist in reaching a differential diagnosis. Technology has advanced greatly and has become more readily available for the average laboratory, passing from the once exotic to the now routine. Techniques once considered to be specialized curiosities are becoming increasingly commonplace. There has also been an increasing desire for speed of diagnosis, leading, in turn, to increased needs for readily available and satisfactory diagnostic reagents. How did all this happen and so relatively quickly—and why not 30 years ago? The answer actually is relatively simple. A time was reached when the various demands upon, and the resulting needs of, the virus laboratory could be met, in part at least, by newly available technology, laboratory equipment, and expertise, often drawn from other fields. Such technology and expertise were combined to make possible, and increasingly feasible, direct methods for diagnosis, such as electron microscopy, microscopic examinations of specifically stained cell cultures, and immunofluorescence techniques. Likewise, serodiagnostic procedures once frequently cumbersome and expensive of reagents have become increasingly routine

with the development of micro techniques. Serological methods, once plagued by nonspecific reactions and unknown optimal reaction conditions, have become more precise and diagnostically significant with more exactly defined methods and improved quality of diagnostic reagents.

In view of the fact that specific treatment and chemotherapeutic agents are not, in general, available at present for viral diseases, the urgency and even the need for a virus diagnostic service could be questioned. If, for the specific treatment of a patient, it was important to distinguish between influenza A and influenza B, the need for a rapid, accurate laboratory diagnosis would be apparent. Is the patient or physician really concerned about an influenza A infection being caused by last year's strain, or this year's current strain, or for that matter, some new strain? For the hospitalized patient, is there an urgency or a need to determine whether the causative agent of infection is an echovirus, a coxsackievirus, or an arbovirus? Is it important to determine whether an ill child is suffering from an infection caused by an adenovirus or respiratory syncytial virus or cytomegalovirus or some other atypical viral infection? From the point of view of direct value to the patient, the answer to most of these questions is probably no. Why then are virological studies being conducted on an increasing scale? Are such diagnostic procedures merely academic exercises to accumulate data or to further research projects?

These and other similar questions are probably valid and deserve considered answers. Broadly speaking, to even determine that an infection is caused by a viral rather than a bacterial agent may be valuable for the treatment of the patient. To differentiate between a viral or bacterial agent as the possible or probable cause of a clinical condition involving, for example, meningitis, has direct implications for antibiotic treatment. Similarly, early identification of a viral or bacterial cause in the still acute phase of an infection will lead to appropriate specific therapy, where available. It is true that in many such cases of separating viral from bacterial causes, the initial implication of a bacterial agent will probably be made first. However, once it is apparent that a virus is the causative agent, the question remains of the value to be gained by a more precise identification.

If the results of diagnostic procedures conducted in the virus laboratory can seldom be translated directly into treatment of the patient, what service can that laboratory supply? For one thing, it can alert the physician to the presence and potential danger of highly infectious and hazardous diseases. For example, there is the obvious benefit to be gained by differentiating between a case of varicella or herpetic lesions and one of smallpox with all its implications for attending staff, contacts, and the community. Since it is recognized that clinical differentiation between

diseases caused by the poxvirus group is frequently difficult or impossible, laboratory diagnosis fills a vital need. When special isolation facilities are scarce, it is important that only those cases identified as requiring them are in fact confined there. Likewise, laborious and expensive searches for patient contacts will be forestalled, if unnecessary, by rapid specific diagnostic procedures. At one time, a physician could expect that the infectious diseases which he encountered would be those which he had been trained to recognize and with which he had become familiar. Today, however, all that has changed. Large numbers of the population are on the move from country to country. With all this international travel and increased personal contact between people of different areas, diseases once thought to be exotic and remote now have the distinct possibility of occurring and being identified anywhere. Tourists, immigrants, and native citizens returning from overseas service may import such diseases into the country while still in the apparently healthy state. Only later does the illness develop and with it the possible urgency for a rapid and specific diagnosis. Cases of Lassa fever, once considered an exotic disease restricted to West Africa, have been identified in London and Washington. Virus diagnostic procedures and reagents are an increasing requirement.

On a still more specific basis, there is the valuable contribution which the virus laboratory can make in the diagnosis of certain diseases in children and infants. For example, respiratory syncytial virus is a major cause of respiratory difficulty in infants, frequently being associated with bronchiolitis or pneumonia and not clinically distinguishable from bacterial infection. Of great concern, is the identification of cytomegalovirus as the cause of an illness in infants, not only because it is also frequently associated with bronchopneumonia, but even more so because it is now known that this infection may cause damage in the central nervous system, appearing later as a speech impairment or mental retardation. Probably one of the most widespread uses of the virus diagnostic laboratory to assess the possible present or future impact of an infection occurs with rubella. While usually of relatively little significance, this infection suddenly creates critical situations when it occurs in the first trimester of pregnancy. The occurrence of congenital lesions in children born following such an infection has thrown great responsibility on the virus laboratory for rapid and accurate answers. Rubella antibodies occur in the IgM immunoglobulin fraction mainly as a result of vaccination or primary infection but infrequently in cases of reinfection. Hence, by precise assessment and differentiation of HI antibodies in IgG and IgM fractions, the diagnostic laboratory can contribute to identifying a primary infection.

Another important function of the virus diagnostic laboratory is partici-

pation in serological surveys. It is the usual purpose of such surveys to assess the so-called immune status or at least the possible resistance to infection in a community or in a country. These data can be applied to identify needs for vaccination programs. Examples are surveys conducted in association with rubella, polio, and influenza vaccine programs. A particularly specialized application of this approach is the assessment of the resistance to infection with rubella virus of women in the child-bearing range. Such surveys are useful for the determination of the numbers of the population presumably susceptible to infection and, therefore, at risk. Moreover, when used in conjunction with vaccine programs, they can provide a valuable indication of the response to the vaccine and the potential success of the total program. A further extension of this type of survey is serological surveillance in which random serum samples from the population at large are tested periodically for specific antibodies. Sudden increases in the number of individuals showing the presence of a new or unexpected antibody to a specific virus, especially when there have been a significant number of seroconversions, is a cause of concern, indicating the possible beginning of an epidemic.

The diagnostic tests performed by the virus laboratory are of two general types, the direct and indirect. Direct tests, where applicable, are usually the more rapid, being based primarily on visualization of the virus particle or cellular inclusions. While very valuable in numerous cases, as, for example, the rapid differentiation between varicalla and smallpox/vaccinia, these procedures do not provide the more precise characterization frequently required. The differentiation between smallpox and vaccinia, influenza type A or influenza type B, the typing of strains, etc., still requires the application of the indirect or serological procedures. The serological procedures are based on a common principle, visualization by means of a biological system of the fact that there has been a specific antigen–antibody combination. The three methods in general use for this purpose are (1) serum–virus neutralization in which the specific antibody blocks the characteristic effect of the virus on cells or animals, (2) complement fixation (CF) in which the antigen–antibody complex adsorbs complement and inhibits hemolysis of the erythrocyte cell suspension which otherwise occurs, and (3) hemagglutination inhibition (HI) in which specific antibody combines with the virus particle, preventing it from causing a visible agglutination of erythrocytes which otherwise occurs. Of course, depending upon the definition used, there are also other methods for demonstration of antigen–antibody reaction, for example, immunodiffusion and immunofluorescence.

The serological procedures in turn can be used for two purposes, by using a reference antiserum to detect a specific virus or antigen or by

using a prepared antigen to detect and assay specific antibodies. It is the purpose of this chapter to describe diagnostic reagents. Therefore, the emphasis will be on specially prepared and available materials. For the purpose of virus isolation and identification, the prepared laboratory reagent is the specifically prepared antiserum. For antibody determinations the reagents will be the prepared antigens, usually used in the complement fixation or hemagglutination inhibition tests. It is recognized that a live virus suspension can be prepared and readily available for use in the neutralization test, but in this chapter it is intended to concentrate primarily on reagents which have been prepared, and usually processed to some degree, for use in the HI and CF tests.

Antisera came into use in virology to identify some infectious agents long before the particular virus was identified as such. That is, when viruses were first detected as infectious agents which passed through regular bacterial filters, antisera prepared against them were used to identify them as the specific cause of disease. Later, when it was found that current influenza A antisera did not neutralize the virus isolated from apparent influenza cases, influenza type B was discovered. The isolation and identification of new viruses is accompanied by the preparation of the homologous antiserum for use in at least one of the serological procedures. Techniques for the preparation of improved antisera of greater activity and specificity have changed relatively little. Modifications which have been introduced involve mainly use of more purified virus inoculum and minor changes in schedules of inoculation. One reason, of course, is the fact that in most cases by the appropriate selection of these, it has been possible to produce antisera considered to be of satisfactory quality. Thus, it will be noted that the various procedures described for the preparation of homologous antiserum have much in common, not only with each other, but also with procedures of years ago. If desired, the immunoglobulin fraction containing the specific antibodies can be partially purified and concentrated using a chromatographic procedure (Levy and Sober, 1960) or the more commonly used precipitation with ammonium sulfate (Kendall, 1937).

A large measure of the success of the virus diagnostic laboratory depends on the reliable quality of its diagnostic reagents. Without these, results will be variable, frequently questionable, and the laboratory loses credibility. Although the preparation of reference antisera has changed relatively little over the years, the quality and the scope of viral antigens have been improving more rapidly. Prior to the tissue culture era, there were relatively few viral antigens which were prepared to be readily available. Today many more diagnostic antigens are prepared, many of these in a partially purified and concentrated form. There are, however, still

large areas of virology not adequately covered by such reagents. A prime requirement of the virus diagnostic laboratory is the ready availability of antigens for use in the HI and CF tests and covering as many viral diseases as possible. These antigens should have the following qualities: (1) adequate potency; (2) proved sensitivity; (3) specificity; and (4) noninfectivity.

Prior to the availability of much of the technical equipment in use today, diagnostic antigens frequently consisted of the crude virus suspensions, such as egg or tissue culture fluids. While in some cases these may be adequate, in many others the potency and specificity were of marginal levels. It is now frequently possible to prepare antigens in a more purified and concentrated form, the examples of which will be presented below. It is important to emphasize that potency and sensitivity are not necessarily synonomous. This is well illustrated in the study by Schmidt and Lennette (1971) in which it was shown that CF antigens of relatively low titer were frequently capable of giving relatively high antibody end points. Thus, in preparing diagnostic antigens to be readily available for routine laboratory use, it is not enough to aim for a high antigen titer alone. An integral part of the evaluation or standardization of the final antigen product should be a measure of its capacity to assay the antibody level of a known human reference serum. All too often such antigen potency levels are determined by titration against hyperimmune animal antisera. The quality of the antigen as measured by its capacity to detect specific antibody at low levels in human serum is often neglected. The specificity of the antigen is equally important. This fact seems obvious, but at times in the course of preparing satisfactory reagents it has been necessary to accept less than optimal specificity, awaiting the development of improved methods of preparation. At one time, cross-reactions between some viruses of a given group or type were considered to be a biological fact when actually improved technology soon led to the preparation of antigens showing clear specific reactions.

Finally, but not least, the preparation of noninfective viral antigens should be an objective for all preparations. Actually, this is important for more than one reason. The first, of course, is the obvious protection afforded to the personnel using such products. Today the total number and range of viral diagnostic antigens being used has greatly increased and includes many in the high hazard category. In most cases, it is possible to prepare these antigens not only in a noninfective form, but also lyophilized for stable storage and shipment. Another important advantage in using inactivated antigens is to minimize cross-contamination between live seed virus stocks in the laboratory, a constant danger when multiple infective products are being used. Furthermore, many virus laboratories

which perform serological diagnostic procedures also engage in virus isolation and identification studies. The introduction of a new antigen in an infected condition into the diagnostic laboratory can only tend to cast some doubt on the reliability of a subsequent first isolation of that virus by that laboratory.

II. HISTORICAL BACKGROUND

It has been said that no branch of science can progress more rapidly than the analytical procedures on which it depends. Certainly virology has followed this pattern, for the major advances in isolation and identification, as well as studies on morphology and biochemical properties, have paralleled advances in physical methods and equipment. With each major advance in physical technology there has been a new surge in virus research and development. Studies on particle size, density, purification, and identity have been dependent upon the availability of increasingly sophisticated centrifuges, filtration equipment, and chromatography systems. The discovery of new viruses, the study of their morphology, and subsequent classification awaited the development of the electron microscope for a significant advance.

It may be considered that virology began with studies based on filtration, using earthenware filters. Although those filters were crude and of unstandardized porosity by present-day standards, they were known to retain the microorganisms under study at that time. However, Iwanowski (1892) found that these filters would not retain an agent causing tobacco mosaic disease. Over the next several years, these results were confirmed by others and extended by Beijerinck (1898). Soon other viral agents, notably smallpox and vaccinia, were discovered using the filtration technique. Progress was slow, however, because these viruses could not be readily visualized by the current light microscopes or concentrated by the centrifuges available. By 1922, however, the centrifuge had been improved to the point that it was shown by MacCallum and Oppenheimer that it could be used to concentrate vaccinia virus. Many subsequent studies conducted at that time involved the vaccinia virus, which is not surprising, since its relatively large size made it amenable to the technology of the day. Unfortunately, because of its relatively large size, it was considered to be a living organism of a similar nature to bacteria, a concept which was not replaced easily.

In the period following the discovery of viruses in 1892 and 1930, viruses associated with a number of diseases of man and animals were discovered. These isolations also resulted largely from the use of the filtra-

tion technique. However, other than demonstrations that the disease could be transmitted by the specific agent isolated from infected tissue, little detailed information was gathered about these viruses, existing as they did beyond the technology of the day. The rate of progress accelerated greatly in the decade between 1930 and 1940 and the general basis for diagnostic virology began to appear.

A significant step was the work of Elford (1931) on the preparation of improved filters composed of collodion membranes of various porosities. These controlled filtration studies indicated that some viruses were of submicroscopic size, much smaller than the vaccinia virus. This caused some doubt about a virus being a complete living organism. This was borne out when it was found that concentrated suspensions of viruses failed to show the usual metabolic properties of bacteria.

Using vaccinia virus, Craigie (1932) showed that in crude extracts of infected tissue there were two antigenic components. One could be precipitated by centrifugation, while the other remained in the supernatant fluid and was not retained by a Seitz EK filter. In similar experiments, Parker and Rivers (1935) showed that in virus-free filtrates of vaccinia preparations there was a soluble agent which could still be precipitated by vaccinia antiserum. Parker and Smythe (1937) showed that in partially purified and concentrated vaccinia virus suspensions there was a relationship between virus activity and the number of virus particles. Hoyle and Fairbrother (1937) found that influenza-infected tissue extracts also contained two antigenic components; the elementary body or viral antigen was precipitated by centrifugation but another antigen remained in the supernatant fluid and was serologically active with complement. This became known as the soluble antigen. With these studies, based on the use of vaccinia and influenza viruses propagated in animal tissues, there was the constant problem of the large amount of foreign substances present per mass of virus.

The introduction of the embryonated egg greatly simplified such studies for there was less tissue protein associated with the virus suspension. Hirst (1941) showed that influenza viruses agglutinate fowl erythrocytes. This observation became the basis of an assay method for virus content, adding fowl erythrocytes to serial dilutions of the virus suspension. This hemagglutination titration technique was soon followed by its companion, the hemagglutination inhibition test, when Hirst, in 1942, showed that hemagglutination by a virus was inhibited by its specific antibody. This formed the basis for an assay procedure for serum influenza antibodies. These procedures were soon adapted for use with other viruses which produced erythrocyte agglutination. The fact that influenza viruses were adsorbed by erythrocytes and would subsequently elute on incubation

was used by Francis and Salk (1942) as a simple method for the concentration and purification of these viral suspensions or antigens.

The above historial account has attempted to describe briefly how virology developed up to the mid-1940's with the propagation of viruses in animal tissues and chick embryos, followed by the early development of virus and antibody assay methods. At this stage, diagnostic virology became a possibility and for the next 10–15 years it was associated with two commonly used terms, soluble antigens and viral antigens.

A. Soluble Antigens

The fact that more than one antigen could be associated with a particular virus was shown by Craigie in 1932. An antigenic component or soluble antigen was shown to exist in the supernatant fluid of vaccinia virus suspensions after the virus particles were removed by centrifugation or Seitz filtration. This vaccinia soluble antigen, in turn, was found to actually consist of two components, one of which was heat-stable and the other heat-labile. These antigens were recognized to be of a protein nature and capable of reacting in complement fixation tests with the homologous antibody, thus constituting one of the first diagnostic test systems. Smadel *et al.* (1942) demonstrated the presence of another antigen, a nucleoprotein, which could be extracted from the elementary body using dilute alkaline solution. Specific antibody can be produced by these antigens and by the hemagglutinating antigen but these antibodies are still different from the neutralizing antibody produced by the whole virus particle.

More progress was made in studies on soluble antigens when the emphasis turned to influenza virus propagated in the chick embryo. In this case, antigen of relatively higher concentration could be obtained, and with less foreign protein, from the infected tissues. When the infected chorioallantoic membranes were extracted with saline and the virus particles precipitated by centrifugation, a soluble antigen free of hemagglutinin remained in the supernatant fluid. As with the vaccinia soluble antigen, the influenza soluble antigen possessed complement-fixing capacity with the homologous antibody.

Wiener *et al.* (1946) using influenza-infected allantoic fluid showed that the virus particles were sedimented by centrifugation at 20,000 rpm for 20 minutes; the soluble antigen which remained in the supernatant fluid could then be sedimented by further centrifugation at 30,000 rpm for 1 hour. The larger particles had a sedimentation constant of about 600 S and possessed the properties associated with the intact virus, notably infectiv-

ity and hemagglutinating capacity. The smaller particles or soluble antigen had a sedimentation constant of about 30 S and lacked the general properties of the virus but it was serologically active in the complement fixation test.

When tested with human convalescent sera, there were serological differences in their reactions. For example, influenza antibodies in humans arising from natural infections reacted with the soluble antigen in the complement fixation test but this reaction was rare following inoculation with vaccine. There was a significant correlation between antibody titers obtained in the hemagglutination inhibition test and the complement fixation test using the viral antigen, providing that the sera did not contain antibody to the soluble antigen. These studies demonstrated that the influenza soluble antigen was useful for the serological diagnosis of influenza.

As with influenza, Henle *et al.* (1948) showed that two distinct complement-fixing antigens could be obtained from the tissues and fluids of chick embryos infected with mumps virus. One antigen was associated with the virus particle and was present primarily in the embryonic fluids; the other antigen could be extracted from the infected chorioallantoic membranes, remaining in the supernatant fluid after precipitation of virus by centrifugation. These two antigens could be clearly differentiated using specific antibody in human convalescent sera; the antibodies to the viral and soluble antigen were separated by cross-adsorption. Thus, the antibodies against the viral antigen were removed by adsorption with the viral antigen, followed by removal of the antigen–antibody complex by centrifugation. In this way a serum containing antibody relatively specific against the soluble antigen could be prepared.

It was found that the soluble antigen detected an antibody response to infection before the viral antigen and these anti-soluble antibodies reached significant levels, sometimes as early as 2 days after the onset of disease. Antibody against the viral antigen appeared a few days later and both antibodies reached significant levels in the convalescent period. The viral antigen, however, detected antibody for a much longer period of time, measured in years. Consequently, it is more useful for the detection of long-standing antibody levels or resistance to infection.

At this time there was some controversy about the origin or status of soluble antigen. Some considered that it arose as a result of the action of the virus on the cellular proteins; others considered that it was a building block in virus synthesis or a unit constituent of the virus particle. However, Hoyle (1952) showed that treatment of influenza virus particles with ether resulted in the liberation of two smaller units, the hemagglutinin and the soluble antigen, indicating that the soluble antigen was in fact a part of

the virus particle. This view was confirmed by the work of Lieff and Henle (1956) who showed that purified influenza virus suspensions gave rise to significant levels of soluble antigen after ether treatment. Furthermore, after the liberation of the soluble antigen there was a disappearance of the properties associated with the intact virus particle.

Early attempts to develop a laboratory method for the diagnosis of herpes simplex infections involved the use of antigens prepared from infected rabbit brain or other tissues. However, there was considerable difficulty due to anticomplementary and nonspecific reactions. In a study of the use of the chick embryo for the preparation of a satisfactory diagnostic antigen. Sosa-Martinez and Lennette (1955) found that an improved antigen could be prepared from the infected chorioallantoic membranes. This tissue extract, however, was not subjected to high-speed centrifugation so that it cannot be considered as a true soluble antigen at this stage.

Most of the antigens prepared up to this time for the serological diagnosis of viral diseases were based on the use of the chick embryo. A notable exception was the soluble antigen prepared by Smadel *et al.* (1939) for the serological diagnosis of lymphocytic choriomeningitis. This antigen was prepared by extracting infected guinea pig spleens with buffered saline, followed by centrifugation at 30,000 rpm for 20 minutes. The supernatant fluid, containing the soluble antigen, was further clarified by Seitz filtration and then stored in the cold. As is the case with mumps infection, here also the soluble antigen detects a complement-fixing antibody before the neutralizing antibody can be demonstrated. The neutralizing antibody remains at significant levels for a much longer period of time.

Soluble antigens for the laboratory diagnosis of influenza A, influenza B, herpes simplex, mumps, and vaccinia have been prepared by this laboratory (Bureau of Virology, Laboratory Centre for Disease Control) for many years. Initially, the method involved maceration of the pooled, infected chorioallantoic membranes with 2.5 ml of saline per membrane, followed by low-speed centrifugation for clarification and then centrifugation of the supernatant fluid at 20,000 rpm for 1 hour (10,000 rpm for herpes and vaccinia). The clarified supernatant fluid is treated with 0.1% formaldehyde at 37°C for 2 hours, followed by storage overnight at 4°C. The excess formaldehyde is neutralized with dibasic ammonium phosphate and the antigen then lyophilized for stable storage (Polley, 1957, 1960).

More recently, an improved method has been developed for the preparation of these soluble or ribonucleoprotein antigens.

1. The infected membranes are harvested, pooled, and centrifuged at 5000 rpm for 10 minutes to remove excess fluid.

2. The membranes are macerated in a cold Waring blender with 2 volumes of 20% acetone in saline.

3. The mixture is centrifuged at 5000 rpm for 10 minutes to remove debris, and then stored at 4°C for 2 days. The mixture is then centrifuged again at 5000 rpm for 10 minutes, after which the supernatant fluid is centrifuged at 20,000 rpm for 1 hour.

4. The supernatant fluid is dialyzed overnight at 4°C against saline and then extraced with an equal volume of chloroform for 15 minutes at 2°C. The mixture is centrifuged at 7000 rpm for 10 minutes to separate the phases. The antigen in the top phase is removed and clarified at 15,000 rpm for 10 minutes.

5. The supernatant fluid is mixed with 3% (w/v) tribasic calcium phosphate for 30 minutes at room temperature to selectively adsorb the antigen, then centrifuged at 3000 rpm for 5 minutes.

6. The precipitate is dissolved in 10 volumes of 10% tetrasodium ethylenediamine tetraacetate in distilled water, pH 7, overnight with stirring.

7. The solution is concentrated to 100 ml by ultrafiltration using a Diaflo cell (Amicon) with a PM-30 membrane, centrifuged at 10,000 rpm for 10 minutes, and then dialyzed overnight at 4°C against phosphate-buffered saline, pH 7.

8. The antigen potency is tested by complement fixation, after which it is diluted to give a titer of about 1:32 and then lyophilized.

One hundred milliliters of packed membranes yields approximately 80 ml of purified antigen. Some typical results are shown in Table I.

B. Viral Antigens

Originally, viruses were identified by isolation from infected hosts, followed by inoculation into normal hosts to reproduce the clinically recognizable disease. This process was simplified with the advent of the use of known human convalescent serum to neutralize the capacity of the homologous virus to infect the host, the original basic serum-virus neutralization test. It was some time later, however, before this test was used in reverse, that is, purified virus suspensions or viral antigens were used to detect and later quantitate antibodies. Diagnostic virology, as a laboratory procedure using prepared viral antigens to detect antibody response to a particular infection, became a practical reality with the observation of Hirst (1941) that influenza virus caused agglutination of chick erythro-

TABLE I
Preparation of Purified Soluble Antigens (Egg Membrane)

Antigen	CF titer		Total N content (mg%)
	Specific	Nonspecific	
Influenza A			
Crude	64	8	320
Purified	32	<2	20
Influenza B			
Crude	64	4	400
Purified	32	<2	35
Mumps			
Crude	32	2	340
Purified	16	<2	50

cytes. This observation led to a method for the assay of the virus content of a fluid by testing serial dilutions for the presence of hemagglutinin. The fact that agglutination of erythrocytes by a given virus was inhibited on mixture with the homologous serum led to the well known hemagglutination inhibition (HI) test (Hirst, 1942), still a basic tool of present-day virology. It is now known that there are a number of different groups of viruses or viral antigens which cause hemagglutination and the HI test is the basic technique for their identification and typing. Such major groups are the poxviruses, adenoviruses, echoviruses, arboviruses, and myxoviruses. In addition, there are an increasing number of individual viruses and subgroups which are capable of agglutinating some cells, under some conditions.

The use of the two terms, viral and soluble, was common until the 1960's when, with the advent of the tissue culture systems, it became more popular to refer to antigens as HA and CF. It is not surprising that some confusion arose concerning the relationships. While the soluble antigens are serologically active in the CF rather than in the HA test and are type- or group-specific, viral antigens are associated with infectivity and erythrocyte agglutination but may also react to varying degrees in the CF test. The situation is not simplified when, as it is now known, various antigens can be separated into further components. With soluble or S antigens another difficulty arose with the discovery of smaller viruses, usually propagated in tissue cultures, where it became increasingly difficult to differentiate between such small viruses and the even smaller soluble antigens, if in fact these antigens existed. These difficulties caused the terms, S and V antigens, to come into disfavor with some workers who preferred CF and HA antigens.

III. INACTIVATION

For use as routine laboratory diagnostic antigens, whether prepared from infected animal tissues or chorioallantoic membranes, many of the early antigens had the disadvantages of limited storage stability, variable specificity, and especially infectivity. The use of live antigens in serology or diagnostic procedures in general is hazardous for personnel and laboratory infections do occur.

A. Heat

Of the various inactivation methods, heat was one of the first to be used and it is still employed in selected cases. Casals (1945) used heating at 60°C for 30 minutes to inactivate a number of arbovirus antigens prepared from infected mouse brains. This treatment did not cause nonspecific reactions or anticomplementary activity but there was considerable loss of antigenicity in some cases. Heat inactivation has never become a popular method of inactivation because it does not have a widespread general application. Different antigens show markedly different sensitivities to it and the margin between inactivation of infectivity and antigenicity is frequently small and variable from batch to batch.

B. Formaldehyde

Formaldehyde came into early use as a more satisfactory procedure of wider application. Although many of the initial methods had in common the use of formaldehyde in the cold, the recommended concentrations varied from 0.01 (Hare *et al.*, 1942) to 0.4% (Salk, 1945). Sever *et al.* (1964) investigated the use of formaldehyde for the inactivation of a number of hemagglutinating antigens but found it to be less satisfactory than β-propiolactone under their conditions of trial. On the other hand, studies were conducted on the formaldehyde inactivation of soluble antigens prepared from infected chorioallantoic membranes (Polley, 1960). The effects of temperature, pH, reagent concentration, and time of treatment were studied. For a given formaldehyde concentration, the best results were obtained by lowering the temperature of treatment and increasing the pH. The method selected to inactivate herpes simplex soluble antigen, which is relatively labile, was 0.01% formaldehyde at 37°C for 2 days at pH 8.5. The method used routinely in this laboratory for many years for other soluble antigens has been 0.1% formaldehyde at 37°C for 2 hours at pH 7, followed by storage overnight at 4°C prior to neutralization and lyophilization.

Although the infectivity of numerous antigens can be destroyed by the addition of formaldehyde, treated antigens frequently lose titer on storage or become anticomplementary. They are also unsuitable for lyophilization with formaldehyde present for the dried product is frequently almost insoluble, suggestive of protein denaturation, and much or all of the antigenicity is lost. The excess formaldehyde can, however, be neutralized by the addition of sodium bisulfite or dibasic ammonium phosphate (0.25 ml of a 30% solution per 10 ml) of a 0.1% treated antigen (Polley, 1957, 1960).

The effect of formaldehyde on viral or hemagglutinating antigens has also been investigated (Polley, 1955, 1959; Sever *et al.*, 1964). Using mumps and influenza viruses, the influence of pH, formaldehyde concentration, temperature, and duration of treatment were studied. For a given formaldehyde concentration, the stability of the hemagglutinating antigen was greatly affected by the pH of the suspending medium, being most stable to treatment at about pH 6 and decreasing as the pH is increased. The complement-fixing activity of these viral antigens was more stable than the hemagglutinin to treatment. For the preparation of noninfective stable myxovirus hemagglutinating antigens, the method of treatment selected was suspension of the purified virus in isotonic phosphate buffered solution at pH 6, treatment with 0.1% formaldehyde at 45°C for 12 hours, followed by the neutralization of excess formaldehyde by the addition of 0.25 ml of 30% dibasic ammonium phosphate solution per 10 ml of treated antigen. Prior to lyophilization, 5% (w/v) of arginine was added as a stabilizing agent (Polley, 1955).

In a subsequent study on the preparation of influenza vaccines using formaldehyde (Polley, 1959), it was found that the infectivity and hemagglutinin were destroyed more rapidly as the pH was increased. By correlating the hemagglutinin titer with the inactivation curve as it approached zero infectivity, it was possible to select a number of reaction conditions whereby the virus suspension could be inactivated with little or no loss of titer. This information was applied to the preparation of stable noninfective influenza viral antigens for typing by hemagglutination inhibition (Polley, 1969). The procedure used was treatment with 0.1% formaldehyde at pH 6 at 37°C for 24 hours. The hemagglutinin in allantoic fluid was slightly more sensitive to treatment than when suspended in buffered saline at pH 6. However, both inactivated antigens gave similar results when tested for specificity in the hemagglutination inhibition test. After inactivation and neutralization of the excess formaldehyde, the purified virus suspensions can be lyophilized and used as required for the typing of influenza antibodies.

C. β-Propiolactone

Another agent in widespread use for the inactivation of virus antigens is β-propiolactone (BPL). Hartman *et al.* (1954) studied it for the treatment of plasma. It was used later for the inactivation of infected mouse brain suspensions (LoGrippo and Hartman, 1955) and allantoic fluid (Mack and Chotisen, 1956). In an investigation of the inactivation of influenza, mumps, and poliomyelitis virus suspensions, it was found that BPL could be used to selectively destroy infectivity rather than hemagglutinin over a wide range of concentrations if the suspending medium was buffered at pH 7. Using influenza virus suspensions, it was found that, as with formaldehyde, the infectivity was destroyed more rapidly as the pH was increased. One advantage of treatment with BPL is that it is rapidly hydrolyzed to hydroxypropionic acid and no treatment is required to remove its excess. Two disadvantages in its use as an inactivating agent are (1) the tendency of the inactivation curves to "tail off," especially at pH 6 and (2) impurities present in suspending media, such as allantoic fluid, impeded inactivation.

Clarke (1964) used BPL to prepare inactivated arbovirus antigens. The mouse brain suspensions were suspended in borate-buffered saline at pH 3 and treated with 0.05% BPL for 18 to 24 hours at 4°C. It was noted, however, that there were variations in the concentrations required to inactivate different antigens.

D. γ-Irradiation

Although chemical agents such as β-propiolactone and formaldehyde are still used extensively for the inactivation of various virus preparations, there remains the practical problem of selecting the optimal concentration to achieve consistent inactivation while preserving maximum antigenicity. The virus particle may be protected in a tissue aggregate or impurities in the suspending medium may reduce the effective concentration of the chemical reagent by consuming some of it in side reactions. Ionizing radiation, on the other hand, can produce inactivation both by direct electron hit and by the production of short-life, but highly reactive agents in the medium such as peroxide, free radicals and a high oxidation potential (Kimball, 1957; Pollard, 1954).

Jordan and Kempe (1956) studied the effect of γ-radiation on four viruses; poliovirus to represent a small virus, St. Louis encephalitis and western encephalomyelitis as medium-sized viruses, and vaccinia as a large virus. All viruses were prepared by differential centrifugation as purified virus suspensions in phosphate buffer, pH 7.2. The samples were

packed in Dry Ice and irradiated, the radiation source being cobalt-60 at a dose rate of 200,000 rad/hour. Under these conditions, purified suspensions were more readily inactivated than crude suspensions; also, the smaller virus required larger doses than the large virus. This might be explained by the direct hit theory, in that the larger the particle the more electron hits it will receive in unit time. Also, however, there is the possibility that the larger virus may have biologically active sites more accessible to the highly reactive agents found in the medium during irradiation. The passage of an ionizing particle through such a biologically sensitive site can cause a local high energy release with destruction of a particular biological function or if that site is near an exposed surface it may be converted chemically. Thus, the infectivity of a virus may be destroyed by action on a site or center which is much smaller than the whole virus. Wilson and Pollard (1958) showed that for Newcastle disease virus there was a spherical radiation-sensitive core of about one-half the radius of the whole particle.

By 1960, soluble antigens were being prepared routinely in this laboratory for the serodiagnosis of influenza, herpes simplex, mumps, and smallpox as vaccinia. They were prepared in a stable, noninfective form using formaldehyde, which, while effective, required careful chemical control and lacked the precision which could be achieved with a procedure such as γ-radiation. A method was then developed for the inactivation of such soluble antigens using γ-irradiation (Polley, 1961). It was found that the radiation dose required for total inactivation was proportional to the initial infectivity titer of the antigen (rate of inactivation is an exponential function of the radiation dose). It was possible to achieve complete destruction of the infectivity while retaining most of the complement-fixing activity. The inactivated antigens could then be lyophilized for stable storage without further processing. A further significant discovery was the fact that it was possible to supply an additional calculated amount of radiation, if required, to destroy residual infectivity. This degree of inactivation control is difficult to attain with chemical agents. The radiation dose routinely used was 1.0 Mrad.

Subsequent studies have been undertaken to improve this method. The soluble antigens were prepared as before but, to remove further virus particles, there was added to the antigen at room temperature, 1 ml of a 10% fowl erythrocyte suspension per 100 ml of antigen. After 1 hour, the mixture was centrifuged at 2500 rpm to remove all erythrocytes. The resulting soluble antigens were used in irradiation experiments. A number of different soluble antigens were then subjected to various doses of γ-radiation at a dose rate of 1.2 Mrads/hour. It is apparent from Table II that it is possible to render these soluble antigens noninfective using a dose of only

TABLE II
Effect of γ-Irradiation on Various Soluble Antigens

Radiation dose (Mrad)	Herpes simplex		Mumps		Influenza A		Influenza B	
	CF	Inf.[a]	CF	Inf.	CF	Inf.	CF	Inf.
0(control)	32	+	64	+	32	+	32	+
0.5	32	–	64	–	32	–	32	–
1.0	16	–	64	–	32	–	16	–
2.0	8	–	32	–	16	–	16	–
3.0	8	–	32	–	16	–	16	–

[a] Inf., infectious.

0.5 Mrad, a process which requires no longer than 30 minutes. This procedure was found to be especially useful for the inactivation of herpes simplex antigen, which is relatively labile to heat and formaldehyde.

γ-Irradiation can also be used for the preparation of noninfective viral or hemagglutinating antigens and for experimental vaccines. Using influenza virus, a study was made of the factors influencing the inactivation of the hemaglutinin and the infectivity (Polley, 1961). Irradiation of purified virus suspensions resulted in the inactivation of both the hemagglutinin and the infectivity at closely similar rates. However, by the addition of certain reagents to the suspensions prior to irradiation, it was possible to confer relative protection to the hemagglutinin. The most effective compounds for this purpose were the sulfur-containing amino acids, such as cystine and methionine, amino acids containing a ring structure, such as histidine and tyrosine, and antioxidants, such as ascorbic acid. Presumably by their action as free radical acceptors, they protect the hemagglutinin against specific chemical action during the irradiation process. Materials such as various carbohydrates or proteins such as serum and albumin had relatively little effect on the inactivation curves. As with the soluble antigens, if the radiation dose applied has been insufficient to produce complete inactivation, a further calculated amount can be administered without destruction of hemagglutinin.

The various applications of γ-radiation for preparing noninfective viral diagnostic reagents will be described below with various examples of the preparation of individual diagnostic antigens.

IV. ADENOVIRUSES

Adenoviruses can be propagated in various cell cultures, such as primary MK, FL, HeLa, and KB but for sensitivity on isolation, human fetal

diploid kidney tissue cultures have been found to be especially useful (Schmidt *et al.*, 1967). In a study by Stevens *et al.* (1967) of adenovirus reference antigens, prototype seed viruses were propagated in KB cell cultures and the clarified fluids stored at −70°C for use as reference viral antigens. Monotypical antisera were prepared in rabbits. In general it was found that the HI titers tended to be lower than those observed in the neutralization test, but cross-reactions were more common in the neutralization test. Five types, 12, 18, 20, 25, and 28 showed no hemagglutinating activity in their study. The agglutination of erythrocytes by adenoviruses is rather complex and varied and the viruses have been divided into three groups based on similarity of their agglutination reactions (Rosen, 1960; Ginsberg, 1962). For example, group I consists of types 3, 7, 11, 14, 16, 20, 21, 25, and 28 which agglutinate rhesus or grivet erythrocytes; group II consists of types 8, 9, 10, 13, 15, 17, 19, 22, 23, 24, 26, 27, 29, and 30 which show agglutination only or to a higher titer with rat erythrocytes; and group III consists of types 1, 2, 4, 5, 6, and 12 which agglutinate rat erythrocytes but require the presence of serum containing antibody to one of the other group III types for optimal agglutination. To type an adenovirus isolate, it may be used as the viral antigen in the HI test with reference antisera but some cross-reactions do occur, for example, between types 7, 11, and 14, between 7 and 9, 10 and 19, and 15 and 22. For more precise and complete typing it is also necessary to use the neutralization test. A further complication with use of reference viral antigens is the fact that the erythrocytes from different animals show considerable variation in their capacity to be agglutinated by the types of a given group. Thus, there is required an available source of erythrocytes known to give optimal agglutination by the members of each group.

A. HA Antigens

For the detection and assay of adenovirus antibodies, both soluble and viral antigens can be prepared. The viral antigens, as above, are type-specific and different types may also react in the CF test to varying degrees. Because of the variation of reactions among the different types, few laboratories routinely prepare noninfective lyophilized viral antigens for use in serodiagnostic tests. When the HI test is to be used for typing an antibody, specifically prepared monotypical virus suspensions, previously prepared and stored at −70°C, are usually employed. For the determination of the particular type of an isolate or the antibody type, the neutralization test is more commonly used. An interesting aid for the identification of adenovirus isolates was developed by Hierholzer (1973). In the study, the thirty-three known prototype strains were tested for their

capacity to agglutinate the erythrocytes of rhesus monkey, man, and rat at 37°C. On the basis of these differential hemagglutination titers, the strains were separated into ten groups.

B. CF Antigen

For routine serological diagnostic purposes, the soluble group antigen is more useful, for it reacts with the anti-S antibodies elicited by infection with the various types. While the anti-S antibodies will react in the CF test with a potent HA antigen, the single soluble group antigen gives superior results. Ginsberg *et al.* (1966) recognized that there are at least three soluble antigens, defined as (1) the hexon antigen, associated with the hexon capsomer, which is the serological group-specific antigen, (2) the penton antigen, which is a complete or incomplete hemagglutinin and not directly serologically active; it can be assayed by its CPE or, in the presence of heterotypical antiserum, by hemagglutination enhancement, and (3) the fiber antigen which is type-specific; that from subgroup I can be assayed by HI consumption, while that from subgroups II and III can be measured by HI. These antigens can also be assayed using the single radial immunodiffusion technique (Grandien and Norrby, 1975), or by the crossed immunoelectrophoresis procedure by which the three antigens were determined within the one test (Martin *et al.*, 1975).

C. Preparation of Routine Diagnostic Antigen

A method has been described for the routine preparation of a purified noninfective adenovirus group antigen in the lyophilized state (Polley and Webb, 1969). The antigen was prepared by propagation of adenovirus type 7a in primary MK cell cultures, followed by inactivation of the pooled fluids with 2.5 Mrads of γ-radiation.

Recently, this method has been modified by the inoculation of approximately 20 roller bottles (690 cm^2) of BSC-1 cell cultures. The cultures are maintained with 150 ml/bottle of medium 199 containing 10% newborn calf serum; for inoculation, the maintenance medium is replaced with 75 ml of medium 199, free of serum, containing 1000 $TCID_{50}$/ml of adenovirus type 7a. The modification decreases the possibility of inclusion of adventitious viruses and increases the volume of infected cells per milliliter of fluid. When the CPE is complete, the bottles are placed in a high-energy ultrasonic bath (Forma) to remove the cell sheet and to prepare a uniform cell suspension. The cell suspensions are pooled and irradiated with 1.5 Mrads of γ-radiation, after which the mixture is clarified at 5000 rpm for 5 minutes. The cellular material is stored at 4°C while

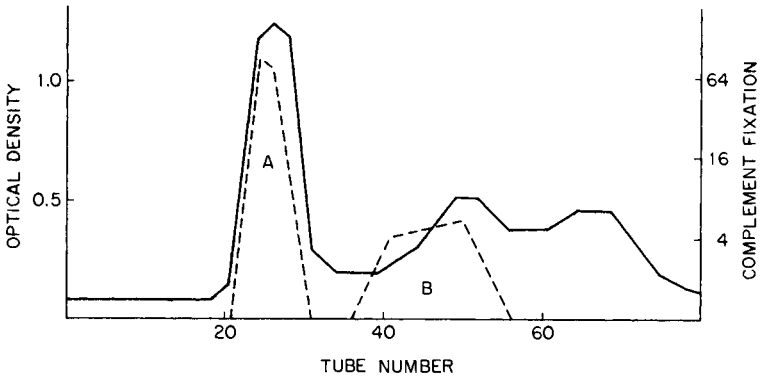

Fig. 1. Adenovirus in BSC-1 fractionated on Sepharose 6B, 5 × 80 cm column. Flow rate, 4–5 ml/cm²/hour. Solid line, optical density at 280 nm; dashed line, complement fixation titer.

the supernatant fluid is concentrated to 50 ml by ultrafiltration using a PM-30 membrane. The concentrated fluid is then added to the cellular material, the mixture ultrasonicated at maximum amplitude for 5 minutes, and then clarified by centrifugation at 10,000 *g* for 5 minutes. The two major antigen components, as identified by CF, are separated as before by gel filtration but using Sepharose 6B for increased resolution. A typical chromatogram is shown in Fig. 1. The fluid samples under peak A contain the hemagglutinin and possess CF activity; the fluids under peak B contain no hemagglutinin but also possess CF serological activity. If the objective is to prepare a CF antigen, the fluids from the two peaks are pooled, now being free of much cellular contamination. If a purified HA antigen is required, just peak A is used; if a purified soluble antigen is required, just peak B is used. In any case, the required fluids are pooled and reconcentrated to about 100 ml, 30% bovine albumin solution is added to a concentration of 1%, and the antigen is dialyzed overnight at 4°C against phosphate-buffered saline, pH 7.5. The purified antigen is then assayed by complement fixation, the volume adjusted to give an antigen titer of approximately 1:32, and the antigen lyophilized for stable storage.

D. Antiserum

For the preparation of strain-specific antisera for use in the neutralization or hemagglutination inhibition tests, rabbits and horses are usually used. The inoculum is obtained by passaging the seed virus as for the preparation of antigen. When the CPE in the cell cultures is complete or after 5–6 days, the cultures are frozen and thawed. The mixture is ultra-

sonicated at full power for 2 minutes at 0°C and then clarified by centrifugation at 3000 rpm for 5 minutes. The clarified fluid is used for inoculum. For the best results, it is advisable to start the immunization with freshly prepared inoculum; aliquots for subsequent inoculation can be stored at −20°C.

For the preparation of rabbit antisera, an initial sample of blood is obtained from each animal and then they are inoculated with 1.0 ml of inoculum intravenously and 0.5 ml intraperitoneally. These inoculations are repeated three to four times at intervals of 1 week. One week later they are bled, but not exsanguinated, by cardiac puncture. Booster inoculations can be given at intervals of 10 days and additional bleeding obtained. The sera are tested for potency and specificity in the designated procedure.

For the preparation of horse antisera, a preliminary bleeding is obtained and the animals then inoculated intravenously with 40 ml of inoculum; the procedure is repeated at weekly intervals for 6 weeks. The animals are bled 1 week later and the sera tested by the neutralization or HI method.

Antiserum for use in the CF test is prepared in this laboratory by using as inoculum part of the infected tissue culture fluid obtained in the production of antigen. Young adult guinea pigs are used. After a preliminary bleeding has been obtained from each animal and tested in the CF test for presence of undesired antibodies, each selected animal receives 0.3 ml intranasally and 1.0 ml intraperitoneally. Eight days later, a second inoculation of 1.0 ml is administered by the intraperitoneal route and the animals are bled after a further 2 weeks. By this procedure the CF titer of the antiserum is 1:32 to 1:64 and is free of heterologous antibodies above 1:2–1:4.

V. *HERPESVIRUS HOMINIS* (SIMPLEX)

The size of this virus has been reported variously from 90 to 230 nm, depending on the procedure used, but it is now generally considered to be about 165–180 nm. There is an outer envelope with a peripheral membrane, an inner spherical core containing deoxyribonucleoprotein, and the capsid which envelops the core. There is considered to be only one immunotype but there are various strains which differ in their antigenic characteristics, most clearly demonstrated in the serum-virus neutralization test. There is a soluble antigen associated with the central core which is serologically active in the complement fixation test. Unlike the myxoviruses, however, the outer envelope is not associated with a viral antigen which usually and reliably produces hemagglutination directly.

The presence of the viral antigen can be demonstrated using the agar double-diffusion technique.

The most common test for the serodiagnosis of infection with herpes virus is complement fixation. The required antigen can be prepared from specifically infected chorioallantoic membranes (CAM) or infected tissue cultures. Suckling mouse brains are seldom used today as a source of antigen.

A. Preparation of Diagnostic Antigens

A soluble antigen can be prepared by macerating the infected CAM with 1 volume of saline, storage at 4°C for 2 days, followed by centrifugation at 10,000 rpm (9000 *g*) for 1 hour. This antigen can be used directly or preferably be prepared in a stable noninfective form. If formaldehyde is used as the inactivating agent, treatment with 0.01% at 37°C for 2 days at pH 8.5 has been found to be a satisfactory procedure (Polley, 1960). However, for the inactivation of this soluble antigen, it was found that γ-irradiation was superior (Polley, 1961).

A diagnostic antigen can also be prepared by propagation of the virus in chick embryo tissue cultures (Guérin and Polley, 1970). The pooled fluids are inactivated using 0.8 Mrad of γ-radiation, after which the fluid is concentrated approximately fortyfold by ultrafiltration to produce a potent diagnostic antigen. After the addition of 5% arginine monohydrochloride or 2% peptone, this inactivated antigen can be lyophilized for stable storage.

A modified method is now being used in this laboratory for the routine production of an efficient diagnostic antigen. The general procedure is similar to that described above for the preparation of vaccinia antigen, with the following exceptions. Approximately 20 roller bottles of Vero cell cultures are inoculated with 1000 $TCID_{50}$/ml of *Herpesvirus hominis* in the form of the infected chorioallantoic membrane extract. The CPE is usually complete in 3 to 4 days and the pooled fluids are inactivated with 0.8 Mrad of γ-radiation. After fractionation on Sepharose 6B, it is found that about 75% of the CF activity is associated with the high molecular weight peak A. For this antigen, the fluids under the two peaks are pooled, reconcentrated to the desired titer, and then lyophilized as for vaccinia.

For many years it has been recognized that there are genital herpetic infections, but the question of venereal transmission was in doubt. However, it was reported by Dowdle *et al.* (1967) that most of the *Herpesvirus hominis* strains recovered from the genital area differed antigenically from the strains usually isolated from other areas. Later, evidence was present

for transmission by the venereal route (Nahmias *et al.*, 1969). With the discovery of the two antigenically different types, 1 and 2, it became necessary to attempt to prepare diagnostic antigens for each one. The determination of the antigenic type of an isolated strain has been performed using various procedures such as CF, plaquing on the chorioallantoic membrane, and quantal microneutralization; similarly, these procedures can be used to identify type 1 and 2 antibodies, if the required antigens are prepared.

Fuccillo *et al.* (1970) described a micro indirect hemagglutination test for the determination of type 1 and 2 antibodies. It was reported that this test was as accurate and as sensitive as the quantal microneutralization test and easier to perform. The antigen used was prepared by inoculation of MA-196 (human skin line) cell cultures with a high multiplicity of the specific type 1 or 2 seed virus. When the CPE was almost complete, the cell lines were removed mechanically from the glass, resuspended as a 10% suspension in phosphate-buffered saline, pH 7.2, frozen and thawed three times, and used as the antigen after clarification by low-speed centrifugation.

Palmer *et al.* (1971) described the preparation of CF antigens against these two types by a procedure involving the alkaline extraction of Vero cells infected by either of the prototype strains. When the CPE was almost complete, the infected cells were removed from the glass and the suspension subjected to ultrasonication. The clarified fluid was used directly as the CF antigen. It was noted that these antigens gave lower titers with human convalescent sera than with rabbit hyperimmune sera.

B. Antiserum

Antisera for use in the neutralization test can be prepared in rabbits but these sera are usually less satisfactory in the CF test. The inoculum is freshly harvested infected tissue culture fluid prepared as for the production of antigen above. The scarified cornea are infected by the introduction of a drop of the inoculum into each eye. One week later, each animal receives an intraperitoneal injection of 2 ml of the inoculum; the intraperitoneal inoculations are repeated at weekly intervals for 3 weeks and the animals are bled 10 days after the final inoculation. The sera are tested for potency and specificity in the neutralization and CF tests.

For the preparation of antiserum for use in the CF test, guinea pigs are usually used. The inoculum used is infected tissue culture fluid, as above. One procedure used is to inoculate each animal by the intraperitoneal route with 2 ml of inoculum. These inoculations are repeated at weekly intervals for 3 weeks; the animals are bled 10 days later. The individual sera

are tested by complement fixation and those found to be satisfactory are pooled.

Another well known procedure is the foot pad inoculation of guinea pigs. By this method the hindfoot pads are scarified and then inoculated with 0.25 ml each of freshly harvested infected tissue culture fluids. At weekly intervals for 3 weeks, each animal receives 2 ml of the inoculum by the intraperitoneal route. One week later, the animals are bled. The individual sera are tested and those found to be suitable are pooled.

The preparation of antiserum for the specific detection of type 1 or 2 antigen is difficult, but a procedure was reported by Jeansson (1975) for increasing the specificity using an immunosorption technique. The antisera for type 1 and 2 were produced in rabbits but there was considerable cross-reaction. Each serum was adsorbed with gluteraldehyde polymerized heterologous virus antigen. The treated antisera showed increased specificity in the CF and neutralization tests.

VI. VARICELLA-ZOSTER VIRUS

The hyphenated name of this virus arises from the fact that it is considered to cause two clinically separate conditions, namely, varicella and herpes zoster. Primary infection appears as varicella, but when the infection occurs in a host with partial immunity, the clinical condition appears as herpes zoster. Clinically, these infections and their interrelationship have been recognized for many years. Although the virus was first isolated in cell cultures over 20 years ago (Weller, 1953), studies on the properties and characteristics of the virus and the preparation of antigens have been hampered by the facts that the virus is relatively heat-labile and is largely cell-associated. However, it is a member of the herpesvirus group and, as such, exhibits a number of the characteristics of other members of the group, such as cytomegaloviruses and *Herpesvirus hominis*. It does not cause hemagglutination but there is a soluble or CF antigen in the infected tissue culture fluid and in vesicle fluid.

The virus can be propagated in primary, diploid, or various continuous cell line cultures. However, the virus is highly labile and the results obtained on antigen assays and infectivity titers are often highly variable from batch to batch. Since it appears that the virus spreads from cell to cell, a high cell density in the cultures is desirable for the preparation of a diagnostic antigen. Inoculation of cultures with a high multiplicity of virus is desirable to ensure infection of as many cells initially as possible, since the antigen is largely cell associated and the potency of the antigen will be related to the total cell volume fully infected at the time of harvest.

The ready serodiagnosis of infections caused by this virus were hampered for a number of years by the difficulty in preparing a satisfactory diagnostic antigen. Schmidt *et al.* (1964) reported a procedure in which the infected cells are harvested at time of 4+ CPE, concentrated 50-fold by resuspension in decreased fluid medium, and the cells disrupted by sonication. By this procedure a crude antigen titer of 1:2 could be increased to 1:32–1:64. This procedure, a minor modification of it, is still used extensively today.

Antiserum

The preparation in animals of satisfactory specific antibody for use in diagnostic virology has been difficult. Frequently, the attained homologous antibody titers have been low and the use of multiple inoculations to increase these levels has resulted in significant levels of nonspecific reactants. Schmidt *et al.* (1965) described a method for the production of specific antiserum in monkeys. The inoculum consisted of a mixture of equal parts of infected monkey kidney tissue culture fluid and adjuvant. Each animal received 4 ml of the inoculum by the intramuscular route for a total of three inoculations at biweekly intervals.

Kissling *et al.* (1968) described a procedure for the preparation of specific antisera in which guinea pigs were inoculated with inoculum obtained by passaging the seed virus in cell cultures of diploid human fibroblasts of foreskin pool origin. Each animal received 0.2 ml of the inoculum by the intraperitoneal route and the injection was repeated on days 2, 4, 7, 9, 11, and 18. The animals were bled 7–10 days after the last inoculation. Typical sera showed CF antibody titers of 1:64–1:256 while the titers in control antisera did not exceed 1:8. Even better results were obtained when semipurified antigen with adjuvant was used.

VII. CYTOMEGALOVIRUSES

The name cytomegalovirus (CMV) was suggested by Weller *et al.* (1960) to replace the long-standing but indefinite terms "salivary glands virus" and "cytomegalic inclusion disease virus." Although these viruses are classed as members of the herpesvirus group, unlike *Herpesvirus hominis*, they have been studied significantly only since their propagation in tissue cultures was described (Rowe *et al.*, 1956; Smith, 1956). After their cultivation in various cell cultures was well established, it became possible to prepare diagnostic antigens. These viruses have presented a rather unique problem to diagnostic virology because subclinical infections ap-

pear to be relatively common and because the viruses are excreted over prolonged periods, despite the fact that there may be circulating antibodies.

These viruses are similar in size and morphology to *Herpesvirus hominis* and their core contains deoxyribonucleic acid, but it is of a different composition than that in herpesvirus (Crawford and Lee, 1964). Although CMV possesses the characteristics and the properties of a member of the herpesvirus group, it differs in that it attacks a larger range of tissues and organs. As with the herpesviruses, the CMV contain a soluble or type-specific antigen which is serologically active in the CF test but the virion or envelope does not cause hemagglutination. The various presently known strains share the common CF antigen, making it the most useful reagent for serological diagnosis and for antibody surveys.

One of the first CF antigens was the direct use of the infected tissue culture fluids by Rowe *et al.* (1956), who reported that the antigen prepared from the AD169 strain appeared to be more sensitive than others for detecting CMV antibodies and for distinguishing them from herpesvirus, varicella, and measles. In young children, the results with the CF antigen were in agreement with those obtained in the neutralization test. In adults, however, a serum negative by CF was frequently positive by neutralization but a positive by CF was positive by neutralization. It was shown by Benyesh-Melnick *et al.* (1966) that a large proportion of the CF antigen could be separated from the virus particles by centrifugation, as with a typical soluble antigen. It was interesting to note that the usual pattern of thermal inactivation of infectivity and CF was reversed, i.e., the CF was inactivated more quickly at 37°C than at 4°C, while the reverse was true of the infectivity. This fact has always presented a significant problem in the preparation of a potent diagnostic antigen.

In addition to the relative difficulty in preparing satisfactory CF antigens, there are other associated problems. Although a CF antigen produced routinely from a standard strain is satisfactory for the detection of antibodies in adults, it is less sensitive for the detection of antibody in infants. While the CF test has been used extensively in serological surveys and in studies on congenital disease, it does not distinguish between antibody produced by the infant and that transferred from the mother. On the other hand, an indirect fluorescent antibody technique which measures IgM antibody (which is not transferred) is useful for indicating active infection (Hanshaw, 1966).

In a serological survey conducted by Deibel *et al.* (1974), using both the CF and indirect hemagglutination (IH) methods, it was found that higher antibody titers were obtained using the IH test. In some cases, the IH test

gave positive results while the CF was negative. A useful observation was the fact that significant levels of IH antibody were frequently found in apparently healthy individuals whereas high CF levels were usually associated with clinical illness. The CF antigen was prepared in the usual manner, that is, it was obtained from the disrupted infected cells and used directly. The IH antigen was prepared similarly except that there was a greater concentration of the infected cells prior to their disruption and the resulting mixture was clarified by centrifugation at 34,000 *g* for 3 hours at 4°C.

Krech and Jung (1969) described the preparation of a CF antigen involving the extraction of the infected cells with glycine buffer, pH 10, at 37°C for 18 hours. The supernatant fluid was used as a CF antigen. Krech *et al.* (1971) showed that this antigen was more sensitive for the detection of antibodies in human sera than the frequently used unprocessed infected cell culture fluids. It was especially useful for detecting antibodies in the sera of children which are frequently negative using the usual antigen.

In addition to the CF test, the precipitation test has also been used for the detection of CMV antibodies but Jung *et al.* (1973) did not find it to be as sensitive.

A. Preparation of Routine Diagnostic Antigen

The CMV virus is propagated in stationary or roller bottle cell cultures of WI-38 or individual cell lines developed from embryonic skin. The cultures are inoculated with a high multiplicity of virus, usually about 1000 $TCID_{50}$/ml. When the CPE is 75–90% complete, the supernatant fluid is decanted and replaced with one-tenth the previous volume of Veronal-buffered saline, pH 7.5. The cultures are frozen and thawed and the pooled fluids then exposed to 0.5 Mrad of γ-radiation. The irradiated suspension is clarified by centrifugation at 5000 rpm, the cell precipitate is resuspended in about 30 ml of the clarified fluid, while the remaining fluid is then concentrated about 20 to 30-fold by ultrafiltration using a Diaflo PM-30 membrane. The two antigen fractions are pooled, ultrasonicated at maximum power for 2 minutes, and then clarified by low-speed centrifugation. This concentrate is then diluted with buffered saline, pH 7.5, to give an estimated CF titer of about 1:80, 30% bovine albumin solution is added to a concentration of 0.5%, and the antigen is lyophilized. This product usually has a maximum titer of 1:64, an optimal titer of at least 1:6 at which it does not react with specific *Herpesvirus hominis* or varicella-zoster antisera.

B. Antiserum

Great difficulty has been experienced in attempts to develop routine procedures for the preparation of specific antisera, especially for use in the CF test. Huang *et al.* (1974) reported the preparation of a satisfactory antiserum in guinea pigs for use in the CF test. The inoculum consisted of virus purified by density gradient centrifugation to free it of cellular materials which in the past have led to the presence of interfering levels of nonspecific reactants in the prepared sera. Each animal received 0.5 ml intraperitoneally and 0.5 ml intramuscularly at weekly intervals for 4 weeks, followed by a final inoculation 2 weeks later. The animals were bled after an additional 2 weeks. Human sera containing satisfactory antibody levels are usually readily available in the diagnostic laboratory. These sera are tested individually by CF and sera are selected which have satisfactory antibody levels against CMV antigen but are as free as possible of antibodies to varicella-zoster and *Herpesvirus hominis*.

Human sera satisfactory for use in the CF test are available commercially. They should, however, be tested in the individual laboratory with the diagnostic antigens in current use.

VIII. POXVIRUSES

The poxviruses were among the first to be studied both for morphology and for their capacity to agglutinate fowl erythrocytes. The hemagglutinin of these viruses, however, is unique in that it can be separated by centrifugation from the virus particle and purified virus suspensions do not contain the hemagglutinin. Probably because of their relatively large size, these viruses have an increased complexity of antigenic structure. It is now known that the viral and soluble antigens of vaccinia, for example, contain several antigenically smaller units. While the hemagglutinin is inhibited by immune serum, there is extensive cross-reaction between variola, vaccinia, and cow pox, rendering the HI test of limited specific diagnostic value, except to distinguish this group from other viruses. A number of secondary observations are frequently used to attempt to differentiate between members of the group and between strains. For example, vaccinia and ectromelia show a degree of serological specificity in that the specific immune serum inhibits the homotypical virus to a higher titer. Of this group, only the ectromelia virus agglutinates mouse erythrocytes. The complement fixation test using a soluble antigen prepared from vaccinia-infected chorioallantoic membranes is suitable to demonstrate antibody to variola-vaccinia but does not distinguish between them. Like-

wise, the specifically prepared vaccinia antiserum can be used to identify a variola-vaccinia isolate. These reagents will, however, serve to differentiate variola-vaccinia from varicella and herpes simplex.

Because of the above diagnostic difficulties, and in the interest of speed of diagnosis, it is not surprising that the initial approach is direct visualization of the agent, if possible, using material from vesicles, pustules, scabs, etc. The secondary approach is attempted isolation of the agent either in tissue culture or on the chick chorioallantoic membrane, which has always been a prime method for the identification of smallpox. However, problems arise from the need to differentiate variola from herpes simplex, varicella, and vaccinia. If the specimen produces pocks on the membrane, varicella is eliminated. If a soluble antigen is prepared by extracting the infected membranes and testing it in the CF test with specific antisera, herpes simplex can be identified but variola and vaccinia are not differentiated. The temperature marker test will differentiate variola from vaccinia; the variola virus will grow on the membranes of eggs incubated at 36°–38°C but not at 38°–40°C, whereas vaccinia virus will grow at both temperatures.

From the remarks above it is understandable that viral antigens for use in the HI test are not in general routine production for these viruses. The most useful serological reagent which is prepared routinely and made available for this purpose is the soluble antigen, prepared using vaccinia virus. The preparation of soluble antigens, in general, from specifically infected chorioallantoic membranes is described separately above.

A. Preparation of Routine Diagnostic Antigen

A serological antigen of the soluble type can be prepared by inoculation of vaccinia virus onto the chorioallantoic membranes of 9-day-old chick embryos, followed by maceration of the pooled infected membranes with 2.5 ml of saline per membrane. The mixture is clarified by centrifugation and can then be inactivated by treatment with formaldehyde or γ-irradiation, as described above.

At the present time, a complement-fixing antigen is being prepared in this laboratory from cell cultures. Stationary cultures of BSC-1 tissue are inoculated with 1000 EID_{50}/ml of vaccinia virus in the form of the infected chorioallantoic membrane extract. After incubation at 36°C for about 4 days, until the CPE is complete, the cultures are frozen and thawed, and the fluids are pooled and inactivated with 1.0 Mrad of γ-radiation at a dose rate of approximately 1 Mrad/hour. The inactivated fluid is then concentrated about thirtyfold by ultrafiltration, clarified by centrifugation at 25,000 rpm for 10 minutes, and the concentrate introduced into a gel fil-

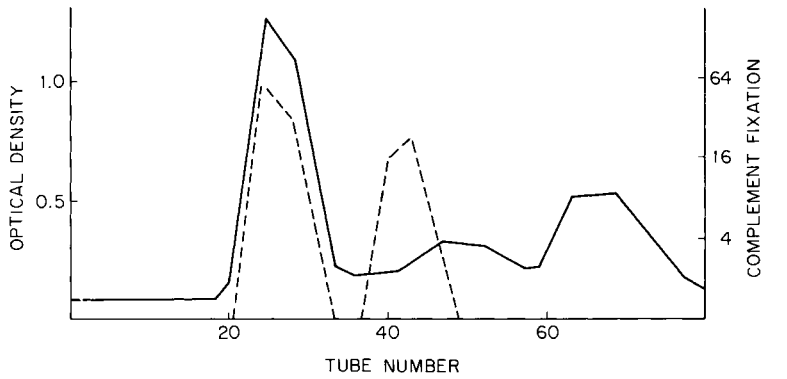

Fig. 2. Vaccinia in BSC-1 fractionated on Sepharose 6B, 5 × 80 cm column. Flow rate, 4–5 ml/cm²/hour. Solid line, optical density at 280 nm; dashed line, complement fixation titer.

tration column packed with Sepharose 6B, as described for the production of adenoviruses antigen (Polley and Webb, 1969). The eluting fractions are tested by complement fixation using rabbit vaccinia antiserum. A typical chromatogram is shown in Fig. 2, from which it can be seen that there are two major peak areas of CF activity. The first peak contains the products of relatively high molecular weight and most of the hemagglutinin, while the second peak of lower molecular weight materials contains the soluble antigen but little or no hemagglutinin. The fractions under these two peaks, relatively free of cell contaminants, are reconcentrated by ultrafiltration to original volume (or to desired titer), 1% bovine albumin is added, and the inactivated purified antigen is lyophilized.

B. Antiserum

Vaccinia antiserum can be prepared in calves, sheep, and rabbits. In laboratories or institutions which prepare vaccine, specific antiserum can be readily prepared by the intramuscular inoculation of 5 ml of vaccinia virus suspension into the animals which have recovered from vaccinia infection. The injections are repeated at weekly intervals for 3 weeks and the animals are bled 10 days after the last injection.

Most laboratories use the adult New Zealand white rabbit for the preparation of antiserum. Seed virus is passaged in primary rabbit kidney tissue culture and 0.5 ml of this infected fluid is inoculated by the intratesticular route. When the animals are obviously ill, the testes are removed and prepared as a 10% suspension in tissue culture medium but without serum. As inoculum, a mixture of equal parts of tissue suspension and Freund's complete adjuvant is prepared. Each rabbit received 3 ml by the intramus-

TABLE III
Preparation of Vaccinia CF Antiserum in Rabbits

Rabbit No.	Day of test	Serum CF titer	
		Vaccinia	Nonspecific
1	0	<2	<2
	24	64	<2
	56	64	2
2	0	<2	<2
	24	16	<2
	56	32	<2
3	0	2	2
	24	64	32 AC[a]
	56	64	16 AC
4	0	2	2
	14	32	4
	25	32	16
	59	16	<2

[a] AC, anticomplementary.

cular route at intervals of 1 week for 4 weeks. The animals are bled 1 week after the last injection.

When smallpox vaccines were assayed for potency using the scarified rabbit skin technique, it was observed in this laboratory that these rabbits frequently developed significant CF antibody levels. For the actual preparation of specific antiserum the following simple procedure was found to be successful. Six shaved scarified skin patches were prepared on the back of each rabbit. Smallpox vaccine diluted 1 : 100 in sterile phosphate-buffered saline was inoculated by light sacrification into each patch. The animals were trial-bled after various periods and the individual sera tested for potency and nonspecific reactions (Heterologous antibody levels and anticomplementary reaction) in the CF test. Typical results are shown in Table III.

This procedure has been found to be simple and to produce satisfactory antiserum in about 50 to 75% of the animals used. It has been noted that nonspecific reactions frequently reach a maximum level at about the 4th week after inoculation and then decrease more rapidly than the vaccinia CF antibody.

IX. ORTHOMYXOVIRUSES (INFLUENZA)

The influenza viruses, A, B, and C are placed in the orthomyxovirus group by virtue of their reactions with certain mucoproteins, notably

those of erythrocytes. The initial attachment to receptor sites on the mucoprotein of the erythrocyte leads to agglutination of the cells but this is followed by elution from the cells due to destruction of the receptor site by the neuraminidase enzyme of the virus. This activity of the viruses is now used as the basis for their identification and classification. The influenza viruses are of medium size, 80–120 nm, and consist of a lipoprotein envelope studded with spikelike projections and a central nucleoprotein core which contains RNA. These two morphological parts are associated with the two broad kinds of antigens which are possessed by influenza viruses. The envelope with its projections comprises the strain-specific viral (V) antigen, with which is associated the capacities for hemagglutination, infectivity, and immunization. The central core contains the type-specific nucleocapsid or soluble (S) antigen which is serologically active in the complement fixation test but does not produce hemagglutination or neutralizing antibody. Actually, on the surface of the influenza virus, there are two virus-coded antigenic structures, the hemagglutinin and the enzyme neuraminidase. It is the hemagglutinin which provokes the production of neutralizing antibody, whereas the neuraminidase produces an antibody which is not neutralizing but which does interfere with the release of virus from the cell in cultures. Following influenza infection there is production of antibody against the type-specific ribonucleoprotein or soluble antigen of the core and the strain-specific hemagglutinin of the envelope.

The serodiagnosis of influenza is based on the observation of 4-fold or greater rise in antibody titer between acute and convalescent phase sera. The two tests usually used are the CF test with the soluble (S) antigen which is type-specific and the HI test with the viral hemagglutinin(V) antigen which is strain-specific. As a general approach, the CF test is usually used since with just the antigens plus a normal control antigen it is possible to identify a rise in antibody level to an influenza type. The S antigen is particularly useful in serological surveys because in a given test it detects infection caused by individual members of its group. If it is desired to identify the actual strain responsible for the infection, the HI or neutralization tests can then be used but at least the tests can be restricted to the correct influenza type. Purified viral suspensions (HA or V antigens) also react serologically in the CF test with homologous antibody. Apart from the fact that sera may contain nonspecific inhibitors of hemagglutination, the HI test also involves the problem that there is a difference in avidity between different strains. Investigating 22 influenza B strains, Chakraverty (1971) showed that the strain-specific CF test was superior for separating them into groups, whereas, the HI test was superior for showing similarities. In addition to the broader scope for detecting

influenza antibodies, the CF tests do not require pretreatment of the serum for removal of nonspecific inhibitors.

A. Preparation of Soluble Antigen

The S antigen can be prepared as a stock reagent in a stable noninfective form, as described above. It can also be prepared by liberation from the virion (Lieff and Henle, 1956). In this process, the virus particles in infected allantoic fluid are partially concentrated and purified by adsorption onto and elution from chick erythrocytes to yield a suspension with a hemagglutination titer of at least 5120 HA units/ml. This suspension is stirred with $\frac{1}{2}$ volume of ether at room temperature for about 2 hours. The aqueous extract is separated, freed of ether by incubation at 37°C or by bubbling nitrogen through the suspension, and is then extracted twice with 10% packed fowl erythrocytes to remove hemagglutinin. The clarified supernatant fluid comprises the S antigen.

B. Preparation of Viral Antigen

For use in the HI test for the typing of antibody produced by a specific strain, it is possible to use (1) live virus suspensions directly, in the form of allantoic fluid or possibly tissue culture fluid, (2) live purified virus suspensions, or (3) either of these, but after inactivation by a chemical agent such as β-propiolactone or formaldehyde or by γ-irradiation. The use of live antigens presents a hazard to personnel and also a problem with possible cross-contamination in a laboratory in which isolation procedures are also being conducted.

Stock lyophilized noninfective influenza viral antigens for use in the HI test are prepared in this laboratory (Polley, 1969). Briefly, the method involves propagation of the specific strain in 10-day-old embryonated eggs, followed by preparation, from the infected allantoic fluid pool, of purified virus suspensions by differential centrifugation. The final product can be prepared by (1) treatment with 0.1% formaldehyde at 37°C for 24 hours, then the addition of 0.25 ml of 30% dibasic ammonium phosphate solution per 10 ml of treated antigen, followed by the addition of 3% arginine hydrochloride as a stabilizing agent, and then lyophilization or (2) by the addition of 0.2% histidine, irradiation with 1.5 Mrad, followed by the addition of 3% arginine hydrochloride and then lyophilization. The method using γ-irradiation is in current use in this laboratory.

Other tests are also available, although less commonly used. For example, Beard (1970) described the use of an agar gel precipitation test on a glass slide for the demonstration of anti-S antibody to types A and B influ-

enza in avian and mammalian sera. This test was not generally satisfactory with swine sera. The antigen used was a crude membrane extract inactivated with formaldehyde and then stored at −20°C.

Influenza type C virus receives relatively little attention today for it is seldom isolated. On the other hand, antibody to it appears to be widespread in the human population. One reason suggested is that it is frequently overlooked with the increasing use of primary rhesus monkey kidney cell cultures for isolation purposes and in which it does not produce a CPE. Also, it does not exhibit hemadsorption with guinea pig erythrocytes which are commonly used. However, it was shown by Chakraverty (1974) that with the use of rat erythrocytes it was possible to produce hemadsorption and on this basis a neutralization test could be conducted in the monkey kidney cell cultures.

An antigen for the serodiagnosis of influenza C is prepared in this laboratory as described above for viral antigens, but is usually used in the complement-fixation test.

C. Antiserum

For influenza serology, there is a need for highly specific HI antisera for the typing of strains and for the specific control antiserum for use in the group-specific CF test.

For the routine production of HI antisera, chickens are usually used. For general use, where the highest degree of strain specificity is not required, the fowl are inoculated slowly intravenously with 3 ml of the freshly harvested infected allantoic fluid. For the best results, this fluid should have an HA titer of at least 1:320. Higher antibody levels can be obtained by partial purification and concentration of the virus inoculum. This can be accomplished by precipitating the virus by centrifugation at 20,000 rpm for 1 hour at 4°C. The virus pellet is resuspended in about $\frac{1}{20}$ the previous volume of physiological saline, clarified by centrifugation at 3000 rpm for 5 minutes, and then diluted to have an HA titer of at least 1:280. The virus can also be purified and concentrated by adsorption onto and elution from fowl erythrocytes. The fowl are bled in 12–14 days and the individual sera assayed in the HI test.

For preparation of the most specific antisera for reference typing purposes, the animal of choice is still the ferret. Each animal receives the installation of 1 ml of the freshly harvested allantoic fluid, usually under light ether anesthesia. In 2 weeks the animals are bled and the individual sera assayed in the HI and CF tests for antibody potency and specificity. The satisfactory sera can be pooled and then lyophilized for stable storage.

For the preparation of antisera for use in the CF test for detection of anti-S antibodies, guinea pigs are usually used. The disadvantage with this method is that the guinea pigs must be carefully tested prior to use to be sure they are free of antibodies to other respiratory agents, especially parainfluenza.

The guinea pigs are inoculated intranasally with 1 ml of freshly harvested infected allantoic fluid. For the preparation of an influenza A CF antiserum, the animals are inoculated with a strain as unrelated as possible to those expected, e.g., A/Equine or A/PR/8/34. For influenza B CF antiserum, influenza B/Lee/40 can be used. In 4 weeks, the animals receive a booster of 1 ml of tissue culture passaged virus by the intraperitoneal route. For influenza A, the cell cultures can be infected with A/Swine/1976/31 strain; for influenza B with B/Maryland/1/59 or B/Canada/66. The infected tissue culture fluid is shaken gently with 10% packed fowl erythrocytes, allowed to stand for 1 hour at room temperature, and the RBC then precipitated by centrifugation at 1800 rpm for 10 minutes. The RBC are resuspended in the original volume of physiological saline, incubated at 37°C for 2 hours, and, after removal of the erythrocytes by low-speed centrifugation, the supernatant fluid is used as inoculum. In 10 days the animals are bled and the individual serum pools tested for potency and specificity. Satisfactory pools can be combined.

An alternative procedure for the preparation of the booster dose is to use purified S antigen, prepared as described above, by treating the virus suspension with ether and then adsorbing residual hemagglutinin with fowl erythrocytes.

A method for the production of influenza CF antibody in mouse ascitic fluid was described by White *et al.* (1975). The general procedure is similar to that described for the preparation of arbovirus antiserum, involving the intraperitoneal injection of 0.5 ml of a 50–50 mixture of purified S antigen and Freund's complete adjuvant at weekly intervals for 4 weeks. On the thirty-fifth day, the mice are injected intraperitoneally with 0.2 ml of 10% suspension of Sarcoma 180/TG cells in 10% glycerol-saline. The ascitic fluid is tapped about 14 days later and additional fluid is removed daily for as long as it continues to accumulate. The individual fluid pools are tested for potency and specificity in the CF test. By this procedure fluid pools with a CF titer of 1:128–1:256 were obtained.

X. PARAMYXOVIRUSES (PARAINFLUENZA)

The paramyxoviruses exhibit basic characteristics of the orthomyxovirus group, such as medium size, chemical composition, and biochemical

properties. In actual size they are somewhat larger than the influenza viruses, frequently up to 200 nm, and the inner ribonucleoprotein core is also larger. As with the influenza viruses there is an outer envelope with spikelike projections, associated with the hemagglutinating property of these viruses. The infectivity is ether-sensitive, reflecting the lipoprotein nature of the outer envelope. The inner core contains ribonucleoprotein in a helical structure.

As with the influenza viruses, these viruses also give rise to both soluble and viral antigens. A soluble CF antigen which does not cause hemagglutination can, in this case also, be prepared by ether treatment of the virus suspension (Cook *et al.*, 1959), followed by removal of hemagglutinins by erythrocyte adsorption. This antigen is also probably associated with the inner core. These soluble antigens are type-specific. The viral (HA) antigen is associated with the outer envelope of the virion, from which it can also be released by ether treatment.

At the present time, four distinct types have been recognized and type 4 contains two subtypes, namely, A and B. Although these types share some antigenic properties and have antigenic similarities, they are distinct from the antigens of the influenza viruses. They can be differentiated on the basis of serological procedures. At one time the differentiation was not always very definite but, as improved methods have been developed for preparing the antigens, the differences have become more distinct. The virus neutralization test and the hemadsorption inhibition test will distinguish all four types and also subtypes 4A and 4B; the CF test will differentiate between the four types but not between the subtypes.

While, in general, the parainfluenza viruses can be propagated in a number of cell cultures from primary to diploid to heteroploid, there are a number of variations in sensitivity with the four types. Primary rhesus monkey kidney cells are satisfactory for the growth of the four types. Types 1, 2, and 3 have been adapted to a number of cell lines, but type 4 has presented more difficulty.

A. Preparation of Routine Diagnostic Antigen

Unpurified antigens for the four types are prepared in a number of laboratories using stationary or roller bottle cultures of primary rhesus monkey kidney cells. As soon as the cell sheet is confluent, the medium is removed and replaced with fresh medium containing about 1000 $TCID_{50}$/ml of seed virus stock. When the CPE is complete (or extensive by hemadsorption on a small flask sample), and this varies with the adaptation of the virus, the cultures are frozen and thawed and the mixture ultrasonicated at maximum amplitude for 5 minutes at 0°C. If required, es-

pecially with type 4, the antigen can be concentrated by centrifugation at about 80,000 *g* for 1 hour, followed by resuspension in $\frac{1}{20}$ the previous volume, or by ultrafiltration. After clarification by centrifugation at 3000 rpm for 5 minutes, the supernatant fluids are used as diagnostic antigens in both the HI and CF tests. It should be noted that with type 4 it is especially difficult to prepare a completely satisfactory HA antigen.

In this laboratory, partially purified antigens are prepared in the noninfective lyophilized state. For parainfluenza type 1, primary rhesus monkey kidney cell cultures are prepared as monolayer cultures, either in flasks or roller bottles. The maintenance medium is M199 plus 10% newborn calf serum. When the cell sheet is confluent, the medium is replaced with fresh medium containing 1000 $TCID_{50}$/ml of seed virus stock but no serum. The cultures are incubated at 35°C and small flask samples are checked daily to determine the time of maximum hemadsorption, at which time the cultures are frozen and thawed, the fluids pooled, and then irradiated with 1.5 Mrad of γ-radiation. The mixture is clarified by centrifugation at 3000 rpm for 5 minutes; the supernatant fluid is concentrated about thirtyfold by ultrafiltration using a Diaflo PM-30 membrane and then recombined with the cellular precipitate. This mixture is ultrasonicated for 5 minutes at 0°C under maximum power, clarified by low-speed centrifugation, and the supernatant fluid fractionated by gel permeation chromatography using Sepharose 6B, as described for adenovirus antigen. The fractions showing CF activity are pooled, reconcentrated to desired titer, and then lyophilized after the addition of 1% bovine albumin.

For the preparation of the type 2 and type 3 antigens, the procedure is similar except that Vero cell cultures are used and usually CPE is quite definite.

B. Antiserum

Antisera for use in serological tests for the assay of parainfluenza antigens can be prepared in horses, rabbits, and guinea pigs. The inoculum is freshly harvested infectious tissue culture fluid.

For the preparation of antiserum in horses, a preimmunization sample of blood is first obtained and then the animal is inoculated intravenously with 30–40 ml of the antigen. The inoculations are repeated at weekly intervals for 6 weeks and the animals are bled 1 week later. These sera are tested serologically for potency and specificity. Satisfactory neutralization titers are usually obtained. Titers of 1:160 are typical for the HI antibody level but results by CF are frequently variable and less reliable.

Rabbits can be used for the preparation of antisera which may be satis-

factory for use in neutralization and HI tests but have variable specificity when tested by CF. After obtaining a preimmunization blood sample, the animals are injected with 1.0 ml of inoculum by the intravenous route. These inoculations are repeated three times at intervals of 4 days and the animals are bled 10 days after the final inoculation.

Antisera of the greatest specificity, especially for use in the CF test, are obtained by the inoculation of guinea pigs. The individual animals must be tested prior to the inoculation procedure to select those free of interfering antibodies. Each animal is given 0.5 ml of inoculum intranasally under light ether anesthesia. A trial bleed is performed after 3 weeks and, if the antibody levels are satisfactory, the animals are exsanguinated. The sera are tested individually for potency and specificity and those found to be satisfactory are pooled. If required, a second intranasal inoculation can be given at this time and the animals bled 3 weeks later. These sera react in the CF test with both virion and soluble antigens and are usually quite specific, clearly differentiating between the other parainfluenza types, mumps and influenza.

XI. MUMPS

As with vaccinia and influenza viruses, the studies on mumps progressed with advances in required technology, involving filtration, improved centrifugation, and electron microscopy. As with influenza, there is an outer envelope with which is associated the viral (V) antigen and its properties of hemagglutination, infectivity, and immunization. The inner core contains ribonucleoprotein in a helical structure and from this is derived the soluble (S) antigen.

The diagnosis of mumps is usually made on a clinical basis but at times the diagnosis may be complicated by the involvement of other organs such as the gonads, pancreas, and the central nervous system. In these cases the laboratory serological procedures are usually very useful in determining the possible role of mumps virus in the illness. The approach may involve isolation and identification of the virus or serodiagnosis using both the S and V antigens. For the isolation of the virus the specimen may be inoculated into chick embryos or various cell cultures, such as primary rhesus monkey kidney or chick embryo, and the infected fluids used as antigens in serological tests with known specific antisera.

For the laboratory diagnosis of mumps, serological methods rather than virus isolation and identification, are usually used. Both the CF and HI tests are employed but the CF test is more useful since it is not influenced by the presence of nonspecific inhibitors of concern in the HI method.

More especially, however, with the CF test there is the capacity for an early diagnosis using the S and V antigens on an early sample of serum. It was shown by Henle *et al.* (1948) that the S antibodies are usually detected first, possibly 2–3 days after the onset of illness; the V antibodies can be demonstrated a few days later. Thus, the finding of S antibodies alone may suggest an early stage of mumps infection but a later serum sample should also be tested for confirmation by showing developing and rising antibody titers to both antigens.

A. Preparation of Routine Diagnostic Antigens

A soluble antigen for use in the CF test is prepared by extracting the specifically infected chorioallantoic membranes. As with influenza, this antigen may be used directly after centrifugation at 20,000 rpm for 1 hour but a stock lyophilized noninfective antigen is prepared in this laboratory for routine use, as described above.

Although not in general or routine use, the HI test can also be used and for this purpose a V antigen is prepared from the infected allantoic fluid by differential centrifugation, after which it can be inactivated and lyophilized, as described above for influenza.

B. Antiserum

Human convalescent serum for use in the CF test to assay or identify antigen is usually readily available in the diagnostic laboratory from serum samples received. Sera which are relatively free of heterologous antibodies are selected and pooled and then standardized against a reference antigen. Such serum is also readily available commercially. Specific CF antisera can be prepared in hamsters and guinea pigs.

With hamsters, young adult animals are inoculated intranasally with 0.3 ml of freshly harvested infectious allantoic fluid, usually with the animals under light ether anesthesia. In 16–18 days the animals are exsanguinated by cardiac puncture and the bloods are pooled. The serum obtained is tested by CF for potency and specificity. This serum usually has a titer of about 1:32 to 1:64 with soluble (S) antigen, but it is lower with the V antigen.

Guinea pigs can also be used but each animal must be tested for the presence of interfering heterologous antibodies, especially to parainfluenza. An anti-S antiserum can be prepared by the intranasal inoculation of 1.0 ml of freshly harvested infectious allantoic fluid, usually with the animals under light ether anesthesia. The animals are bled in 14–16 days. A serum containing both anti-S and anti-V antibodies can be pre-

pared by inoculating the animals with 0.5 ml intranasally plus 1.0 ml intraperitoneally. After 1 week the intraperitoneal injection is repeated and the animals are bled 10–12 days later. A serum containing mostly anti-V antibodies can be prepared by inoculating the animals with 1.0 ml of inactivated viral antigen, repeating the inoculation 1 week later. The animals are bled in 2 weeks.

XII. MEASLES

The routine propagation of measles virus, leading subsequently to the preparation of diagnostic antigens and vaccines, has only been possible for about 20 years since Enders and Peebles (1954) succeeded in propagating it in tissue culture systems. For purposes of isolation and initial passages, human amnion and primary monkey kidney cell cultures are especially susceptible, but the virus can soon be adapted to various continuous cell lines.

The size of the measles viruses in cell cultures has been reported as varying between 120 and 230 nm. The virion contains an outer envelope which encloses the internal nucleocapsid and the helical ribonucleic acid. The envelope of the virus consists of a lipid membrane covered by a layer of projections which are composed of glycoproteins and these in turn are associated with the hemagglutinating capacity. Although in these regards the measles virus has similarities to other members of the myxovirus group, only one major type is known to date. This fact has greatly simplified the preparation of diagnostic antigens and vaccines. Here also, there is a CF or soluble antigen associated with the inner core (Numazaki and Karzon, 1966) while the whole virion or outer envelope is associated with hemagglutination. The hemagglutinin is only demonstrated, however, with certain erythrocytes, notably those of the monkey. The hemagglutination is temperature-dependent, being optimal at 37°C, but, unlike influenza viruses, elution does not follow.

Antibodies against measles virus can be detected by various methods such as virus neutralization, complement fixation, immunofluorescence, and hemagglutination inhibition. The HI test can be performed using the intact virions as antigen or the virions can be disrupted by treatment with Tween 80-ether (Norrby, 1962), which is reported to give higher HI titers.

With measles there is a greater correlation of titers obtained by CF, HI, and neutralization than is the case with numerous other viral infections. The CF and HI antibodies appear at about the same time, although the HI test may detect them sooner because of a greater sensitivity. Attenuated measles vaccine stimulates the formation of CF antibodies, presumably

against the ribonucleoprotein or S antigen and also HI antibodies against the hemagglutinin or envelope component. If the virions of the measles vaccine are split by Tween-80 ether treatment, the CF response is greatly reduced (Enders-Ruckle, 1967). In nearly all cases of measles tested, the split antigen gave HI titers which were 4- to 8-fold higher than obtained with the usual virion antigen and this factor was still greater in some cases of subacute sclerosing panencephalitis (Thiry *et al.*, 1969).

For the past several years there has been an increasing interest in the relationship and significance of antibodies to measles virus in the sera of some patients with multiple sclerosis. The cerebrospinal fluids (CSF) of these patients may contain measles antibodies to unusually high titers. Norrby *et al.* (1974) using different measles virus components as antigens, tested serum and CSF from multiple sclerosis patients and reported that there was a reduced ratio of antibodies in serum to CSF in some patients. This suggests that measles antibodies were being produced in the central nervous system.

When measles virus suspensions are extracted with ether there is a partial disruption of the virion with the release of a smaller particle hemagglutinin. When ether was used alone, Norrby (1962) found that there was not the expected increase in HA titer. However, by extracting the virus suspension first with 0.125% Tween-80 in the cold for a few minutes, followed by extraction with one-half volume of ether in the cold, there was an increase of almost fourfold in the HA titer of the virus suspension. Of equal significance was the fact that this antigen showed increased sensitivity in the HI test.

A. Preparation of Routine Diagnostic Antigens

For the serodiagnosis of measles, antigens can be prepared for use in the HI and CF tests. Most work in the past has been conducted using a whole virion antigen which is serologically active in both the CF and HI tests. Usually this antigen has been prepared simply by freezing and thawing the infected cell cultures, followed by clarification of the resulting suspension by centrifugation. In some cases this antigen has been treated with Tween-80 ether.

Unpurified diagnostic antigens can be prepared by inoculating stationary monolayer cultures of primary human amnion or BSC-1 cells with the seed virus stock at a multiplicity of between 1 and 10. After incubation at 35°C for 2 days, the fluid is harvested and replaced with fresh medium containing 5% inactivated chicken serum. The HA titer of the fluid is determined twice daily and when it reaches a peak, the fluid is harvested, stored at 4°C, and the medium is replaced. This process is repeated until

cell degeneration has become extensive, at which time the final harvest of fluid plus cells is obtained. The various pools are clarified by centrifugation at 3000 rpm for 5 minutes, after which they are assayed for HA and CF content and then combined to include the maximum content of whichever antigen is desired. For HA antigen, the pool can be extracted with Tween-80 ether for a possible increase in titer, as described above. One problem with this procedure is the fact that the serum content poses a serious restriction on concentration by ultrafiltration or by the hydrophilic agent method.

The method currently used in this laboratory for the preparation of these antigens involves inoculation of approximately 20 roller bottles (690 cm^2) of BSC-1 cell cultures with 1000 $TCID_{50}$/ml of seed virus stock. When the CPE is complete, the bottles are placed in a high-energy ultrasonic bath to remove the remaining cell sheet and to prepare a uniform cell suspension. The cell suspensions are pooled and irradiated with 0.8 Mrads of γ-irradiation, after which the mixture is clarified at 5000 rpm for 5 minutes. The cellular material is stored at 4°C while the supernatant fluid is concentrated to about 50 ml by ultrafiltration using a Diaflo cell with a PM-30 membrane. The concentrated fluid is then added to the cellular material; the mixture is ultrasonicated at maximum amplitude for 5 minutes and then clarified by centrifugation at 10,000 *g* for 5 minutes. The two major antigen components, as identified by CF, are separated by gel filtration using Sepharose 6B, as described above for adenovirus antigen. Two antigenic peaks are obtained here also but in this case the first peak (A) contains only the remaining CF antigen. These peaks are selected, processed, and lyophilized as described for adenovirus antigen.

B. Antiserum

Antiserum for measles is seldom prepared in the diagnostic laboratory since human positive serum is usually readily available or can be obtained commercially. However, it can be prepared in young adult rhesus monkeys. For inoculum, the seed virus is propagated in primary rhesus monkey kidney or BSC-1 cell cultures and harvested as in the preparation of diagnostic antigen. The freshly harvested, clarified infectious fluid in the amount of 2 ml is inoculated intravenously into each animal and the inoculation repeated in 1 week. After a further 10 days the animals are trial-bled and the individual sera assessed in the HI and CF tests. If required, a further inoculation can be administered with the final bleeding 10 days later.

XIII. RESPIRATORY SYNCYTIAL VIRUS

This virus was isolated from chimpanzees with apparent respiratory infection (Morris *et al.*, 1956) and later it was also shown to be a pathogen of humans. The "syncytial" part of the name stems from the type of pathology observed in cell cultures (Chanock *et al.*, 1957). While it has properties of size and structure which resemble a paramyxovirus, it does not cause hemagglutination, at least not under presently known conditions. Although the size of the particles varies greatly, the general structure includes an outer envelope of significant lipid content and surface projections and an inner component of a filamentous nature. This virus is relatively heat-labile, as is the case with cytomegalovirus, both being inactivated relatively rapidly at 37°C. Ribonucleic acid is contained in the inner component.

Although in the virus neutralization test there is some individual specificity of strains, in the CF test the strains appear to share a common antigen. The virus can be propagated in continuous cell lines such as Hep-2 and in human diploid fibroblast cells such as the WI-38 line. Although some virus appears in the fluid after propagation, a significant proportion remains cell-associated. The CF antigen in infected cellular fluid can be separated from the virus particles indicating a soluble antigen. If the cell culture fluid is fractionated by gel permeation chromatography using Sepharose 4B or 6B, the eluting fractions can be monitored for antigen content by CF only, since RSV does not produce hemagglutination. Two antigen peaks are obtained; the first peak of high molecular weight materials contains infectious virus and CF activity, presumably representing the virion antigen, while a second peak of lower molecular weight substance elutes later and possesses only CF activity.

As the virus propagates in the cells, virus begins to be released into the culture fluid and also a CF antigen which can be separated from the virus particle by centrifugation at approximately 100,000 *g* for 1 hour.

RSV is a major cause of respiratory illness in infants, frequently requiring hospitalization; in adults the illness is less severe. For serological diagnosis the neutralization test is the one of choice in infants but the CF test can be used in older children and adults.

For the preparation of a CF antigen, stationary monolayer cultures of Hep-2 or Vero cells are inoculated by replacing the growth medium with fresh medium containing 2% inactivated fowl serum and 1000 $TCID_{50}$/ml of seed virus stock. When the CPE is almost complete, the cultures are frozen and thawed, the pooled fluids are clarified by centrifugation at 3000 rpm for 5 minutes, and the supernatant fluid used as the antigen.

In this laboratory a noninfective lyophilized diagnostic antigen is prepared by the inoculation of either roller bottle or stationary monolayer Vero cell cultures with 1000 $TCID_{50}$/ml of seed virus stock. When the CPE is almost complete the cultures are frozen and thawed or ultrasonicated and the pooled suspensions irradiated with 0.8 Mrad of γ-radiation. After clarification by centrifugation for 5 minutes at 5000 rpm, the cellular material is stored at 4°C while the supernatant fluid is concentrated to about 50 ml by ultrafiltration using a Diaflo PM-30 membrane. The concentrated fluid is then added to the cellular material, the mixture ultrasonicated at maximum amplitude for 5 minutes, and then clarified by centrifugation at 5000 rpm for 5 minutes. The supernatant fluid is then partially purified by gel filtration using Sepharose 6B, as described for adenovirus antigen. Two peaks of CF activity are obtained and these fluids are pooled, reconcentrated to desired titer, and then lyophilized after the addition of 1% bovine albumin as stabilizing agent.

Antiserum

For the preparation of reference antiserum for use in the CF and neutralization tests, ferrets and guinea pigs can be used. The inoculum used is usually virus suspension freshly harvested from Hep-2, WI-38, or Vero cell cultures. If guinea pigs are used, it is necessary to select animals free of paramyxovirus antibodies.

The procedure used in this laboratory is to inoculate each animal intranasally with 0.5 ml of freshly harvested, clarified infectious Vero tissue culture fluid, usually obtained at the time of preparing the diagnostic antigen. The intranasal inoculation is repeated after 3 weeks; 3 weeks later, a trial bleeding is obtained. If the antibody level is satisfactory, usually 1:32–1:64, the animals are bled at once; if necessary, the inoculation can be repeated. The individual sera are tested for potency and specificity by CF and satisfactory sera are pooled and lyophilized.

XIV. ARBOVIRUSES

The arbovirus classification contains a widely divergent group of viruses which have been grouped together on the general basis that they are maintained in nature through a cycle from an arthropod vector to a vertebrate. When the virus is multiplying in the vertebrate a viremia is produced and this blood is infectious to other arthropod vectors when the vertebrate is used for a blood meal. Man is not an essential link in the maintenance and propagation of arboviruses but is rather an incidental

and accidental victim in the biological chain. Many viruses have been included in this group primarily on the basis of serological studies which have shown similarities to other known or accepted members of the groups. Some, such as Junin and Machupo, have already been removed to the arenavirus group. Further reclassifications will doubtless occur as more information is collected on the over 200 present members of the arboviruses.

With so many viruses being included in this group, it is not surprising that they present a variety of chemical and physical properties. Although the majority are relatively small, 20–60 nm, a few are considerably larger, up to 180 nm. They share the properties of possessing a lipid envelope with a central core of ribonucleoprotein. The fact that they are relatively heat-labile and sensitive to numerous solvents has complicated procedures for the preparation of satisfactory diagnostic antigens.

For diagnostic purposes the neutralization, complement fixation, and hemagglutination inhibition tests are used. The most widely used procedure is the CF and its value has increased with improved methods for the preparation of purified diagnostic antigens. In addition, satisfactory CF antigens can be prepared in cases where it is difficult or impossible to prepare an equally efficient HA antigen. The arboviruses can be propagated in a variety of tissues or cells and with varying results. The suckling mouse is still probably the most widely used and reliable host tissue, although the chick embryo and cell cultures such as Vero, BHK-21, and chick embryo are also used.

A. Preparation of Routine HA Antigens

As with many other classes of viruses, these antigens are basically suspensions of the virus in varying states of purity. As with the myxoviruses, many sera, both normal and those containing the antibodies in question, also contain nonspecific inhibitors of hemagglutination. These inhibitors are usually of the lipid and lipoprotein type. Extraction of the sera is therefore required to remove such inhibitors, but unfortunately it frequently also produces some loss of titer, a critical factor in the event of a low natural antibody level.

The most common tissue in use for the preparation of HA antigens is suckling mouse brain which has the advantage of possessing lower lipid content than that found in older animals. Most procedures used today are based on the sucrose-acetone extraction method developed by Clarke and Casals (1958) with relatively minor modifications. These antigens can be usually used in both the HA and CF tests.

1. Suckling mice 2–3 days old are inoculated intracerebrally with

0.02 ml of stock seed virus diluted 1:100–1:1000 in phosphate-buffered saline containing 1% bovine albumin plus 200 units of penicillin and 200 μg of streptomycin sulfate/milliliter.

2. When the mice are sick and beginning to die, the infected brains are removed, usually by aspiration, into a tube in an alcohol–Dry Ice bath.

3. To this brain material is added four volumes of 8.5% sucrose at 4°C; the mixture is homogenized and then added quickly to 20 volumes of acetone at −20°C.

4. After thorough shaking, the supernatant fluid is removed and the acetone extraction of the precipitate is repeated. The supernatant fluid is removed and the precipitate dried at 0°C under vacuum.

5. The dried powder is resuspended in physiological saline to a volume of about one-tenth of the original homogenate volume, clarified by centrifugation at 10,000 *g* for 1 hour at 4°C, and is then assayed by HA and/or CF. The suspension is diluted to desired titer and then lyophilized after the addition of 0.5% bovine albumin.

While the suckling mouse brain is probably still the most widely used tissue for the preparation of arbovirus antigens, especially for use in the HI test, cell cultures are also used. Schmidt and Lennette (1971) made a detailed study of the preparation of serological antigens from infected cultures and found that, in general, for the release of CF and HA antigen from infected cells, freezing and thawing or sonication were the procedures of choice rather than alkaline extraction. Although satisfactory arbovirus CF antigens could be prepared in a number of cases, the HA activity was minimal. The best yield of antigen was obtained when the cultures were infected with a high concentration of virus, the multiplicity being greater than one. Of particular interest also was the observation that a high titer of a CF antigen did not necessarily indicate an equally high capacity to detect antibody.

A method has been described for the preparation of a noninfective lyophilized diagnostic antigen for western equine encephalomyelitis (W.E.E.) using the chick embryo (Polley and Guérin, 1968). By a modified procedure this antigen is prepared by inoculating 10-day-old chick embryos by the allantoic route with a 10^{-3} dilution of infected allantoic fluid. After incubation at 35°C for 2 days, the extraembryonic fluids are harvested from dead embryos, the pooled fluids irradiated with 2.0 Mrads of γ-radiation, and then concentrated approximately twentyfold by ultrafiltration using a PM-30 membrane in a Diaflo cell (Amicon). After clarification by centrifugation at 5000 rpm for 5 minutes, the supernatant fluid is assayed by CF and/or HA, diluted to desired titer, and lyophilized. This antigen can be used in both the CF and HI procedures.

More recently, the above method was modified by the use of chick embryo cell cultures (Polley and Guérin, 1969). The method involves the use of stationary monolayer cultures of chick embryo cells infected by the replacement of the growth medium with infected allantoic fluid seed virus stock ($TCID_{50} > 10^{-6}$) diluted 1:5000 in medium 199. When the CPE is complete, usually after incubation for 3 to 4 days at 37°C, the cultures are frozen and thawed, the fluids are pooled, and then irradiated with 1.5 Mrads of γ-radiation. The irradiated fluid is concentrated twentyfold by ultrafiltration using a PM-30 membrane in a Diaflo cell. After the addition of 1% bovine albumin or 5% peptone for stabilization, the antigen is lyophilized. This antigen usually has a CF titer of about 1:64, is sensitive for the detection of antibodies of W.E.E. virus, but the HA activity is usually low.

B. Antiserum

Specific antisera can be produced in a number of animal species, the most common being guinea pigs, suckling or weanling mice, and horses.

For the preparation in mice, the inoculum is a 10% suspension in phosphate-buffered saline of suckling mouse brain infected with the specific seed virus. This suspension in the amount of 0.2 ml is inoculated by the intraperitoneal route into female mice of about 5 weeks of age. If the inoculum causes early death of the mice due to pathogenicity of the virus, it is necessary to use virus which has been completely or largely inactivated for at least the first inoculation. This can be achieved by treatment with 0.1% β-propiolactone at 4°C at pH 7 or by 1.0 Mrad of γ-radiation. The inoculations are repeated at weekly intervals for 4 to 5 weeks and the animals then bled 1 week after the last inoculation. The serum, if found to be satisfactory for potency and specificity in the HI and/or neutralization test, can then be lyophilized for stable storage.

Larger volumes of antibody per mouse can be obtained by using the immune ascitic fluid technique. In this procedure the virus inoculum is mixed with an adjuvant and/or an ascites cell line such as Sarcoma 180/TG. On the first day, the female mice 5–6 weeks old are inoculated with a mixture of equal parts of 10% infected suckling mouse brain suspension and Freund's complete adjuvant; each animal receives 0.2 ml by the intraperitoneal route. These inoculations are repeated at weekly intervals for 3 weeks. Three days later each mouse is inoculated with 0.2 ml of fresh Sarcoma 180/TG ascitic fluid. One week later each animal receives 0.5 ml of the same inoculum as day 1. In about 10 days, the abdomens appear larger and fluid can be removed with a syringe, the process being repeated on successive days as long as fluid is produced.

Each pool is tested by the designated procedure (CF, HI, or neutralization), and satisfactory pools are combined and stored frozen or lyophilized. If the particular virus used is highly pathogenic for mice, it is necessary to use inactivated virus for the first one or two inoculations.

Antiserum for use in the CF test can be prepared with variable success in guinea pigs. If possible, the inoculum is infected tissue culture fluid obtained by passaging the virus in BHK-21 or Vero cell cultures; infected allantoic fluid or 10% suspensions of infected guinea pig brain tissue can be also used. A preimmunization bleeding is obtained from each animal, after which each animal receives an intraperitoneal inoculation of 0.5 ml of seed virus inoculum diluted in saline to contain 1000 ID_{50}/ml. Subsequently, at weekly intervals for 6 weeks each animal receives 0.1 ml of the same inoculum by the intravenous route. One week later, the animals are bled and the serum tested for potency and specificity.

For the preparation of antiserum in horses, specifically infected tissue culture fluid can be used, the first inoculation consisting of 10 ml subcutaneously and 40 ml intravenously. The subcutaneous doses are repeated at weekly intervals for two inoculations, with a final inoculation 1 week later with 40 ml by the intravenous route. Two weeks after the final inoculation the animals are bled and the sera tested for potency and specificity.

XV. RUBELLA

Rubella virus is the causative agent of, in most cases, a relatively minor infection in children and young adults. Under ordinary conditions it may not be considered to be highly infectious and many people reach adult life without being infected. However, in crowded situations the infection can spread rapidly and show a high attack rate (Buescher, 1965). From serological studies it is known that there are large numbers of adults without immunity and immunological status varies in different areas (Schiff and Sever, 1966). These facts would be of relatively little importance in what is usually a minor illness of childhood except that there has developed an increasing awareness and concern with the fact that in the early state of pregnancy it is teratogenic. While in an epidemic the probability of an accurate clinical diagnosis of rubella is high; in isolated cases this is clinically more difficult. Laboratory serological procedures are of great importance in rubella. In the case of current infection the tests can implicate rubella virus as the causative agent. In pregnant women the serological tests can serve to differentiate between a primary infection and a case of reinfection, which is of less serious impact. The diagnostic procedures can also be applied in serological and epidemiological surveys to assess the

so-called immune status of the population, probably in practical terms an indication of resistance to reinfection, since reinfections do occur. Furthermore, the diagnostic laboratory can assist in assessing the antibody response to vaccine in selected groups or surveys.

The size of the virus has been reported variously between 50–200 nm but it probably is in the 60–70 nm range (Best *et al.*, 1969). Because the infectivity is readily destroyed by ether, it is assumed that lipid is a significant component of the outer envelope. The inner core contains ribonucleoprotein. The virus causes hemagglutination and there are two CF antigens associated with it. The one CF antigen, probably associated directly with the virion, can be sedimented by centrifugation and the infectivity associated with it is destroyed by ether. This CF antigen also appears in the high molecular weight or void volume peak on gel permeation chromatography using Sepharose 4B. The second CF antigen is of the soluble type, remaining in the supernatant fluid after centrifugation at 10,000 *g* for 1 hour and appearing as a lower molecular weight material on gel permeation chromatography. Because of these various similarities to a myxovirus, it was at one time considered to be a member of this group. However, rubella virus does not attack mucoproteins in a similar manner.

In primary infections, antibodies as measured by the neutralization, complement fixation, and hemagglutination inhibition tests, are present in both the IgM and IgG immunoglobulin fractions. In the IgM fraction they arise within 1 to 2 days after the appearance of the rash, reach a peak within 1 to 2 weeks, and then decline relatively rapidly, usually disappearing within an additional 2 weeks. The antibodies in the IgG fraction appear 2 to 3 days after those in the IgM fraction but reach higher levels and persist longer; in the case of HI and neutralizing antibodies this may be for life, even if at low levels.

It is generally considered that the HI antibodies appear first, during the period of the rash, followed closely by the neutralizing antibodies. The HI antibody appears first in the IgM fraction, followed in 2 to 3 days by its appearance in the IgG fraction. The peak is usually reached about a month after the appearance of the rash and then slowly declines but usually remains at detectable levels for many years.

In general, the appearance and persistence of the neutralizing antibody levels parallel that of the HI, although at somewhat lower levels.

The CF antibodies appear about 2 weeks after the onset of the rash, reach a peak in about 6 weeks, and then slowly decline, usually disappearing after 3 or 4 years.

In rubella, the differentiation of antibodies in IgM and IgG immunoglobulin fractions is of special importance. In the IgG fraction, rubella antibodies arise as a result of primary infection, reinfection, and administra-

tion of vaccine. On the other hand, they do not arise to a significant extent in cases of reinfection. Thus, the presence of significant levels of rubella antibodies in the IgM fraction of serum from a pregnant woman in the first trimester presents the serious problem attending a primary infection.

With rubella, as with other viral infections, the significance of serological tests is increased when there is a demonstration of an antibody increase in a convalescent phase serum sample. However, in this case, the results on a single specimen can be significant if related to the presence of antibodies in the IgM fraction. If treatment of the single serum sample with 2-mercaptoethanol causes a significant decrease in the HI titer, it is considered to be a component largely of the IgM fraction (Cooper *et al.*, 1969) and so suggestive of primary infection. While the HI test is the one in most extensive use in routine serology, the CF test can also serve a useful purpose. The HI titers rise rapidly and if the levels are high in two successive serum samples it is difficult to assess the complete significance. However, the CF titer rises more slowly and it may be more readily possible to demonstrate a diagnostically significant change. Unfortunately, although satisfactory HA antigens can be produced in the laboratory or obtained commercially, the routine preparation of a satisfactory CF antigen has proved to be much more difficult and is seldom readily available commercially.

Production of Routine Diagnostic Antigen

In the laboratory, partially purified lyophilized diagnostic antigens for rubella are prepared from specifically infected Vero cell cultures. Approximately 30 roller bottles (690 cm^2) of Vero cell cultures are prepared using medium M199 + 10% newborn calf serum, with incubation at 35°C. When the cell sheet is complete, the medium is decanted from each bottle and the cell sheet washed once with 50 ml of medium to remove residual serum. Then 10 ml of freshly passaged seed virus, HPV strain, is added and adsorption is allowed to proceed with rolling for 2 hours, after which 65 ml of medium without serum is added. After 3 days, the medium is decanted and discarded, being replaced with 50 ml of fresh medium. Two days later the cells and fluid are harvested by rapidly rotating the bottles in a 50-liter ultrasonic tank (Forma) for about 20 seconds. The fluids are pooled and the virus inactivated with 1.0 Mrad of γ-radiation. The suspension is then clarified by centrifugation at 3000 rpm for 5 minutes; the supernatant fluid is concentrated approximately thirtyfold by ultrafiltration at 4°C using a Diaflo cell with a PM-30 membrane. The concentrated fluid is recombined with the sedimented cells and the mixture subjected to ultrasonication for 1 minute. The suspension is clarified

by centrifugation at 10,000 rpm for 5 minutes. The supernatant fluid is fractionated by gel filtration using a column packed with Sepharose 4B. All fractions in the void volume peak are recombined, reconcentrated to desired titer, and then lyophilized after the addition of 1% bovine albumin. A typical antigen has an HA titer = 1:160 and a CF titer = 1:32 in a volume of 400 ml.

XVI. ROTAVIRUSES (INFANTILE GASTROENTERITIS VIRUS)

Until recently the causative agent of nonbacterial infantile gastroenteritis escaped isolation and identification. Then Kapikian *et al.* (1972) identified a small particle by immune electron microscopy as the probable causative agent. In addition, antibody to this viral agent was demonstrated in a convalescent phase sera. These virus particles, which possess similarities to the reoviruses, were found to be similar to the viral agent causing acute diarrhea in newborn calves by Flewett *et al.* (1974) who suggested the name "rotaviruses." They have also been variously called reo-like, duoviruses, and orbi; of these, orbi is in the more common use at present. Later, Kapikian *et al.* (1975) described the development of a diagnostic complement fixation test, based on the use of Nebraska calf diarrhea virus (NCDV) as antigen.

For the preparation of antigen, the NCDV was passaged in primary bovine embryonic kidney cell cultures. Six days after inoculation, when the CPE was nearly complete, the cultures were frozen and thawed. The mixture was concentrated 25-fold by ultrafiltration using a Diaflo XM-100A membrane. This fluid concentrate was used as the CF antigen. The antiserum used was obtained from an NCDV convalescent calf.

Antigens for use in the CF test have also been prepared and reported by Spence *et al.* (1975), using 2% stool filtrates from stool specimens from infected human patients and shown to be positive by electron microscopy. It was found that this CF test appeared to be as sensitive as electron microscopy for detection of rotavirus in stools and false positive reactions were not encountered. In addition, the diagnosis was possible in the acute stage of illness.

More purified antigens have also been prepared (Petric *et al.*, 1975; Middleton *et al.*, 1975) starting from pooled diaper extracts which were clarified by centrifugation at 5000 rpm for 10 minutes, followed by centrifugation at 25,000 rpm for 2 hours. The precipitate was resuspended in one-fortieth of the previous volume of phosphate-buffered saline, then extracted with one-quarter volume of Freon 113. This partially purified antigen could be purified further if desired by density gradient centrifu-

gation using CsCl of starting density 1.36 g/ml. Before use, the purified virus suspension was precipitated once again by centrifugation and resuspended in phosphate-buffered saline.

REFERENCES

Beard, C. W. (1970). *Bull. W. H. O.* **42,** 779–785.

Beijerinck, M. (1898). *Verh. Akad. Wet. Amsterdam II* **6,** No. 5, 1–21.

Benyesh-Melnick, M., Vonka, V., Probstmeyer, F., and Wimberley, I. (1966). *J. Immunol.* **96,** 261–267.

Best, J. M., Banatvala, J. E., Almeida, J. D., and Waterson, A. P. (1967). *Lancet* **2,** 237—239.

Buescher, E. L. (1965). *Arch. Gesamte Virusforsch.* **16,** 470–476.

Casals, J. (1945). *Science* **102,** 618–619.

Chakraverty, P. (1971). *Bull. W. H. O.* **45,** 755–766.

Chakraverty, P. (1974). *J. Gen. Virol.* **25,** 421–425.

Chanock, R., Roizman, B., and Myers, R. (1957). *Am. J. Hyg.* **66,** 291–300.

Clarke, D. H. (1964). *Bull. W.H.O.* **31,** 45–56.

Clarke, D. H., and Casals J. (1958). *Am. J. Trop. Med. Hyg.* **7,** 561–673.

Cook, M. K., Andrews, B. E., Fox, H. H., Turner, H. C., James, W. D., and Chanock, R. M. (1959). *Am. J. Hyg.* **69,** 250–264.

Cooper, L. Z., Matters, B., Rosenblum, J. K., and Krugman, S. (1969). *J. Am. Med. Assoc.* **207,** 89–93.

Craigie, J. (1932). *Br. J. Exp. Pathol.* **13,** 259–268.

Crawford, L. V., and Lee, A. J. (1964). *Virology* **23,** 105–107.

Deibel, R., Smith, R., Clarke, L. M., Decker, W., and Jacobs, J. (1974). *N. Y. State J. Med.* **74,** 785–791.

Dowdle, W. R., Nahmias, A. J., Haswell, R. W., and Pauls, F. P. (1967). *J. Immunol.* **99,** 974–980.

Elford, W. J. (1931). *J. Pathol. Bacteriol.* **34,** 505–421.

Enders, J. F., and Peebles, T. C. (1954). *Proc. Soc. Exp. Biol. Med.* **86,** 277–286.

Enders-Ruckle, G. (1967). *Arch. Gesamte Virusforsch,* **22,** 23–34.

Flewett, T. H., Bryden, A. S., Davies, H., Woode, G. N., Bridger, J. C., and Derrick, J. M. (1974). *Lancet* **2,** 61–63.

Francis, J., Jr., and Salk, J. E. (1942). *Science* **96,** 499–500.

Fuccillo, D. A., Moder, F. L., Catalano, L. W. Vincent, M. M., and Sever, J. L. (1970). *Proc. Soc. Exp. Biol. Med.* **133,** 735–739.

Ginsberg, H. S. (1962). *Virology* **18,** 312–319.

Ginsberg, H. S., Pereira, H. G., Valentine, R. C., and Wilcox, W. C. (1966). *Virology* **28,** 782–783.

Grandien, M., and Norrby, E. (1975). *J. Gen. Virol.* **27,** 343–353.

Guerin, M. M., and Polley, J. (1970). *Can. J. Microbiol.* **16,** 53–56.

Hanshaw, J. B. (1966). *N. Engl. J. Med.* **275,** 476–479.

Hare, R., McClelland, L., and Morgan, J. (1942). *Can Public Health J.* **33,** 325–331.

Hartman, F. W., LoGrippo, G., and Kelly, A. R. (1954). *Am. J. Clin. Pathol.* **24,** 339–348.

Henle, G., Harris, S., and Henle, W. (1948). *J. Exp. Med.* **88,** 133–147.

Hierholzer, J. C. (1973). *J. Infect. Dis.* **128,** 541–550.

Hirst, G. K. (1941). *Science* **94,** 22–23.

Hirst, G. K. (1942). *J. Exp. Med.* **75,** 49–64.
Hoyle, L. (1952). *J. Hyg.* **50,** 229–245.
Hoyle, L., and Fairbrother, R. W. (1937). *J. Hyg.* **37,** 512–520.
Huang, Y.-T., Huang, E.-S., and Pagano, J. S. (1974). *J. Immunol.* **112,** 528–532.
Iwanowski, D. (1892). *Bull. Acad. Imp. Sci., St. Petersbourg* [N.S.] **3,** 67–70.
Jeansson, S. (1975). *Acta Pathol. Microbiol. Scand., Sect. B* **83,** 48–54.
Jordan, R. T., and Kempe, L. L. (1956). *Proc. Soc. Exp. Biol. Med.* **91,** 212–215.
Jung, M., Price, P. C., Kistler, G. S., and Krech, U. (1973). *Z. Immunitaetsforsch. Exp. Klin. Immunol.* **145,** 191–198.
Kapikian, A. Z., Almeida, J. D., and Stott, E. J. (1972). *J. Virol.* **10,** 142–146.
Kapikian, A. Z., Cline, W. L., Mebus, C. A., Wyatt, R. G., Kalica, A. R., James, H. D., Van Kirk, D., Chanock, R. M., and Kim, H. W. (1975). *Lancet* **1,** 1056–1061.
Kendall, F. E. (1937). *J. Clin. Invest.* **16,** 921–931.
Kimball, R. F. (1957). *Ann. Rev. Microbiol.* **11,** 199–220.
Kissling, R. E., Casey, H. L., and Palmer, E. L. (1968). *Appl. Microbiol.* **16,** 160–162.
Krech, U., and Jung, M. (1969). *Arch. Gesamte Virusforsch.* **28,** 248–250.
Krech, U., Jung, M., and Sonnabend, W. (1971). *Z. Immunitacts-forsch., Exp. Klin. Immunol.* **141,** 411–429.
Levy, H. B., and Sober, H. A. (1960). *Proc. Soc. Exp. Biol. Med.* **103,** 250–252.
Lieff, F. S. (1963). *J. Immunol.* **90,** 172–177.
Lieff, F. S., and Henle, W. (1956). *Virology* **2,** 753–771.
LoGrippo, G. A., and Hartman, F. W. (1955). *J. Immunol.* **75,** 123–128.
MacCallum, W. G., and Oppenheimer, E. H. (1922). *J. Am. Med. Assoc.* **78,** 410–411.
Mack, W. N., and Chotisen, A. (1956). *Proc. Soc. Exp. Biol. Med.* **91,** 288–290.
Martin, G. R., Warocquier, R., and Boulanger, P. A. (1975). *Int. Virol.* **5,** 162–172.
Middleton, P. J., Petric, M., Hewitt, C. M., Szymanski, M. T., and Tam, J. S. (1976). *J. Clin. Pathol.* **29,** 191–197.
Morris, J. A., Blaunt, R. E., Jr., and Savage, R. E. (1956). *Proc. Soc. Exp. Biol. Med.* **92,** 544–549.
Nahmias, A. J., Dowdle, W. R., Naib, Z. M., Josey, W. E., McLane, D. and Domescik, G. (1969). *Bull. J. Vener. Dis.* **45,** 294–298.
Norrby, E. (1962). *Proc. Soc. Exp. Biol. Med.* **111,** 814–818.
Norrby, E., Link, H., and Olsson, J. (1974). *Arch. Neurol.* **30,** 285–292.
Numazaki, Y., and Karzon, D. T. (1966). *J. Immunol.* **97,** 470–476.
Palmer, E. L., Martin, M. L., and Warfield, D. T. (1971). *Appl. Microbiol.* **22,** 925–927.
Parker, R. F., and Rivers, T. M. (1935). *J. Exp. Med.* **62,** 65–72.
Parker, R. F., and Smythe, C. V. (1937). *J. Exp. Med.* **65,** 109–120.
Petric, M., Szymanski, M. J., and Middleton, P. J. (1975). *Inter. Virology* **5,** 233–238.
Pollard, E. (1954). *Adv. Virus Res.* **2,** 109–151.
Polley, J. (1955). *Can. J. Microbiol.* **1,** 256–261.
Polley, J. (1957). *Can J. Microbiol.* **3,** 715–719.
Polley, J. (1959). *Can. J. Microbiol.* **5,** 237–244.
Polley, J. (1960). *Can. J. Microbiol.* **6,** 515–518.
Polley, J. (1961). *Can. J. Microbiol.* **7,** 135–139.
Polley, J. (1969). *Can. J. Microbiol.* **15,** 203–207.
Polley, J., and Guérin, M. M. (1968). *Can J. Microbiol.* **14,** 1017–1020.
Polley, J., and Guérin, M. M. (1969). *Can. J. Microbiol.* **15,** 1091–1094.
Polley, J., and Webb, T. S. (1969). *Can J. Microbiol.* **15,** 1167–1171.
Polley, J., Burr, M., and Gillen, A. (1951). *Proc. Soc. Exp. Biol. Med.* **76,** 330–332.
Rosen, L. A. (1960). *Am. J. Hyg.* **71,** 120–128.

Rowe, W. P., Hartley, J. W., Waterman, S., Turner, H. C., and Huebner, R. J. (1956). *Proc. Soc. Exp. Biol. Med.* **92,** 418–424.
Salk, J. E. (1945). *Science* **101,** 122–124.
Schiff, G. M., and Sever, J. L. (1966). *Virology* **8,** 30–61.
Schmidt, N. J., and Lennette, E. H. (1971) *Appl. Microbiol.* **21,** 217–226.
Schmidt, N. J., Lennette, E. H., Shon, C. W., and Shinomoto, T. T. (1964). *Proc. Soc. Exp. Biol. Med.* **116** 144–149.
Schmidt, N. J., Lennette, E. H., Woodie, J. D., and Ho, H. H. (1965a). *J. Lab. Clin. Med.* **66,** 403–412.
Schmidt, N. J., Ho, H. H., and Lennette, E. H. (1965b). *Am. J. Clin. Pathol.* **43,** 297–301.
Sever, J. L., Castellano, G. A., Pelon, W., Huebner, R. J., and Walman, F. (1964). *J. Lab Clin. Med.* **64,** 983–988.
Smadel, J. E., Baird, R. D., and Wall, M. J. (1939). *J. Exp. Med.* **70,** 53–66.
Smadel, J. E., Rivers, T. M., and Hoagland, C. L. (1942). *Arch. Pathol.* **34,** 275–285.
Smith, M. G. (1956). *Proc. Soc. Exp. Biol. Med.* **92,** 424–430.
Sosa-Martinez, J., and Lennette, E. H. (1955). *J. Bacteriol.* **70,** 205–215.
Spence, L., Fauvel, M., Bouchard, S., Babiuk, L., and Saunders, J. R. (1975). *Lancet* **2,** 322.
Stevens, D. A., Schaeffer, M., Fox, J. P., Brandt, C. D., and Romano, M. (1967). *Am. J. Epidemiol.* **86,** 617–633.
Thiry, L., Dachy, A., and Lowenthal, A. (1969). *Arch. Gesamte Virusforsch.* **28,** 278–284.
Weller, T. H. (1953). *Proc. Soc. Exp. Biol. Med.* **83,** 340–346.
Weller, T. H., Hanshaw, J. B., and Scott, D. E. (1960). *Virology* **12,** 130–132.
White, L. A., Gamble, W. C., and Chappell, W. A. (1975). *J. Clin. Microbiol.* **2,** 287–291.
Wiener, M., Henle, W., and Henle, G. (1946). *J. Exp. Med.* **83,** 259–279.
Wilson, D., and Pollard, E. (1958). *Radiat. Res.* **8,** 131–141.

Chapter 9

Immunoperoxidase Technique in Diagnostic Virology and Research: Principles and Applications

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I. INTRODUCTION

A. Development of Immunohistochemistry

During the past 25 years immunology has assumed a vital role in diagnostic techniques and in the analysis of biological problems because of the precise discriminatory power of antibodies and of the development of increasingly sophisticated techniques. The specific interactions between antibodies and their homologous antigens are widely used for the localization of antigens in cells or tissues, as well as for the titration of circulating free antigens or antibodies.

Since 1950 it has become possible to localize various antigens in cells by the use of immunofluorescent techniques. Although these techniques have numerous important applications, they cannot be used for electron microscopic studies. Therefore, around 1960, the fluorochrome was replaced by an electron-dense marker: ferritin. This marker, however, has the disadvantage of having a large molecular size, MW 650,000, and thus prevents the penetration of this probe into the cell. The main applications of the immunoferritin technique are, therefore, in fundamental studies of membranes. In addition, it is only applicable in electron microscopy.

It was recognized around 1970 that enzymes, which can be visualized at both optic and electron microscopic levels, can be conjugated to antibodies and that the use of such labeled antibodies would overcome these problems. Among the different enzymes used, peroxidase has been shown to have a great potential, because of its stability, high enzymatic activity, and its rather simple conjugation to antibodies. Furthermore, peroxidase-labeled antibodies can be used both for histochemical studies in light microscopy and ultrastructural studies in electron microscopy.

B. Principles of the Immunoperoxidase Technique

The principles of the immunoperoxidase technique are very similar to those of the immunofluorescence technique. Antibodies are covalently labeled with peroxidase without imparing the immunological activity of the antibody or the enzymatic activity of the peroxidase. This probe is

brought in contact with the corresponding antigen in the specimen and the antigen–antibody complex formed is localized by the cytochemical demonstration of the enzyme. Best results are obtained by using a soluble substrate, the ensuing reaction product of which precipitates spontaneously, is visible (or can be rendered visible in a separate step) and amorphous. Since peroxidase catalyzes the oxidative polymerization of the soluble hydrogen donor 3,3′-diaminobenzidine tetrahydrochloride to an insoluble, visible (brown) product (a phenazine polymer), the popularity of this enzyme is easily understandable. Although the reaction product is not visible at the ultrastructural level, it can be rendered visible by the addition of osmium tetroxide. Osmium tetroxide is rapidly reduced and chelated by this osmiophilic polymer, resulting in an electron-opaque product.

Other enzymes can also be used for the labeling of antibodies but they are less useful for various reasons. For example, cytochrome *c* is much cheaper and smaller than peroxidase, but its enzymatic activity is much lower (about 500 times) and is active only at low pH, which might dissociate the antigen–antibody complexes.

The methods for the detection of antigens by the immunoperoxidase technique are very similar to those of the immunofluorescence techniques (see Figs. 2–4).

Since the application of the immunoperoxidase technique in virology (Kurstak *et al.*, 1969; Kurstak, 1971; Abelson *et al.*, 1969; Leduc *et al.*, 1969; Wicker and Avrameas, 1969), the exploitation of this technique has been hampered by serious problems, due mainly to the low efficiency of the conjugation methods. Therefore, it is not surprising that alternative methods have been explored. Sternberger (1974) took advantage of the fact that the enzymatic activity of peroxidase is not abolished after its reaction with antiperoxidase antibodies. In this modification, peroxidase is not coupled chemically but through an immunological reaction with specific antibodies (see Fig. 4).

At present, highly efficient labeling techniques are available which will be discussed later. The immunoperoxidase technique has very important advantages over other methods: (1) its high sensitivity; Petrali *et al.* (1974) showed that immunoperoxidase is up to 100,000 times more sensitive than radioimmunoassay; (2) it can be applied for ultrastructural studies; (3) specimens can be stored infinitely; and (4) no special equipment is required.

C. Scope of This Chapter

This chapter is intended to give some details of the immunoperoxidase technique for its application for both diagnostic and research purposes

and to describe some recent developments and perspectives. The limitations of the methods will also be discussed. In order to avoid an excessively lengthy chapter, with much duplication of material already published in other books or reviews, only a cursory survey of the widely known and applied techniques will be given. The emphasis will be placed on specific topics related to the immunoperoxidase technique and its pitfalls. For example, nucleic acids as antigens deserve more attention than generally given.

This powerful tool would certainly have a great potential in the study of autoimmune diseases in which nucleic acids presumably play a role, e.g., systemic lupus erythematosus (SLE). The preparation of enzymes and active groups of enzymes are also of importance and are discussed in detail. The methods of conjugation which have been improved greatly in this and other laboratories during the last few years are also presented in some detail. In the section dealing with the procedures for the detection of viral antigens by light and electron microscopy, special attention is drawn to nonspecific staining. Finally, a list of applications in virology is also given and some recent developments are discussed.

II. REAGENTS

A. Preparation of Antigens

1. Viruses or Virus-Related Proteins as Antigens

For reviews on the problem of obtaining satisfactory antigen preparations, the reader is referred elsewhere (Williams and Chase, 1967; Sela, 1973).

Even if the purified antigen is not prepared experimentally, one should be familiar with the physicochemical characterization methods which permit the evaluation of the purity and homogeneity of proteins. Antigens are often contaminated with unwanted proteins with similar physicochemical properties. If such impurities are very immunogenic, disproportionately high amounts of antibodies may be produced against these contaminants.

A wide spectrum of techniques used in protein chemistry may be employed for the purification of antigens and for the evaluation of the purified preparations. Viruses may contain host-specific proteins, making it necessary to purify and/or dissociate virus and to prepare antibodies only against the desired structural proteins. On the other hand, one may be interested in the so-called "soluble" antigens which are smaller in size and do not occur in virions but play a role in the viral morphogenesis. The

intended use of the antigen determines the requirements for its concentration and/or purity.

If it is necessary to destroy the viral infectivity (rabies, arboviruses) β-propiolactone has the advantage that it is less detrimental to antigenicity and immunogenicity than many other chemical or physical treatments (LoGrippo and Hartman, 1955). A method to avoid the production of some of the nonspecific antibodies may be the use of homologous host systems, e.g., immunizing mice with coxsackievirus propagated in mouse muscle or brain. However, the adverse effects of anti-host antibodies should be circumvented.

The first step in the purification of viral antigens often involves concentration of the antigenic material. This is best achieved by differential ultracentrifugation. For effective pelleting of small viruses, it is important to include an unrelated protein (e.g., 0.06% gelatin) in the suspending medium (Baron, 1957).

2. Nucleic Acids as Antigens

It is well established that nucleic acids can react with antibodies, despite the earlier conclusion that nucleic acids are not immunogenic (Stollar, 1973; Lacour, 1968; Plescia *et al.*, 1968). Most of the effective immunogens are in fact complex antigens in which a macromolecular nucleic acid or an oligonucleotide plays a haptenic role. Therefore, a variety of methods have been developed for conjugation of nucleic acids to protein or synthetic polypeptide carriers (Levine *et al.*, 1971; Steiner *et al.*, 1972; Plescia *et al.*, 1968).

Methylated bovine serum albumin (MBSA) has been often used for the formation of noncovalent immunogenic complexes with single-stranded (Plescia *et al.*, 1964) or with double-stranded and triple-stranded nucleic acids (Nahon *et al.*, 1967).

One of the most intriguing aspects of nucleic acid immunocytochemistry is the spontaneous occurrence of anti-nucleic acid antibodies in patients with SLE (Pincus and Kaplan, 1970). Immunoperoxidase, using anti-nucleic acid antibodies, has also been applied to such studies (Fritzler *et al.*, 1974). Surprisingly, no studies of delayed hypersensitivity or other aspects of cellular responses have been carried out with nucleic acid antigens.

B. Preparation of Immunoglobulins

1. General Remarks

The successful location of antigens or the adaptation of the immunoperoxidase technique for diagnostic purposes is dependent on the nature

and quality of the antibody preparation used. In the ideal situation, a preparation of high specificity, high titer, high avidity, and good quality should be obtained.

It is preferable to use purified antibodies for the immunoperoxidase technique for the following reasons: (1) the extent of nonspecific staining decreases with increasing purity of the antibody; (2) the inactive proteins, which are a possible source for nonspecific staining and for the wasteful loss of enzyme, are eliminated; and (3) the concentration of immunoglobulins can be easily established without interference by other proteins.

For ultrastructural studies in order to overcome the difficulty of penetration in fixed cells, the use of smaller tracers is recommended. These smaller tracers consist of univalent antibody fragments (Fab fragments) conjugated with purified active sites of cytochrome *c* (microperoxidase). In other cases, particularly in light microscopy, it is not necessary to prepare such microperoxidase–Fab conjugates since the use of peroxidase–antibody conjugates gives satisfactory results.

2. Immunization

Numerous methods have been described for the immunization of various species. For best results, two important points should be kept in mind: (1) possible impurities in the antigen preparation could be prevented from inducing large amounts of antibodies by diluting the antigen and (2) the dose of the antigen should be sufficiently large to provoke high titers of antibodies. As a compromise, it is preferable to immunize with small quantities of antigen, as low as 25 μg/kg body weight in a small volume (0.05 ml) emulsified with an equal volume of Freund's adjuvant (Harboe and Ingild, 1973). For the production of polyspecific antibodies, the dose should be increased (up to ten times). Farr and Dixon (1960) have presented evidence that the concentration of the antigen in the adjuvant–antigen mixture is more important than the total, absolute amount of antigen injected. Complete virions are strongly immunogenic, therefore, only microgram quantities are needed. Soluble viral proteins sometimes have questionable purity. Prior to injection, they may be alum-precipitated; the use of alum significantly increases their immunogenicity and is less likely to induce antibodies against quantitatively minor proteins than that of Freund's adjuvant (Chase, 1967).

Preparation of antiglobulins deserves special attention since it is so frequently used in the indirect staining method. It should be mentioned that high-titered antisera may cross-react with γ-globulins of other species (Henle and Henle, 1965). Different methods have been described for the preparation of antiglobulins (Fahey and Horbett, 1959; Niel and Fribourg-Blanc, 1962) which vary in their injection schedules.

The exact specificity of a given antiserum cannot be predicted but must be evaluated experimentally (Horwitz and Scharff, 1969). Preliminary testing is most effectively carried out using the method of double diffusion in agar and immunoelectrophoresis.

The selection of a suitable host for immunization is dependent on a number of factors. For herpesviruses, precautions should be taken to minimize the possibility of the host already having antibodies to an indigenous virus which cross-reacts with the virus under study (Hampar and Martos, 1973). Furthermore, the immunizing virus or antigen may be fatal for the host, such as in the case of pseudorabies virus for rabbits (Kaplan, 1969). Other factors, such as age, route of inoculation, and immunizing dose may also affect the animal's response.

3. Isolation of Immunoglobulins or Antibodies

Methods for the isolation of pure antibodies are often quite tedious and the loss of specific antibodies may be significant. On the other hand, fractionation of immune sera by physicochemical or chemical methods (nonspecific methods) enhances considerably the ratio of antibody to nonspecific proteins, without important losses.

Immunoglobulins of different species may have quite different physicochemical properties. Orlans *et al.* (1961) reported that fractionation with ethanol, which is very satisfactory in general, gives poor yields for chicken immunoglobulins. Fahey *et al.* (1964) obtained poor yields of mouse immunoglobulins after fractionation by chromatography on DEAE–cellulose. Many standard methods are available for the purification of immunoglobulins (fractionation with ethanol, precipitation by neutral salts, formation of complexes, ion-exchange chromatography, gel filtration). It is beyond the scope of this chapter to present the details of these nonspecific techniques which can be found in immunochemistry handbooks, such as, Kabat and Mayer (1971), Williams and Chase (1967), Haurowitz (1968), Nezlin (1970), and Kurstak and Morisset (1974).

The isolation of "serologically pure" antibodies (not necessarily pure with respect to chemical properties) involves, in the first place, the purification of the antigen to the highest possible degree. Subsequently, this antigen preparation is used for the preparation of immune complexes. The antigen–antibody complexes are then separated from the other proteins, followed by their dissociation and the recovery of the reactants. Various procedures can be used for this purpose (Haurowitz, 1968; Nezlin, 1970) and the results vary widely with the method used, the type of antibody, and the antigen–antibody system involved.

Immunosorbents have become extremely valuable for such experiments since the antigen is fixed to an insoluble carrier conferring to this

technique the following advantages: (1) the antigen is not detached from the support during the dissociation of the antigen–antibody complexes; (2) insoluble supports with minimal nonspecific adsorbing capacity can be employed; (3) the immunosorbents may be used repeatedly; and (4) several types of insoluble matrices are commercially available.

Immunosorbents can also be prepared by cross-linking the antigens with glutaraldehyde (Avrameas and Ternynck, 1969) or ethyl chloroformate (Stanislawski and Mathieu, 1973), without impairing their antigenicity. After incubation with the antiserum and thorough washing, the retained specific antibodies can be eluted at pH 2.8 with 0.2 *M* glycine-HCl (Nezlin, 1970; Kurstak and Kurstak, 1974; Tijssen and Kurstak, 1974). It is evident that the purity of the antigen is one of the limiting factors for the possibilities of these techniques. One should also be aware of the possible nonspecific retention of immunoglobulins on immunosorbents.

4. Preparation of Fab Fragments

It is essential to start with pure γ-immunoglobulins for the preparation of Fab fragments. The immunoglobulin preparation can be digested with papain by Porter's method (1959). In short, the IgG preparation is incubated with papain for 4 hours (1.5 mg papain per 150 mg immunoglobulins in 0.1 *M* sodium phosphate buffer, pH 7.0, containing 0.01 *M* cysteine and 2 m*M* EDTA). Prolongation of the digestion gives good yields of sheep Fab fragments (Heimer *et al.*, 1969). After the removal of undigested immunoglobulins by chromatography, the Fab fragments may be purified by block electrophoresis, affinity chromatography, or chromatography on CM-cellulose using a gradient from 0.1 to 0.9 *M* sodium acetate, pH 5.5.

5. Evaluation and Estimation of the Concentration of Immunoglobulins or Antibodies

Several methods are available for the analysis of the quality of immunoglobulins, both for their chemical and immunological purity, but immunoelectrophoresis is one of the most powerful and convenient methods (Scheidegger, 1955).

An important parameter for the characterization of an antibody preparation is the titer. However, comparisons made using different titration methods or antigen preparations can lead to contradictory or confusing results. Harboe and Ingild (1973) compared four different titration methods (quantitative precipitin test, Sewell titration, single radial immunodiffusion, and rocket immunoelectrophoresis) and found wide discrepancies. Nevertheless, with a careful estimation of the titer, the efficiency of purification and the biological activity can be established.

The concentration of immunoglobulins can be easily estimated by standard methods used for proteins, such as by the reaction with the Folin-phenol reagent (Lowry *et al.*, 1951).

Spectrophotometric analysis is based on the typical absorption spectrum of IgG; its absorbance at 278 nm is at least 2.5 times higher than at 251 nm which makes it different from other proteins. This property provides a rapid method for establishing which fractions contain IgG. Moreover, the concentration of IgG may be estimated from these optical data; all IgG preparation so far analyzed have an extinction coefficient ($A^{1\%}_{278\ \mathrm{nm};\ 1\ \mathrm{cm}}$) of 14.6 ± 1.5 (Little and Donahue, 1967).

C. Preparation of Enzymes or Enzymatically Active Groups

1. Synopsis of Some Problems

Horseradish peroxidase (HRP) is the most frequently used enzyme in immunoenzymatic techniques for light and electron microscopy. The occurrence of a strongly immunogenic nonperoxidatic glycoprotein in commercial HRP preparations deserves special attention, particularly in studies using the noncovalent antibody–enzyme method. This glycoprotein contaminant is present in most commercial preparations of HRP and can be distinguished from HRP by its immunochemical nonidentity, electrophoretic mobility, amino acid composition, and marked tendency to aggregation, as well as by the absence of peroxidase activity and the spectrophotometric Soret band in this contaminant (Moroz *et al.*, 1974). The commercially available "purified" peroxidase (type VI) is considerably more expensive (about 20 times) than the unpurified preparation which contains only about 35% HRP. The purification of HRP in the laboratory is relatively simple to perform (see below).

For ultrastructural studies it may be important to use smaller tracers but which fully retain their enzymatic and antibody activities. This decrease in the size of the antibody–enzyme conjugate is evidently desirable since an enzyme-labeled antibody requires 2 days to penetrate reproducibly tissue sections with a thickness of 0.02 to 0.04 mm. This problem could be circumvented by localizing the viral antigens directly on ultrathin sections, but this poses very special problems such as the nonspecific adsorption of proteins to the embedding material. Therefore, other ways had to be found, such as the conjugation of the active group of cytochrome *c* (with molecular weight ~ 1500) to the univalent Fab fragments. This method has a great potential for electron microscopic studies.

In next sections the physicochemical properties of peroxidase will be described, followed by a presentation of the purification procedures of the

enzyme from commercial preparations or from horseradish roots and of the preparation of microperoxidase.

2. *Physicochemical Characteristics of Horseradish Peroxidase*

The properties of peroxidase (EC 1.11.1.7) are given in the handbook on this group of enzymes (Saunders *et al.*, 1964) and in the Worthington Enzyme Manual (Worthington Biochemical Corp., Freehold, New Jersey). Horseradish peroxidase (HRP) consists of seven isozymes (Shannon *et al.*, 1966) with a molecular weight of 40,000. The enzyme contains approximately 18% of neutral and amino sugars. This characteristic is very important for the conjugation procedure. All isozymes contain the prosthetic group protohemin IX in their active site (Weinryb, 1966). The enzyme exhibits a high specificity (Ljunggren, 1966):

$$\text{Peroxidase} + H_2O_2 \longrightarrow \text{compound}$$
$$\text{Compound} + AH_2(\text{donor}) \longrightarrow \text{peroxidase} + H_2O + (AA)_n$$

The fact that the activity of peroxidase can be inhibited by various chemicals makes it possible to inactivate the endogenous enzyme activity in the specimens (see Section IV,C,2). The activity of the enzyme in an aqueous solution at a concentration of 1 mg/ml does not diminish over 1 year at 5°C. As lyophilized powder, it may be stored several years at 4°C.

The quality of the enzyme preparation can be evaluated on the basis of two characteristics, namely, the purity of the protein and its enzymatic activity.

The *RZ* value (Reinheits Zahl = degree of purity) which corresponds with the absorbance ratio $A_{403\ nm}/A_{275\ nm}$, is generally used as an indication of purity. The commercially available peroxidase is considered pure if the $RZ \geqslant 3.0$. Shannon *et al.* (1966) showed that the different isozymes have considerably different *RZ* values (Table I) However, for convenience, this *RZ* is often used and the concentration of HRP (in milligrams per milliliter) in an aqueous solution at pH 6–7 can be calculated from its absorbance at 403 nm for 1 cm optical pathway by simply multiplying it by 0.4.

The enzymatic activity of peroxidase can be assayed by numerous methods (Maehly and Chance, 1954). The most commonly used method, the catalyzed formation of purpurogallin from pyrogallol, consists in dissolving pyrogallol (5 g) in water (2 liter) containing H_2O_2 (50 mg) at pH 6 and 20°C. Five minutes after the addition of peroxidase, the reaction is stopped with 50 ml of 10% sulfuric acid. The yellow product is extracted with ether and estimated colorimetrically (Saunders *et al.*, 1964).

Another satisfactory and easy assay is described in the Worthington

TABLE I
Characteristics of Horseradish Peroxidase Isozymes

Isozyme	Retention on ion-exchange column: CM-cellulose 0.005 *M* acetate, pH 4.4	Retention on ion-exchange column: DEAE-cellulose 0.005 Tris-HCl, pH 8.4	Absorbance ratio (A_{401}/A_{275})
A-1	–	+	4.19
A-2	–	+	4.12
A-3	–	+	3.71
B	+	–	3.37
C	+	–	3.42
D	+	–	2.57
E	+	–	2.50

Enzyme Manual which, however, has the drawback that single oxidation products are not obtained. This method is based on the measurement of the rate of decomposition of hydrogen peroxide with *o*-dianisidine as hydrogen donor. This rate is established by measuring the color development at 460 nm. One unit of peroxidase activity is defined as the amount of enzyme decomposing 1 μmole of peroxide per minute at 25°C. In this assay, the enzyme preparation (0.1 mg) is diluted with 250 ml water just before use. The substrate stock consists of 1 ml of 30% H_2O_2 diluted to 100 ml with water. For use, 1 ml of stock H_2O_2 is diluted to 100 ml with 0.01 *M* phosphate buffer, pH 6.0 (made fresh daily). The dye, 1% *o*-dianisidine in methanol, is prepared fresh and kept in an amber bottle. Experimentally, 0.05 ml of dye is added to 106.0 ml of substrate. From this mixture 2.9 ml are transferred to the test cuvette and 3 ml into the control cuvette. At zero time, 0.1 ml of diluted enzyme is introduced into the cuvette with a 0.1-ml pipette, immersing the tip below the surface. The cuvette, covered with Parafilm, is then inverted for thorough mixing. The absorban of the sample is recorded at 15-second intervals for 1 to 2 minutes and the rate of change per minute is determined. If the *RZ* value of the enzyme preparation approaches 2, less enzyme should be used. After the test the cuvette should be rinsed with 5 *N* HCl. The activity of the enzyme is calculated with the equation

$$\text{Units/mg} = A_{460\text{nm/min}}/(11.3 \times \text{mg enzyme/ml reaction mixture})$$

based on the fact that 1 mole of hydrogen peroxide causes a change in the absorbance of 1.13×10^4 cm^{-1} at 460 nm.

Commercial purified HRP (*RZ* 3.0; Worthington) has a minimal activity of 3000 units/mg.

3. *Purification of Peroxidase from Horseradish*

The method described by Shannon *et al.* (1966) is presented here which provides the separation of the isozymes with good yields. Horseradish roots are cut into small cubes and homogenized in a minimal volume of 0.1 *M* K_2HPO_4 in a Waring blender. After filtering through a cheesecloth, $(NH_4)_2SO_4$ is added to 35% saturation. The supernatant is collected and brought to 90% with respect to $(NH_4)_2SO_4$. The precipitate is collected by centrifugation after standing overnight, redissolved in a minimal volume of 0.05 *M* Tris buffer, pH 7.0, and dialyzed against 0.05 *M* Tris buffer, pH 8.0, containing 0.1 *M* KCl. The dialyzed solution is centrifuged and the supernatant, which contains 100% of the peroxidase activity of the crude extract, is collected.

The isozymes may now be purified from this preparation. For this purpose, the solution is dialyzed against 0.005 *M* acetate buffer, pH 4.4, and applied to a CM-cellulose column equilibrated with the same buffer. The A isozymes are not retained, while the other isozymes can be recovered progressively with a linear gradient made of 500 ml 0.005 *M* acetate buffer, pH 4.4, and 500 ml 0.1 *M* acetate buffer, pH 4.4. At the end of this gradient, a second system consisting of 300 ml of 0.1 *M* acetate buffer, pH 4.4, and 300 ml 0.25 *M* acetate buffer, pH 4.9, is applied. The effluent fractions are designated as B, C, D, and E isozyme.

The A isozymes are purified on a DEAE-cellulose column equilibrated with 0.005 *M* Tris buffer, pH 8.4. The A isozymes are eluted from the column with a linear gradient consisting of 500 ml of 0.005 *M* Tris buffer, pH 8.4, and 500 ml 0.005 *M* Tris buffer, pH 8.4, containing 0.1 *M* NaCl. The characteristics of the seven isozymes are given in Table I.

Subsequent studies showed that the seven isozymes have quite similar catalytic properties although the A isozymes differ markedly from the other four in pH optimum, specific activity, apparent K_m value, and affinity toward inhibitors (Kay *et al.*, 1967).

The purity of the A isozymes can be verified by electrophoresis on alkaline gels (Davis, 1964) and the B,C,D, and E isozymes on acidic gels (Reisfeld *et al.*, 1962). The sedimentation coefficient of these isozymes is about 3.5 S.

4. *Purification of Peroxidase from Crude Commercial Preparation*

The purification of HRP from commercial preparations with an *RZ* of about 1 was described by Moroz *et al.* (1974). The crude HRP is suspended in PBS. The suspension is clarified by centrifugation (10,000 *g*, 10 minutes) and fractionated on a Sephadex G-100 column with a bed volume

50–100 times that of the sample. The absorbance at 403 nm reveals the presence of the HRP. Contaminating proteins are precipated by the addition of $(NH_4)_2SO_4$ to 50% saturation. After centrifugation (10,000 *g*, 10 minutes) the supernatant is saturated to 75% with $(NH_4)_2SO_4$. The precipitate collected after centrifugation (10,000 *g*, 10 minutes) is suspended in a small volume of distilled water, dialyzed against water overnight, followed by an additional 48-hour dialysis against 0.005 *M* phosphate buffer, pH 8. The HRP is clarified by centrifugation and further purified on a DEAE-cellulose column equilibrated with the same buffer. The peroxidase thus isolated is free of the contaminating glycoprotein frequently found in commercial preparations of HRP.

5. Preparation of Microperoxidase from Cytochrome c

The so-called 11-MP (microperoxidase: porphyrin with 11 amino acids), derived from cytochrome *c* from horse heart, has been known for a long time in biochemical research (Harbury and Loach, 1959). Only recently (Feder, 1971) has it been used as a tracer for permeability studies at the electron microscopic level. A still smaller probe, 8-MP has been prepared more recently (Kraehenbuhl *et al.*, 1974; Tijssen and Kurstak, 1974). This heme octapeptide is obtained with a good yield by successive digestion of cytochrome *c* with pepsin (in 0.1 *N* HCl, 24 hours at room temperature) and trypsin (in 0.1 *M* NH_4HCO_3, pH 8, 36 hours at 37°C), followed by gel filtration on Biogel P6 (4 × 90 cm). This crude 8-MP preparation is further purified by countercurrent distribution on a two-phase system consisting of butanol, pyridine, and 0.1% acetic acid (5:3:11) using 3 ml for the lower phase and 5 ml for the upper phase. The peroxidatic activity of the preparation is evaluated as described earlier for HRP.

III. METHODS OF CONJUGATION

A. Requirements for Ideal Conjugation

In the ideal situation, the antibody should retain its biological activity and each antibody should be labeled with an active anzyme molecule. In order to minimize the loss of immunological activity, an antibody molecule should be conjugated covalently with only one enzyme molecule. A higher ratio of enzyme molecules to antibody would (1) lower the immunological activity because of the increase of the probability that enzyme molecules couple to the amino groups situated in the antigen-binding site and of the increasing steric hindrance with larger conjugates, (2) give rise to stronger nonspecific staining since the lowered immunological activity

of the conjugate will not allow the use of higher dilutions (a means of decreasing possible background staining) and the nonspecifically trapped antibodies cannot be removed efficiently from the cells during the considerably shorter washing than incubation times, and (3) hamper the penetration of the labeled antibodies into the cells. On the other hand, the conjugation of one enzyme molecule per antibody would leave at least one antigen binding site free for the immunological reaction. Intramolecular and intermolecular cross-linking producing homopolymers should also be avoided. Furthermore, the conjugation should not abolish enzyme activity, although some impairment may be acceptable. Until recently, the conjugation methods were either not very efficient, giving only a low yield (less than 1%) of peroxidase–antibody conjugates, or impaired the immunological activity of the antibody in the case of more efficient coupling. These methods used "connector" molecules, which are mostly bifunctional agents (see below). Two more efficient approaches are currently favored for the fixation of peroxidase to the antibodies: (1) the utilization of the carbohydrate moiety of the enzyme as a bridge between the enzyme and IgG molecules and (2) the use of soluble peroxidase–antiperoxidase complexes.

B. The Use of Bifunctional Agents for Coupling

Bifunctional reagents contain two reactive groups which are capable of reacting with, and forming bridges between, the polypeptide chains of two proteins.

For controlling the conjugation reaction the important parameters are the concentration of the proteins, the protein/reagent ratio, and the pH and ionic strength of the solution. If one desires to link like molecules, i.e., to form homopolymers, the protein should be used at a high concentration, as close as possible to its pI. In contrast, for linking of different proteins, e.g., the enzyme to the antibody, the pH should be maintained between the p*I* values of the two proteins and the ionic strength adjusted to provide maximum opposite net charges to favor their interactions.

The most widely used reagent is glutaraldehyde, an aliphatic dialdehyde. In recent years much information became available on the nature and mode of action of this reagent. Alternative reagents are difluorodinitrodiphenyl sulfone (FNPS), carbodiimide, and dimethyl suberimidate. Although these methods are becoming less popular, they are presented here in some detail. A very promising new approach is the use of heterobifunctional reagents with photoactive groups, such as diazoalkyl derivatives (Hexter and Westheimer, 1971) and aryl azides (Fleet *et al.*, 1969; Kiefer *et al.*, 1970). With these reagents the first step of coupling is per-

formed in the dark and the second is conducted in the presence of activating light.

1. Glutaraldehyde

Commercial glutaraldehyde is seldom or never pure. Richards and Knowles (1968) demonstrated by nuclear magnetic resonance studies that commercial preparations of aqueous glutaraldehyde contained virtually no free glutaraldehyde but rather a complex mixture of polymeric material rich in unsaturated aldehydes. However, Rasmussen and Albrechtsen (1974) as well as others claimed that only a relatively low amount of polymeric material (with absorption maximum at 235 nm) was present in commercial preparations, together with pure glutaraldehyde (absorbing light at 280 nm). The influence of pH, temperature, and other factors on the polymerization rate was also investigated. The absorbance ratio A_{235}/A_{280} for commercial preparations was frequently observed in this laboratory to be between 2 and 4 (P. Tijssen, unpublished data). However, since the polymeric material absorbs very strongly, it may represent only a low percentage of impurities.

Little is known about the mechanism of the reaction of glutaraldehyde with proteins. The specificity of this coupling agent for the amino group of lysyl residues is lower than generally assumed in immunocytochemistry and its specificity for any particular type of functional group in a protein is probably low. Glutaraldehyde may react with amino groups, sulfhydryl groups, and the phenolic or imidazole rings of tyrosine or histidine (Habeeb and Hiramoto, 1968; Wold, 1972). Korn *et al.* (1972) showed that an average of four glytaraldehyde residues react with one lysyl residue. For conjugation two methods are generally used, a single-step (Avrameas, 1969) and a two-step procedure (Avrameas and Ternynck, 1971).

In the single-step procedure, 6 mg of peroxidase and 5 mg of the immunoglobulin preparation are suspended in 1 ml of 0.05 *M* phosphate buffer, pH 6.9, containing 0.1 *M* NaCl. A solution of glutaraldehyde is slowly added with gentle stirring to a final cocentration of 0.05%. The reaction mixture is rotated for 2 hours. The residual active groups of glutaraldehyde are blocked with 5 mg of L-lysine per milliliter of solution. The mixture is rotated for another 45 minutes and then purified on a Sephadex G-150 superfine column (Kurstak and Kurstak, 1974; Tijssen and Kurstak, 1974).

The two-step procedure is based on the observation that glutaraldehyde is relatively unreactive with peroxidase. In the first step, therefore, peroxidase (10 mg in 0.2 ml of 0.1 *M* phosphate buffer, pH 6.8) is incubated overnight with an excess of glutaraldehyde (1.25%). Under these conditions practically no intra- or intermolecular cross-links occur. This

mixture is then passed through a Sephadex G-25 column to remove free glutaraldehyde. The activated peroxidase is further incubated for 24 hours at 4°C with 5 mg immunoglobulins in 0.05 *M* carbonate buffer, pH 9.5. Free reactive groups are blocked with L-lysine and the conjugate is finally purified by chromatography on a Sephadex G-150 superfine column.

2. p,p'-Difluoro-m,m'-dinitrodiphenyl Sulfone (FNPS)

Nakane and Pierce (1966, 1967) used FNPS for the labeling of γ-globulins with peroxidase. For this purpose, 50 mg of peroxidase and 50 mg of immunoglobulins were dissolved in 2 ml cold 0.5 *M* carbonate buffer, pH 10, and 0.25 ml of 0.5% FNPS in acetone were added. The mixture was agitated for 6 hours at 4°C and then dialyzed overnight against phosphate-buffered saline (PBS). The immunoglobulins were separated from free peroxidase by precipitation with ammonium sulfate at 50% saturation.

3. Carbodiimide

In this procedure (Avrameas and Uriel, 1966; Kurstak *et al.*, 1969) 15 mg of peroxidase and 5 mg of the specific anti-viral antibody preparation are suspended in 1 ml of 0.05 *M* Tris-acetate buffer, pH 7.2. To this solution 40 mg of 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-paratoluene sulfonate (MCDI) dissolved in the same buffer are added. The mixture is stirred for 6 hours at 4°C. The complex is purified by chromatography on Sephadex.

C. Operational Details for an Effective Coupling Using the Carbohydrate Moiety of the Enzyme

The method of Nakane and Kawaoi (1974) with some modifications (Tijssen and Kurstak, 1977) is presented here. This method is based on the oxidation of the carbohydrate portion of peroxidase to aldehyde groups with sodium *m*-periodate, after blocking of the amino and hydroxy groups with fluorodinitrobenzene (FDNB), followed by the formation of Schiff bases between this activated enzyme and the free amino groups of the antibodies. Subsequently, these Schiff bases are stabilized by reduction with sodium borohydride. During these manipulations the enzymatic activity remains virtually unchanged and up to six peroxidase molecules can be coupled to one antibody molecule. Such a high labeling, however, may impair the immunological activity of the antibody.

In a study on the kinetics of the conjugation reaction (Tijssen and Kurstak, 1977), it was shown that the number of peroxidase (n) molecules at-

tached to one antibody molecule can be established by measuring the quantity of free peroxidase at equilibrium $(PO)_e$. For a particular batch of peroxidase (111C-9340, Sigma Chemical Company) n was calculated to be 5.73, using the empirical formula

$$n = \frac{16.67(PO)_e}{1 + 2.91(PO)_e}$$

Evidently, for high values of $(PO)_e$, n becomes constant (saturation) This equation also allows an estimation of the quantity of peroxidase that will not be coupled with a certain input quantity (conservation law). However, in order to have a certain number of peroxidase molecules attached to one antibody, it is necessary to use a certain excess of peroxidase (Tijssen and Kurstak, 1977). Figure 1 illustrates the results obtained with the above-mentioned batch of peroxidase. The theoretical values calculated with the formula were in close agreement with the experimental data obtained in this and other laboratories.

Another important aspect to consider is the fact that this method, as well as all other methods, does not produce a uniform labeling. At very

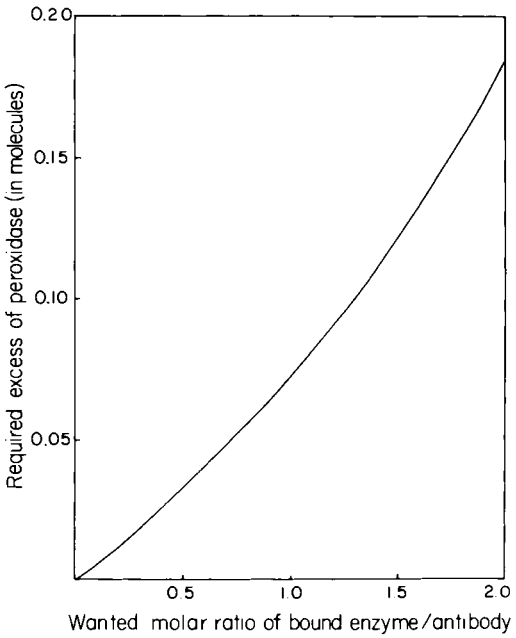

Fig. 1. The required excess of peroxidase molecules, in order to have a certain quantity of peroxidase molecules conjugated to an antibody molecule, increases exponentially with the ratio of bound enzyme/antibody.

low enzyme to IgG ratios, one enzyme molecule will be coupled only to a small number of antibody molecules and, with increasing amounts of activated peroxidase, certain antibody molecules will have more than one enzyme marker, whereas other antibody molecules will have no enzyme at all. It has been shown recently (Tijssen and Kurstak, 1977) that irrespective of the amount of peroxidase added, only about one-third of the antibodies have the ideal conjugation with one enzyme molecule. It was also shown that in the original conjugation procedure (Nakane and Kawaoi, 1974) a large fraction of the antibody preparation was immunologically inactivated since too much enzyme was used (input ratio: four enzyme molecules per antibody). If this ratio is lowered to about 0.8 enzyme molecules per antibody, about 30 to 33% of the antibodies become labeled with one enzyme molecule and only a very low percentage of larger complexes are obtained. Accordingly, the procedure currently used in this laboratory is the following: 5 mg of peroxidase (either purified in the laboratory or purchased: type VI, *RZ* 3.0) are dissolved in 1.0 ml of freshly made 0.3 *M* sodium bicarbonate buffer, pH 8.1, and its possible reactive sites are blocked with 0.1 ml of 1% FDNB in absolute ethanol by gentle mixing for 1 hour at room temperature. The carbohydrates are oxidized with 1 ml of 0.04 *M* $NaIO_4$ in distilled water for 30 minutes at room temperature by the method of Rothfus and Smith (1963). To this yellow-green solution, 1.0 ml of 0.16 *M* ethylene glycol in distilled water is added and mixed for 1 hour at room temperature. This mixture is, at 4°C, passed through a Sephadex G-25 column (bed volume 15 ml) equilibrated in 0.01 *M* sodium carbonate buffer, pH 9.5.

The activated peroxidase is then added to the antibody preparation (also in carbonate buffer) in amounts to obtain a suitable conjugation (0.6 to 2 molecules of enzyme per antibody). This mixture is rotated at room temperature for 3 hours. In order to stabilize the Schiff bases, $NaBH_4$ (1 mg/ml) is added to the mixture which is incubated overnight at 4°C. The preparation is then passed through a Sephadex G-25 column equilibrated with PBS. A final chromatography on Sephadex G-150 in PBS separates the free peroxidase from the conjugated antibodies. Bovine serum albumin (10 mg/ml) can be added to the preparation to prevent aggregation. The enzyme-conjugated antibodies should be kept in sealed vials at 4°C under sterile conditions and should not be frozen.

D. Noncovalent Antibody–Enzyme Method

This method avoids the problems associated with the specificity and sensitivity of other techniques, inherent to ineffective labeling (Stern-

berger, 1974). The principle of this technique is illustrated in Fig. 2 and is composed of four steps: (1) free antibody reacts with its antigen in the smear, (2) antiglobulin is allowed to react with this fixed antibody, (3) antiperoxidase antibody prepared in the same species as the first antibody reacts with the other binding site of the anti-globulin antibody, and (4) this antiperoxidase antibody fixes peroxidase, without abolishing its enzymatic activity, and is revealed histochemically.

Advantages of this technique over the labeled antibody techniques are the elimination of nonspecific reactions resulting from the labeling process itself, of the loss of antibody due to labeling, and of the interference caused by unlabeled antibodies. Unfortunately, this method has also several drawbacks. With human antisera, the anti-peroxidase must also be from the same species, which is seldom or never available. Therefore, this technique has a diagnostic value only if antisera can be prepared in animals. A modification could make this technique applicable for human antisera. One possibility would be the preparation of hybrid antibodies with two specificities: one directed against the antigen in question, the other against peroxidase. Experimentally, this could be achieved through re-

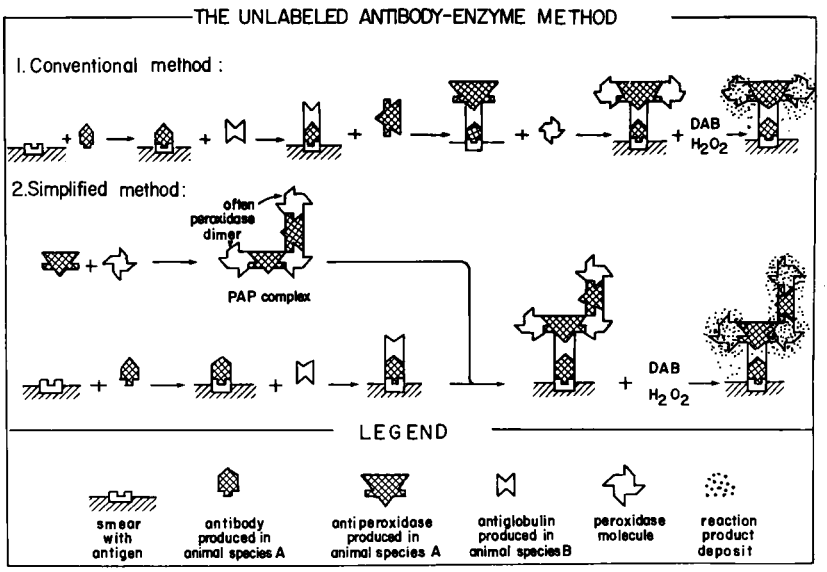

Fig. 2. Schematic description of the noncovalent antibody–enzyme method. Each reagent is applied separately in the conventional method (1), whereas in the simplified method (2) a peroxidase–antiperoxidase complex is prepared in advance. These very sensitive methods result afterward in considerable background staining.

ductive cleavage and reoxidization of the appropriate antibodies. Another disadvantage of this technique is that large complexes may hamper its application in ultrastructural studies. The difficulty of using direct detection of antigens on ultrathin sections will be discussed in Section IV,B,3. Perhaps, the main criticism of this technique is that antibodies with high avidity are selectively bound whereas antibodies with low avidity remain in solution. Although the reaction between the anti-globulin and anti-peroxidase, incurred in the third step, is of high avidity, that occurring between anti-peroxidase and peroxidase is of variable avidity. As an excess of soluble antibody is used in this step, antibodies with low avidity may be lost during washing. The same consideration applies for the binding of peroxidase if anti-peroxidase with low avidity is complexed to the anti-globulin. A way to avoid the latter complication is to use soluble peroxidase–anti-peroxidase complexes (Fig. 2, simplified method)

As mentioned earlier (Section II,C,1), "purified" commercial peroxidase is frequently contaminated with a strongly immunogenic, nonperoxidatic glycoprotein. This mostly ignored fact should be well remembered when preparing antisera to peroxidase.

The important technical details of this procedure (Fig. 2, conventional method) are the following: the specific antiserum from species A should be applied to the smear at a reasonably high dilution, followed by the subsequent application of an excess of antiglobulin (from species B). These conditions will favor the reaction with only one of the combining sites of the anti-globulin antibodies. A further incubation with anti-peroxidase, purified from an antiserum produced in species A, will saturate the remaining free combining sites of the anti-globulin antibodies. The use of whole antiserum at this step considerably lowers the sensitivity of the method. Incubation with peroxidase and the histochemical staining of the enzyme completes this procedure (see Sections IV,A,3 and 4).

A modified version of this procedure (Sternberger *et al.*, 1970) uses soluble peroxidase-antiperoxidase (PAP) complexes (Fig. 2, simplified method). In this method, the first steps are similar to those of the original procedure, but instead of applying anti-peroxidase and peroxidase separately they are applied as a complex. For the preparation of the soluble PAP complexes it is necessary to determine the equivalence zone of the anti-peroxidase serum. About 1.5 to 2 times higher concentration of peroxidase will yield the best results. In the experimental procedure, first all anti-peroxidase antibodies are precipitated with the antigen at equivalence, the precipitate is washed thoroughly, and the washed precipitate is incubated with an excess of peroxidase at pH 2.3 for 2 minutes. The solution is then neutralized and the soluble complexes are separated from the remaining free peroxidase by precipitation with ammonium sulfate at

50% saturation. The preparation of PAP complexes from chicken antisera requires special conditions (Dougherty *et al.*, 1972). On the average, PAP complexes consist of three peroxidase and two antiperoxidase molecules (as shown in Fig. 2, simplified method) with a particle weight of more than 400,000. It is, therefore, evident that such complexes are useful in ultrastructural studies only if applied directly on ultrathin sections, with all the problems inherent to this technique. The sensitivity of this technique, however, is unequaled by other methods (see for discussion Section III,F). This technique may also be extended to a molecular level (molecular immunocytochemistry), as suggested by Hinton *et al.* (1973).

E. Conjugation of Microperoxidase to Fab

This conjugation procedure consists of two steps (Kraehenbuhl *et al.*, 1974): (1) the preparation of *N*-hydroxysuccimide ester of *p*-formylbenzoic acid and the coupling of this complex to the N-terminal amino group of the heme octapeptide with dicyclohexylcarbodiimide and (2) the formation of a Schiff base between the aldehyde group of the derivatized octapeptide and the amino group of the Fab fragment. The first step is carried out in pyridine, the second in borate buffer, pH 9.5. The possible reduction of disulfide bridges in the Fab fragment during the stabilization of the Schiff bases through reduction with sodium borohydride usually does not inactivate the Fab fragments; upon removal of the reducing agent, these disulfide bridges are reoxidized spontaneously. By this method (see Kraehenbuhl *et al.*, 1974; Tijssen and Kurstak, 1974), on the average of two molecules of microperoxidase are attached to each Fab fragment.

F. Properties of Conjugated Sera

The distribution profile of the conjugate after chromatography on Sephadex can be readily determined from the optical densities at 251, 278, and 402 nm and/or by immunoelectrophoresis (Kurstak and Kurstak, 1974; Tijssen and Kurstak, 1977). On electrophoresis (Scheidegger, 1955), the free enzyme and the free antibody move in opposite directions from the application site, whereas the conjugate remains at the point of application. The position of the enzyme can be determined by applying 0.02 *M* guaiacol and 0.1% H_2O_2 in the central trough; in a few minutes, a brown color appears at the position of the enzyme.

The immunological quality of the conjugate can be verified by affinity chromatography (Kraehenbuhl *et al.*, 1974). Inactivation of the immunoglobulins during labeling may be prevented by use of the so-called solid

phase conjugation technique (Kraehenbuhl *et al.*, 1971). In short, the antigen is coupled to Sepharose 4B and the specific antibody is allowed to react with this insolubilized antigen. The antigen-binding sites of the antibodies are blocked by this reaction and thus will not be the target for the subsequent labeling.

The sensitivity of the staining obtained with conjugates prepared by the single-step or two-step procedure is about equal. Higher sensitivities can be obtained by conjugation through the carbohydrate moiety or by the noncovalent labeling method. Conjugation of peroxidase with Fab fragments also enhances the sensitivity, since the size of the complex is smaller. Moreover, conjugated Fab is more easily separated from unconjugated Fab than in the case of intact antibodies.

The properties of PAP complexes are highly dependent on the method of their preparation. The average complex with three peroxidase and two antibody molecules has a sedimentation coefficient of 11.5 S (Sternberger, 1974) and has an association constant of 10^8 moles/liter.

In a comparative study, the sensitivity of the PAP method was compared with that of the peroxidase-labeled antibody method (Burns, 1975) and the PAP procedure was found to be twenty times more sensitive, but at the same time the background staining was also considerably higher. At a dilution of 1:5120, the PAP method still gave a moderate background staining, whereas in the peroxidase-labeled antibody method background staining disappeared at a dilution of 1:20. It is evident that the best improvements for the immunoperoxidase methods can be brought about by decreasing or inhibiting the background staining (see also Section IV,C).

The properties of microperoxidase–Fab complexes were determined by Kraehenbuhl *et al.* (1974). These complexes consisted mainly of two molecules of microperoxidase attached to one Fab molecule. The molecular weight of these complexes varied from 40,000 to 50,000, depending on the origin of the antibody. The apparent V_{max} of the conjugate was 0.4 mmole/minute/mg of attached heme octapeptide and its K_m was 0.4 M. The pH optimum of the conjugate was 7.0.

IV. PROCEDURES FOR DETECTION OF VIRAL ANTIGENS IN CELLS

A. Light Microscopy

1. Preparation of the Slides

Dividing the microscope slides with water-repellent rings is very useful and practical for the easy localization of the often transparent and colorless smears. Furthermore, this pretreatment also prevents the mixing of

samples and of the varions reagents used in the histochemical technique. This might be achieved with hydrophobic inks or paints (Mark-Tex Corp.). In the author's laboratory, slides are prepared in advance the following way: clean slides are lined up on a horizontal surface, preferentially in a hood, and a Plexiglas mold composed of eight cylindrical blocks is placed on each slide. Alternatively, eight drops of 50% glycerol in water may be used instead of the mold. The slides are then sprayed with a Teflon-like compound (Fluoro-Glide, Chemplast, Inc.) and the molds are removed or the glycerol is rinsed off under tap water a few minutes later. The slides thus treated acquire a ground-glasslike appearance, except for the clear areas protected by the mold or the glycerol.

2. Fixation

Fixation of virus-infected cells in tissue cultures, smears, or frozen sections is almost universally accomplished with acetone. Dried preparations have been stained successfully after fixation in acetone at temperatures from 37° to −50°C for periods ranging from 10 minutes to several days (Kundin and Liu, 1963). Other fixatives have also been used, such as cold ethanol (Williamson and Blattner, 1965), ethanol–acetic acid (Sokolov *et al.*, 1963), formol–saline (Kovacs *et al.*, 1963), and methanol–acetone (Reedman and Klein, 1973). Prior to fixation, the cells are rinsed with PBS and, in the case of lymphocytes or hemocytes, with a hypotonic buffer (0.95% trisodium citrate). The antiserum is applied after air drying.

Sectioning of unfixed, frozen tissues offers an attractive means for the studying of antigens in their native state (Pearse, 1960). Artifacts due to diffusion may occur during thawing. Freeze substition (dehydration by organic solvents at low temperatures) offers some advantages (Balfour, 1961).

3. Reaction on Smears

a. Direct Method. This technique (Fig. 3, top) is extremely simple. The labeled antibody preparation is applied to the cell smear supposedly containing the antigen, and incubated in a wet chamber (a closed petri dish with wet cotton or paper) during 1 hour at 25° or 37°C. This step is followed by repeated washings, staining, and mounting (Section IV,A,4).

The main advantages of the direct method are its simplicity (only one reagent is needed) and the minimum of controls required. However, its drawbacks are that for each antigen to be localized separate preparations of antibodies should be conjugated and that its sensitivity is lower than of most other methods. The direct method is often used in the study of autoimmune diseases [Fig. 3, top (2)].

b. Indirect Method. This method is based on the fact that antibody

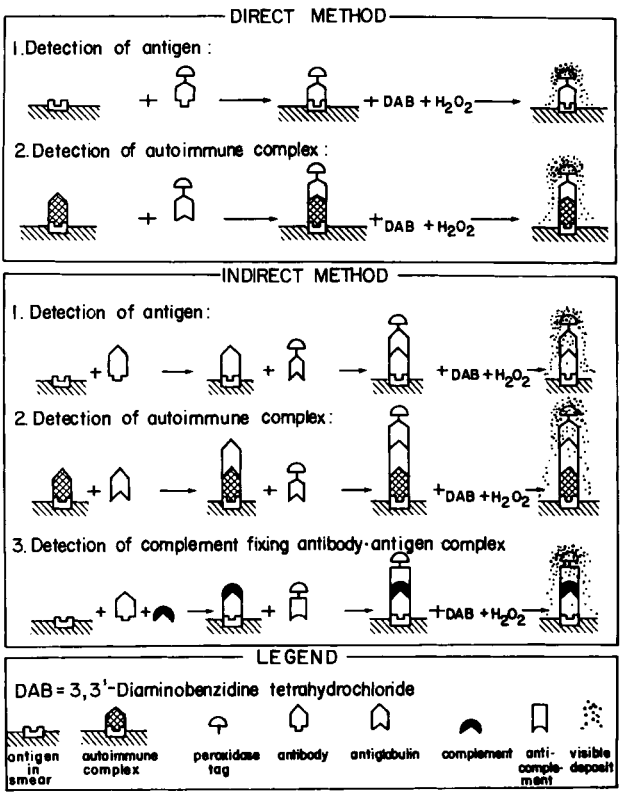

Fig. 3. Schematic representation of the direct and indirect immunoperoxidase methods. The anti-complement technique allows the detection of many different mammalian complement fixing antibody–antigen complexes with a single conjugated anti-complement preparation (for example, anti-guinea pig complement).

molecules themselves are capable of serving as antigen. Thus, peroxidase-labeled antibodies against immunoglobulins will detect antigen–antibody complexes *in situ* [Fig. 3, middle (1)] The unlabeled antibody is generally applied in the form of whole, unfractionated antiserum, since purification often provokes aggregation or denaturation which might cause nonspecific adsorption on the tissue or cell preparation. Antibodies to normal tissue constituents may be removed prior to the test by absorption with normal noninfected tissue. The conditions for the incubation are the same as in the direct method. In the second step, the labeled anti-immunoglobulin antibodies are overlaid on the sections or smears and the incubation is repeated. Recommendations for the washing, staining, and mounting are given in Section IV,A,4.

The greater complexity of the indirect method consequently requires more controls. The concentration of control sera should be similar to that of the antisera. Preliminary titrations of the specific antiserum and of the conjugated anti-globulins are also necessary for the determination of the optimum concentration for each reagent.

The main advantage of this technique is that a single preparation of labeled anti-immunoglobulins can be used for detection of antibodies of different specificities from a single species. A second advantage is the increase in sensitivity which is due to the higher molecular combining ratio of the anti-immunoglobulins to antigen than in the case of the specific antibodies; the increase in sensitivity is often 10-fold. A third advantage is the prevention of the denaturation of the specific antibodies during purification. For best results, this purification should be avoided.

c. Anti-complement Method. Goldwasser and Shepard (1958) were the first to suggest that complement is itself antigenic. Therefore, fluorescein-labeled antibodies to complement could be used to detect antigen-antibody complexes in histological preparations. Guinea pig serum is the most frequently used source of complement. Although complement may be present in the antisera, it is preferable to heat-inactive (30 minute, 56°C) the anti-serum and mix it with an adequate amount of active guinea pig complement prior to the detection of the antigen. The most widely used anti-complement sera are directed against the C3 component.

The principle of the technique is illustrated in [Fig. 3, middle (3)]. The specific antibody, after its reaction with the homologous antigen in the section, tissue, or smear, fixes complement and the labeled anti-complement anti-body reacts readily with this complex. The concentration of each reagent should be kept within certain limits by appropriate dilutions and the amount of complement should be very low (Hinuma and Hummeler, 1962). Generally the anti-complement conjugate is used at a higher concentration.

The ability of the bound immunoglobulin to fix complement is important. Only IgM and certain subclasses of IgG are capable of fixing complement. Whereas a single IgM molecule bound to the antigen is sufficient for the complement fixation, IgG must be aggregated. Such aggregation may be brought about using a sufficiently high concentration of antibody to bind at two adjacent antigenic sites.

Another way to circumvent this problem is illustrated with the example for the detection of the Epstein-Barr nuclear antigen (EBNA) in human lymphocytes (Kurstak *et al.*, 1976). In this system, the antibodies are of the IgG class. In order to aggregate IgG after its reaction with the EBNA, an excess of sheep anti-human IgG can be added. In the subsequent steps, guinea pig complement, followed by the conjugated rabbit antibodies to

guinea pig complement are applied. To minimize nonspecific reactions, the latter is first absorbed with normal sheep serum.

The applicability of this technique for ultrastructural studies is restricted, since this method gives incorrect localization of the antigens. It has been shown that both with the immunoferritin (Mardiney *et al.*, 1968) and the noncovalent antibody–enzyme method (Sternberger, 1974) C3 binds over a wide area of contiguity on the cell surface, even if the antigen sites are widely dispersed.

The anti-complement method has definite advantages over the other methods, since with a single preparation of conjugated antibodies to complement, antisera from various mammalian sources can be used and compared. The sensitivity of this method is higher than that of the indirect method.

The main drawback of the method is the inaccurate localization of the antigen at the level of electron microscopy.

4. Enzymatic Revelation and Subsequent Mounting

Any neutral buffer or even unbuffered saline is suitable for washing. However, at the last washing step, 0.05 *M* Tris-HCl buffer, pH 7.6, must be used. The washing time depends on the incubation time. For the localization of peroxidase, the substrate solution is prepared in the following manner: 3,3′-diaminobenzidine tetrahydrochloride (DAB) is dissolved in 0.05 *M* Tris-HCl buffer, pH 7.6, at a concentration of 0.05% and filtered. Since DAB is carcinogenic, it should be handled with care. The specimen is immersed in this solution for 1 minute and, after the addition of hydrogen peroxide (to a final concentration of 0.01%), the specimen is immersed again for 2 or 3 minutes, followed by several washings.

The simplest and most commonly used mounting medium consists of nine parts of glycerol and one part of PBS. The advantage of this hygroscopic mixture is that it has a clearing effect on stained preparations and does not dry out even on prolonged storage. Alternatively, the smears can be serially dehydrated with xylol–alcohol (1 : 1) and xylol and mounted in Eukitt. The product of the peroxidatic reaction, however, is soluble in alcohol. Thus, alcohol should be used only in cases where clearing of a very heavy staining is required; in all other cases, alcohol should be replaced by acetone.

5. Control, Specificity, Blocking, and Neutralization

The controls to be used with the immunoperoxidase technique can be divided into three groups, namely controls for the antigen, the antibody, and the performance of the technique. For the control at the antigen level, cells known to be devoid of the specific antigen should remain unstained

upon exposure to the specific antibodies. This control should be performed in the direct method (Fig. 4, top), in the first and second steps of the indirect method (Fig. 4, middle), and in the antibody and anticomplement steps of the anticomplement method. Absorption of the immune serum with certain purified antigens may make it possible to detect which antigen is present (neutralizing method, Fig. 5). It should be established that none of the reagents other than the specific antibody in question reacts with the antigen in the smear, section, or tissue.

The controls at the antibody level provide the tests for the specificity of the reaction. The antigen exposed to normal immunoglobulin conjugate should remain unstained [Fig. 4, middle (1)]. The same principle also applies for autoimmune complexes [Fig. 4, middle (2)]. In the indirect methods, these controls have to be performed at both the specific antibody and

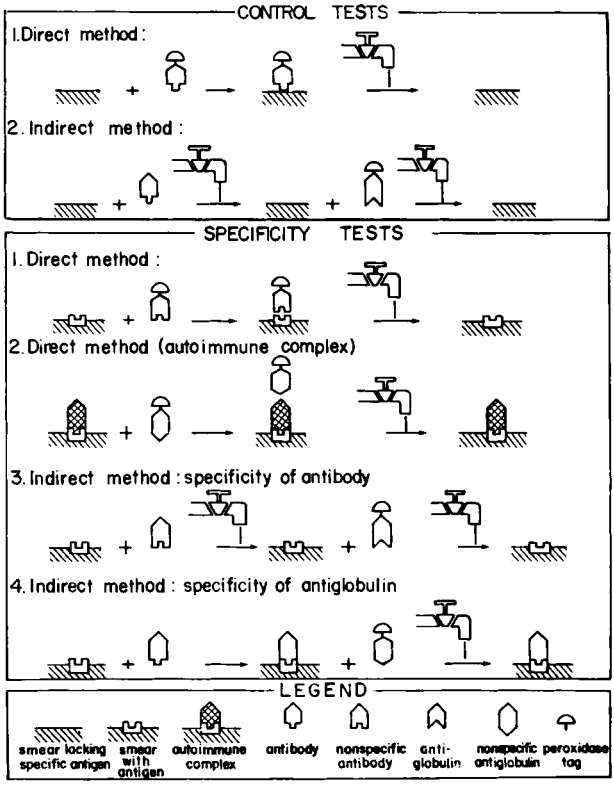

Fig. 4. The control and specificity tests concern all steps of the immunoperoxidase technique. More tests than represented here are mentioned in the text.

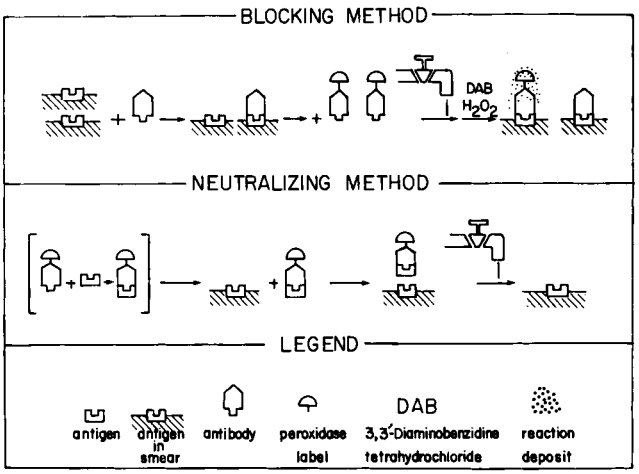

Fig. 5. Both the blocking method and the neutralizing method evaluate the specificity of the antibody–antigen reaction. In the blocking method, the staining is reduced by blocking the antigenic sites with specific nonlabeled antibodies. In the neutralizing method purified antigen is mixed with labeled antibodies, thus preventing the detection of that specific antigen in the specimen.

anti-globulin steps [Fig. 4, middle (3) and (4)]. The specificity of the anti-globulin step has to be verified both by using labeled normal serum as reagent in step 2 or saline in step 1. It is evident that the number of controls required increases with the number of steps.

Technical controls include tests for the endogenous peroxidatic activity of the cell or tissue preparations (see also Section IV,C,2), and for the nonspecific uptake of peroxidase by cells (incubation with free peroxidase). The quality of antigen preservation should also be established by the use of different fixatives. When purified antibodies are used, their immunological activity should be compared with that of the original antiserum.

B. Electron Microscopy

1. General Considerations

The principles for the detection of antigens by immunoperoxidase at the electron microscopic level are similar to those in light microscopy. As mentioned earlier, one should be aware of the fact that the anti-complement technique using antibodies against C3 cannot be used at the ultrastructural level; instead, antibodies against C1q should be used.

Many different procedures have been reported for the application of the

immunoperoxidase technique for electron microscopic studies (Abelson *et al.*, 1969; Kurstak and Kurstak, 1974; Kurstak *et al.*, 1969; Leduc *et al.*, 1969; McLean and Nakane, 1974; Petrali *et al.*, 1974).

Almost all procedures consist of the following steps: (1) prefixation, (2) incubation with conjugate, (3) fixation, (4) incubation with the substrate for the enzymatic revelation, (5) postfixation with OsO_4 and, (6) dehydration and embedding. Extensive washings are included between every step. The reason for the multiple fixation steps is a compromise between the best preservation of cellular morphology and the minimal loss of antigenicity.

The noncovalent antibody–peroxidase method differs from these methods. The large PAP complexes (diameter 205 Å) penetrate the cell or tissue preparations very poorly. This method, therefore, is used only on ultrathin sections.

2. Labeled Antibody Technique

a. Prefixation and Antigen Preservation. There are several requirements which must be fulfilled in this step. It is crucial that the antigenicity of the substances under investigation should not be destroyed. However, these antigens must be stabilized with the best possible preservation of tissue and cell morphology. Most conventional fixatives (mostly aldehyde derivatives, such as glutaraldehyde and paraformaldehyde) interact strongly with proteins, often causing denaturation.

Glutaraldehyde is a superior fixative (Sabatini *et al.*, 1964) for the preservation of morphology but has several drawbacks in immunohistochemistry, namely that the immunological properties of the tissue preparations are often altered (Habeeb and Hiramoto, 1968) and that Schiff-positive aldehyde groups are introduced into the tissue preparation which may be a possible cause for background staining. On the other hand, paraformaldehyde preserves the antigenicity of some antigens, but its ability to preserve tissue morphology is inferior to that of glutaraldehyde (Leduc *et al.*, 1969). Among others, Singer (1974) showed that the mode of fixation of the preparation may profoundly influence the results of the subsequent immunocytochemical reaction. For example, for the H-1 virus, fixation with 4% paraformaldehyde or with glutaraldehyde at a concentration of 0.25% or higher never, or only very rarely, gave a positive reaction. Hassell and Hand (1974) demonstrated that diimidoesters, such as dimethyl suberimidate, represent a very useful alternative to the aldehydes. McLean and Nakane (1974) developed a new fixative for immunoelectron microscopy which utilizes the carbohydrate moieties of the cells. Good results have been obtained with this technique in this and other laboratories. This fixative contains periodate, lysine, and paraform-

aldehyde. The periodate oxidizes the carbohydrates to aldehydes and the lysine cross-links these groups. This fixation preserves the ultrastructure as well or better than glutaraldehyde, without destroying the antigens.

The fixation conditions have to be defined for every system before a proper immunodiagnosis of a virus may be made. Because of the superiority of glutaraldehyde for the preservation of structure, this fixative should be tested first for any new antigen. Satisfactory fixation can often be obtained with as little as 0.125% glutaraldehyde in 5% sucrose at room temperature for 3 hours. A review on fixation techniques is given by Miller (1972).

b. Incubation with Conjugate, Fixation, and Staining. The incubation time of the prefixed thoroughly washed cells with the antiserum may vary considerably over the range of 1 to 48 hours. The optimum incubation time depends on the site of the target, the penetrability of the cells, and the size of the conjugate. The time should be kept as short as possible.

The accessibility of the antigens can be improved by several methods. Fixation itself aids penetration of antibodies. Although, freezing and thawing also increases penetrability, it affects ultrastructure to a considerable extent. This damage may be reduced by the use of a cryobiological technique. In the presence of dimethylsulfoxide (10% in PBS, 30 minutes), glycerol (Andres *et al.*, 1966), or a mixture of both, cell morphology is affected very little when the temperature is lowered slowly. The tube with the sample is placed in a container with a sieve bottom which is placed in another container with alcohol. Dry Ice is added to the outer bath, at a rate to decrease the temperature in the inner bath at a speed of 1°C per minute. The addition of a small amount of digitonin, immediately prior to fixation, also improves the accessibility of antigens (Dales *et al.*, 1965).

Shortening of the incubation time can also be achieved by the use of Fab–microperoxidase conjugates (Section III,E).

After completion of the antigen–antibody reaction, the cells are fixed with glutaraldehyde to prevent further deterioration of the morphology. This step is possible since peroxidase activity itself is not affected by glutaraldehyde. This fixation also protects the tissue against possible damage by H_2O_2. Since the penetration of diaminobenzidine (DAB) is slower than that of H_2O_2, DAB is first applied for 30 minutes, followed by a second incubation for 30 minutes with the complete medium. It is absolutely necessary to apply the substrate under continuous agitation in order to prevent the accumulation of large, nonspecific deposits.

c. Osmification, Dehydration, and Embedding. The reaction product

of the peroxidatic reaction is not visible by electron microscopy but, due to its osmiophilic nature, it can be rendered visible with OsO_4.

Dehydration is usually performed with graded ethanol. However, as pointed out earlier (Section IV,A,4), special care should be taken when using ethanol, as the phenazine polymers are quickly solubilized in this solvent.

Embedding of the cell or tissue preparation in plastic is easily performed by techniques described elsewhere. Araldite 502 (Glauert and Glauert, 1958; Luft, 1961), Epon-Araldite (Abelson *et al.*, 1969; Hayat, 1970), and Epon or Spurr (Kurstak and Kurstak, 1974) are the most frequently used embedding materials.

d. Operational Details. Although some of the experimental details may vary slightly for the different systems, the general procedure for the indirect immunoperoxidase technique, as applied for cultured cells, is described here. The cells are pelleted at low speed and fixed with periodate–lysine–paraformaldehyde (PLP) (McLean and Nakane, 1974) for 1 to 3 hours at 0°C. PLP is prepared by adjusting the pH of a 0.15 *M* solution of lysine-HCl in distilled water to 7.4 with 0.1 *M* dibasic sodium phosphate and by completing the solution with 0.1 *M* sodium phosphate buffer, pH 7.4, to twice the volume of the lysine-HCl solution. Just before use, one part of an 8% paraformaldehyde solution is combined with three parts of the lysine–phosphate buffer and solid sodium *m*-periodate is added to a final concentration of 0.01 *M*.

The prefixed preparation is washed four times with PBS and once overnight with PBS containing 0.3% sucrose. The cells are then incubated with the antiserum and washed overnight with PBS. The labeled anti-globulin is then added to the cells and the mixture is incubated at room temperature with gentle rotation. After five washings with PBS with gentle shaking, the cells are fixed with 2–2.5% glutaraldehyde for 1 hour. After extensive washing with PBS, the cells are incubated with a 0.05% solution of DAB in 0.05 *M* Tris-HCl buffer, pH 7.6, for 30 minutes with continuous shaking, followed by an incubation with 0.02% DAB and 0.005% H_2O_2 in the same buffer. The cells are then washed extensively and treated with 1% OsO_4 in PBS for 2 hours. After thorough washing, the cells are dehydrated with graded acetone and embedded.

3. Noncovalent Antibody–Enzyme Technique

The principles of the noncovalent antibody–enzyme technique are discussed in Section III,D. A severe drawback for its application in electron microscopy is the large size of PAP complexes. Although penetrability can be enhanced by various ways as indicated in Section IV,B,2,b, such

treatments may deteriorate the ultrastructure preservation. Therefore, this technique is applied directly on ultrathin sections.

Fixation of tissue, dehydration, and embedding are necessary in order to obtain mechanical stability of the sample for ultrathin sectioning. Antigenicity must be maintained, the antigen should be accessible for antibody, and the plastic embedding material should not adsorb the antibody and/or peroxidase reagents. This problem cannot be readily overcome by substituting the common embedding material with media made from hydrophilic or charged monomers (Sternberger, 1974). Therefore, staining must be done either prior to embedding or the plastic material be saturated each time with normal serum. Using the latter technique. Petrali *et al.* (1974) developed a very sensitive procedure, although the background (Burns, 1975) was considerable. Operational details of this technique are given by Petrali *et al.* (1974).

C. Nonspecific Staining

1. General Remarks

The term nonspecific staining is used here to designate any staining after incubation with the substrate which is not due to the specific antigen–antibody reaction involving the labeled antibodies. This phenomenon might be due to the presence of endogenous peroxidase in the cells, cross-reactions given by the antisera used, nonspecific uptake of labeled antibodies by the cells, and adsorption of nonspecific proteins to the embedding material. Lowest levels of background staining can be achieved by controlling these factors.

2. Endogenous Peroxidase

The presence of endogenous peroxidase in the tissue or cell preparation would obscure any specific staining. Therefore, this peroxidatic activity must be eliminated prior to the test. It has been recognized that fixation in methanol is often accompanied by a decrease in peroxidatic activity. Using 1% sodium nitroferricyanide, 1% acetic acid, and methanol, Straus (1971) observed the destruction of the enzyme activity but the antibody reaction was also weakened. Streefkerk (1972) showed that posttreatment with H_2O_2 at low concentrations strongly enhanced the inhibitory effect of methanol. It is evident that while the enzyme activity should be destroyed, the antigenic activity of the preparation should not be affected. Since methanol might destroy some cellular antigens, Weir *et al.* (1974) replaced it by a mixture of ethanol and 0.075% HCI for fixation at room temperature for 15 minutes.

3. *Cross-Reactions*

The widespread phenomenon of immunological cross-reactions might be considerably reduced or completely eliminated by the purification of the specific antibodies or by the judicious absorption of the antisera.

Redys *et al.* (1963) used an ingenious approach to eliminate cross-reactions in a streptococcal system. Antisera to group A streptococci remained cross-reactive with group C organisms even after absorption. To render this antiserum completely specific for group A streptococci in their test, a small amount of unlabeled group C antiserum was added to the labeled group A antiserum.

4. *Nonspecific Uptake of Labeled Proteins by Cells*

Many factors may influence the nonspecific uptake of the labeled antisera by cells. It has been shown by several investigators that the major cause of nonspecific staining in the immunofluorescence techniques is not an immunological but rather a charge effect (Nairn *et al.*, 1960; Riggs *et al.*, 1960; Goldstein *et al.*, 1961). Nor corrective technique is absolutely effective to eliminate completely these charge effects and to restrict the reaction of the labeled antibodies only with the specific antigen.

Simple dilution of the antiserum, if the titer is sufficiently high, will eliminate most of the nonspecific staining.

The pH of the medium has the strongest influence on the net charge of the macromolecules in the smears and of the labeled antibodies. However, a pH too far from neutrality will cause dissociation of the antigen–antibody complexes, particularly for antibodies with low avidity.

Absorption of conjugated antibodies with acetone-precipitated liver powder is widely used in immunofluorescence techniques (Coons and Kaplan, 1950), despite the excessive loss of specific proteins.

A more sophisticated method to eliminate nonspecific reactions consists of the use of immunosorbents, particularly if the specific antigen is available in pure form. However, the disadvantage of this method is that antibodies with high avidity will be recovered with a poor yield. These purification methods depend on the antigen–antibody system under investigation and the proper experimental conditions must be established for each system.

5. *Nonspecific Adsorption of Proteins to the Embedding Material*

It has been suggested (Kawarai and Nakane, 1970) that the problem of penetration of large tracer complexes into tissue sections could be overcome by localizing the viral antigens directly on ultrathin sections. How-

ever, fixation, dehydration, and embedding of the tissue in plastic are necessary in order to confer the required mechanical stability upon the tissue. During these processes the antigenicity of the preparation must be maintained and the accessibility of these antigens must be assured. Although some good results have been obtained with this method, nonspecific reactions due to the binding of antibodies to the plastic embedding material usually overshadow any specific localization. This interference could not be overcome with hydrophilic or charged monomers (Sternberger, 1974) but could be minimized by the saturation of the thin sections with normal sheep globulins prior to each step of incubation with specific antibodies (Petrali *et al.*, 1974).

V. APPLICATIONS AND PERSPECTIVES IN DIAGNOSIS AND RESEARCH

A. Applications in Virology

Reviews on this subject were published recently by Kurstak and Kurstak (1974) and Kurstak *et al.* (1975). Figures 6–10 obtained in the authors' laboratory demonstrate the applicability of this technique for the detection and localization of viral antigens in infected cells, both in light and electron microscopy. In Table II, the various applications of immunoperoxidase in virology are listed, up to the beginning of 1976. The viruses concerned are grouped as recently proposed by Fenner (1976). The virus families occurring only in invertebrates, plants, and bacteria are not within the scope of this treatise and have been omitted.

B. Perspectives in Titration Using Agarose Bead Model Systems

Titration of antisera with the immunoperoxidase technique is currently performed by applying serial dilutions of the serum concerned on fixed infected cells. The presence, and quantity of antibodies is then detected with labeled anti-globulin or anti-complement antibodies. The results of the immunoperoxidase and immunofluorescence techniques are comparable in such titrations of Epstein-Barr virus (EBV)-related antigens. (E. Kurstak *et al.*, 1977, unpublished data). The stable lymphocyte cell lines known to contain EBV can serve as reference in this system. However, for most other viruses such references are completely lacking and titers determined in different laboratories are quantitatively not comparable.

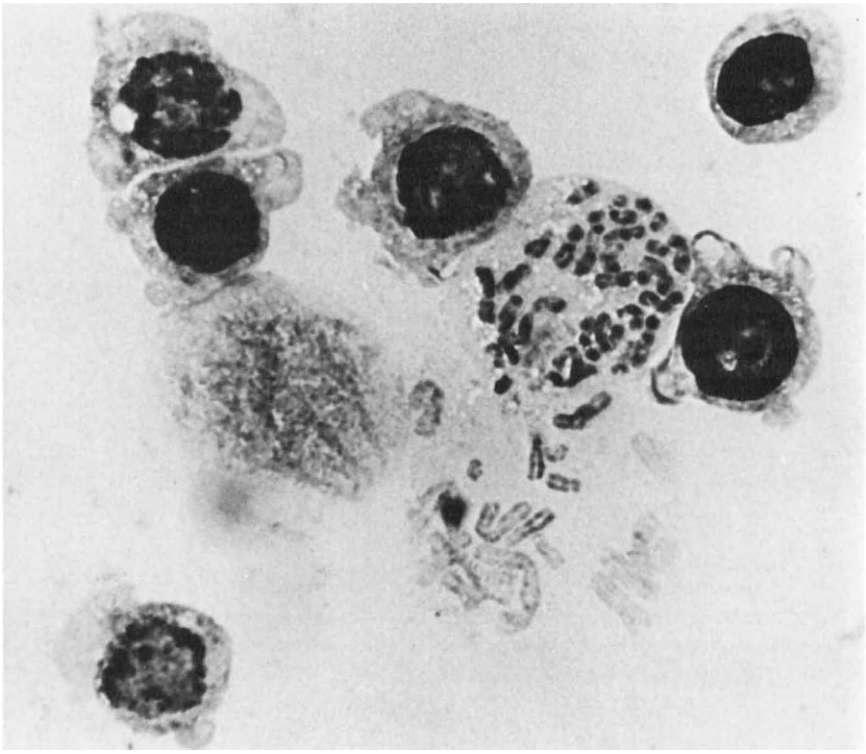

Fig. 6. Epstein-Barr nuclear antigen (EBNA) detection by the anti-complement immunoperoxidase (ACIP) method in Raji cells treated with colchicine. EBNA is demonstrated in the nucleus of the cells transformed by the EB virus. Note detection of EBNA on the chromosomes in the cell during metaphase.

Antigens covalently bound to agarose beads (such as Sepharose 4B, Pharmacia) can overcome this problem. This technique was originally developed for the microfluorometric evaluation of fluorochrome-labeled antibodies and proved to be a sensitive and reproducible method for the detection of antibodies in the nanogram range (van Dalen *et al.*, 1973; Capel 1974; Knapp *et al.*, 1975). This system, the Defined Antigen Substrate Spheres (DASS) system, is based on the principle that antigens (or antibodies) coupled to Sepharose bind the homologous antibodies (or antigens) which can be detected by the immunofluorescence or immunoperoxidase method. The application of this system for the determination of reaginic antibodies in the sera of allergic individuals gave results comparable to those of the radioallergosorbent test (RAST) (Wide *et al.*,

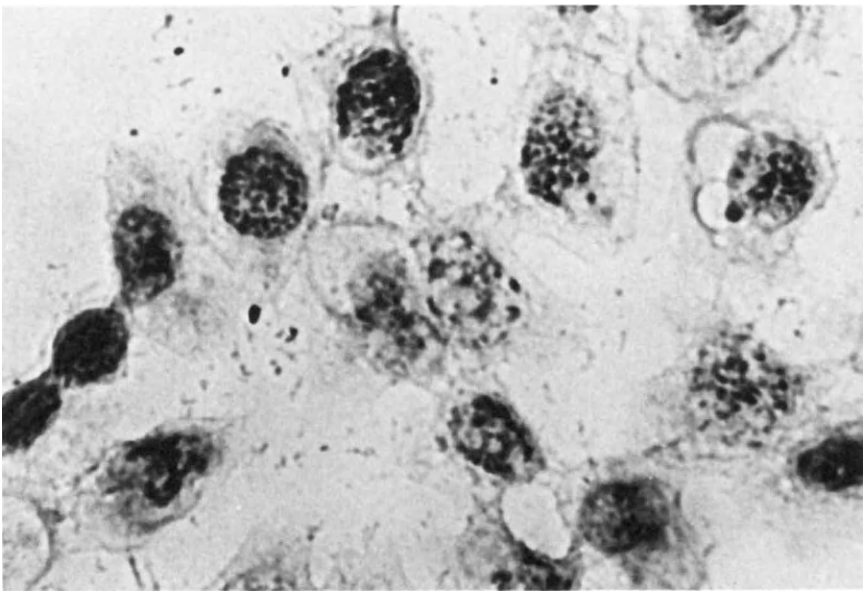

Fig. 7. Intranuclear localization of herpes simplex type 2 antigens in infected Hep-2 cells by direct immunoperoxidase technique. Note the strong granular staining.

1967). Some applications of the DASS system for the quantitative determination of antibodies or antigens and for the immunodiagnosis of helminth infections were given by Capel (1975) and Deelder *et al.* (1975).

A serious drawback of this method, however, is that for accurate quantitation of the antigen–antibody reaction the volume of the beads should be known. Since the size of beads varies considerably, this problem could be overcome by attaching the antigen only to the surface of the beads. This has been achieved by increasing the CNBr concentration 10-fold for the activation of the beads (Streefkerk *et al.*, 1975a). Under these circumstances, the quantitative and general stoichiometric aspects of the immunohistoperoxidase procedure could be studied.

Using the indirect immunoperoxidase technique, this method proved to be specific and highly sensitive. As an example of the diagnostic applicability of this technique, the study on the detection of antibodies against the trematode *Schistosoma mansoni* (Streefkerk and Deelder, 1975) can be mentioned. For serodiagnostic purposes, agarose beads can be fixed to microscope slides with gelatin on which well defined immunohistochemical procedures can be performed. The sensitivity of the technique increases with decreasing quantities of antigen-coated beads) (Streefkerk

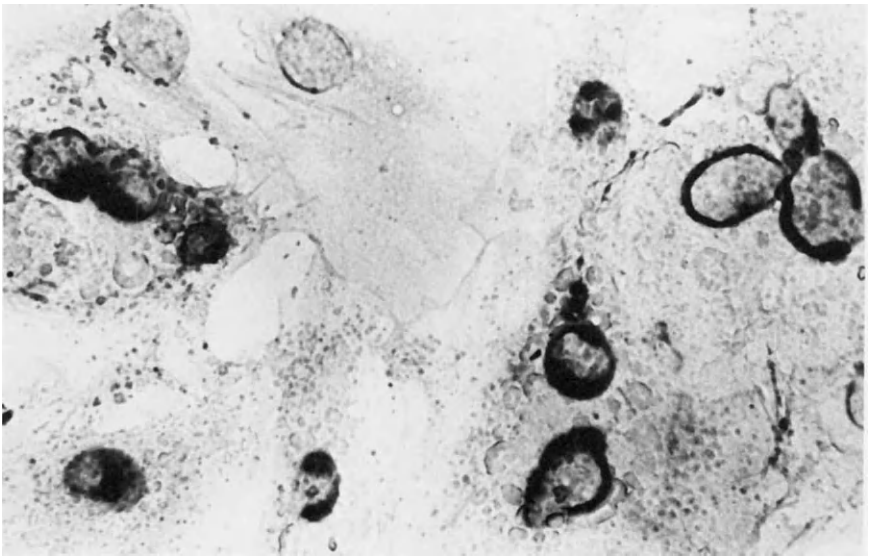

Fig. 8. Parainfluenza virus 2 antigens detected in PMK infected cells by the indirect immunoperoxidase technique. Note the strong perinuclear staining and the globular intracytoplasmic inclusions.

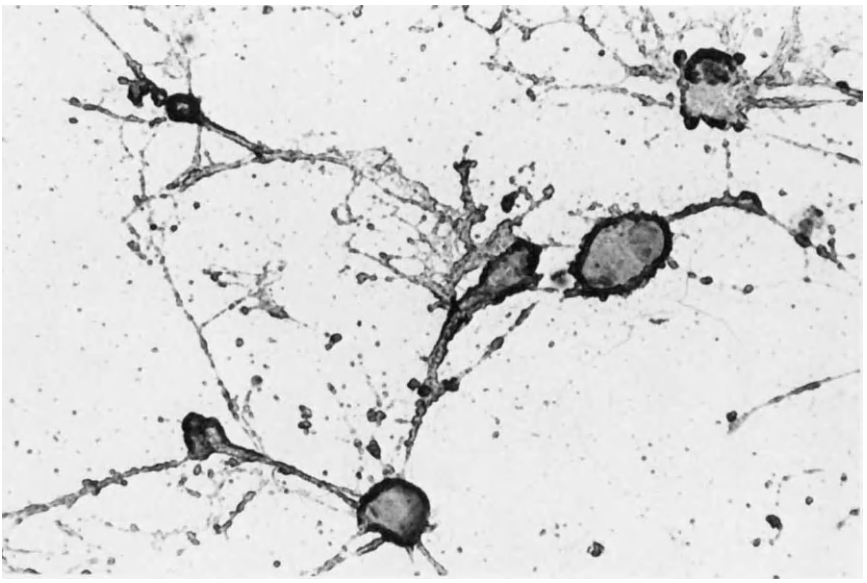

Fig. 9. Measles virus antigens detection in BS-C-1 cells by indirect immunoperoxidase staining. Spindle cells with protuberances containing viral antigens are demonstrated.

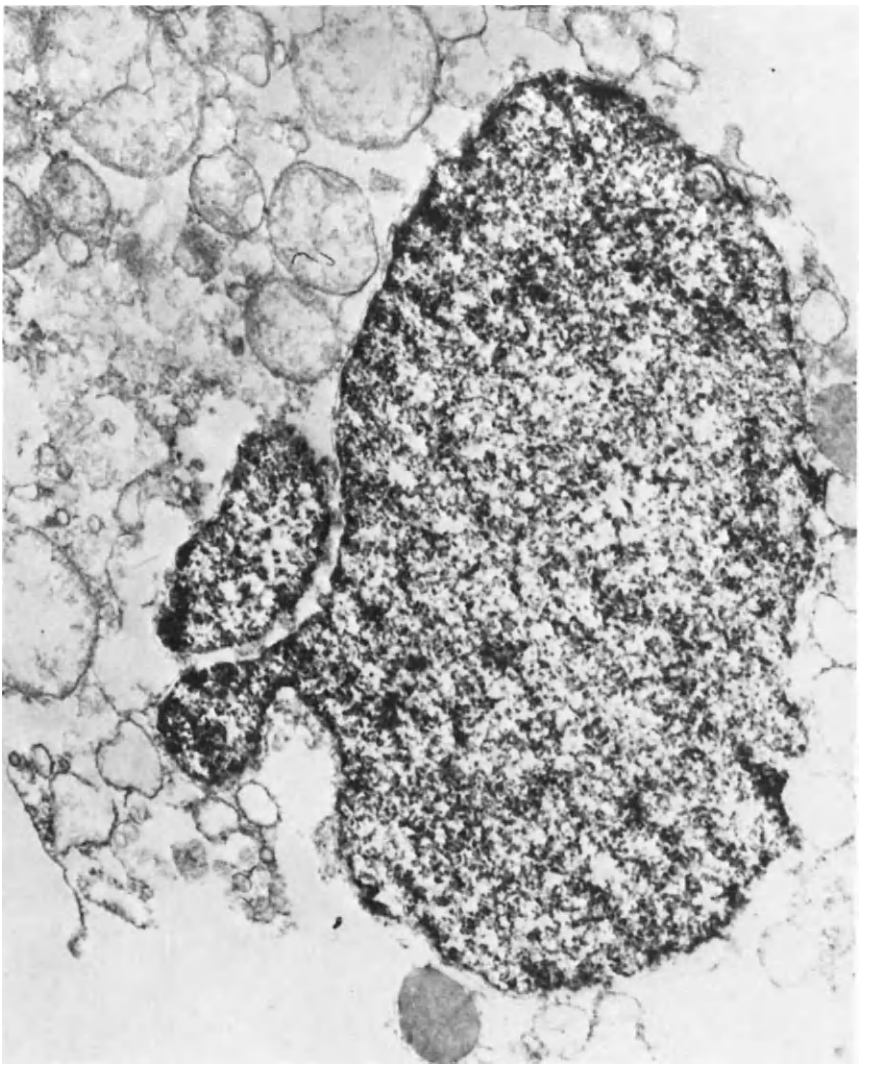

Fig. 10. Raji cell carrying Epstein-Barr virus genome shows clearly EBNA in the nucleus after anticomplement immunoperoxidase (ACIP) staining in electron microscopy. ×13,680.

TABLE II
Immunoperoxidase Applications in Virology

Families	References[a]
Families involving viruses of vertebrates and other hosts	
Poxviridae	64, 65, 70, 72
Parvoviridae	43–45, 49, 64
Reoviridae	73
Rhabdoviridae	3, 4, 41, 54
Families involving viruses of vertebrates	
Herpetoviridae	7, 9, 12, 13, 26, 27, 31, 42, 46, 48–52, 58, 59, 61–63, 66, 68
Adenoviridae	33, 35, 74, 75
Papovaviridae	6, 37, 53, 57, 62, 74, 75
Retroviridae	5, 15, 17, 18, 23, 28, 38, 69, 71
Paramyxoviridae	8, 19–22, 32, 39, 40
Orthomyxoviridae	8, 14, 16, 29, 30
Togaviridae	11, 24, 25, 56
Coronaviridae	—
Arenaviridae	1
Picornaviridae	34, 36, 55, 67
Others	
Hepatitis	2, 10, 60

[a] Key to references: (1) Abelson *et al.* (1969); (2) Afrondakis *et al.* (1976); (3) Atanasiu *et al.* (1971); (4) Atanasiu *et al.* (1975); (5) Aupoix *et al.* (1973); (6) Baba *et al.* (1973); (7) Benjamin (1974); (8) Benjamin and Ray (1974); (9) Benjamin and Ray (1975); (10) Burns (1975); (11) Catanzaro *et al.*, (1974); (12) Cauchy (1973); (13) Cauchy (1974); (14) Čiampor *et al.* (1974); (15) Dmochowski *et al.* (1974); (16) Dobardzic *et al.* (1973); (17) Dougherty *et al.* (1972); (18) Dougherty *et al.* (1974); (19) Dubois-Dalcq and Barbosa (1973); (20) Dubois-Dalcq, M., et al. (1973); (21) Dubois-Dalcq (1974); (22) Dubois-Dalcq *et al.* (1975); (23) François *et al.* (1972); (24) Gerna (1975); (25) Gerna and Chambers (1976); (26) Gerna *et al.* (1976a); (27) Gerna *et al.* (1967b); (28) Girardi *et al.* (1973); (29) Hahon and Eckert (1972); (30) Hahon *et al.* (1975); (31) Hampar and Martos (1973); (32) Herndon *et al.* (1975); (33) Herrmann and Herrmann (1973); (34) Herrmann and Morse (1973); (35) Herrmann *et al.* (1973); (36) Herrmann *et al.* (1974); (37) Horan *et al.* (1975); (38) Hoshino and Dmochowski (1973); (39) Hoshino and Maeno (1971); (40) Hoshino *et al.* (1972); (41) Jentzsch and Zipper (1974); (42) Kurstak and Kurstak (1974); (43) Kurstak *et al.* (1969); (44) Kurstak *et al.* (1970); (45) Kurstak *et al.* (1970); (46) Kurstak (1971); (47) Kurstak *et al.* (1972a); (48) Kurstak *et al.* (1972b); (49) Kurstak *et al.* (1975); (50) Kurstak *et al.* (1976); (51) Laflamme *et al.* (1976); (52) Leary *et al.* (1976); (53) Leduc *et al.* (1969); (54) Levadite *et al.* (1973); (55) Metiani *et al.* (1975); (56) McLean *et al.* (1975); (57) Miller *et al.* (1974); (58) Montplaisir *et al.* (1972); (59) Morisset *et al.* (1974); (60) Nayak and Sachdeva (1975); (61) Rapp (1973); (62) Shabo *et al.* (1972); (63) Shabo *et al.* (1973); (64) Singer (1974); (65) Siverd and Sharon (1969); (66) Storch and Sandow (1973); (67) Sutmoller and Cowan (1974); (68) Suzuki and Hoshino (1971); (69) Thomas *et al.* (1973a); (70) Thomas *et al.* (1973b); (71) Thomas *et al.* (1974); (72) Tripathy (1973); (73) Ubertini *et al.* (1971); (74) Wicker (1971); (75) Wicker and Avrameas (1969).

et al., 1975b). The enzyme-linked immunosorbent assay method, ELISA, is based on the same principle (see Chapter 10).

C. Perspectives of Immunoperoxidase in Laser Flow Cytophotometry for Diagnostic Purposes

The adaptation of the immunoperoxidase technique for the rapid diagnostic screening of herpes simplex type 2 (HSV-2) antigens in gynecological specimens was recently described by Leary *et al.* (1976). A high-speed flow system for laser cytophotometric analysis of large populations of cells was used. The infected cells were stained in suspension with 4-chloro-1-naphthol as hydrogen donor and introduced at a speed of approximately 500 cells/second into the laminar flow of a Bio/Physics Systems Cytograf (Model 6300A) apparatus. The cells were scanned with a 632.8 nm He-Ne laser and the stained cells were found to scatter and absorb light differently from the unstained cells. Separate simultaneous measurements of scatter and absorbance for each cell were made by an array of three silicon photodetectors, two detectors (S) measuring the intensity of light scattered at 1°–19° from the axial line of the undeflected laser beam, while a third detector (A) detecting light at 0.5°–1.0° from this axis. The detected light was electronically inverted to generate pulses and these simultaneously produced A and S pulses were electronically paired to produce a two-dimensional scattergram (the S signals on the x input and the A signal on the y input). The large-angle detectors were more important for most stain effects, as the square of the change in the refractive index was inversely proportional to the large-angle scattered light.

D. Immunoenzymatic Radial Diffusion Methods

Guesdon *et al.* (1976) reported a simple immunoenzymatic method for measuring IgE in human sera. This method is based on the technique of simple radial immunodiffusion described by Mancini *et al.* (1965). The sensitivity of this immunodiffusion technique has been shown to increase twentyfold and allowed measurement of IgE concentrations ranging from 20 to 700 IU/ml.

VI. CONCLUSIONS

Recent improvements in the immunoperoxidase technique revealed new possibilities for the application of this method to its full potential. The many advantages of immunoperoxidase over immunofluorescence,

immunoferritin, and radioimmunological techniques, combined with its simplicity, make it the technique of choice for immunodiagnostic and research purposes.

The sensitivity and specificity of this method, however, is limited by the titer, purity, and specificity of the antisera used. It is likely that in the near future the immunoperoxidase technique will be applied on a much larger scale in medical and veterinary diagnosis and that automated procedures will become available.

ACKNOWLEDGMENTS

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REFERENCES

Abelson, H. T., Smith, G. H., Hoffman, H. A., and Rowe, W. P. (1969). *J. Natl. Cancer Inst.* **42,** 497.

Afrondakis, A. P., Liew, C. T., and Peters, R. L. (1976). *Am. J. Clin. Pathol.* **65,** 533.

Andres, G. A., Accinni, L., Hsu, K. C., Zabriskie, J. B., and Seegal, B. C. (1966). *J. Exp. Med.* **123,** 399.

Atanasiu, P., Dragonas, P., Tsiang, H., and Harbi, A. (1971). *Ann. Inst. Pasteur, Paris* **121,** 247.

Atanasiu, P., Tsiang, H., Perrin, P., and Favre, S. (1975). *Ann. Microbiol.* **126 B,** 69.

Aupoix, M. C., Šimkovič, D., and Gazzolo, L. (1973). *Neoplasma* **20,** 597.

Avrameas, S. (1969). *Immunochemistry* **6,** 825.

Avrameas, S., and Ternynck, T. (1969). *Immunochemistry* **6,** 53.

Avrameas, S., and Ternynck, T. (1971). *Immunochemistry* **8,** 1175.

Avrameas, S., and Uriel, J. (1966). *C. R. Hebd. Seances Acad. Sci.* **262,** 2543.

Baba, T., Yamaguchi, N., Ishida, R., and Suzuki, I. (1973). *Gann* **64,** 173.

Balfour, B. M. (1961). *Immunology* **4,** 206.

Baron, S. (1957). *Proc. Soc. Exp. Biol. Med.* **95,** 760.

Benjamin, D. R. (1974). *Appl. Microbiol.* **28,** 568.

Benjamin, D. R., and Ray, C. G. (1974). *Appl. Microbiol.* **28,** 47.

Benjamin, D. R., and Ray, C. G. (1975). *Am. J. Clin. Pathol.* **64,** 472.

Burns, J. (1975). *Histochemistry* **43,** 291.

Capel, P. J. A. (1974). *J. Immunol. Methods* **5,** 165.

Capel, P. J. A. (1975). *Ann. N. Y. Acad. Sci.* **254,** 108.

Catanzaro, P. J., Brandt, W. E., Hogrefe, W. R., and Russell, P. K. (1974). *Infect. Immun.* **10,** 381.

Cauchy, L. (1973). *C. R. Hebd. Seances Acad. Sci.* **277,** 2093.

Cauchy, L. (1974). *In* "Viral Immunodiagnosis" (E. Kurstak and R. Morisset, eds.), p. 77. Academic Press, New York.
Chase, M. W. (1967). *Methods Immunol. Immunochem.* **1,** 197.
Čiampor, F., Bystricka, M., and Rajcani, J. (1974). *Arch. Gesamte Virusforsch.* **46,** 341.
Coons, A. H., and Kaplan, M. H. (1950). *J. Exp. Med.* **91,** 1.
Dales, S., Gomatos, P. J., and Hsu, K. C. (1965). *Virology* **25,** 193.
Davis, B. J. (1964). *Ann. N.Y. Acad. Sci.* **121,** 404.
Deelder, A. M., Snoijink, J. J., and Ploem, J. S. (1975). *Ann. N.Y. Acad. Sci.* **254,** 119.
Dmochowski, L., Hoshino, M., Shigematsu, T., Hiraki, S., and Priori, E. S. (1974). *In* "Viral Immunodiagnosis" (E. Kurstak and R. Morisset, eds.), p. 183. Academic Press, New York.
Dobardzic, R., Boudreault, A., and Pavilanis, V. (1973). *Can. J. Microbiol.* **19,** 147.
Dougherty, R. M., Marucci, A. A., and DiStefano, H. S. (1972). *J. Gen. Virol.* **15,** 149.
Dougherty, R. M., DiStefano, H. S., and Marucci, A. A. (1974). *In* "Viral Immunodiagnosis" (E. Kurstak and R. Morisset, eds.), p. 89. Academic Press, New York.
Dubois-Dalcq, M., and Barbosa, L. H. (1973). *J. Virol.* **12,** 909.
Dubois-Dalcq, M., Schumacher G., and Sever J. L. (1973). *Lancet* **2,** 1408.
Dubois-Dalcq, M., Barbosa, L. H., Hamilton, R., and Sever, J. L. (1974). *Lab. Invest.* **30,** 241.
Dubois-Dalcq, M., Worthington, K., Gutenson, O., and Barbosa, L. H. (1975). *Lab. Invest.* **32,** 518.
Fahey, J. L., and Horbett, A. P. (1959). *J. Biol. Chem.* **234,** 2645.
Fahey, J. L., Wunderlich, J., and Mishell, R. (1964). *J. Exp. Med.* **120,** 223.
Farr, R. S., and Dixon, F. J. (1960). *J. Immunol.* **85,** 250.
Feder, N. (1971). *J. Cell Biol.* **51,** 339.
Fenner, F. (1976). *Virology* **71,** 371.
Fleet, G. W. J., Porter, R. R., and Knowles, J. R. (1969). *Nature (London)* **224,** 511.
François, D., Vu Van Tuyen, Febvre, H., and Haguenau, F. (1972). *C. R. Hebd. Seances Acad. Sci.* **274,** 1981.
Fritzler, M. J., Watson, J. I., and Church, R. B. (1974). *J. Immunol. Methods* **5,** 21.
Gerna, G. (1975). *Arch. Virol.* **49,** 291.
Gerna, G., and Chambers, R. W. (1976). *J. Infect. Dis.* **133,** 469.
Gerna, G., McCloud, C. J., and Chambers, R. W. (1976a). *J. Clin. Microbiol.* **3,** 364.
Gerna, G., Vasquez, A., McCloud, C. J., and Chambers, R. W. (1976b). *Arch. Virol.* **50,** 311.
Girardi, A., Hampar, B., Hsu, K. C., Oroszlan, S., Hornberber, E., Kelloff, G., and Gilden, R. V. (1973). *J. Immunol.* **111,** 152.
Glauert, A. M., and Glauert, R. H. (1958). *J. Biophys. Biochem. Cytol.* **4,** 191.
Goldstein, G., Slizys, I. S., and Chase, M. W. (1961). *J. Exp. Med.* **114,** 89.
Goldwasser, R. A., and Shepard, C. C. (1958). *J. Immunol.* **80,** 122.
Guesdon, J. L., Thierry, R., and Avrameas, S. (1976). *Clin. Exp. Immunol.* **25,** 180.
Habeeb, A. F. S. A., and Hiramoto, R. (1968). *Arch. Biochem. Biophys.* **126,** 16.
Hahon, N., and Eckert, H. L. (1972). *Infect. Immun.* **6,** 730.
Hahon, N., Simpson, J., and Eckert, H. L. (1975). *J. Clin. Microbiol.* **1,** 324.
Hampar, B., and Martos, C. M. (1973). *In* "The Herpesviruses" (A. S. Kaplan, ed.), p. 221. Academic Press, New York.
Harboe, N., and Ingild, A. (1973). *Scand. J. Immunol.* **2,** Suppl., 161.
Harbury, H. A., and Loach, P. A. (1959). *Proc. Natl. Acad. Sci. U.S.A.* **45,** 1344.
Hassel, J., and Hand, A. R. (1974). *J. Histochem. Cytochem.* **22,** 223.

Haurowitz, F. (1968). "Immunochemistry and the Biosynthesis of Antibodies." Wiley (Interscience), New York.
Hayat, M. A. (1970). *Princ. Tech. Electron Microsc.* **1,** 131.
Heimer, R., Clark, L. G., and Maurer, P. H. (1969). *Arch. Biochem. Biophys.* **131,** 9.
Henle, G., and Henle, W. (1965). *J. Immunol.* **95,** 118.
Herndon, R. M., Rena-Descalzi, L., Griffin, D. E., and Coyle, P. K. (1975). *Lab. Invest.* **33,** 544.
Herrmann, B., and Herrmann, H. (1973). *Zentralbl. Bakteriol. Hyg., Abt. 1: Orig., Reihe A* **224,** 273.
Herrmann, B., Herrmann, H., Döhner, L., and Schmidt, J. (1973). *Zentralbl. Baktriol., Hyg., Parasitenkd., Infektinskr. Abt. 1: Orig., Reihe A* **224,** 285.
Herrmann, J. E., and Morse, S. A. (1973). *Infect. Immun.* **8,** 645.
Herrmann, J. E., Morse, S. A., and Collins, M. F. (1974). *Infect. Immun.* **10,** 220.
Hexter, C. S., and Westheimer, F. H. (1971). *J. Biol. Chem.* **246,** 3928.
Hinton, D. M., Petrali, J. P., Meyer, H. G., and Sternberger, L. A. (1973). *J. Histochem. Cytochem.* **21,** 978.
Hinuma, Y., and Hummeler, K. (1962). *J. Immunol.* **87,** 367.
Horan, M., Horan, P. K., and Williams, C. A. (1975). *Exp. Cell Res.* **91,** 247.
Horwitz, M. S., and Scharff, M. D. (1969). *Fundam. Tech. Virol.* pp. 253 and 297.
Hoshino, M., and Dmochowski, L. (1973). *Cancer Res.* **33,** 2551.
Hoshino, M., and Maeno, K. (1971). *J. Electron Microsc.* **20,** 49.
Hoshino, M., Maeno, K., and Inuma, M. (1972). *Experientia* **28,** 611.
Jentzsch, K. D., and Zipper, J. (1974). *Exp. Pathol.* **9,** 163.
Kabat, E. A., and Mayer, M. M. (1971). "Experimental Immunochemistry," 2nd ed. Thomas, Springfield, Illinois.
Kaplan, A. S. (1969). "Herpes Simplex and Pseudorabies Virus." Monographs in Virology, Vol. 5, p. 1. Springer-Verlag, New York.
Kawarai, Y., and Nakane, P. K. (1970). *J. Histochem. Cytochem.* **18,** 161.
Kay, E., Shannon, L. M., and Lew, J. Y. (1967). *J. Biol. Chem.* **242,** 2470.
Kiefer, H., Lindstrom, J., Lennox, E. S., and Singer, S. J. (1970). *Proc. Natl. Acad. Sci. U.S.A.* **67,** 1688.
Knapp, W., Haaijman, J. J., Schuit, H. R. E., Rádl, J., van den Berg, P., Ploem, J. S., and Hijmans, W. (1975). *Ann. N.Y. Acad. Sci.* **254,** 94.
Korn, A. H., Feairheller, S. H., and Filachione, E. M. (1972). *J. Mol. Biol.* **65,** 525.
Kovacs, E., Baratawidjaja, R. K., and Labzoffsky, N. A. (1963). *Nature (London)* **200,** 497.
Kraehenbuhl, J. P., De Grandi, P. B., and Campiche, M. A. (1971). *J. Cell Bio.* **50,** 432.
Kraehenbuhl, J. P., Galardy, R. E., and Jamieson, J. D. (1974). *J. Exp. Med.* **139,** 208.
Kundin, W. D., and Liu, C. (1963). *Proc. Soc. Exp. Biol. Med.* **144,** 359.
Kurstak, E. (1971). *In* "Methods in Virology" (K. Maramorosch and H. Koprowski, eds.), Vol. 5, p. 423. Academic Press, New York.
Kurstak, E., and Kurstak, C. (1974). *In* "Viral Immunodiagnosis" (E. Kurstak and R. Morisset, eds.), p. 3. Academic Press, New York.
Kurstak, E., and Morisset, R., eds. (1974). "Viral Immunodiagnosis." Academic Press, New York.
Kurstak, E., Côté, J. R., and Belloncik, S. (1969). *C. R. Hebd. Seances Acad. Sci.* **268,** 2309.
Kurstak, E., Belloncik, S., and Garzon, S. (1970). *C. R. Hebd. Seances Acad. Sci.* **271,** 2426.
Kurstak, E., Belloncik, S., Onji, P. A., Montplaisir, S., and Martineau, B. (1972a). *Arch. Gesamte Virusforsch.* **38,** 67.

Kurstak, E., Lauzon, M., Kurstak, C., and Morisset, R. (1972b). *C. R. Hebd. Seances Acad. Sci.* **274,** 3145.
Kurstak, E., Tijssen, P., Kurstak, C., and Morisset, R. (1975). *Ann. N.Y. Acad. Sci.* **254,** 369.
Kurstak, E., de Thé, G., Tijssen, P., van den Hurk, J., and Charpentier, G. (1976). *Microsc. Soc. Can.* **3,** 116.
Lacour, F. (1968). *In* "Methods in Enzymology" (L. Grossman and K. Moldave, eds.), Vol. 12, Part B, p. 903. Academic Press, New York.
Laflamme, M. Y., Kurstak, C., Kurstak, E., and Morisset, R. (1976). *Can. J. Ophthalmol.* **11,** 217.
Leary, J. F., Notter, M. F. D., and Todd, P. (1976). *J. Histochem. Cytochem.* **24,** 1249.
Leduc, E. H., Wicker, R., Avrameas, S., and Bernhard, W. (1969). *J. Gen. Virol.* **4,** 609.
Levadite, J. C., Atanasiu, P., Gamet, A., and Guillon, J. C. (1973). *Bull. Soc. Pathol. Exot.* **66,** 12.
Levine, L., Van Vunakis, H., and Gallo, R. C. (1971). *Biochemistry* **10,** 2009.
Ljunggren, J. G. (1966). *Biochim. Biophys. Acta* **133,** 71.
Little, J. R. and Donahue, H. (1967). *Methods Immunol. Immunochem.* **2,** 343.
LoGrippo. G. A., and Hartman, F. W. (1955). *J. Immunol.* **75,** 123.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951). *J. Biol. Chem.* **193,** 265.
Luft, J. H. (1961). *J. Biophys. Biochem. Cytol.* **9,** 409.
McLean, D. M., Gubash, S. M., Grass, P. N., Miller, M. A., Petric, M., and Walters, T. E. (1975). *Can. J. Microbiol.* **21,** 453.
McLean, I. M., and Nakane, P. K. (1974). *J. Histochem. Cytochem.* **22,** 1077:
Maehly, A. C., and Chance, B. (1954). *Methods Biochem. Anal.* **1,** 357.
Mancini, G., Carbonara, A. O., and Heremans, J. F. (1965). *Immunochemistry* **2,** 235.
Mardiney, M. R., Müller-Eberhard, H. J., and Feldman, J. D. (1968). *Am. J. Pathol.* **53,** 253.
Metiani, T., Virat, J., and Cuong, T. (1975). *Recl. Med. Vet.* **151,** 491.
Miller, H. R. P. (1972). *Histochem. J.* **4,** 305.
Miller, M. H., Karnovsky, M. J., and Diamandopoulos, G. T. (1974). *Proc. Soc. Exp. Biol. Med.* **146,** 432.
Montplaisir, S., Belloncik, S., Leduc, N. P., Onji, P. A., Martineau, B., and Kurstak, E. (1972). *J. Infect. Dis.* **125,** 533.
Morisset, R., Kurstak, C., and Kurstak, E. (1974). *In* "Viral Immunodiagnosis" (E. Kurstak and R. Morisset, eds.), p. 31. Academic Press, New York.
Moroz, L. A., Joubert, J. R., and Hogg, J. C. (1974). *J. Immunol.* **112,** 1094.
Nahon, E., Lacour, F., and Michelson, M. (1967). *C. R. Hebd. Seances Acad. Sci.* **264,** 515.
Nairn, R. C., Richmond, H. G., and Fothergill, J. E. (1960). *Br. Med. J.* **2,** 1341.
Nakane, P. K., and Kawaoi, A. (1974). *J. Histochem. Cytochem.* **22,** 1084.
Nakane, P. K., and Pierce, G. B. (1966). *J. Histochem. Cytochem.* **14,** 929.
Nakane, P. K., and Pierce, G. B. (1967). *J. Cell Biol.* **33,** 307.
Nayak, N. C., and Sachdeva, R. (1975). *Am. J. Pathol.* **81,** 479.
Nezlin, R. S. (1970). "Biochemistry of Antibodies." Plenum, New York.
Niel, G., and Fribourg-Blanc, A. (1962). *Ann. Inst. Pasteur, Paris* **102,** 616.
Orlans, E., Rose, M. E., and Marrack, J. R. (1961). *Immunology* **4,** 262.
Pearse, A. G. E. (1960). "Histochemistry: Theorical and Applied." Churchill, London.

Petrali, J. P., Hinton, D. N., Moriarty, G. C. and Sternberger, L. A. (1974). *J. Histochem. Cytochem.* **22,** 782.
Pincus, T., and Kaplan, A. P. (1970). *Nature (London)* **227,** 394.
Plescia, O. L., Braun, W., and Palczuk, N. C. (1964). *Proc. Natl. Acad. Sci. U.S.A.* **52,** 279.
Plescia, O. L., Braun, W., Imperato, S., Cora-Block, E., Jaroskova, L., and Schembor, C. (1968). *In* "Nucleic Acids in Immunology" (O. L. Plescia and W. Braun, eds.), p. 5. Springer-Verlag, Berlin and New York.
Porter, R. R. (1959). *Biochem. J.* **73,** 119.
Rapp, F. (1973). *J. Natl. Cancer Inst.* **50,** 825.
Rasmussen, K. E., and Albrechtsen, J. (1974). *Histochemistry* **38,** 19.
Redys, J. J., Parzick, A. B., and Borman, E. K. (1963). *Public Health Rep.* **78,** 222.
Reedman, B. M., and Klein, G. (1973). *Int. J. Cancer* **11,** 499.
Reisfeld, R. A., Lewis, U. J., and Williams, D. E. (1962). *Nature (London)* **195,** 281.
Richards, F. M., and Knowles, J. R. (1968). *J. Mol. Biol.* **37,** 231.
Riggs, J. L., Loh, P. C., and Eveland, W. C. (1960). *Proc. Soc. Exp. Biol. Med.* **105,** 655.
Rothfus, J. A., and Smith, E. L. (1963). *J. Biol. Chem.* **238,** 1402.
Sabatini, D. D., Miller, F., and Barnett, R. J. (1964). *J. Histochem. Cytochem.* **12,** 57.
Saunders, B. C., Holmes-Siedle, A. G., and Stark, B. P. (1964). "Peroxidase." Butterworth, London.
Scheidegger, J. J. (1955). *Int. Arch. Allergy Appl. Immunol.* **7,** 103.
Sela, M. (1973). "The Antigens." Vols. I and II. Academic Press, New York.
Shabo, A. L., Petricianni, J. C., and Kirschstein, R. L. (1972). *Appl. Microbiol.* **23,** 1001.
Shabo, A. L., Petricciani, J. C., and Kirschstein, R. L. (1973). *Invest. Ophthalmol.* **12,** 839.
Shannon, L. M., Kay, E., and Lew, J. Y. (1966). *J. Biol. Chem.* **241,** 2166.
Singer, J. J. (1974). *In* "Viral Immunodiagnosis" (E. Kurstak and R. Morisset, eds.), p. 101. Academic Press, New York.
Siverd, N. J., and Sharon, N. (1969). *Proc. Soc. Exp. Biol. Med.* **131,** 939.
Sokolov, N. N., Parfonavich, M. I., and Mekler, L. B. (1963). *Acta Virol. (Engl. Ed.)* **7,** 217.
Stanislawski, M., and Mathieu, C. (1973). *J. Immunol. Methods* **3,** 25.
Steiner, A. L., Ferrendelli, J. A., and Kipnis, D. M. (1972). *J. Biol. Chem.* **247,** 1121.
Sternberger, L. A. (1974). "Immunochemistry." Prentice-Hall, Englewood Cliffs, New Jersey.
Sternberger, L. A., Hardy, P. H., Cuculis, J. J., and Meyer, H. G. (1970). *J. Histochem. Cytochem.* **18,** 315.
Stollar, B. D. (1973). *In* "The Antigens" (M. Sela, ed.), Vol. 1, p. 1. Academic Press, New York.
Storch, H., and Sandow, D. (1973). *Dtsch. Gesundheitswes.* **28,** 1614.
Straus, W. (1971). *J. Histochem. Cytochem.* **19,** 682.
Streefkerk, J. G. (1972). *J. Histochem. Cytochem.* **20,** 829.
Streefkerk, J. G., and Deelder, A. M. (1975). *J. Immunol. Methods* **7,** 225.
Streefkerk, J. G., van der Ploeg, M., and Kors, N. (1975a). *Ann. N.Y. Acad. Sci.* **254,** 212.
Streefkerk, J. G., Deelder, A. M., Kors, N., and Kornelis, D. (1975b). *J. Immunol. Methods* **8,** 251.
Sutmoller, P., and Cowan, K. M. (1974). *J. Gen. Virol.* **22,** 287.
Suzuki, I., and Hoshino, M. (1971). *In* "Recent Advances in Human Tumor Virology and Immunology" (W. Nakane *et al.,* ed.), p. 489. Tokyo Univ. Press, Tokyo.
Thomas, J. A., Garzon, S., and Lambré, C. (1973a). *C. R. Hebd. Seances Acad. Sci.* **277,** 457.
Thomas, J. A., Lambré, C., and Garzon, S. (1973b). *C. R. Hebd. Seances Acad. Sci.* **277,** 2573.

Thomas, J. A., Garzon, S., Lambré, C., Hollande, E., Henry, M., Kurstak, C., and Kurstak, E. (1974). *In* "Viral Immunodiagnosis" (E. Kurstak and R. Morisset, eds.), p. 41. Academic Press, New York.

Tijssen, P., and Kurstak, E. (1974). *In* "Viral Immunodiagnosis" (E. Kurstak and R. Morisset, eds.), p. 125. Academic Press, New York.

Tijssen, P., and Kurstak, E. (1977). Manuscript in preparation.

Tripathy, D. N. (1973). *Avian Dis.* **17,** 274.

Ubertini, T., Wilkie, B. N., and Noronha, F. (1971). *Appl. Microbiol.* **21,** 534.

van Dalen, J. P. R., Knapp, W., and Ploem, J. S. (1973). *J. Immunol. Methods* **2,** 383.

Weinryb, I. (1966). *Biochemistry* **5,** 2003.

Weir, E. E., Pretlow, T. G., Pitts, A., and Williams, E. E. (1974). *J. Histochem. Cytochem.* **22,** 51.

Wicker, R. (1971). *Ann. N.Y. Acad. Sci.* **177,** 490.

Wicker, R., and Avrameas, S. (1969). *J. Gen. Virol.* **4,** 465.

Wide, L., Bennich, H., and Johansson, S. G. O. (1967). *Lancet* **2,** 1105.

Williams, C. A., and Chase, M. E., eds. (1967). "Methods in Immunology and Immunochemistry," Vol. 1. Academic Press, New York.

Williamson, A. P., and Blattner, R. J. (1965). *Proc. Soc. Exp. Biol. Med.* **118,** 576.

Wold, F. (1972). *In* "Methods in Enzymology" (C. H. W. Hirs and S. N. Timasheff, eds.), Vol. 25, p. 623. Academic Press, New York.

Chapter 10

Enzyme Immunoassays and Their Potential in Diagnostic Virology

A. VOLLER AND D. E. BIDWELL

I. INTRODUCTION

The enzyme-linked immunosorbent assay (ELISA) first described by Engvall and Perlmann (1971, 1972) and van Weemen and Schuurs (1971) appears to have great promise in virology as a serological tool and is com-

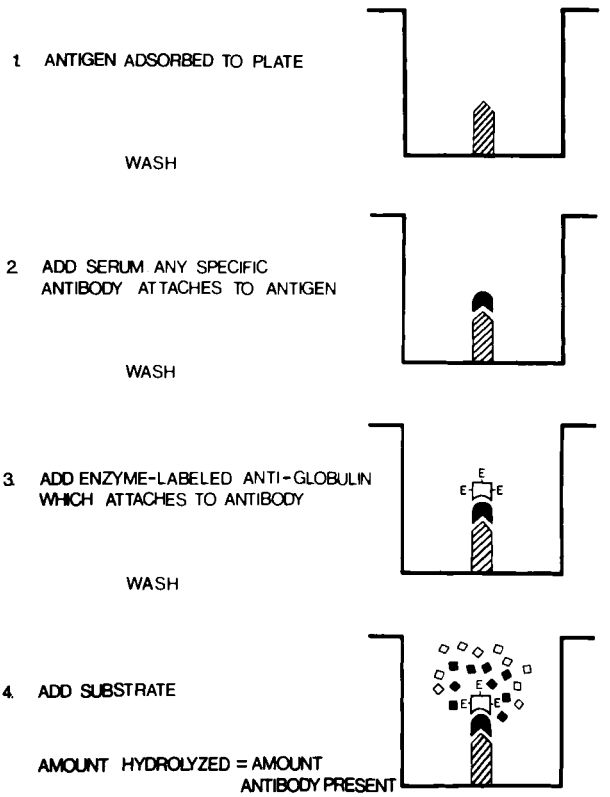

Fig. 1. The indirect ELISA for antibody quantitation. (1) A solid phase support, e.g., polystyrene or polyvinyl tubes, beads or microhemagglutination plates, are sensitized with the antigen (usually by passive adsorption). The sensitized surface is then washed. (2) The diluted test sera are incubated for some hours with the antigen-sensitized solid phase support. Washing is repeated. (3) The conjugate (e.g., enzyme-labeled anti-human immunoglobulin is incubated with the solid phase support for some hours. Washing is repeated. (4) The enzyme–substrate is incubated with the solid phase support. A substrate is chosen which changes color on degradation. The rate of color change is proportional to the antibody content of the serum tested (in step 2). Reproduced with permission from Voller *et al.* (1976a).

plementary to the immunoperoxidase method reviewed by Kurstak and Kurstak (1974) and Kurstak, Tijssen, and Kurstak in Chapter 9.

ELISA can be used to detect and assay both the virus and the antibody produced against the virus. For the detection of antibody, the indirect method (Fig. 1) is most appropriate. For asssaying viruses or their antigenic constituents, the double antibody sandwich method (Fig. 2) is best employed.

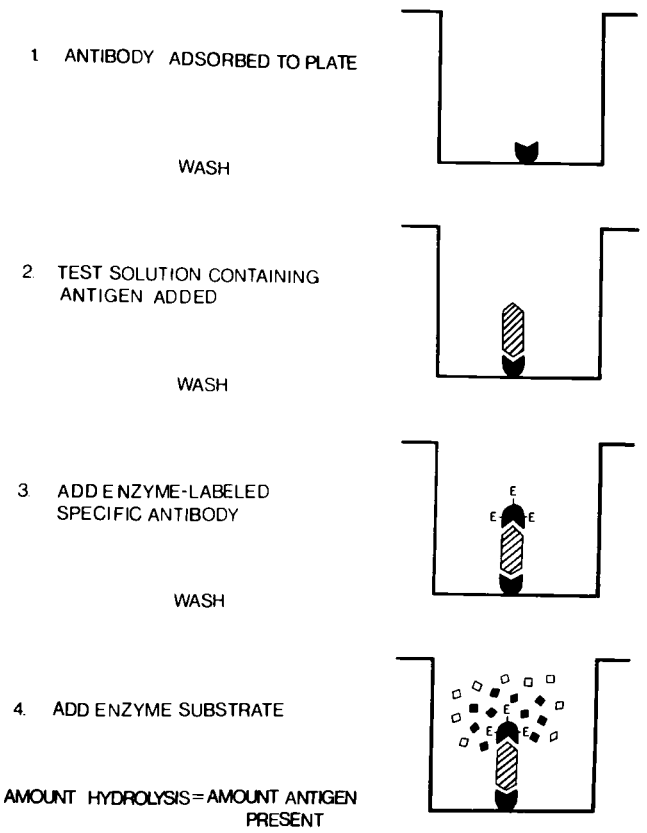

Fig. 2. The double antibody sandwich method for assaying antigens or viruses. (1) The solid phase is coated with immunoglobulin containing specific antibody to the antigen which is to be assayed. Sensitized surface is then washed. (2) The test samples submitted for antigen (or virus) assay are incubated with the sensitized surface for some hours. Washing is repeated. (3) An enzyme-labeled conjugate of specific antibody or immunoglobulin containing specific antibody is then incubated for some hours. Washing is repeated. (4) The enzyme substrate is then added. Its rate of degradation measured by a color change is proportional to the amount of antigen (or virus) in the test sample (step 2). Reprinted with permission from Voller *et al.* (1976a).

II. MATERIALS AND METHODS

A. Solid Phase Support

Polystyrene tubes or beads can be used but these require large volumes of reagents in the case of the former or special washing apparatus for the former. Microhemagglutination plates of polystyrene (Dynatech M29AR)

or polyvinyl chloride (Dynatech 220-29) have proved to be the most suitable type of carrier. These plates can be used with volumes as low as 0.2 ml and because of the large numbers of wells (96 per plate) are convenient for large scale processing.

B. Washing Steps

Between each step of the test it is necessary to wash the solid phase support. In the case of microhemagglutination plates this is done as follows: The plate is emptied. Each well is refilled with PBS Tween and the plate is left for 3 minutes then is shaken dry. This operation is repeated 3 times.

C. Antigens

At the present time antigens are passively adsorbed on to the wells of the microhemmaglutination plates. This is achieved by making up the stock antigen in coating buffer (pH 9.6, 1.59 g Na_2CO_3, 2.93 g $NaHCO_3$, and 0.2 g NaN_3 in 1 liter of distilled water) and adding 200 μl of the diluted material to each well of the microhemagglutination plate (see example) and leaving at 4°C overnight.

All incubation steps are carried out under humid conditions. This can be achieved by placing the plates in plastic bags or boxes containing dampened cotton wool.

The dilution of the antigen for use must be determined by a checkerboard titration against positive and negative reference sera. Commercially available antigens previously prepared for tests can be used. We have successfully employed the following commercial antigens*: rubella, cytomegalovirus, herpesvirus, mumps, measles, adenovirus, and picornavirus. In addition we have prepared our own antigens for Epstein-Barr virus, Japanese B, encephalitis, Oropouche virus, and yellow fever virus from extracts of tissue culture. Control antigens from uninfected tissue cultures must also be tested in each set of assays.

D. Test Sera

The sera to be tested are diluted in PBS Tween (0.15 *M* pH 7.4 phosphate-buffered saline containing 0.5 ml Tween 20 and 0.2 g NaN_3 per liter). For most purposes only a single dilution of the test serum is required. This dilution is determined by checkerboard titrations in ELISA with a reference positive and a reference negative serum. That dilution (usually

* Obtained from Microbiological Associates, Bethesda, Maryland.

between 1/100 and 1/1000) giving maximal separation between these reference samples is used in all subsequent tests. Similarly the best serum incubation times are determined initially by trial and error using the reference positive and negative sera. Usually serum incubation times of 2–5 hours at room temperature are satisfactory, although overnight incubation at 4°C may sometimes be advantageous from the point of convenience.

E. Conjugates

A variety of enzymes can be used as labels. Peroxidase is cheap and is readily available, and conjugates of peroxidase-labeled anti-globulin are commercially available. We have, however, usually used alkaline phosphatase conjugates. This enzyme is more expensive than peroxidase, but the substrate is particularly convenient to use. For most purposes it is sufficient to label the globulin fraction of the anti-species serum (e.g., alkaline phosphatase-labeled sheep globulin containing antibodies against human immunoglobulin). However for some applications it is better to separate and label the specific antibodies. The conjugates are stored in the concentrated form at +4°C in Tris buffer (pH 8.0, 0.05 mole/liter) containing 1% bovine serum albumin and 0.02% NaN_3. Stored in this way the conjugates are stable for many months. Immediately before use, the conjugate is diluted to working strength in PBS-Tween following the manufacturers instructions or the optimal dilution is determined by checkerboard titrations with the positive and negative reference sera.

F. Substrate

For alkaline phosphatase conjugates a convenient substrate is *p*-nitrophenyl phosphate. One tablet of 5 mg Sigma 104 phosphate is dissolved in 5 ml of 10% diethanolamine buffer (97 ml diethanolamine, 800 ml H_2O, 0.2 g NaN_3, and 100 mg $MgCl_2 \cdot 6\ H_2O$); 1 *M* HCl is added to give a solution with pH 9.8, finally made up to 1 liter with H_2O.

This substrate is initially colorless but changes to yellow on degradation. The substrate reaction is stopped by addition of 3 *M* NaOH either after a specified time or when the reference sample reaches a certain value (read on the spectrophotometer).

III RESULTS

The results are either read visually (those wells with a yellow color being considered as positive) or the contents of each well are transferred

to a microcuvette in a spectrophotometer and the absorbance read at 400 nm for *p*-nitrophenyl phosphate. The results can then be expressed as absorbance (E_{400}) units or can be expressed in relation to the reference serum. For each viral system it is necessary to determine the range of values on sera from an uninfected group of individuals. Values above that level can then be considered as positive. Values on the "negative" antigen must be substracted from the viral antigen to give true readings.

A. Indirect ELISA for Measles Antibody

1. Add 200 μl of measles antigen (Microbiological Associates 30–850 diluted 1:100 in coating-buffer) to each well in microhemagglutination plate (Dynatech M29AR). Incubate overnight at +4°C.
2. Wash plate.
3. Add 200 μl of each test serum (diluted 1:100 in PBS Tween) to separate wells of the plate. Incubate 2 hours at room temperature.
4. Wash plate.
5. Add 200 μl of diluted conjugate (alkaline phosphatase-labeled sheep anti-human immunoglobulin). Incubate 2 hours at room temperature.
6. Wash plate.
7. Add 200 μl of *p*-nitrophenyl phosphate substrate to each well. Read absorbance of the reference sample at 400 nm at intervals in a spectrophotometer.
8. When absorbance of reference sample reaches 1.0 add 50 μl 3 *M* NaOH to each well.
9. As soon as possible read absorbance of contents of each well at 400 nm.

B. Applications of Indirect ELISA in Virology

This method has wide possibilities for the detection and measurement of antibodies to viruses in both man and animals. The initial studies in human virology using this method were undertaken by Voller and Bidwell (1975, 1976) on rubella virus. They found that there was a good overall correlation between HI titers and ELISA values, although there was a wide range of individual ELISA values in any HI titer group. Some sera with high rubella virus ELISA values also reacted with the control antigen. By means of an enzyme labeled anti-human IgM conjugate it was possible to detect IgM antibody to rubella in a person who had been vaccinated against rubella. The ELISA was used to measure rubella antibody

in an epidemiological survey in New Guinea. It was found that about three-quarters of the population were positive by the age of 9 years and over 90% by the age of 30. The mean titers of rubella antibody rose sharply between 2 and 9 years then stayed at a high plateau level thereafter (Voller *et al.*, 1976b).

Care must be taken to ascertain that the antigen used is suitable for ELISA, since many of the commercially available materials which are quite satisfactory for HI or CFT are unreactive in ELISA.

Similar methods have been employed for the detection of antibody to cytomegalovirus (Voller *et al.*, 1976b). In this instance a CFT antigen was used, and in general the ELISA results correlated closely with those of CFT. However some of the sera with CFT titers of 1:8 or less were recorded as negative by the ELISA.

The herpesvirus group are important from a serological standpoint, and the potential of ELISA has been investigated to a limited extent. Using the techniques described above Voller *et al.* (1976a) could easily discriminate between reference positive and negative sera for herpesvirus simplex. By a comparison of values obtained with homologous and heterologous antigens it is possible to differentiate between herpesvirus 1 and herpesvirus 2 antibody. Docherty (1977) used plastic beads as the antigen carrier, and his ELISA results correlated well with immunofluorescence but showed an even higher level of sensitivity.

The value of ELISA has been investigated for Epstein-Barr virus serology using a crude extract of infected P3HR1 cells as antigen source. The ELISA values correlated with the immunofluorescent titers with the viral capsid antigen.

C. Other Human Viruses

The same basic method has been used by us to assess the antibody response to measles, adenovirus, coxsackie B, and mumps and to a variety of arboviruses, including Japanese B encephalitis, Oropouche, and yellow fever viruses. The work on measles has shown that ELISA is very suitable for epidemiological investigations, since only small volumes of sera are required and many hundreds of samples can be processed simultaneously. ELISA can also be used on individual patients and on the study on Japanese B encephalitis. Voller *et al.* (1976a) showed that the titer changes in HI or neutralization from acute to convalescent sera are equally well reflected by increases in ELISA values. Leinikki and Päsillä (1977) found that ELISA was more sensitive than other techniques for early detection of influenza antibody.

D. Veterinary Applications

Saunders and Wilder (1974) pioneered the use of the indirect ELISA in veterinary work using hog cholera virus as the model system. The ELISA results correlated well with those obtained by neutralization tests. The ELISA was sensitive and rapid, and in a modified form (Saunders and Clinard, 1976) was suitable as a field screening procedure using plates which were presensitized with antigen.

In our laboratory indirect ELISA tests have been set up on Newcastle disease virus (NDV) infections in chickens and a comparison made with hemmaglutination inhibition tests. Chickens were vaccinated with NDV and it was found that the ELISA detected antibody in all those which had HI titers of 1:4 or greater. The ELISA values correlated with HI titers between 1:4 and 1:4096. HI titers above 1:4096 showed a plateau with the ELISA. We have also established the ELISA for respiratory syncytical virus disease in cattle. The enzyme assay was very sensitive and specific at detecting antibody in cattle with experimentally induced infections. The test has not yet been assessed under field conditions.

E. The Double Antibody Sandwich Method for Detection of Viruses or Their Antigens

The principle of this method has been illustrated in Fig. 2. It can be used for the detection and measurement of virutally any soluble antigen and small particles (such as viruses) which carry antigenic determinants.

This method was employed by Wolters *et al.* (1976) in their microplate enzyme immunoassay for hepatitis B surface antigen (HBsAg). Even when the end result was assessed visually, the sensitivity approached that of the radioimmunoassay (RIA) methods at present in use. The specificity was also equal to that of RIA especially when a confirmatory procedure was used in the ELISA. The enzyme immunoassay* has now been produced in a commercial kit form, and satisfactory results have been obtained on over 50,000 tests to date. Voller *et al.* (1976c) described a similar method for the assay of viruses in tissue extracts. They illustrated this by giving examples of the detection and measurement of two plant viruses. The ELISA was far more sensitive than the other serological methods currently used. It has also been possible to detect and assay EBV in tissue culture extracts using this procedure.

Tenoso (1977) also reported satisfactory preliminary attempts to detect and measure herpes virus in clinical specimens.

It is probable that this method, because of its sensitivity, will become

* Hepanostika, Organon-Teknika, Oss, Holland.

widely used in the assay of viruses in clinical samples and for the quality control in therapeutic products, especially those originating from tissue culture and from blood products.

IV CONCLUSIONS

The simplicity, safety and economy of enzyme immunoassays suggests that these will become routine in viral laboratories both at the research level and for clinical diagnosis. Improvements in techniques and reagents, especially in the conjugates, should enable these methods to achieve the precision, reliability, and sensitivity without many of the problems associated with assays using isotopes.

REFERENCES

Docherty, J. (1977). *J. Infec. Dis.* (in press).
Engvall, E., and Perlmann, P. (1971). *Immunochemistry* **8,** 871.
Engvall, E., and Perlmann, P. (1972). *J. Immunol.* **109,** 129.
Kurstak, E., and Kurstak, C. (1974). *In* "Viral Immunodiagnosis" (E. Kurstak and R. Morisset, ed.), p. 3. Academic Press, New York.
Leinikki, P., and Pässilä, S. (1977). *J. Clin. Pathol.* **29,** 1116.
Saunders, G. C., and Clinard, E. H. (1976). *J. Clin. Microbiol.* **3,** 604.
Saunders, G. C., and Wilder, M. E. (1974). *J. Infect. Dis.* **129,** 362.
Tenoso, H. (1977). *J. Infec. Dis.* (in press).
van Weemen, B. K., and Schuurs, A. H. W. M. (1971). *FEBS Lett.* **15,** 232.
Voller, A., and Bidwell, D. E. (1975). *Brit. J. Exp. Pathol.* **56,** 338.
Voller, A., and Bidwell, D. E. (1976). *Brit. J. Exp. Pathol.* **57,** 243.
Voller, A., Bidwell, D. E., and Bartlett, A. (1976a). *Bull. WHO* **53,** 55.
Voller, A., Bidwell, D. E., and Bartlett, A. (1976b). *In* "Manual of Clinical Immunology" (N. Rose and H. Friedmann, eds), p. 506. American Society of Microbiology, Washington, D.C.
Voller, A., Bartlett, A., and Bidwell, D. E. (1976c). *J. Gen. Virol.* **33,** 165.
Wolters, G., Kuijpers, L., Kacaki, J., and Schuurs, A. H. W. M. (1976). *J. Clin Pathol.* **29,** 873.

Chapter 11

Radioimmunoassay in Viral Diagnosis

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I. INTRODUCTION

Radioimmunoassay (RIA) was first developed to measure minute quantities of hormones (Berson and Yalow, 1968). Recent adaptations have been useful for measuring viral antigens and antibodies. In early applications, Fazekas de St. Groth and Webster (1963) used RIA methods to determine the kinetics of the interaction of influenza virus with specific antibodies, and Gerloff *et al.* (1962) developed a precipitin assay for poliovirus antibodies. Each of these procedures provided precise, quantitative measurements of specific immunological reactions and provided models for applications of RIA in other areas of virology.

There are many forms of RIA; however, the primary immunological reaction in RIA consists of the binding of an antigen by a specific antibody. The reaction may be quantitated by radiolabeling one of the primary reactants and measuring the radioactivity bound in the antigen–antibody complex. Alternatively, the reaction may be quantitated by a secondary reaction in which a radiolabeled indicator reacts with the primary immune complex. In any RIA procedure, separation of unbound (free) labeled reagent in either the primary or secondary reaction is essential for the success of the method. All reactions of liquid phase RIA take place in a fluid suspension, and separations are accomplished by procedures such as centrifugation, membrane filtration, and fractional precipitation. The reactions of solid phase RIA take place on a polymeric surface to which one of the reactants is attached, and unbound reactants are removed by rinsing. Thus, the versatility of RIA permits adaptations to detect and measure a variety of viral antigens and antibodies.

Many of the conventional serological procedures adequately provide diagnostic analyses in specific applications; therefore, alternate methods are not needed. However, some procedures have characteristic shortcomings, and alternate methods such as RIA should therefore be considered. RIA is analogous to fluorescent antibody (FA) techniques in concept and sensitivity, but measurement of radioactivity offers clear-cut advantages over measurement of fluorochrome excitation by FA. Quantitation by RIA is accomplished automatically by scintillation counters, whereas FA determinations require visual judgments by trained observers who determine quantitative end points. Other serological tests require a constant supply of viable cell cultures and infectious viruses, and are tedious and costly. Thus, alternate analytic procedures, such as RIA, should be considered.

The value of RIA in virological research has been amply demonstrated, and RIA is now a standard technique in many laboratories. Adoption of the procedure will be contingent upon the availability of a gamma scintillation spectrometer. In spite of this requirement, RIA methods are operationally simple, yet the radioactive labeling provides objective and quantitative determination of end-point titers. The RIA procedure can be used for the accurate determination of antibodies to numerous infectious agents. In its present form it is less time-consuming than most neutralization tests and eliminates the need for trained observers to visually judge titration end points. RIA will be especially advantageous for diagnostic laboratories that are considering automating serological procedures in order to process large numbers of specimens. The numerical data generated by RIA can be automatically recorded on magnetic tape or punch

tapes and transferred directly to relatively inexpensive electronic calculators for determination of titers.

In this review we shall present basic descriptions of RIA procedures which have been used successfully in research and diagnostic virology. Presentation of operational details for specific procedures is not within the scope of the review. However, we shall give examples of significant uses of the technique which may provide models for the prospective user of RIA.

II. METHODS AND REAGENTS

A. Liquid Phase RIA

Numerous forms of RIA for measuring various biological materials have been described; however, most RIA procedures may be classified as either liquid phase or solid phase systems. A liquid phase RIA is characterized by the fact that all immunological reactions occur between materials in a liquid suspension. In a solid phase system one of the immunological reactants is bound to the surface of a solid material at which site all reactions occur.

Liquid phase RIA is versatile in that any conventional *in vitro* antigen–antibody reaction can provide the basis of an RIA procedure. Moreover, the primary immunological reactions may be detected either directly, if one of the primary reactants is radiolabeled, or indirectly, if a radiolabeled indicator binds to the primary immune complex by a secondary reaction.

1. Direct RIA

In a direct noncompetitive RIA (Fig. 1) the only reaction is that which occurs between a purified viral antigen (Kalmakoff and Parkinson, 1973) or cellular material containing viral antigenic determinants (Klein *et al.*, 1972) and specific antibody. If the antigen is to be determined, the antibody is labeled with a radioisotope and incubated with various dilutions of the antigen-containing specimens. If antibody is to be determined, the labeled antigen is incubated with various amounts of antibody-containing specimens. Procedures for measuring either antigen or antibody by direct liquid phase RIA are listed in Table I. Typically, in a direct RIA for antibody, virus concentrations of 1 to 10 ng labeled with ^{125}I equivalent to approximately 10,000 cpm are reacted with serial dilutions of unlabeled antibody (Tronick *et al.*, 1975). The bound ^{125}I-labeled virus is then sep-

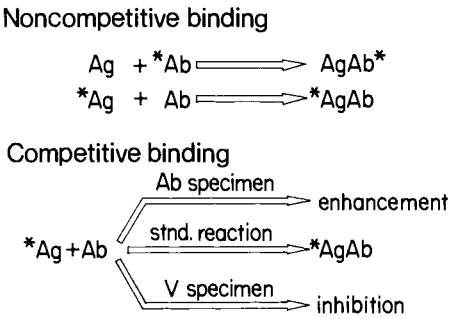

Fig. 1. Liquid phase RIA. Most common variations in quantitating viral antigen (V) or antibody (Ab) with either the Ag or Ab radiolabeled (*) are indicated.

arated from the mixture by centrifugation or other physical means. Endpoint concentrations of the antibody specimen are the dilutions which precipitate 50% of the labeled viral preparation.

2. Indirect Liquid Phase RIA

A variation of the double antibody technique has been used to measure either antigen or antibody. In this procedure neither of the primary reactants is radiolabeled; instead, a secondary antibody (species-specific for the Ig in the primary reaction) is radiosotope-labeled. Thus, the primary reaction is measured indirectly by a secondary immunological reaction (Daugharty, 1971; Daugharty *et al.*, 1972b). If viral antibody is measured, antibody-containing specimens are reacted with a standard quantity of unlabeled antigen in the primary reaction. If viral antigen is to be measured, a standard quantity of unlabeled antibody is used. In either assay the reaction is quantitated indirectly by determining the amount of radiolabeled antiglobulin binding to the primary antigen–antibody complex.

3. Competitive Inhibition RIA

A useful adaptation of the direct RIA procedure is based upon competition between an unlabeled reactant (antigen or antibody) and the same reactant which is radiolabeled (Fig. 1). Several sensitive assays are listed (Table I). In a model assay for antigen, the specimen is preincubated with a standard concentration of virus-specific antibody. The specimen will react to form antigen–antibody complexes in proportion to the concentration of antigen in the specimen. After the preincubation of antigen-containing specimens and standard antibody, a standard amount of labeled antigen is then added to the mixture and incubated. This competitive procedure then permits labeled antigen to react only with the remaining antibody sites not occupied by unlabeled antigen of the speci-

TABLE I
Liquid Phase RIA by Double Antibody Competitive Inhibition

Viral antigen	Antigen	Antibody
Swine influenza	Nath and Rodkey (1975)	
Rabies		Wiktor (1973); Tadeusz *et al.* (1972)
Hepatitis B core	Moritsugu *et al.* (1975); Robinson and Greenman (1974)	Moritsugu *et al.* (1975)
Hepatitis B surface	Lander *et al.* (1971); Aach *et al.* (1971)	Lander *et al.* (1971); Aach *et al.* (1971)
Adenovirus		Scott *et al.* (1975); Stinski and Ginsberg (1974)
Herpesvirus	Anzai *et al.* (1975)	
Poliovirus		Gerloff *et al.* (1962)
Arbovirus	Dalrymple *et al.* (1972), (1973)	
Vaccinia	Lander *et al.* (1972)	Felsenfeld *et al.* (1971)
Type C (general)	Scolnick *et al.* (1972); Sherr and Todaro (1974); Tronick *et al.* (1973); Szmuness *et al.* (1974); Strickland *et al.* (1974); Stephenson and Aaronson (1973b); Parks and Scolnick (1972); Todaro *et al.* (1974); Mickey *et al.* (1975); Attias *et al.* (1973); Strand (1973); Stephenson and Aaronson (1973a)	Scolnick *et al.* (1972); Stephenson and Aaronson (1973b); Parks and Scolnick (1972); Strand (1973); Attias *et al.* (1973)
Murine leukemia	Ihle *et al.* (1973); Hersh *et al.* (1974b); Grant *et al.* (1974)	Hersh *et al.* (1974b)
Murine sarcoma	Spira *et al.* (1974)	Spira *et al.* (1974)
Murine mammary tumor	Lo Gerfo *et al.* (1974)	Parks and Scolnick (1973)
Avian leukemia	Stephenson *et al.* (1973); Fritz and Qualtiere (1973)	Ruoslahti *et al.* (1973); Vaheri and Rouslahti (1973); Sandelin *et al.* (1974)

men. The amount of labeled antigen bound in the reaction with antibody is inversely related to the amount of unlabeled antigen in the specimen. Thus a comparison of the reduced amounts of radioactivity precipitated by the specimens with amounts of radioactivity precipitated by the controls provides a measure of the amount of antigen in the specimens. Modifications of the competitive inhibition assay have been reported (Coller *et al.*, 1971; Daugharty *et al.*, 1974) which permit detection of either antigen or antibody in a single determination.

4. Separation of Immune Complexes from Unbound Reagents

Most liquid phase RIA procedures have used centrifugation to separate the bound labeled reagents from the unbound or free reagents. This is accomplished by sedimentation in the range of 40 to 120 $\times$ $10^3 g$ (Kalmakoff and Parkinson, 1973; Tadeusz *et al.*, 1972). More complete sedimentation is assured when anti-Ig binds, in a secondary reaction with the primary antigen–antibody complex, to form a double antibody complex (antigen–antibody–anti-Ig). The formation of larger aggregates facilitates sedimentation of the radiolabeled reactants bound in the primary complex from the unbound reactants.

In addition to double antibody reactions, unreacted reagents have also been separated by precipitation of carrier proteins. The carrier precipitate is formed by the reaction of bovine serum albumin (BSA) with anti-BSA (Daugharty, 1971; Daugharty *et al.*, 1972a) or other protein–anti-protein systems and precipitation with $(NH_4)_2SO_4$ at a final concentration of 25% (Coller *et al.*, 1971; Daugharty *et al.*, 1974) or 40% (Charman *et al.*, 1974) or by Na_2SO_4 at a final concentration of 15% (Oroszlan *et al.*, 1972).

An effective method for separating the bound radiolabeled reagent in liquid phase assays from unbound reactants uses filtration on cellulose acetate filters. Antibody against ^{3}H-labeled reovirus (Attias *et al.*, 1973) or synthetic DNA (Sylvester *et al.*, 1973) has been quantitated by RIA in which complexes were collected by filtration, washed, dried, and counted directly with the entrapped materials remaining on the filters. An assay for rabies antibody, likewise, uses membrane filtration (0.45 μm Millipore filters) to separate complexes from unbound reactants (Neurath *et al.*, 1973). Separation of unbound labeled reactants from labeled materials by gel filtration (McPherson and Carnegie, 1968) has been used successfully; however, the technical difficulty of the method has precluded its use in routine procedures.

A cumbersome but satisfactory RIA for hepatitis B surface antigen (HB_s) used a combination chromatographic-electrophoretic procedure for separating HB_s antigen–antibody complexes from unbound elements of the reaction mixture (Walsh *et al.*, 1970). Aliquots of the reaction mixture

were spotted on suitable filter paper, chromatographed, and finally electrophoresed at a right angle to the chromatographic development to separate bound from free ^{125}I-HB_s. The labeled fractions were located by scanning for radioactivity. An assay utilizing staphylococcal strains rich in Jensen's protein antigen A is used to aid sedimentation of HB_s antibody complexed with radiolabeled HB_s antigen (Figenschau and Ulstrup, 1974). This is accomplished through the interaction of the protein A with IgG in the HB_s antigen–antibody complex.

Complexes may be separated from unbound reactants in gel diffusion systems. Although lack of sensitivity is often a hindrance, radioimmunodiffusion (RID) with autoradiography has been used in epidemiological surveys of viral antibody. RID for viral antibody is performed in a 1.0% agar medium with dilutions of test serum in peripheral wells and a species-specific Ig antiserum in the central well. After incubation, the globulin of the test specimen reacts with the anti-Ig and unbound reagents are removed by rinsing. Immobilized complexes are then allowed to react with ^{32}P- or ^{14}C-labeled viral antigen. Labeled complexes formed in the gels are thoroughly washed to remove unreacted labeled reagents. The labeled antigen bound to specific antibody provides a measure of antiviral activity of the test sera. The amount of isotope bound is measured by exposure of photographic film for 2 to 4 weeks. The titer of viral antibody in test serum is taken as the highest dilution of serum giving detectable lines on the developed film. RID methods have been applied to assays with respiratory syncytial virus (Bellanti *et al.*, 1971), poliovirus (Ainbender *et al.*, 1965; Ogra *et al.*, 1968, 1973), rubella (Ogra *et al.*, 1971), and echovirus (Ogra, 1970).

A conceptually different RIA for Shope fibroma virus antibody involves a complement-dependent cytolytic reaction (Singh *et al.*, 1972), with infected cell cultures labeled with ^{51}Cr. In this procedure the labeled cells are suspended in a liquid maintenance medium, incubated with the test serum and guinea pig complement, and, finally, the remaining cells are sedimented. The percentage of ^{51}Cr release by the cytolytic reaction is an index of viral antibody present in serum specimens.

B. Solid Phase RIA

Recently, RIA with reactants conjugated physically or chemically to an insoluble solid carrier (solid phase) were shown to be rapid, sensitive, reproducible, and inexpensive. Most of the solid phase methods to be discussed were developed for measuring hormones, specific serum proteins, and polypeptides; however, these methods can be readily adapted to measure viral antigens and antibodies. Selected examples of published solid

TABLE II
Solid Phase RIA

Virus antigen or antibody	Polymeric support	Reference
Newcastle disease	Microtiter plates	Cleland *et al.* (1975)
Herpesvirus type 1	Imitation pearl beads	Smith *et al.* (1974a); Patterson and Smith (1975)
Herpesvirus types 1 and 2	Microtiter plates	Forghani *et al.* (1974); Rosenthal *et al.* (1973a)
Smallpox	Microtiter plates	Ziegler *et al.* (1975)
Iridescent viruses	Glass cover slips	Bilimoria *et al.* (1974)
Epstein-Barr virus	Microtiter plates	Hutchinson *et al.* (1975)
Polyomavirus	Glass cover slips	Volkers and Pitts (1973)
Influenza virus	Plastic tubes	Daugharty *et al.* (1972b)
Hepatitis A	Microtiter plates	Hollinger *et al.* (1975)
Hepatitis B	Plastic tubes	Ling and Overby (1972); Ginsberg *et al.* (1973)
Hepatitis B	Microtiter plates	Purcell *et al.* (1973)
RNA tumor virus	Glass cover slips	Olsen *et al.* (1975)
Avian tumor virus	Glass cover slips	Weber and Yohn (1972)
Simian virus 40	Microtiter plates	Soriano *et al.* (1974)
Tobacco mosaic virus	Plastic tubes	Ball (1973)

phase RIA procedures for determination of either viral antigens or antibodies are listed in Table II.

Solid phase RIA in virology is characterized by the attachment of viral antigen or antibody to the surface of an inert, insoluble material on which all immunological reactions occur. In direct RIA, labeled antibody can be used to quantitate antigen attached to the solid phase medium, or labeled antigen can be used to quantitate antibody attached to the solid phase. Alternatively, in an indirect RIA (Fig. 2) for antibody, specific viral antibody reacting with antigen bound to the solid phase can be quantitated by a secondary immunological reaction in which radiolabeled anti-Ig is allowed to bind to the primary antigen–antibody complexes attached to the solid matrix. The unbound radiolabeled indicator is separated by rinsing.

Several polymeric materials, including diazotized polystyrene, *p*-aminobenzyl cellulose, bromacetyl cellulose, polytetrafluoroethylene, and copolymers of styrene and polytetrafluorethylene, were evaluated for use as solid phase support material for RIA (Catt *et al.*, 1966, 1967). Various polymers in the form of beads, plastic tubes, and small discs were activated by appropriate chemical treatment to produce protein binding sites on the polymer surface. Proteins or polypeptides are easily bound by covalent reactions between the active sites on the polymer and reactive groups of amino acid moieties.

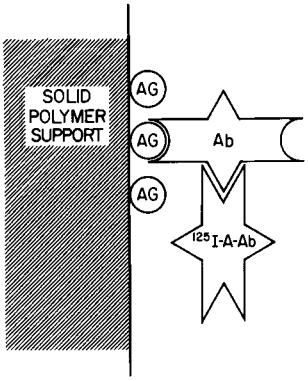

Fig. 2. Solid phase RIA for antibody. Indirect method consists of primary reaction between antigen (Ag), fixed to solid support, and sepecific antibody (Ab) in specimen. The amount of specific antibody bound to antigen is measured by the binding of radioisotope-labeled anti-globulin (^{125}I-A-Ab) to Ag–Ab complex.

One adaptation used antigen linked to disposable plastic tubes or isothiocyanate-substituted plastic discs for measuring immunoglobulins with a sensitivity in the nanogram to microgram range (Salmon *et al.*, 1969). Pure antigen was bound to the polymer; antibody reacted with the bound antigen, and finally labeled or unlabeled Ag was bound to the antibody in a competitive reaction. Because of the sequential binding of the reactants to the solid support, the method was designated "sandwich" solid phase RIA (Fig. 3).

A "sandwich" system of polystyrene tubes coated, sequentially, with rabbit anti-influenza globulin, influenza virus, serum specimen, and

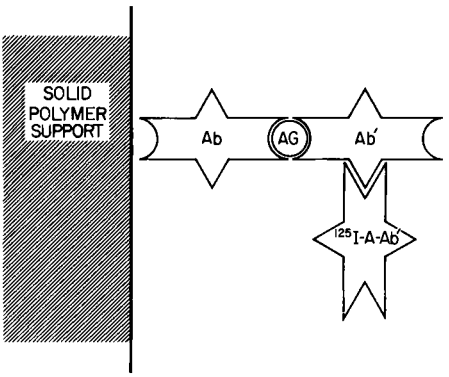

Fig. 3. Solid phase RIA. Indirect technique with reactants "sandwiched" onto solid support. Ab, antibody (nonhuman) specific for antigen; AG, viral antigen; Ab', antibody (human) specific for antigen; ^{125}I-A-Ab', radioisotope-labeled anti-human globulin.

finally with ^{125}I-labeled anti-human globulin was successfully developed for measuring antibodies to influenza virus (Daugharty *et al.*, 1972b). The titers obtained by RIA correlated well with titers obtained by a hemagglutination inhibition test. The sensitivity of the solid phase RIA system was reported by Allen *et al.* (1973) to be 50-fold greater than the sensitivity of the HI test.

Similar assays for hepatitis B antigens have been developed and used extensively in many clinical laboratories (Ling and Overby, 1972; Purcell *et al.*, 1973; Ginsberg *et al.*, 1973; Prince *et al.*, 1973; Irwin *et al.*, 1974; Hoofnagle *et al.*, 1974). In each of these RIA procedures both the primary reaction between an antigen and its specific antibody and the secondary reaction of this antigen–antibody complex with radioisotope-labeled indicator take place on the surface of plastic tubes or microtiter plates. In another application, the solid phase medium is Sepharose (Thamer and Kommerell, 1975). These procedures were found to be more sensitive than counterelectrophoresis and other conventional serological tests. The RIA test was observed to give some false positive reactions and the false positives had to be eliminated by the use of appropriate controls.

Recently, an RIA for hepatitis A antigen (HAV-Ag) was reported (Hollinger *et al.*, 1975). In this procedure unlabeled guinea pig hepatitis A antibody (HAV-Ab) was coupled to the wells of microtiter plates. Antigen-containing specimens were added to the coated wells and incubated for 2 hours at 45°C. Finally, ^{125}I-IgG anti-HAV-Ag was added to the wells and incubated. The amount of HAV-Ag in the specimens was determined by measuring the amount of radioactivity bound to each well of the microtiter plate. The specificity of the test was assured by a blocking reaction with known reagents. The development of this test offers promise that hepatitis A viral antigen may be routinely detected.

Solid phase systems have been further simplified, permitting them to be considered as practical alternatives for some conventional serological tests. In these simplified procedures antigenic materials are affixed to planar glass or plastic surfaces by histological fixatives or by simply drying. With this procedure a variety of relatively crude viral antigens, virus-infected cell homogenates, and tissue extracts have been successfully used in sensitive tests. These include procedures for antibodies to herpesvirus (Rosenthal *et al.*, 1972; Hutchinson and Ziegler, 1972), Epstein-Barr virus (EBV) (Hutchinson *et al.*, 1975), Newcastle disease virus (Cleland *et al.*, 1975), and iridescent virus (Bilimoria *et al.*, 1974).

RIA procedures have also been developed in which the viral antigens were prepared by infecting monolayer cells growing directly on cover slips (Volkers and Pitts, 1973) or in small glass vials (Forghani *et al.*, 1974). With each of these procedures the infected cell cultures were al-

lowed to dry or were histologically fixed to the planar surface in preparation for the subsequent immunological reactions of the RIA procedures.

In another form of a solid phase RIA, immune lysis of herpesvirus-infected cell monolayers (Smith *et al.*, 1972) or vaccinia-infected cells (Gipson and Daniels, 1974) have been used to measure viral antibodies. Instead of measuring direct binding of a labeled reagent, immune lysis of ^{51}Cr-labeled cells with release of ^{51}Cr into tissue culture fluid quantitates the amount of specific viral antibodies contained in the test sera.

C. Reagents

1. Antigens

The development of an RIA system is dependent on the availability of antigens and immunoglobulins of appropriate specificity. Generally, reagents of the highest purity are the most satisfactory. However, purity of antigens used in RIA depends upon the nature of the antigen and whether it is to be radiolabeled. If the antigen is radiolabeled it should possess a high degree of purity. If it is used unlabeled as a reagent reacting with labeled antibody, a less pure antigen may be used, and the antibody instead of the antigen should be highly purified.

Cultures infected with 20 egg infective doses (EID_{50}) of influenza virus per cell have been used in a successful RIA for influenza antibody (Smith *et al.*, 1972). BHK cells on cover slips infected with adenoviruses (Evans and Yohn, 1970; McCammon and Yohn, 1971), herpesviruses (Smith *et al.*, 1972; Hayashi *et al.*, 1974), and polyomaviruses (Volkers and Pitts, 1973) have also been used. Monolayer cultures grown on the bottom of 1-dram vials infected with 0.1 to 0.3 infectious particles of herpesvirus per cell provided a satisfactory antigen for determining herpesvirus antibody (Irwin *et al.*, 1974). Antigenic material from infected monolayers have also been prepared for herpes and vaccinia RIA by freezing and thawing, or sonication, and then dessication and fixation on microtiter plates (Hutchinson and Zeigler, 1972; Felsenfeld *et al.*, 1971; Rosenthal *et al.*, 1973a; Ziegler *et al.*, 1975). Influenza and herpes have likewise been fixed on imitation pearl beads as a solid phase (Smith *et al.*, 1974b). Cell suspensions infected with EB virus have been used successfully in liquid phase assays (Hewetson *et al.*, 1972). Alternatively, in solid phase systems, mumps (Daugharty *et al.*, 1973) and influenza (Daugharty *et al.*, 1972b) viral antigens from crude viral suspensions have been adsorbed onto polystyrene surfaces of centrifuge tubes by adherent viral-specific globulin (Daugharty *et al.*, 1972b). In this procedure some purification is effected by the selective adsorption and subsequent rinsing of the tubes.

Liquid phase RIA generally uses viral antigen of relatively high purity, since extraneous proteins may inhibit efficient binding. The hepatitis B surface antigen used in both liquid and solid phase RIA is usually purified by rate zonal centrifugation or by combinations of CsCl and sucrose density gradient centrifugation (Coller *et al.*, 1971; Purcell *et al.*, 1974; Daugharty *et al.*, 1974; Lander *et al.*, 1971). Poliovirus antigen has been purified similarly (Gerloff *et al.*, 1962). In addition to centrifugation, various purification procedures have been used advantageously to prepare specific antigens. Rabies viral antigen, especially the glycoprotein, has been obtained from tissue culture cells disrupted with tri-(*n*-butyl) phosphate followed by zinc acetate precipitation and ultracentrifugation (Wiktor, 1973; Neurath *et al.*, 1973). Influenza virus can be purified and concentrated from allantoic fluid by adsorption and elution from chicken red blood cells and further purified by ultracentrifugation (Daugharty, 1971). In some RIA procedures, such as radioimmunoprecipitation, highly purified subviral components may have to be used; therefore, additional steps are required to dissociate viral particles and to resolve the various components into purified fractions. For example, purified intact influenza virus (Nath and Rodkey, 1975) or avian leukosis virus (Fritz and Qualtiere, 1973) purified by ultracentrifugation is further dissociated with deoxycholate and other detergents preparatory to fractionation of subviral components. The preparation of each antigen must be considered separately. Hence, various purification steps have been reported. HSV antigen eluted from Sepharose 2B may also be further fractionated by dissociation and subsequent isolation of subviral components by density gradient centrifugation (Aach *et al.*, 1971). Tween–ether disruption of avian myeloblastosis virus produced a satisfactory antigen for RIA. Many of the type-C viruses purified by Ficoll gradient centrifugation provided a satisfactory antigen for RIA (Verstraeten *et al.*, 1975). Preparation of specific subviral fractions as antigens frequently requires a combination of two or more methods of fractionation to obtain the desired purity. Specifically reactive viral components were produced in several laboratories (Tronick *et al.*, 1975; Attias *et al.*, 1973; Parks *et al.*, 1973; Ruoslahti *et al.*, 1973; Parks and Scolnick, 1972) by a combination of procedures, including sucrose gradient centrifugation, gel filtration in 6 *M* guanidine, and isoelectric focusing.

Herpes simplex viral (HSV) antigens have been radiolabeled in tissue culture by uptake of isotope-containing metabolic precursors. Labeling by this method is inefficient; however, it has been used advantageously for specific research purposes. The metabolically labeled virus can be purified for use in RIA by hypotonic disruption of the cells to release viral antigens and then by ion-exchange chromatography, cesium chloride gra-

dient centrifugation, or gel filtration. Both HSV (Anzai *et al.*, 1975) and adenovirus type 5 hexon antigen (Stinski and Ginsberg, 1974) have been prepared by one or more of these procedures. The Dane particles of hepatitis B virus for use in immune precipitation with anti-HB_c and anti-human γ-globulin require digestion with a DNA poylmerase (Robinson and Greenman, 1974).

2. Test Specimens

Native material, such as whole serum, spinal fluid, tissue extracts, or secretory fluids, can be used in RIA. Each of these materials, however, should be clarified by low-speed centrifugation and, frequently, special procedures are necessary to release antigenic materials. In the routine assay of tumor tissues for type-C viral peptide antigens, hypotonic disruption and homogenization are used to release antigen. Some native materials must be treated with a detergent or by reduction with 2-mercaptoethanol to release viral antigens capable of competitively inhibiting immunological reactions in RIA (Vaheri and Ruoslahti, 1973; Mickey *et al.*, 1975). In some procedures, disrupted tumor tissues are also sonicated, RNase treated, or precipitated with salt to concentrate the viral antigen. The precipitated antigen may be solubilized before testing in RIA (Soriano *et al.*, 1974). In other procedures crude antigens, such as smears of virus-containing tissues made in vials, are satisfactory for RIA (Irwin *et al.*, 1974). With other pathological tissues, specific viral antibody may be eluted with low pH buffers.

3. Globulins

The purity of globulins required in RIA depends upon the specific requirements of the analytical systems. Globulins in double antibody assays may be used in the form of whole serum with species specificity for globulins bound in immune complexes (Hollinger *et al.*, 1971; Scolnick *et al.*, 1972; Park and Scolnick, 1972) or for adherence to solid phase media (Ling *et al.*, 1973; Prince *et al.*, 1973; Daugharty *et al.*, 1972b). If the globulin is used as a radiolabeled indicator in direct assays, a purer preparation should be used. Radiolabeled globulins are generally satisfactory when the globulins are prepared by sodium sulfate (Daugharty, 1971; Hutchinson and Ziegler, 1972) or ammonium sulfate (Rosenthal *et al.*, 1973b) perciptitation. Additional purification of salt-precipitated globulins by ion-exchange chromatography yields partially purified globulin fractions having increased reactivity in RIA (Heweston *et al.*, 1972; Fazekas de St. Groth and Webster, 1963; Purcell *et al.*, 1974). All classes of viral-specific globulin can be indirectly quantitated with a radiolabeled indicator of sulfate-precipitated globulin of combined specificity for IgG, IgA, and

IgM (Daugharty *et al.*, 1972a; Hutchinson and Ziegler, 1972). Detection and quantitation of antibody of a particular Ig class, however, requires globulin that is specific for a single Ig class. Ig-class-specific antisera are prepared by immunizing animals with an appropriate Ig heavy chain protein. In preparation of the globulin protein, specific Ig-class globulins are isolated by salt precipitations and ion-exchange chromatography. Then heavy chain protein is prepared from purified globulins by reductive cleavage, alkylation of the cleaved products, and isolation by gel filtration (Daugharty *et al.*, 1972a; 1973; McCammon and Yohn, 1971). Alternatively, Ig class specificity has been attained by selective adsorption to remove immunoglobulins of unrelated specificity.

D. Calculation of RIA Titers

Characteristically, RIA generates data which permit comparison of amounts of radiolabeled reagents specifically bound in an immunological reaction with amounts of the same reagents nonspecifically bound to negative control materials. In any RIA procedure the concentrations of the materials being assayed may be calculated by one of several realtively simple mathematical methods.

1. Statistical Method

The simplest method of determining positivity of specimens by RIA consists of comparing the radioactivity (cpm) bound by the test specimen with the radioactivity bound by negative controls (Daugharty *et al.*, 1972a). The comparison may be accomplished statistically by first determining the standard deviation of radioactivity among an appropriate number of replicates of negative controls. This accounts for variations of the technique and variations among negative control materials. Then the radioactivity bound by specimens is compared with the control values. Test values which vary significantly from the controls are considered positive (Daugharty *et al.*, 1974). Titers may be designated as the highest dilution factor at which the bound radioactivity deviates significantly from the radioactivity of the controls (Purcell *et al.*, 1973). Similar comparisons of test specimens with known concentrations of a standard material may be used to express concentrations (Parks and Scolnick, 1972).

2. Plot of Dose–Response Relationships

Dose–response curves may be established by plotting bound radioactivity versus dilution factors of the specimens. Generally, the variables must be transformed to obtain linear curves [e.g., log-log plot (Hutchinson *et al.*, 1975); logit-log plot (Hollinger *et al.*, 1971)]. Titers may be

determined by interpolation or extrapolation of specimen dilution factors causing binding of radiolabeled indicators in amounts equivalent to a standard multiple of the radioactivity bound by background controls (Rosenthal *et al.*, 1973a; Soriano *et al.*, 1974).

3. Paired Radioiodine-Labeled Antibody Technique (PRILAT)

Nonspecific binding of radiolabeled indicator-globulins to antigens in all RIA procedures must be recognized, and appropriate controls must be included in the procedure to obviate its effect. The PRILAT procedure of RIA (Pressman and Roholt, 1961; Tanigaki *et al.*, 1967; Evans and Yohn, 1970) was designed to eliminate, in a single assay, contributions due to nonspecific binding reactions. In this procedure ^{125}I-labeled IgG from specific antiserum is mixed with ^{131}I-labeled IgG from normal serum, and the paired serum mixture is then reacted with both test specimens and control material. The ratios of ^{125}I(cpm)/^{131}I(cpm) for the specimen and control permit calculation of specific binding, expressed as "specific uptake quotient" (SUQ) (Evans and Yohn, 1970). Titers may be expressed as the dilution factor of a specimen yielding a specified SUQ.

E. Radiolabeling

In addition to the requirement for reagents of immunological specificity, successful development of sensitive RIA methods also requires radioisotope-labeled reagents, either labeled antigen or labeled antibody, with high specific radioactivity. Isotopes of value in RIA with suitably long half-lives include ^{3}H, ^{14}C, ^{125}I, and ^{131}I. Relatively simple methods for labeling proteins with these elements will be described.

1. Iodination

Because of the simplicty of chemical iodination methods, reagents labeled with ^{125}I or ^{131}I are used in most of the RIA procedures. These radioisotopes can be used interchangably; both isotopes have sufficiently energetic gamma emissions to permit detection by an automatic scintillation spectrometer equipped with a crystal detector. The half-life of ^{131}I (8.05 days) is shorter than the half-life of ^{125}I (60 days); therefore, more frequent iodination of reagents is necessary when ^{131}I is used. Iodine atoms of each of these isotopes are efficiently incorporated into protein molecules by several methods, with only minimal loss of immunological or biological specificity.

The simplicity of the chloramine-T iodination procedure (Greenwood *et al.*, 1963) has led to adoption of the method for labeling viral antigens and antibodies (Hutchinson and Ziegler, 1972; Rosenthal *et al.*, 1973a; Spira

et al., 1974; Daugharty *et al.*, 1974). Hutchinson and Ziegler (1974) have described precise conditions yielding radioisotope-labeled Ig of both high specific radioactivity and immunospecificity. In each of the adaptations of the Greenwood *et al.* (1963) method, $Na^{125}I$ or $Na^{131}I$ is added, with stirring, to a protein solution. The oxidizing reagent, chloramine-T, is then rapidly added to the mixture and allowed to react for 5 minutes or less. The reaction is terminated by the rapid addition of a reducing agent, sodium metabisulfite.

A similar iodination procedure (McFarlane, 1956), with iodine monochloride as an oxidizing agent, has been used successfully in viral RIA (Daugharty, 1971). With both the chloramine-T and iodine monochloride methods, unreacted iodine is separated from the labeled proteins by dialysis or column chromatography.

Several other methods of iodination may have special advantages for some applications. Lactoperoxidase and relatively low concentrations of hydrogen peroxide are used in an enzymatic procedure to create mild oxidizing conditions and thus label proteins (Stanley and Haslam, 1971; Witte *et al.*, 1973; Sefton *et al.*, 1973; Fritz, 1974). Soluble proteins are efficiently iodinated by this method; however, because of the molecular size of lactoperoxidase, only surface protein of intact virions and cell membranes are labeled. Selective labeling of external proteins has been used advantageously in investigations of membrane antigens. With this method of iodination there is little or no denaturation of the protein; however, the iodinated protein reagents will be contaminated with small amounts of enzyme protein. Moreover, the protein preparations to be iodinated must be free of azide which interferes with the labeling reaction.

A method described by Bolton and Hunter (1973) may have special application for iodinating labile materials which are denatured by other methods. With this procedure, labeling is accomplished by first iodinating an alkylating agent and then coupling the iodinated ligand to the protein through free amino groups. This method eliminates exposure of the protein to oxidizing reagents. Other methods, including an electrolytic method (Rosa *et al.*, 1964) and a microdiffusion technique (Gruber and Wright, 1967), have been reported, but have not been widely used.

2. *Labeling with β-Emitters*

Although γ-emitting isotopes of iodine are commonly used in RIA, β-emitters (^{14}C and ^{3}H) have been used. In spite of early attempts in which only low efficiency was attained, recently described methods may be of value. Viruses were adequately labeled with ^{14}C or ^{3}H by reductive alkylation with [^{14}C]formaldehyde or [^{3}H]formaldehyde and sodium borohydride. With this procedure, polyomavirus (McMillen and Consigli, 1974),

Newcastle disease virus (McMillen and Consigli, 1975), and feline leukemia virus (Velicer and Graves, 1974) were effectively labeled without alteration of their biophysical characteristics. Membrane surface proteins of influenza viruses were labeled by [^{3}H]borohydride reduction of a Schiff's base formed by pyridoxal phosphate and protein amino groups (Rifkin *et al.*, 1972).

Acetylation has been used in other procedures to incorporate either ^{3}H or ^{14}C into viral protein antigens or antibodies. Montelaro and Rueckert (1975) used either ^{3}H- or ^{14}C-labeled acetic anhydride to label viral antigens and, with a similar procedure, immunoglobulins may be acetylated with [^{3}H]iodoacetate (Kalmakoff and Parkinson, 1973).

Although reagents labeled with ^{3}H or ^{14}C are advantageous because of their long half-lives, determinations of weak β-emitters require the use of liquid scintillation counters. Thus, ^{3}H- or ^{14}C-containing specimens must be solubilized in a toluene-base scintillation fluid. On the other hand, materials labeled with γ-emitters can be counted directly, without preparation, in crystal detectors.

III. APPLICATIONS

A. Characterization of Viral Antibodies

RIA procedures for determination of specific viral antigen and antibodies were listed in Tables I and II. In addition to procedures for measuring viral antigen or antibody in specimens, some of the procedures listed were specifically designed to determine Ig class-specific antibodies, the kinetics of immune responses, and the unique characteristics of immunological factors. Likewise, some of the procedures for measuring antigens are designed to differentiate related antigens by detecting subviral components and to characterize antigens and their appearance in viral infections. Selected examples of RIA procedures designed to detect and measure specific factors are described.

1. Sensitivity of RIA

RIA tests are usually more sensitive than conventional serological methods. RIA for HB_s antigen has been shown to detect as little as 0.01 μg/ml (Ling and Overby, 1972). This represents a sensitivity 1000 times that of gel diffusion, 500 times that of cross-over electrophoresis, and 250 times that of complement fixation. RIA for adenovirus antibody was a 1000-fold more sensitive than the conventional neutralization test, and 300-fold more sensitive than the HI test (Scott *et al.*, 1975). RIA tests for

antibodies to influenza (Daugharty *et al.*, 1972a) and mumps virus (Daugharty *et al.*, 1973) were shown to correlate closely with hemagglutination inhibition or neutralization tests. Studies have indicated that RIA for EB viral antibody is equally (Hutchinson *et al.*, 1975) or about four times (Hewetson *et al.*, 1972) as sensitive as the indirect membrane fluorescence test. Similarly, RIA for the group A arboviruses (Dalrymple *et al.*, 1972) is about as sensitive as the plaque reduction neutralization test. RIA for smallpox antibody in vaccinees, on the other hand, readily detected responses which were not always detected by plaque reduction neutralization (Ziegler *et al.*, 1975).

2. *Determination of Immunoglobulin Classes*

A potentially important application of RIA is in measuring the Ig class of viral antibody. This is done by using radiolabeled Ig class-specific antibody to measure only a specific Ig class of antibodies reacting with viral antigens. Investigations of antibodies against influenza (Daugharty, 1971; Daugharty *et al.*, 1972a) and mumps viruses (Daugharty *et al.*, 1973) (Fig. 4) showed that serum specimens collected early during the acute phase of illness were more reactive with specific ^{125}I-anti-IgM than with ^{125}I-anti-IgG. Conversely, convalescent specimens were more reactive with ^{125}I-anti-IgG. In similar studies, class-specific antibodies arising from both immunization and natural infections were reported for other viruses. Ogra (1971) successfully characterized the Ig classes in persons immunized with polio vaccine. In a related study (Ogra *et al.*, 1971) the Ig class-

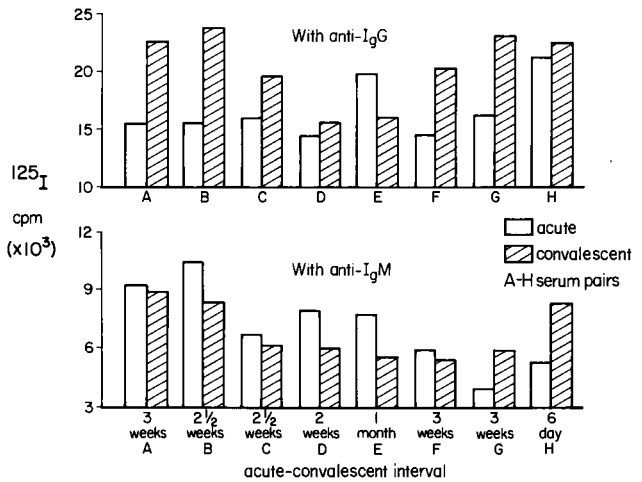

Fig. 4. Binding values for ^{125}I-anti-IgG and ^{125}I-anti-IgM indicators in solid phase RIA for mumps virus-bound antibodies in paired acute and convalescent sera.

specific response to both natural rubella infections and rubella immunization by different routes was determined by RIA. The Ig class antibody response to echovirus T6 (Ogra, 1970) was likewise determined. The sensitivity of the RIA procedure has permitted determination and comparison of the IgA class of antibodies with the concentrations of the other classes of immunoglobulins (Ainbender *et al.*, 1965).

Other studies were useful in determining virus-specific immunoglobulin subfractions. Generally, indirect methods of RIA for total IgG or virus-specific IgG have proved to be more sensitive than direct methods (Daugharty *et al.*, 1972b). Virus-specific antibody fractions of IgG, as measured by indirect RIA, were shown to be 10–12% of the total IgG in rabies hyperimmune sera (Tadeusz *et al.*, 1972). In a related study, subfractions of immunoglobulins were characterized by determining the relative avidities of Ig classes (Daugharty, 1973). Heterogeneity of the antibodies was indicated by inhibition of 19 S antibodies when 7 S globulin was added in amounts giving a ratio (19 S:7 S) of 5 or less. The reverse effect, inhibition of 7 S globulin by 19 S globulin, was not observed.

Flexibility and sensitivity of RIA has permitted studies of closely related populations of viral antibodies. Dalrymple *et al.* (1972) investigated the relationships of antibodies produced by immunizing animals with eastern equine encephalitis (EEE), western equine encephalitis (WEE), and Sindbis (SIN) encephalitis viral antigens. In a radioimmunoprecipitation system with homologous and heterologous reactions, WEE antibodies were shown to consist of three populations, namely, those with (1) type specificity, (2) broad reactivity at intermediate dilutions, and (3) intragroup (WEE–SIN) specificity.

3. Detection of Immune Responses to Viral Infections

RIA procedures have been used extensively to characterize immune responses to viral infections. In many studies they have provided both qualitative and quantitative determinations which were not possible with conventional serological procedures. The sequential appearance of immunoglobulins in hepatitis B infections was investigated by Lander *et al.* (1971). These studies revealed that maximal titers were attained about 2 weeks after transaminase enzyme levels increased and that a secondary HB infection could be distinguished from a primary response. In other studies temporal responses to both immunization and natural infections with polio were described (Ogra *et al.*, 1968). Polio antibodies measured by RIA were detectable long after neutralizing antibodies disappeared. Similar investigations showed that responses to poliovirus immunization depend upon the mode of immunization. Most systemic responses are readily determined by conventional serological methods. However, more

senstive RIA procedures revealed that antibodies produced at local sites depended upon the route of immunization. Antibody production varied when the immunogen was inoculated parenterally, intranasally, or intrathecally (Ogra *et al.*, 1973).

Characterization of the immune responses of other viruses has likewise been described. Comparisons of the RIA titers of antibodies against respiratory syncytial virus (Bellanti *et al.*, 1971) and vaccinia virus (Felsenfeld *et al.*, 1971) with the titers obtained by neutralization tests indicated that antibodies measured by RIA persisted longer. Neutralizing antibodies for vaccinia subsided after approximately 1.5 years, whereas antibodies measured by RIA lasted up to 3 years. The prolonged existence of antibodies indicated by RIA may reflect either a greater sensitivity of RIA or detection of qualitatively different antibodies.

RIA has been used extensively in investigations of virus-induced tumors and the possible immunotherapy of tumors. Determination of Ig class-specific antibodies by RIA has been used to describe the immune responses to oncogenic viruses. In rabbits inoculated with Shope fibroma virus, tumors had formed by day 13, and by day 23 they had completely regressed. Seven days after infection with the virus, antibodies were detected by RIA. The specific antibody titers increased through day 23 and persisted for at least 50 days (Singh *et al.*, 1972). On day 7 the antibodies were primarily of the IgM class (19 S) and by day 13 the concentration of IgM-class antibodies was maximal. In related studies in human patients with malignancies, immunization with Rauscher's murine leukemia virus antigen produced an immune response in 50–60% of the patients (Hersh *et al.*, 1974a,b). Antibody first appeared about 2 weeks after immunization and reached maximal levels in about 8 weeks. Thus, antibodies against oncogenic viruses were produced in many patients and, in some, regression of the tumor was coincident with the immune response.

Patterns of immune responses to herpesviruses were measured by a cytolytic ^{51}Cr-release RIA (Smith *et al.*, 1972). Because of the antigenic relationship of HSV-1 and HSV-2, selective absorption of immune sera was used successfully to distinguish between the responses to each of the viruses. Absorption of sera with HSV-1-infected cells removed all homologous cytolytic antibodies, leaving cytolytic antibodies specific only for HSV-2 by RIA. HSV-2 antibodies in sera collected 3–28 days after onset of illness were found in the IgM fractions. The cytolytic activity in sera collected after 8 months was in the IgG fraction.

4. Autoimmune Reactions

The possible significance of specific anti-viral antibodies in the sera of patients with autoimmune diseases has led to extensive examination of

sera from persons with systemic lupus erythematosis (SLE). In these studies with an RIA procedure, reovirus RNA labeled with tritium was used to detect specific RNA-binding antibodies. A high incidence of anti-RNA reactivity was found in many lupus sera (Sylvester *et al.*, 1973; Attias *et al.*, 1973). The binding of antibodies in lupus sera by the reovirus antigen was inhibited by reovirus RNA as well as phage RNA (Sylvester *et al.*, 1973) and RNA from mycoplasma (Talal, 1972). Although the heterogeneous reactivity shown by RIA is not fully explained, the results suggest that RNA antibody was induced by viral RNA.

Immunological factors which interfere with the determination of antibodies by conventional serological methods may also interfere with RIA procedures. Two of these factors, rheumatoid factor (RF) and immune complexes, have been investigated. With a complement-dependent cytolytic system using ^{51}Cr-labeled vaccinia-infected cells, RF was shown to inhibit immune lysis (Gipson and Daniels, 1974). The inhibition was attributed to the competition of RF with complement for binding sites to the Fc region of vaccinia antibody. Thus, RF may alter the cytolysis of the vaccinia-infected cells, and as a consequence may give false negative results. On the other hand, in an RIA method for determining IgM-class anti-CMV antibodies, RF in a serum specimen may react with CMV-specific IgG bound to the solid phase CMV test antigen and give false positive results (V. Knez *et al.*, 1976). The "apparent" indication of CMV-specific IgM was due, however, to interaction of RF with the CMV-IgG complex. Thus, IgM-RF was measured as well as IgM-CMV antibodies.

5. Immune Complexes

Viral antigen and antibody in complex have been detected either by RIA methods specific for complexes or by RIA for viral antigen or antibody after the complexes have been physically separated. Quantitative interaction of influenza virus with antibody results in the formation of complexes which, when separated by gradient centrifugation, react with radiolabeled anti-Ig proportional to the concentration of virus or viral antibody (Daugharty, 1971).

The quantitative relationships of HB_s antigen and antibody in the formation of complexes were also evaluated (Daugharty *et al.*, 1974). Complexes formed *in vitro* from reaction of HB_s antigen or antibody were indicated by the absence of either excess antigen or antibody; the complexes did not have to be separated physically. In another investigation of the quantitative aspects of complex formation, antibodies existing as complexes were eluted from aortic tissue and measured. The detection of antibodies against measles and herpesviruses suggests that viral antigens and

antibodies were bound in complexes in approximatlely 30% of a group of specimens examined by RIA (Smith *et al.*, 1974b).

B. Characterization of Viral Antigens

1. Detection of Viral Antigens

The procedures cited in Tables I and II represent radioimmunoassays of various configurations, each adapted to a specific application. Because of the versatility of RIA, conventional serological analyses may provide models for development of RIA procedures to fulfil the needs of specific projects. Examples of useful applications of RIA to detect viral antigens are described.

In a study by Fazekas de St. Groth and Webster (1963), designed to relate antigenic determinants of influenza virus with infectious particles, the specific binding of ^{131}I-anti-influenza globulin was measured. They determined that there are approximately 2000 antigenic sites per infectious influenza virus particle.

The sensitivity of RIA permits early detection of viral antigens arising from *de novo* synthesis. Newly synthesized pox antigen (Hayashi *et al.*, 1974) and tumor-specific transplantation antigen of polyomavirus (Volkers and Pitts, 1973) were shown to appear as early as 2–6 hours after infection. Adeno 12 tumor antigens, on the other hand, do not reach maximal titers until 36 hours postinfection (Evans and Yohn, 1970).

Superinfection of lymphoblastoid cell lines with EBV was investigated by Kein *et al.* (1972). They found that 18 of 29 cell lines produced either virus or viral antigens. RIA procedures were used to determine the mechanism of resistance to infection by the "nonproducers." After each of the cell lines was exposed to the EBV inoculum, absorption of virus by the cells was determined indirectly by measuring the amount of ^{125}I-labeled anti-EBV globulin the cells adsorbed. Quantitative examination of the binding of radiolabel to the cells clearly showed that "nonproducing" cell lines did not adsorb virus and that resistance probably was established at the receptor level.

2. Viral Strain Differentiation

RIA has been especially useful for investigation of antigenic relationships among related viral strains. In an early application of RIA in influenza virus studies (Fazekas de St. Groth and Webster, 1963), cross-reactivity among heterologous viral strains was evaluated. The cross-reactivity of swine influenza viruses SW31 and SW73 was demonstrated in binding studies. Heterologous strain SW31 partially inhibited the bind-

ing of ^{125}I-labeled SW73 by SW73 antiserum. The partial cross-reactivity was demonstrated by the fact that SW31 required larger amounts of antigen than inhibition by the homologous unlabeled SW73. Among related viruses the amount of inhibition by heterologous strains is an indication of the degree of antigenic relatedness.

Differentiation of various plant mosaic virus strains by RIA techniques has also been reported (Ball, 1973). These viruses were differentiated by measuring the inhibition by heterologous viruses of the binding of a homologous virus with its homologous antibody.

Development of RIA was closely associated with the early research on hepatitis, and during this developmental period the specificity of RIA procedures was frequently considered. The specificity was confirmed by studies in which HB antigen–HB antibody reactions were blocked by partially purified fractions from convalescent hepatitis sera (Hollinger *et al.*, 1975). Furthermore, the infectivity of blood from donors was shown to be directly related to the quantity of HB core (HB_c) and HB surface (HB_s) antigens detected by RIA (Hoofnagle *et al.*, 1974). It was concluded that either HB_s or HB_c antigens, which probably arise from persistent viral replication, are responsible for transmission of the disease.

In later investigations HB antigens were efficiently subtyped according to group and type specificity by RIA. HB_s antigenic groups can be ascertained on the basis of binding curves resulting from the reaction of antigens in specimens with standard antisera. If the ^{125}I-anti-HB is of a predominant type specificity (i.e., d or y) levels of saturation (binding) and dose-response curves, characteristic of that type, can be demonstrated (Ginsberg *et al.*, 1972). Thus, saturation analysis with standard reagents provides a method for differentiating ad and ay specificity. Definitive antigenic analysis requires highly specific antisera prepared by absorption with specific antigens. Absorption of HB_s antisera with either ad- or ay-specific antigen yields antisera of type specificity. With these type-specific reagents, the HB_s antigen in specimens may be efficiently determined (Ling *et al.*, 1973).

Closely related viruses in many specimens may be identified by characterizing antigen–antibody reactions. Usually, characterization is based upon the slopes of RIA binding curves. Curves with the greatest slopes are indicative of binding reactions of high affinity (Tronick *et al.*, 1973). Thus, with standard antisera the binding affinity, as expressed by the slopes of binding curves, may be used to identify or distinguish closely related antigens. This technique has been used to characterize various strains of mammary tumor virus (Verstraeten *et al.*, 1973). Temperature mutant strains of the Raucher MLV could be separated into three distinct classes on the basis of differential slopes of RIA binding curves. Like-

wise, the use of specifically adsorbed anti-herpes globulins in RIA has provided definitive reagents for distinguishing HSV-1 and HSV-2 (Irwin *et al.*, 1974).

3. Identification of Antigens of Type-C Viruses

Many investigators have used RIA to characterize the antigenic structure of type-C RNA viruses. The relationship of virus-associated antigens with tumor development was confirmed by RIA in investigations of the mouse mammary tumor virus (LoGerfo *et al.*, 1974). RIA procedures were especially useful for identifying and characterizing the antigenic relationships of the endogenous cellular antigens isolated from tumors of various animals. In these procedures the inhibition by a heterologous test antigen of the binding reaction between an oncornaviral antigen and its homologous antiserum yields inhibition curves characteristic of the test antigen. Thus comparisons of inhibition curves permit identification and characterization of the endogenous group antigens induced by oncornaviruses. In each of the investigations component antigens were isolated and purified in preparation for antigenic characterization. Antigens of the murine mammary tumor virus (Parks *et al.*, 1974) were characterized in a competitive RIA system with specific antisera prepared in rabbits. The peptide antigens, P27 and P52 (MW 27×10^3 and 52×10^3 daltons, respectively), were found to be different from each other and from other major murine type-C viral polypetides.

In similar investigations of type-C viruses of primates, the major viral component polypeptides were similarly examined. A glycopolypeptide of about 30,000 daltons was isolated from tumors of both woolly monkeys and gibbons (Parks *et al.*, 1973). Comparisons by RIA established that the 30,000-dalton peptides from each of the species were indistinguishable. Therefore, investigators concluded that the viruses from each of the species were closely related. They further concluded that there was little immunological relationship between the type-C viruses of primates and other mammals. Tronick *et al.* (1974) also isolated a 12,000-dalton polypeptide from a woolly monkey type-C virus. In characterization studies the 12,000-dalton antigen, unlike the 30,000-dalton antigen, was clearly distinguishable from the same peptide isolated from the gibbon tumor virus. Thus, by comparing the 12,000-dalton peptide antigens, the type-C viruses of woolly monkeys and gibbons can be distinguished.

Investigations have established that type-C viruses of mammalian origin share common antigens, or interspecies antigens. Interspecies specificity was shown for murine- and feline- derived antigen, P3 (Parks and Scolnick, 1972; Strand, 1973); likewise, interspecies specificity was confirmed and extended for antigens P3 and P30 produced in cats, hamsters, rats,

and mice (Charman *et al.*, 1974). Interspecies specificity was further confirmed in studies with cats infected with feline leukemia virus (FeLV) (Olsen *et al.*, 1975). Infection with FeLV produced antibodies to mammalian interspecies antigenic determinant common to type-C mammalian tumor viruses. RIA procedures were used in each of these investigations to characterize the antigenic relationship of these tumor-inducing viruses.

4. Identification of Subviral Antigenic Components

Characterization of the antigenic specificities of subviral components has been a valuable technique for establishing the relationships of viruses of specific groups. This was demonstrated in radioimmunoprecipitation tests with three arboviruses (Dalrymple *et al.*, 1973). Nucleocapsid antigens prepared from SIN, WEE, and EEE viruses were cross-reactive to a degree that precluded differentiation of homologous and heterologous reactions. On the other hand, with envelope antigens homologous reactions were easily distinguished from heterologous reactions.

Likewise, the specificity of surface antigen of another group of viruses was demonstrated. Examination of the antigenic specificity of mouse mammary tumor virus showed that in an RIA inhibition test the slopes of the inhibition curves were altered when the intact virus was treated with detergent (LoGerfo *et al.*, 1974). The change of specificity suggests that the envelope antigen was altered or removed by the detergent treatment. Furthermore, acetone fixation of infected cells for use in RIA permitted characterization of antibodies against cytoplasmic antigens, whereas the use of unfixed cells permitted measurement of surface antigens (Hayashi *et al.*, 1974). Fractionation of antigenic materials from infected cells or tissues and identification by RIA have been used to reveal association of specific antigens with cell structures. The group antigen, P30, of type-C viruses has been detected on the surface of sarcoma cells infected with murine leukemia virus (Grant *et al.*, 1974) and in lymphoid cell extracts from cases of acute leukemia (Sherr and Todaro, 1975). RIA has also been useful in detecting antigen in certain clones of cells. Feline type-C virus group-specific antigens in cat cell clones were shown to exist within the cells as an endogenous entity (Livingston and Todaro, 1973).

5. Detection of Virus-Induced Physiological Changes

In most RIA procedures the binding of radiolabeled indicators, either labeled antibodies or antigens, provides the basis for quantitation of immunological reactions indicative of viral infections. However, detection and quantitation of abnormal physiological reactions in animals or cell cultures have also been used to indicate viral infections. These procedures

usually involve measurement of physiological reactions or distribution of radiotracers.

Infection with hepatitis B is generally confirmed by identification of HB antigens or antibody in the serum. However, investigators have observed that DNA polymerase levels, as measured by the uptake of radiolabeled precursors, are elevated at the time antigen production is initiated and that these elevations precede HB antibody production (Bradley *et al.*, 1974; Moritsugu *et al.*, 1975; Krugman *et al.*, 1974).

Detection of characteristic metabolic activities in tissue culture cells provides another useful index of viral activity. Some viruses, upon infection of susceptible cells, induce *de novo* synthesis of thymidine kinase, which is easily measured in thymidine kinase deficient cells. A serum neutralization test was described in which the amount of unneutralized vaccinia virus was determined by measuring the virus-induced uptake by cells of [^{3}H]thymidine and incorporation into ^{3}H-DNA (Ziegler and Hutchinson, 1969). Only sensitive RIA methods can detect such subtle metabolic changes.

REFERENCES

Aach, R. D., Grisham, J. W., and Parker, C. W. (1971). *Proc. Natl. Acad. Sci. U.S.A.* **68,** 1056.

Ainbender, E., Berger, R., Hevizy, M., Zepp, H. D., and Hodes, H. L. (1965). *Proc. Soc. Exp. Biol. Med.* **119,** 1166.

Allen, T. R. Buckland, R. A., and Tyrrell, D. A. J. (1973). *Virusforsch.* **41,** 394.

Anzai, T., Dreesman, G. R., Courtney, R. J., Adam, E., Rawls, W. E., and Benyesh-Melnick, M. (1975). *J. Natl. Cancer Inst.* **54,** 1051.

Attias, M. R., Sylvester, R. A., and Talal, N. (1973). *Arthritis Rheum.* **16,** 719.

Ball, E. M. (1973). *Virology* **55,** 516.

Bellanti, J. A., Smith, C. B., Kim, H. W., Mills, J., Gerin, J. L., Parrot, R. H., and Chanock, R. M. (1971). *Secretory Immuno. Syst. Proc. Conf., 1969* pp. 183–194.

Berson, S. A., and Yalow, R. S. (1968). *Clin. Chim. Acta* **22,** 51.

Bilimoria, S. L., Parkinson, A. J., and Kalmakoff, J. (1974). *Appl. Microbiol.* **28,** 133.

Bolton, A. E., and Hunter, W. M. (1973). *Biochem. J.* **133,** 529.

Bradley, D. W., Maynard, J. E., Berquist, K. R., and Krushak, D. H. (1974). *Nature (London)* **251,** 356.

Catt, K., Niall, H. D., and Tregear, G. W. (1966). *Biochem. J.* **100,** 31c.

Catt, K., Niall, H. D., and Tregear, G. W. (1967). *Nature (London)* **213,** 825.

Charman, H. P., Kim, N., and Gilden, R. V. (1974). *J. Virol.* **14,** 910.

Cleland, G. B., Perey, D. Y., and Dent, P. B. (1975). *J. Immunol.* **114,** 422.

Coller, J. A., Millman, I., Halbherr, T. C., and Blumberg, B. S. (1971). *Proc. Soc. Exp. Biol. Med.* **138,** 249.

Dalrymple, J. M., Teramoto, A. Y., Cardiff, R. D., and Russell, P. K. (1972). *J. Immunol.* **109,** 426.

Dalrymple, J. M., Vogel, S. N., Teramoto, A. Y., and Russell, P. K. (1973). *J. Virol.* **12,** 1034.
Daugharty, H. (1971). *J. Immunol.* **107,** 802.
Daugharty, H. (1973). *J. Immunol.* **111,** 404.
Daugharty, H., Davis, M. L., and Kaye, H. S. (1972a). *J. Immunol.* **109,** 849.
Daugharty, H., Warfield, D. T., and Davis, M. L. (1972b). *Appl. Microbiol.* **23,** 360.
Daugharty, H., Warfield, D. T., Hemingway, W. D., and Casey, H. L. (1973). *Infect. Immun.* **7,** 380.
Daugharty, H., Young, R. G., Hatch, M. H., and Ziegler, D. W. (1974). *Appl. Microbiol.* **27,** 756.
Evans, M. J., and Yohn, D. S. (1970). *J. Immunol.* **104,** 1132.
Fazekas de St. Groth, S., and Webster, R. G. (1963). *J. Immunol.* **90,** 151.
Felsenfeld, O., Dutta, N. K., Felsenfeld, A. D., and Brannon, R. B. (1971). *Proc. Soc. Exp. Biol. Med.* **138,** 149.
Figenschau, K. J., and Ulstrup, J. C. (1974). *Acta Pathol. Microbiol. Scand., Sect. B* **82,** 422.
Forghani, B., Schmidt, N. S., and Lennette, E. H. (1974). *Appl. Microbiol.* **28,** 661.
Fritz, R. B. (1974). *J. Virol.* **13,** 42.
Fritz, R. B., and Qualtiere, L. F. (1973). *J. Virol.* **11,** 736.
Gerloff, P. K., Hoyer, B. H., and McLaren, L. C. (1962). *J. Immunol.* **89,** 559.
Ginsberg, A. L., Bancroft, W. H., and Conrad, M. E. (1972). *J. Lab. Clin. Med.* **80,** 291.
Ginsberg, A. L., Conroad, M. E., Bancroft, W. H., Ling, C. M., and Overby, L. R. (1973). *J. Lab. Clin. Med.* **82,** 317.
Gipson, T. G., and Daniels, C. A. (1974). *Clin. Immunol. Immunopathol.* **4,** 16.
Grant, J. P., Bigner, D. D., Fischinger, P. J., and Bolognesi, D. P. (1974). *Proc. Natl. Acad. Sci. U.S.A.* **71,** 5037.
Greenwood, F. C., Hunter, W. M., and Glover, J. S. (1963). *Biochem. J.* **89,** 114.
Gruber, J., and Wright, G. G. (1967). *Proc. Soc. Exp. Biol. Med.* **126,** 282.
Hayashi, K. , Niwa, A., Rosenthal, J., and Notkins, A. L. (1974). *Intervirology* **2,** 48.
Hersh, E. M., Gutterman, J. U., Marligit, G., Gschwind, C. R., and Hanna, M. G. (1974a). *Haematol. Bluttransfus.* **14,** 119.
Hersh, E. M., Hanna, M. G., Jr., Gutterman, J. U., Marligit, G., Yurconic, M., Jr., and Gschwind, C. R. (1974b). *J. Natl. Cancer Inst.* **53,** 327.
Hewetson, J. F., Gothoskar, B., and Kline, G. (1972). *J. Natl. Cancer Inst.* **48,** 87.
Hollinger, F. B., Vorndam, V., and Dreesman, F. R. (1971). *J. Immunol.* **107,** 1099.
Hollinger, F. B., Bradley, D. W., Maynard, J. E., Dreesmen, G. R., and Melnick, J. L. (1975). *J. Immunol.* **115,** 1464.
Hoofnagle, J. H., Gerety, R. J., Ni, L. Y., and Barker, L. F. (1974). *N. Engl. J. Med.* **290,** 1336.
Hutchinson, H. D., and Ziegler, D. W. (1972). *Appl. Microbiol.* **24,** 742.
Hutchinson, H. D., and Ziegler, D. W. (1974). *Appl. Microbiol.* **28,** 935.
Hutchinson, H. D., Feorino, P. M., and Ziegler, D. W. (1975). *J. Clin. Microbiol.* **1,** 429.
Ihle, J. N., Yurconic, M., Jr., and Hanna, M. G., Jr. (1973). *J. Exp. Med.* **138,** 194.
Irwin, G. R., Allen, A. M., Bancroft, W. H., Willhight, M., and Russell, P. K. (1974). *Appl. Microbiol.* **28,** 604.
Kalmakoff, J., and Parkinson, A. J. (1973). *Appl. Microbiol.* **26,** 624.
Klein, G., Dombos, L., and Gothoskar, B. (1972). *Int. J. Cancer* **10,** 44.
Knez, V., Stewart, J. A., and Ziegler, D. W. (1976). *J. Immunol.* **117,** 2006.
Krugman, S., Hoofnagle, J. H., Gerety, R. J., Kaplan, P. M., and Gerin, J. L. (1974). *N. Engl. J. Med.* **290,** 1331.

Lander, J. J., Alter, H. J., and Purcell, R. H. (1971). *J. Immunol.* **106,** 1166.
Lander, J. J., Holland, P. V., Alter, H. J., Chanock, R. M., and Purcell, R. H. (1972). *J. Am. Med. Assoc.* **220,** 1079.
Ling, C. M., and Overby, R. L. (1972). *J. Immunol.* **109,** 834.
Ling, C. M., Irace, H., Decker, R., and Overby, L. R. (1973). *Science* **180,** 203.
Livingston, D. M., and Todaro, G. J. (1973). *Virology* **53,** 142.
LoGerfo, P., Silverstein, G., and Charney, J. (1974). *Surgery* **76,** 16.
McCammon, J. R., and Yohn, D. S. (1971). *J. Natl. Cancer Inst.* **47,** 447.
McFarlane, A. S. (1956). *Biochem. J.* **62,** 135.
McMillen, J., and Consigli, R. A. (1974). *J. Virol.* **14,** 1627.
McMillen, J., and Consigli, R. A. (1975). *Infect. Immun.* **12,** 1411.
McPherson, T. A., and Carnegie, P. R. (1968). *J. Lab. Clin. Med.* **72,** 824.
Mickey, D. D., Seal, E., Jr., and Paulson, D. F. (1975). *J. Urol.* **113,** 658.
Montelaro, R. C. and Rueckert, R. R. (1975). *J. Biol. Chem.* **250,** 1413.
Moritsugu, Y., Gold, J. W., Wagner, J., Dodd, R. Y., and Purcell, R. H. (1975). *J. Immunol.* **114,** 1792.
Nath, D. M., and Rodkey, L. S. (1975). *Arch. Virol.* **48,** 245.
Neurath, A. R., Vernon, S. K., Wiener, F. P., Hartzell, R. W., and Rubin, B. A. (1973). *Microbios* **7,** 7.
Ogra, P. L. (1970). *Infect. Immun.* **2,** 150.
Ogra, P. L. (1971) *N. Engl. J. Med.* **284,** 59.
Ogra, P. L., Karzon, P. T., Righthand, F., and MacGillivray, M. (1968). *N. Engl. J. Med.* **279,** 893.
Ogra, P. L., Kerr-Grant, D., Umana, G., Dzierba, J., and Weintraub, D. (1971). *N. Engl. J. Med.* **285,** 1333.
Ogra, P. L., Ogra, S. S., al-Nakeeb, S., and Coppola, P. R. (1973). *Infect. Immun.* **8,** 931.
Olsen, R. G., Mathes, L. E., and Yohn, D. S. (1975). *Cancer Res.* **35,** 2580.
Oroszlan, S., White, M. M., Gilden, R. V., and Charman, H. P. (1972) *Virology* **50,** 294.
Parks, W. P., and Scolnick, E. M. (1972). *Proc. Natl. Acad. Sci. U.S.A.* **69,** 1766.
Parks, W. P., and Scolnick E. M. (1973). *Virology* **55,** 163.
Parks, W. P., Scolnick, E. M., Noon, M. C., Watson, C. J., and Kawakami, T. G. (1973). *Int. J. Cancer* **12,** 129.
Parks, W. P., Howk, R. S., Scolnick, E. M., Oroszlan, S., and Gilden, R. V. (1974). *J. Virol.* **13,** 1200.
Patterson, W. R., and Smith, K. O. (1975). *J. Clin. Microbiol.* **2,** 130.
Pressman, D., and Roholt, O. (1961). *Proc. Natl. Acad. Sci. U.S.A.* **47,** 1606.
Prince, A. M., Brotman, B., Jass, D., and Ikram, H. (1973). *Lancet* **1,** 1346.
Purcell, R. H., Wong, D. C., Alter, H. J., and Holland, P. V. (1973). *Appl. Microbiol.* **26,** 478.
Purcell, R. H., Gerin, J. L., Almeida, J. B., and Holland, P. V. (1974). *Intervirology* **2,** 231.
Rifkin, D. B., Compans, R. W., and Reich, E. (1972). *J. Biol. Chem.* **247,** 6432.
Robinson, W. S., and Greenman, R. L. (1974). *J. Virol.* **13,** 1231.
Rosa, U., Scassellati, G. A., Pennsi, F., Riccioni, N., Giagnoni, P., and Giordani, R. (1964). *Biochim. Biophys. Acta* **86,** 519.
Rosenthal, J. D., Hayashi, K., and Notkins, A. L. (1972). *J. Immunol.* **109,** 171.
Rosenthal, J. D., Hayashi, K., and Notkins, A. L. (1973a). *Appl. Microbiol.* **25,** 567.
Rosenthal, J., Hayashi, K., and Notkins, A. L. (1973b). *J. Gen. Virol.* **18,** 195.
Ruoslahti, E., Vaheri, A., Estola, T., and Sandelin, J. (1973). *Int. J. Cancer* **11,** 595.
Salmon, S. E., Mackey, G., and Fudenberg, H. N. (1969). *J. Immunol.* **103,** 129.

Sandelin, J., Estola, T., Ristimsaki, S., Ruoslahti, E., and Vaheri, A. (1974). *J. Gen. Virol.* **25,** 415.
Scolnick, E. M., Parks, W. P., and Livingston, D. M. (1972). *J. Immunol.* **109,** 570.
Scott, J. V., Dreesman, G. R., Spira, G., and Kasel, J. A. (1975). *J. Immunol.* **115,** 124.
Sefton, B. M., Wickus, G. G., and Burge, B. (1973). *J. Virol.* **11,** 730.
Sherr, C. J., and Todaro, G. J. (1974). *Proc. Natl. Acad. Sci. U.S.A.* **71,** 4703.
Sherr, C. J., and Todaro, G. J. (1975). *Science* **187,** 855.
Singh, S. B., Smith, J. B., Rawls, W. E., and Tevethia, S. S. (1972). *Infect. Immun.* **5,** 352.
Smith, J. W., Adam, E. Melnick, J. L., and Rawls, M. E. (1972). *J. Immunol.* **109,** 554:
Smith, K. O., Gehle, W. D., and McCracken, A. W. (1974a). *J. Immunol. Methods* **5,** 337.
Smith, K. O., Gehle, W. D., and Sanford, B. A. (1974b). *Proc. Soc. Exp. Biol. Med.* **147,** 357.
Soriano, F., Shelburne, G. E., and Gokcen, M. (1974). *Nature (London)* **249,** 421.
Spira, G., Biswal, N., and Dreesman, G. R. (1974). *Appl. Microbiol.* **28,** 239.
Stanley, P., and Haslam, E. A. (1971). *Virology* **46,** 764.
Stephenson, J. R., and Aaronson, S. A. (1973a). *J. Virol.* **12,** 564.
Stephenson, J. R., and Aaronson, S. A. (1973b). *Virology* **54,** 53.
Stephenson, J. R., Wilsnack, R. E., and Aaronson, S. A. (1973). *J. Virol.* **11,** 893.
Stinski, M. F., and Ginsberg, H. S. (1974). *Intervirology* **4,** 226.
Strand, M. (1973). *J. Biol. Chem.* **248,** 5627.
Strickland, J. E., Kind, P. D., Fowler, A. K., and Hellman, A. (1974). *J. Natl. Cancer Inst.* **52,** 1161.
Sylvester, R. A., Attias, M., Talal, N., and Tuffanelli, D. L. (1973). *Arthritis Rheum.* **16,** 383.
Szmuness, W., Prince, A. M., Goodman, M., Ehrich, C., Pick, R., and Ansari, M. (1974). *N. Engl. J. Med.* **290,** 701.
Tadeusz, J. W., Koprowski, H., and Dixon, F. (1972). *J. Immunol.* **109,** 464.
Talal, N. (1972). *Clin. Immunol. Immunopathol.* **1,** 230.
Tanigaki, N., Yagi, Y., Moore, G. E., and Pressman, D. (1967). *J. Immunol* **98,** 274.
Thamer, G., and Kommerell, B. (1975). *Clin. Chim. Acta* **61,** 273.
Todaro, G. J., Benveniste, R. E., Lieber, M. M. and Sherr, C. J. (1974). *Virology* **58,** 65.
Tronick, S. R., Stephenson, J. R., and Aaronson, S. A. (1973). *Virology* **54,** 199.
Tronick, S. R., Stephenson, J. R., and Aaronson, S. A. (1974). *Virology* **57,** 347.
Tronick, S. R., Stephenson, J. R., Aaronson, S. A., and Kawakani, T. G. (1975). *J. Virol.* **15,** 115.
Vaheri, A., and Ruoslahti, E. (1973). *Int. J. Cancer* **12,** 361.
Velicer, L. F., and Graves, D. C. (1974). *J. Virol.* **14,** 700.
Verstraeten, A. A., Hageman, P. C., and Kwa, H. G. (1973). *Eur. J. Cancer* **9,** 155.
Verstraeten, A. A., Van Nie, R., Kwa, H. G., and Hageman, P. C. (1975). *Int. J. Cancer* **15,** 270.
Volkers, S. A. S., and Pitts, J. D. (1973). *Nature (London), New Biol.* **244,** 274.
Walsh, J. H., Yalow, R., and Berson, S. A. (1970). *J. Infect. Dis.* **121,** 550.
Weber, J., and Yohn, D. S. (1972). *J. Virol.* **9,** 244.
Wiktor, T. J. (1973). *WHO Monogr. Ser* **23,** 182.
Witte, D. N., Weissman, I. L., and Kaplan, H. S. (1973). *Proc. Natl. Acad. Sci. U.S.A.* **70,** 36.
Ziegler, D. W., and Hutchinson, H. D. (1969). *Appl. Microbiol.* **17,** 41.
Ziegler, D. W., Hutchinson, H. D., Koplan, J. P. and Nakano, J. H. (1975). *J. Clin. Microbiol.* **1,** 311.

Chapter 12

Cytohybridization Techniques in Virology

P. TIJSSEN AND E. KURSTAK

I. INTRODUCTION

Before the immunocytochemical techniques (immunofluorescence, immunoferritin, and immunoperoxidase) became available, localization of

antigens in cells or tissues was performed by assaying various subcellular fractions with specific antibodies.

Parallel to this development, many sophisticated *in vitro* methods have been developed in nucleic acid research for the recognition of certain sequences at the molecular level. These techniques are based on the observations that denatured nucleic acids can be reannealed specifically and that the hybrid molecules containing complementary strands can be isolated.

Hybridization has recently been shown to be an extremely useful method in light and electron microscopic histochemical studies. Applications of this method may vary from the detection of certain nucleic acid sequences in cells to the localization of genes. Such studies may be particularly important in the detection of latent viruses or tumor viruses in cells. Such latent viral genomes may express some of its genes or remain without gene expression (see Chapters 2 and 6, Volume I). For the latter group of latent viruses, hybridization provides an important tool for establishing the possible presence, quantity, and exact localization of this genome, as well as the number of cells harboring this genome. A review on various methods of hybridization used for the study of tumor viruses was given by Nonoyama (1975). In this chapter, only the cytohybridization technique is discussed. The sensitivity of the cytohybridization technique is less than that of the hybridization on membrane filters. In the latter technique, a large excess of total DNA, extracted and pooled from a number of cells, is used, whereas in the case of cytohybridization, each cell is assayed individually.

The formation of hybrids between radioactive-labeled and denatured nucleic acids can be detected *in situ* in cytological preparations, such as squashes, wax-embedded tissue, whole air-dried cells, and ultrathin sections (Buongiorno-Nardelli and Amaldi, 1970; Jacob *et al.*, 1971; Jones and Robertson, 1970).

II. BASIC PRINCIPLES OF HYBRIDIZATION

The basic tenet underlying hybridization is, first, the denaturation of DNA, i.e., the separation of the two strands. This is generally achieved by elevated temperatures or chemicals, such as NaOH, and must be derived from the entropy increase which follows the dissociation of the complementary base pairs (Fixman, 1963).

The concept that the bases of the two strands are held together only by hydrogen bonds is an oversimplification, since other in-plane interactions can play a major role in the stacking of the bases (Pullman *et al.*, 1966).

In the hybridization technique, the complementary strands of a mixture of denatured nucleic acids are reannealed and the hybrid DNA–RNA duplexes isolated.

Hybridization starts at several sites along the complementary strands, unlike the duplex formation during DNA replication where the new DNA helix grows in one direction only. Hybridization is not just the reverse of melting. The slow kinetics of hybridization can be attributed to the formation of many faulty annealings where noncomplementary sequences are first located in loops, before a true duplex is formed. To accelerate this process, hybridization is performed at a relatively high temperature (about 25°C below the melting point). The formation of artifactual duplexes can be minimized by digesting the hybrids with a nuclease specific for single-stranded nucleic acids. It should be borne in mind that hybridization is an equilibrium process and the presence of minor components in the reaction mixture may give erroneous results.

Different approaches have been used to describe the kinetics of the hybridization, the most commonly used method being the so-called "Cot method" (Britten and Kohne, 1968). A very thorough discussion was provided by Kennell (1971), based on the applications of the hybridization technique in various areas of nucleic acid research, e.g., the determination of the extent of redundancy in the DNA sequence and of the proportion of the chromosome which is transcribed *in vivo* (Kennell, 1968; Gelderman *et al.*, 1971).

III. REQUIREMENTS IN CYTOHYBRIDIZATION

In the cytohybridization technique, cells, squashes, or sections can be used. These preparations are fixed and the nucleic acids of the cells are denatured *in situ*, preventing, as much as possible, morphological damage. Hybridization is then performed, which is followed by autoradiography.

The overall, absolute requirement in this technique is that the nucleic acid which is to be labeled or used as a template for the transcription of the labeled probe must be pure.

A second requirement is the high specific activity of the labeled probe. Closely related to this requirement is the need for a sufficient number of complementary nucleotides in one region to bind detectable amounts of the probe. The higher the specific activity, the smaller the template which may be detected. For the detection of sequences which are not highly redundant, several months of autoradiographic exposure may be necessary to obtain significant numbers of silver grains over the background. Iodine-125 has a half-life of 60 days which is about 70 times shorter than that of ^{3}H.

It decays by electron capture and resultant Auger electrons (see Glossary) have varying energies. Resolution in the autoradiographs produced by ^{125}I is inferior to that by ^{3}H (Ertl *et al.*, 1970) and 1 silver grain was estimated to be produced for each five disintegrations (Ada *et al.*, 1966).

The autoradiographic efficiency of ^{125}I appears to be twice that of ^{3}H (Prensky *et al.*, 1973). For the more exact localization of the molecular hybrid, slides should be examined after exposure for different lengths of time. For diagnostic purposes it is highly impractical to wait several months for the results; Prensky *et al.* (1973) shortened the length of exposure to 2 days by using ^{125}I of high specific activity.

IV. PREPARATION OF THE RADIOACTIVE PROBES*

A. General Comments

The most important factor for the success of the hybridization technique is the use of labeled nucleic acids with high specific activity (at least 10^6 cpm/μg). Labeled nucleic acids can be prepared both *in vivo* and *in vitro,* the latter yielding, by far, the best preparations. In virology, the nucleic acid used for hybridization is generally the viral genome. A disadvantage of *in vivo* labeling experiments is the possible induction of radiation damage, due to the administration of high doses of radioactivity, which may complicate the subsequent purification of the specific nucleic acid. For the *in vitro* labeling of isolated nucleic acids, numerous methods are available, such as the incorporation of labeled nucleotides into the nucleic acids, their insertion by scission, and *in vitro* repair and *in vitro* synthesis by replication, transcription, or reverse transcription.

B. *In Vivo* Labeling of Nucleic Acids with Tritium (^{3}H)

The specific activity of nucleic acid prepared by this method is quite low. In general, 1 mCi of [^{3}H]uridine (10–20 Ci/mmole) or 100 μCi of [^{3}H]thymidine (10–20 Ci/mmole) are added to 20 ml of culture for 5 days. The cultures are then "chased" with nonradioactive medium for 12 hours, the nucleic acids extracted and purified by standard methods (Kirby, 1968). Examples of *in vivo* labeling are provided by Gall and Pardue (1971) and Jacob *et al.* (1971).

* See Section VIII (Glossary) for definition of terms used in discussion of radioactive probes.

C. *In Vitro* Labeling of DNA with ^3H

The *in vitro* synthesis of DNA was realized in several laboratories a few years ago (Kacian *et al.*, 1972; Verma *et al.*, 1972; Ross *et al.*, 1972). The experiments described by these authors are essentially identical, with some small variations.

The 10 S RNA from reticulocytes, used as a template, was incubated with reverse transcriptase (from avian myeloblastosis virus particles), the four deoxyribonucleoside triphosphates, suitable concentrations of Mg^{2+}, and a primer (oligo-dT chains of about twelve bases). The addition of the primer is essential since reverse transcriptase initiates the synthesis of DNA by forming a phosphodiester bond between the 3′-hydroxy group of the short primer chain, which is hydrogen bonded to the template RNA and the 5′α-phosphate of the first deoxyribonucleotide. Since these initial studies, this technique has undergone several refinements. For Rous sarcoma virus, a small RNA molecule was identified as the primer. This small RNA primer was shown to be present in noninfected avian, mouse, and human cells and is in all likelihood a cell-specified molecule (Faras and Dibble, 1975). The *in vitro* DNA synthesis was considerably enhanced with the addition of a low concentration of Triton X-100 and full length DNA transcripts of Rous sarcoma virus RNA were obtained (Junghans *et al.*, 1975).

For the preparation of labeled DNA by the replication *in vivo*, the reader is referred to Probst *et al.* (1975).

D. *In Vitro* Labeling of RNA with ^3H

RNA polymerase is commercially available, but it is preferable to prepare it in the laboratory by the method of Burgess (1969; Burgess and Travers, 1971). This enzyme should be stored at −20°C (never in liquid nitrogen!), in 50% glycerol, in the presence of highly purified bovine serum albumin (1 mg/ml). The glassware to be used should be absolutely free of RNase. Briefly, template DNA (5 μg in 0.12 ml), *E. coli* RNA polymerase (5 units in 0.03 ml), and 0.1 ml of a solution containing 0.1 *M* Tris-HCl, pH 7.9, 0.025 *M* $MgCl_2$, 0.375 *M* KCl, 0.01 *M* $MnCl_2$, 0.25 m*M* EDTA, 0.25 m*M* dithiotreitol (or β-mercaptoethanol), and 0.375 m*M* each of ATP, GTP, and CTP and 0.5 mCi of ^3H-UTP (specific activity 30 Ci/mmole) are mixed and incubated at 37°C for 1 to 2 hours (Gall and Pardue, 1971; Huang *et al.*, 1973; Pagano and Huang, 1974). The DNA is then digested with pancreatic DNase I (40 μg/ml, RNase-free) for 30 minutes. Nonradioactive *E. coli* RNA is added as carrier and the mixture is extracted with SDS-phenol. The aqueous phase is passed through a Sephadex G-50 column (20 ml bed

volume); the first peak is collected, heated at 85°C for 3 minutes, and cooled and forced through a nitrocellulose filter (0.45 μm pore size). Approximately 2 μg of radioactive RNA is thus produced.

E. *In Vitro* Labeling of Purified Nucleic Acids with Iodine (^{125}I)

Commerford (1971) described a method for the *in vitro* labeling of nucleic acids with iodine. In this technique, essentially all iodine is utilized to form 5-iodocytosine and it is widely used for labeling nucleic acids. However, it suffers from the inconvenience that it involves treatment with oxidizing and reducing agents at elevated temperatures and extreme pH values.

Steinert *et al.* (1976) modified this technique: 1 mCi of ^{125}I (carrier-free in NaOH solution at pH 8–11) was mixed with 2 μl of KI (10 mg/100 ml H_2O), 15 μl $TlCl_3$ (1.6 m*M* in 0.15 sodium acetate, pH 4.5), and 4 μl of RNA solution (4 μg). The reaction mixture was heated in a sealed vial at 65°C for 20 minutes. The reaction was stopped by dipping the vial in ice and by adding 200 μl of 2 × SET buffer (1 × SET h 0.15 *M* NaCl, 0.005 EDTA, and 0.05 *M* Tris-HCl, pH 8) and 0.1 volume of 0.1 *M* Na_2SO_3.

Unreacted iodine was removed by passing the mixture through Sephadex G-50 (in 2 × SET). The RNA was then made 0.01 *M* with respect to Na_2SO_3, heated at 70°C for 30 minutes, precipitated with ethanol, and redissolved and further purified on Sephadex G-50. The specific activity of the preparation was about 2.5×10^7 cpm/μg.

F. Labeling of Nucleic Acids with ^{125}I by *in Vitro* Repair Synthesis

Shaw *et al.* (1975) reported a simple procedure for the preparation of iodinated herpes simplex DNA of high activity.

This technique consists of three steps: (1) iodination of dCTP, (2) purification of the ^{125}I-dCTP by chromatography on DEAE-cellulose, and (3) synthesis of ^{125}I-labeled DNA by *in vitro* repair. The specific activity of the preparation exceeded 10^8 cpm, whereas probes prepared with ^{3}H-labeled TTP had activities in the range of 1 to 5×10^6 cpm/μg.

The number of silver grains in the radioautograph was four times higher than given by the ^{3}H-labeled DNA, indicating that the increase in the sensitivity was accompanied by some loss in resolution.

For the preparation of ^{125}I-labeled 5-iodo-dCTP, a 0.08 ml volume of 0.1 *M* sodium acetate–0.04 *M* acetic acid buffer, pH 5, contained 40 nmoles of dCTP, 1.7 nmoles of $Na^{125}I$ (3.8 mCi), and 17 nmoles of $TlCl_3$

(prepared from freshly made 0.02 *M* solution). The mixture was heated at 60°C for 15 minutes and chilled to 0°C. The excess of thallium chloride was reduced with 0.02 ml of a freshly prepared 80 m*M* sodium sulfite solution. After 2 minutes, 0.01 ml of 1 *M* ammonium acetate–0.5 *M* NH_4OH buffer, pH 9, was added and the mixture was heated at 40°C for 15 minutes to dissociate unstable intermediates (Commerford, 1971).

For the purification of the ^{125}I-dCTP, the solution was diluted to contain less than 0.01 *M* salt and applied to a DEAE-cellulose (DE 52, Whatman) column (bed volume about 15 ml) which had been washed previously with three volumes of cold 1 *M* TEABC (triethylammonium bicarbonate, pH 8.0) and equilibrated with 0.01 *M* TEABC. The column was eluted with a 1-liter linear gradient of 0.01 to 0.5 *M* TEABC. The peak fractions containing the labeled dCTP were pooled and evaporated to dryness, dissolved in 0.5 ml water, and stored at −20°C.

The *in vitro* repair synthesis of HSV-DNA is described elsewhere (Kelly *et al.*, 1970).

Iodinated CTP can also be prepared in a similar manner and used for the *in vitro* synthesis of complementary RNA (Huang *et al.*, 1973). In the latter procedure, the radioactive uridine is replaced by cold uridine and 40 μCi of ^{125}I-labeled 5-iodo-CTP replaces the cold CTP.

The nucleic acid sample prepared by this method is then made 1% with respect to Sarkosyl 97 and 0.01 *M* with respect to EDTA and filtered through Sephadex G-50 equilibrated with 0.01 *M* Tris-HCl buffer, pH 7.4, containing 0.001 *M* EDTA and 0.1% Sarkosyl. Calf thymus DNA (1 mg) and SDS to a final concentration of 0.5% are added to the fraction recovered in the excluded volume. The solution is then extracted once with an equal volume of saturated phenol and the nucleic acid preparation is recovered by precipitation with ethanol. Finally, the labeled nucleic acid is dissolved in Tris-buffered saline (0.05 *M* Tris-HCl, pH 7.4, 0.15 *M* NaCl, and 0.001 *M* EDTA) and sheared by sonic treatment (12 minutes at 0°C). After extensive dialysis, the preparation is stored at −20°C.

V. PROCEDURES FOR *IN SITU* HYBRIDIZATION

A. General Remarks

It is well known that basic proteins interfere with the nucleic acids in the filter hybridization technique of Gillespie and Spiegelman (1965). The conventional fixation with ethanol–acetic acid, however, eliminates this interference in cytohybridization.

Gall and Pardue (1971) noticed that treatment of the smear with RNase

improved the specific hybridization, presumably by removing competing unlabeled RNA from the tissue. The fear that some RNase might remain in the tissue and destroy the RNA probe (Das and Alfert, 1969) proved to be groundless.

Since the DNA to be hybridized with the RNA probe is double-stranded, it is necessary to denature the nucleic acid in the smear. This should be performed without causing cytological damage.

B. Light Microscopy

For cytohybridization with cell preparations, the cells are fixed with freshly prepared absolute ethanol and acetic acid (3:1) for 5 to 10 minutes. After centrifugation, the cells are resuspended in 0.2 ml of fixative, applied onto a precooled slide, and air dried. In order to prevent detachment of the cells, the slides can be precoated with a thin layer of gelatin and chrome alum (Gall and Pardue, 1971) or dipped into 0.4% agarose at 60°C after the application of the cells (Pagano and Huang, 1974). Procedures for squashing the cells were described by Gall and Pardue (1971). In this technique, an additional step is inserted for the removal of RNA and residual proteins.

For denaturation, the slide is placed in 0.07 *N* NaOH for 2 minutes, washed with 70% ethanol (twice, 4 minutes), and rinsed twice in 95% and once in absolute ethanol (3 minutes each). In the next step, the radioactive probe, in 0.1 ml of 2 × SSC (0.15 *M* NaCl + 0.015 *M* trisodium citrate), is applied to each slide, covered with a slip, and placed in a wet chamber for 24 hours (at 65°C).

After hybridization, the slides are washed three times with 2 × SSC, placed in RNase solution (20 μg/ml in 2 × SSC) for 1 hour at 37°C, washed three times with 2 × SSC, twice with 70% ethanol, twice with 95% ethanol, and three times with absolute ethanol. After drying, autoradiography is performed (see Section V,D).

C. Electron Microscopy

The technique described here is the one of Jacob *et al.* (1971). The tissue fragment is fixed with 2.5% cold glutaraldehyde in 0.05 *M* phosphate buffer, pH 7.2, for 20 minutes and embedded in glycol methacrylate (Leduc *et al.*, 1963).

"Gold" sections, about 120 nm in thickness, are collected on Formvar-carbon coated grids. RNA is removed from the tissue sections by floating the grids, with the section facing down, on a 0.2% RNase solution in 2 × SSC for 1 hour at 37°C. The grids are then washed with a

large volume of distilled water and subjected to proteolytic treatment for 30 minutes at 37°C with 0.01% protease, followed by thorough washing. DNA is denatured by floating the grids on 0.1 *M* NaOH for 2 hours at room temperature. The girds are then washed with 2 × SSC.

For tritium-labeled RNA (specific activity, 1.5×10^6 cpm/μg) the preparations are incubated at 70°C for 5 hours. Prior to the incubation, the labeled RNA is treated with 0.1 *M* NaOH in order to reduce its molecular weight to approximately 20,000.

After annealing, the grids are washed with 2 × SSC and treated with RNase (0.05% in 2 × SSC) for 15 minutes at room temperature, followed by thorough washing with 2 × SSC. The grids are taken through 70% ethanol and stained for 15 minutes at room temperature in freshly prepared 1% uranyl acetate in 70% ethanol. The grids are then dried and carbon coated. Coating of the grids (attached to glass slides) with a layer of Ilford-L4 can be performed with the wire loop method (Caro and van Tubergen, 1962).

After exposure (which can last up to several months for RNA with low specific activity), the preparations are developed in Kodak-D19b for 3 minutes at 18°C, fixed in Kodak F24, and stained again with uranyl acetate and lead citrate.

Very useful alternatives to this method are given in Section V,D,3 (particularly the method of Hayashi and Telstad, 1976).

D. Autoradiography

1. General Remarks

In view of the short half-life of ^{125}I, the probe should not be prepared too far in advance. The percentage of the original activity of the label can be conveniently estimated graphically from a semilogarithmic plot of the activity versus time (Fig. 1). The amount of radioactivity remaining after a given time, i.e., the radioactive decay, can also be calculated from the equation

$$N_t = N_0 e^{-\lambda t}$$

where N_t and N_0 are the number of radioactive atoms (N) present at times t and 0, respectively, and λ is the decay constant.

From the emission spectrum of tritium and the range of the electrons, the percentage of particles emitted from the specimen and reaching the surface was computed by Pelc and Welton (1967). Since one-half of the particles is emitted toward the back, the maximal yield on the pho-

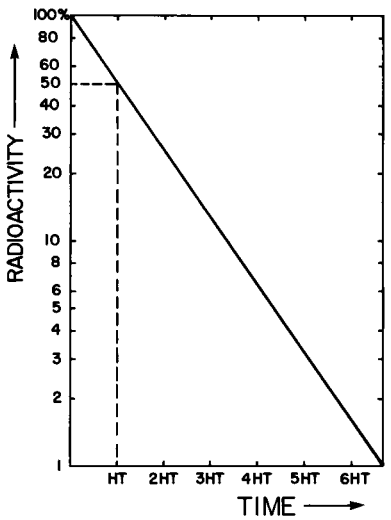

Fig. 1. The calculation of the amount of radionuclides, expressed in % of the initial radioactivity, present at various times after their production. Radioactivity is plotted on a log scale on the ordinate, and the time, expressed in half-life (HT) units, on a linear scale on the abscissa. The remaining radioactivity can be read off the graph using the straight line drawn from 100% through 50%, where the half-life (HT) corresponds to: ^{125}I, 60 days; ^{32}P, 14.3 days; ^{33}P, 25.2 days. The half-lives for ^{14}C (thousands of years) and ^{3}H (12.3 years) are sufficiently long to regard their activity as relatively constant. However, chemical instability of more complex molecules such as nucleotides should be taken in consideration.

tographic emulsion should be 50%. However, in general, the sources are not evenly loaded. Moreover, a layer of protein of 0.15 mg/cm^2, i.e., 1 μm thickness with a density of 1.5, transmits only 4% of the particles emitted from ^{3}H in the forward direction (Pelc and Welton, 1967). Thus, the gain in the radiographic resolving power obtained with tritium, the beta particles of which have a short range, is at a considerable cost of activity. These low-energy particles may be absorbed in the histological section. As mentioned earlier, the extent of this self-absorption depends on the thickness of the histological section and the reduction in the number of silver grains for a given amount of tritium may be considerable (Maurer and Primbsch, 1964; Maurer and Schultze, 1969). In whole cells, this self-absorption should be even higher.

Since the number of the reducible silver grains per unit of the emulsion is limited, overexposure could lead to false conclusions concerning the relative distribution of radioactivity in the various cell compartments (Maurer and Schultze, 1969).

A very useful and clearly written book on autoradiography, including various procedures and experimental details, was edited by Gahan (1972).

2. *Microautoradiography (Light Microscopy)*

The autoradiographic technique consists essentially of four steps: coating with emulsion, exposure, developing, and staining. In microautoradiography, coating of the slides can be performed by two techniques, namely, the stripping film technique and the liquid emulsion technique. The stripping film technique, which is simple and yields an emulsion layer of uniform thickness, was described very clearly and thoroughly by Appleton (1972). Kodak AR-10 plates, with the emulsion side facing down, are placed on the surface of a dish of water and allowed to swell for 3 minutes. Rectangular strips of the emulsion are then peeled off slowly. The slide to be coated is immersed under the floating piece of film which is lifted out, without trapping air bubbles. The slides are then drained vertically for 30 minutes. In the liquid emulsion technique, the commercial available bulk emulsion is first melted in a water bath at 43°–47°C for 30 minutes. To remove trapped air bubbles, the melted emulsion can be filtered through cheesecloth. The slides, preferably dry and at the same temperature, are dipped into the emulsion for 5 seconds and withdrawn slowly.

The dry coated slides are placed horizontally on a tray in a light-tight box and kept at 4°C during the exposure, which may vary up to 6 months. It is preferable to transfer the light-tight boxes to a vacuum desiccator filled with N_2, which reduces the possibilitity of image fading. At various intervals, a slide should be developed to check if exposure had proceeded long enough.

The instructions given for the emulsion used should be followed for the development and fixing. Kodak-D19b is usually developed for 2 to 5 minutes at 18°C, rinsed for 30 seconds in distilled water, fixed with 30% sodium thiosulfate for 10 minutes or Kodak rapid fixer for 4 minutes, and washed thoroughly in running tap water ($\frac{1}{2}$ hour) and then with distilled water. Staining can be performed conveniently at this stage with Giemsa (30 minutes). Additional details of this technique were given by Perry (1969) and Bogoroch (1972).

Both Ilford and Kodak emulsions are suitable for microautoradiography. The Ilford K2 emulsion is recommedned for B particles (^3H and ^{125}I), whereas the L4 is used mainly in electron microscopy. The Kodak NTB (nuclear track beta) emulsions vary in their sensitivity to beta emission: the type 2 is sensitive for the lower-energy β particles, whereas the type 3 is

sensitive for all charged particles. In contrast to the NTB emulsions, Ilford emulsions should never be remelted.

3. *Electron Microscope Autoradiography*

Uniformity of the emulsion is very important for electron microscopy. Two techniques are currently in use: the loop method and the flat substrate method (Salpeter and Bachmann, 1964). An excellent review of these techniques is given by Budd (1972). Ilford L4 emulsions can be used for both techniques, whereas the high resolution Kodak NTE is convenient only for the flat substrate technique.

In the loop method (Caro and van Tubergen, 1962), a loop of thin wire (4 cm in diameter) is dipped in the emulsion and withdrawn slowly, forming a thin film in the loop. If the preparation is correct, this film gels almost immediately. The loop is then touched to the surface of the slide and the film falls on the grids and adheres to them very firmly.

The flat substrate method is well described by Kopriwa (1973) and involves the following steps: (1) mounting of the sections on a flat collodion film supported by a glass plate; (2) coating the specimen with liquid emulsion (dipping or pipetting); (3) exposing and processing; and (4) transferring the emulsion, specimen, and film to a grid. This last step often leads to damage or loss of the specimen.

Hayashi and Trelstad (1976) recently modified this step in order to ameliorate the flat substrate technique. In this modification, the glass slides are cleaned with 100% ethanol and the grids (400 mesh) are placed on the dry slides. A 0.4–0.5% collodion solution in amyl acetate is gently pipetted over the grids. A few seconds later, when the grids are settled well, the slides are gradually tilted to drain off the excess collodion with a filter paper. The slides are then air dried in a vertical position. The sections floating on the convex water surface of the microtome boat are picked up with the coated slides by touching carefully with the collodion coat facing down. The preparations are then stained and a thin layer of carbon is evaporated onto the slides. The photographic emulsion is applied to the slides which are dryed in a vertical position. After the appropriate exposure, the slides are developed, washed, and the specimen detached from the slide by cutting the collodion around the grid with a fine forceps. With this technique, the damage or loss of the specimen is greatly reduced.

4. *Discrimination of Different Isotopes by Autoradiography*

Schultze *et al.* (1976) described a two-emulsion autoradiographic technique for double labeling experiments with [^{3}H]- and [^{14}C]thymidine

which permits a discrimination of the different types of labels. This opens up new perspectives for differential hybridization.

VI. APPLICATIONS IN RESEARCH AND DIAGNOSIS

Several elegant studies using this technique have been reported in virology, genetics, and other related fields, both at the level of light and electron microscopy.

Cytohybridization in electron microscopic preparations is an extremely powerful and attractive technique since particular stretches of DNA within the genome or subcellular organelles can be localized with great precision.

Jacob *et al.* (1971) localized with ^{3}H-labeled 28 S RNA the ribosomal DNA complements in ultrathin sections of oocytes of *Xenopus laevis* after brief glutaraldehyde fixation and glycol-methacrylate embedding.

Very recently, using ^{125}I-labeled ribosomal RNA, Steinert *et al.* (1976) showed that ribosomal DNA was present in the Feulgen-positive bodies which appeared in the cytoplasm of the *Xenopus laevis* oocytes during maturation. These bodies proved to be in close association with the mitochondria.

The feasibility of the localization of genes with this technique was also shown by Prensky *et al.* (1973), Price *et al.* (1973), Kuo *et al.* (1975), and Lindquist McKenzie *et al.* (1975).

Prensky *et al.* (1973) localized by this method the genes coding for the 5 S ribosomal RNA in *Drosophila*. Using ^{125}I, this localization required only 2 days, whereas with ^{3}H-RNA a 2-month exposure was needed.

Lindquist McKenzie *et al.* (1975) used cultured *Drosophila melanogaster* cells and investigated the drastic changes in the pattern of protein synthesis after heat treatment. The heat-shock induced RNA's coding for new proteins were purified and the site of these genes was localized on the chromosome by means of the *in situ* hybridization technique. In human chromosomes, the sites of repeated sequences of cytoplasmic membrane-associated DNA were determined by this technique by Kuo *et al.* (1975).

Price *et al.* (1973) used *in situ* hybridization to detect regions of human metaphase chromosomes exhibiting complementarity with radioactively labeled RNA isolated from RD114-virus. The region of homology was confined to one D-group chromosome.

Other applications of the cytohybridization technique in virology have been reviewed by Pagano and Huang (1974). These experiments illustrate the *in situ* hybridization with cytomegalovirus, Epstein-Barr virus, and SV40.

VII. CONCLUSIONS AND PROSPECTS

The technique described here permits us to envisage the utilization of cytohybridization for viral diagnostic purposes, particularly in tumor virology and in the field of slow virus infections. With the use of ^{125}I-labeled probes, exposure time can be as short as 2 days.

The recent developments in the preparation of radioactive probes with very high specific activity render this method highly sensitive and relatively simple to perform. However, the preparation of the probe is still the most time-consuming step. Unfortunately, the decay of the radioactive tracer limits somewhat the storage life of the labeled probe. On the other hand, once the experimental conditions are established for a particular virus–cell system, minimal amounts of materials are sufficient for the test.

The use of RNA-dependent RNA polymerase, e.g., plant virus replicase, might be a useful tool for the preparation of the labeled probes for RNA viral genomes. Reassociation in such systems, however, occurs only at relative high "Cot" values, particularly when the viral genomes are present at low concentrations.

VIII. GLOSSARY

Auger effect: A process by which excess energy, stored by one or more electrons in higher orbits, may be released from an atom. This process competes with fluorescence for the release of such energy. The energy, which is liberated by one electron making a transition from a higher to a lower orbit, is transferred to another electron in a higher orbit. This energy overcomes the binding energy of the electron and confers on it an additional kinetic energy. Auger electrons are electrons emerging from an atom as a result of the excitation by the Auger effect

Background radiation: Radiation from all sources other than the one being measured, such as naturally occurring radioactive sources, cosmic radiation

Beta particle: Electron ejected from a nucleus during some radioactive transformation

Curie (Ci): See radioactive decay

Electron capture: A mechanism of radioactivity in which an orbital electron disappears and a neutrino (a hypothetical elementary particle with an extremely small mass and carrying no electric charge) and one or more γ rays are emitted from an atomic nucleus

Exposure: The act of irradiating the emulsion layer

Isotope: Chemical elements having the same atomic number but possessing a different atomic mass

Radioactive decay: Radioactive disintegration. Rate of disintegration of nuclei of a radionuclide, expressed in Curie (Ci), one Curie being 3.7×10^{10} disintegrations per second. Specific activity is the amount of Ci per unit quantity of molecules or atoms. Half-life is the time required for the disintegration of one-half the atoms of a radionuclide. The fraction of the total nuclides which undergoes radioactive decay per unit time is the disintegration constant (λ)

Radionuclide: An atomic nucleus which decays spontaneously into some other nuclear species with the liberation of energy. A nuclear species which is radioactive has either too many or too few neutrons for the number of protons. Approximately 1500 such nuclides have been identified

REFERENCES

Ada, G. L., Humphrey, J. H., Askonas, B. A., McDevitt, H. O., and Nossall, G. J. V. (1966). *Exp. Cell Res.* **41,** 557.

Appleton, T. C. (1972). *In* "Autoradiography for Biologists" (P. B. Gahan, ed.), p. 33. Academic Press, New York.

Bogoroch, R. (1972). *In* "Autoradiography for Biologist" (P. B. Gahan, ed.), p. 66. Academic Press, New York.

Britten, R. J., and Kohne, D. E. (1968). *Science* **161,** 529.

Budd, G. C. (1972). *In* "Autoradiography for Biologists" (P. B. Gahan, ed.), p. 95. Academic Press, New York.

Buongiorno-Nardelli, M., and Amaldi, F. (1970). *Nature (London)* **225,** 946.

Burgess, R. R. (1969). *J. Biol. Chem.* **244,** 6160.

Burgess, R. R., and Travers, A. A. (1971). *In* "Methods in Enzymology" (K. Moldave and L. Grossman, eds.), Vol. 21, Part D, p. 500. Academic Press, New York.

Caro, L. G., and van Tubergen, R. P. (1962). *J. Cell Biol.* **15,** 173.

Commerford, S. L. (1971). *Biochemistry* **10,** 1993.

Das, N. K., and Alfert, M. (1969). *J. Histochem. Cytochem.* **17,** 418.

Ertl, H. H., Feinendegen, L. E., and Heiniger, H. J. (1970). *Phys. Med. & Biol.* **15,** 447.

Faras, A. J., and Dibble, N. A. (1975). *Proc. Natl. Acad. Sci. U.S.A.* **72,** 859.

Fixman, M. (1963). *J. Mol. Biol.* **6,** 39.

Gahan, P. B., ed. (1972). "Autoradiography for Biologists." Academic Press, New York.

Gall, J. G., and Pardue, M. L. (1971). *In* "Methods in Enzymology" (K. Moldave and L. Grossman, eds.), Vol. 21, Part D, p. 470. Academic Press, New York.

Gelderman, A. H., Rake, A. V., and Britten, R. J. (1971). *Proc. Natl. Acad. Sci. U.S.A.* **68,** 172.

Gillespie, D., and Spiegelman, S. (1965). *J. Mol. Biol.* **12,** 829.

Hayashi, K., and Trelstad, R. L. (1976). *Stain Technol.* **51,** 68.

Huang, E.-S., Chen, S.-T., and Pagano, J. S. (1973). *J. Virol.* **12,** 1473.

Jacob, J., Todd, K., Birnstiel, M. L., and Bird, A. (1971). *Biochim. Biophys. Acta* **228,** 761.
Jones, K. W., and Robertson, F. W. (1970). *Chromosoma* **31,** 331.
Junghans, R. P., Duesberg, P. H., and Knight, C. A. (1975). *Proc. Natl. Acad. Sci. U.S.A.* **72,** 4895.
Kacian, D. L., Spiegelman, S., Bank, A., Terąda, M., Metafora, S., Dow, L., and Marks, P. A. (1972). *Nature (London), New Biol.* **235,** 167.
Kelly, R. B., Cozzarelli, N. R., Deutscher, M. P., Lehman, I. R., and Kornberg, A. (1970). *J. Biol. Chem.* **245,** 39.
Kennell, D. (1968). *J. Mol. Biol.* **34,** 85.
Kennell, D. E. (1971). *Prog. Nucleic Acid Res. Mol. Biol.* **11,** 259.
Kirby, K. S. (1968). *In* "Methods in Enzymology" (L. Grossman and K. Moldave, eds.), Vol. 12, Part B, p. 87. Academic Press, New York.
Kopriwa, B. M. (1973). *Histochemie,* **37,** 1
Kuo, M. T., Meinke, W., and Saunders, G. F. (1975). *Proc. Natl. Acad. Sci. U.S.A.* **72,** 5004.
Leduc, E., Marinozzi, V.,and Bernhard W. (1963). *J. R. Microsc. Soc.* [3] **81,** 119.
Lindquist McKenzie, S., Henikoff, S., and Meselson, M. (1975). *Proc. Natl. Acad. Sci. U.S.A.* **72,** 1117.
Maurer, W., and Primbsch, E. (1964). *Exp. Cell Res.* **33,** 8.
Maurer, W., and Schultze, B. (1969). *In* "Autoradiography of Diffussible Substances" (L. J. Roth and W. E. Stumpf, eds.), p. 15. Academic Press, New York.
Nonoyama, M. (1975). *Int. Rev. Exp. Pathol.* **14,** 69.
Pagano, J. S., and Huang, E.-S. (1974). *In* "Viral Immunodiagnosis" (E. Kurstak and R. Morisset, eds.), p. 279. Academic Press, New York.
Pelc, S. R., and Welton, M. G. E. (1967). *Nature (London)* **216,** 925.
Perry, R. P. (1969). *In* "Fundamental Techniques in Virology" (K. Habel and N. P. Salzman, eds.), Vol. 1, p. 242. Academic Press, New York.
Prensky, W., Steffensen, D. M., and Hughes, W. L. (1973). *Proc. Natl. Acad. Sci. U.S.A.* **70,** 1860.
Price, P. M., Hirschhorn, K., Gabelman, N., and Waxman, S. (1973). *Proc. Natl. Acad. Sci. U.S.A.* **70,** 11.
Probst, G. S., Stalker, D. M., Mosbaugh, D. W., and Meyer, R. R. (1975). *Proc. Natl. Acad. Sci. U.S.A.* **72,** 1171.
Pullman, B., Claverie, P., and Caillet, J. (1966). *Proc. Natl. Acad. Sci. U.S.A.* **55,** 904.
Ross, J., Aviv, H., Scolnick, E., and Leder, P. (1972). *Proc. Natl. Acad. Sci. U.S.A.* **69,** 264.
Salpeter, M. M., and Bachmann, L. (1964). *J. Cell Biol.* **22,** 469.
Schultze, B., Maurer, W., and Hagenbusch, H. (1976). *Cell Tissue Kinet.* **9,** 245.
Shaw, J. E., Huang, E.-S., and Pagano, J. S. (1975). *J. Virol.* **16,** 132.
Steinert, G., Thomas, C., and Bracket, J. (1976). *Proc. Natl. Acad. Sci. U.S.A.* **73,** 833.
Verma, I. M., Temple, G. F., Fan, H., and Baltimore, D. (1972). *Nature (London), New Biol.* **235,** 163.

Chapter 13

Electron and Immunoelectron Microscopic Procedures for Diagnosis of Viral Infections

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I. INTRODUCTION

The small size of viruses places them beyond the limit of resolution of the light microscope (~250 nm). Thus, they can be visualized only with the electron microscope, which provides a resolving power of approximately 0.3 nm. Although the first electron microscope was developed in the 1930's, the complexity of the instrument and the slow development of preparative methods for examining biological material delayed its application in the study of viruses. The first use of the electron microscope as a diagnostic instrument in virology was reported by Nagler and Rake (1948) and van Rooyen and Scott (1948), who demonstrated that virus particles could be identified in clinical specimens from patients affected with smallpox, vaccinia, and varicella. Virologists were slow to adopt this approach, and it was more than a decade before the electron microscope was finally being used in the differential diagnosis of smallpox and chicken pox (Peters *et al.*, 1962; Nagington, 1964; Cruickshank *et al.*, 1966).

Only recently has the electron microscope (EM) become generally recognized by virologists as more than a research instrument, but rather as an important tool in the rapid diagnosis of virus infections (Banatvala *et al.*, 1975). There now exists ample evidence that viruses can be detected by EM in clinical specimens from a wide variety of human and veterinary infections (Doane *et al.*, 1967, 1969; Joncas *et al.*, 1969; Spradbrow and Francis, 1969; Pavilanis *et al.*, 1971; McFerran *et al.*, 1971).

Direct visualization of virus in clinical specimens obviously provides the most rapid diagnostic method. For reasons discussed later, it is not always practical to examine by EM every specimen received in a diagnostic laboratory, and many laboratories continue to carry out virus isolation in a host cell system, but identify the viral isolate by electron microscopy (Doane *et al.*, 1969; Spradbrow and Francis, 1969; Pennington *et al.*, 1975).

Identification of a virus on the basis of its morphology bypasses the usual problem of loss of viral infectivity in a clinical specimen prior to isolation. Accordingly, by electron microscopy, it has been possible to demonstrate for the first time a number of elusive viruses that are difficult or

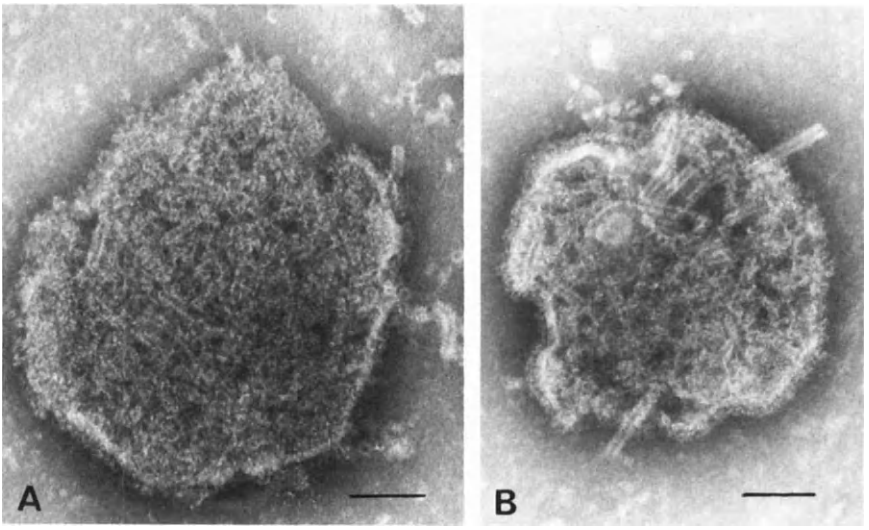

Fig. 1. Paramyxoviruses found by EM in negatively stained clinical specimens. Each virus particle exhibits the typical paramyxovirus morphology of pleomorphic enveloped virion with helical nucleocapsid. (A) Mumps virus in CSF. (B) Parainfluenza virus in nasopharyngeal secretions. Scale, 100 nm. (From Doane *et al.*, 1967.)

impossible to culture, notably the viruses of subacute sclerosing panencephalitis (Bouteille *et al.*, 1965; Tellez-Nagel and Harter, 1966), progressive multifocal leukoencephalopathy (ZuRhein and Chou, 1965; Howatson *et al.*, 1965), rubella (Best *et al.*, 1967), hepatitis (Bayer *et al.*, 1968), and acute gastroenteritis (Bishop *et al.*, 1973; Flewett *et al.*, 1973).

By morphology alone, most viruses can be identified only to the level of their taxonomic family or genus. For example, enteroviruses cannot be distinguished from rhinoviruses, or mumps virus from parainfluenza virus (Fig. 1). Techniques are gradually being developed, however, within the field of immunoelectron microscopy, that offer a means of specific differentiation by serological typing directly on the EM specimen grid.

II. ESTABLISHING AN EM UNIT FOR VIROLOGY

Ideally, an active virus laboratory should have its own electron microscopy facilities. In Toronto, three of the largest hospitals, as well as the Central Public Health Laboratories, have an EM Unit as an integral component of the virus diagnostic laboratory. The microscopes are in continuous use, both for direct examination of clinical specimens and as backup

for identifying viruses isolated in cell culture. In the pediatric hospital, as many as 30–40 clinical specimens might be examined daily by EM during the height of the infantile gastroenteritis season.

To be useful for identifying viruses, the electron microscope should be capable of resolving better that 10–15 Å. It should also be possible for the operator to discern clearly ultrastructural details at a viewing magnification of 30,000 to 40,000 times, which provides a sufficiently enlarged field of view for detection of even small viruses (Doane *et al.*, 1969; McFerran *et al.*, 1971).

The electron microscopist should have a basic technical knowledge of the operation and routine maintenance of the instrument. Thus, he should be capable of keeping the microscope optically aligned and at a maximum performance level. He should perform, or supervise, filament changes and routine cleaning of apertures and specimen holders. Preferably the instrument will be covered by a service contract, thereby reducing to a minimum the time lost for repairs and general servicing.

It is, of course, vital that the operator should be able to recognize viral ultrastructure. Most negatively stained specimens contain large quantities of cellular debris and small quantities of virus. An experienced eye is needed to differentiate between the two.

Where negative staining is the principle technique being used, it is advisable to have a simple vacuum evaporation unit for preparing carbon films on Formvar-coated specimen grids. As quantities of these can be prepared 2 to 3 weeks before use, it is not essential to have an evaporator located in the laboratory itself.

If thin sectioning techniques are to be used in the EM Unit, several major items of equipment are necessary. These include an ultramicrotome, a knife breaker, and an embedding oven. It should be recognized that thin sectioning techniques require a considerable amount of technical skill and experience, and it may be advisable to have this phase of the work performed by a service laboratory, if one is available. The only task then remaining for the electron microscopist is to examine the prepared thin sections.

Photography is an essential adjunct to the electron microscope, and photographic facilities should be readily available for the virology EM Unit. The microscopist should personally process his own films, plates, and micrographs, or at least supervise their processing. (Because of the low cost, ease of handling and storing, and high resolution of roll films, this format is well-suited to the diagnostic laboratory). A photographic enlarger should also be available; it can be equipped with contrast filters if the convenient polycontrast photographic paper is used. It is our experience that photographic processors greatly reduce the work load in the EM

Unit, and yet these instruments produce micrographs of high quality. In our own laboratory, all micrographs are prepared by processors (15 seconds from enlarger to print!), rather than by the standard tray method of development.

The scanning electron microscope (as opposed to the better known transmission electron microscope) is still in its infancy with respect to its application to diagnostic virology. It appears to be useful in monitoring cell cultures for mycoplasma contamination (Section VI), but at present it is essentially a research instrument in virology.

III. DIRECT EXAMINATION OF CLINICAL SPECIMENS BY NEGATIVE STAINING

A. Introduction

Negative staining of clinical specimens provides the simplest and most rapid method for virus detection. The main limitation to this approach relates to the low concentration of virus particles in many clinical specimens. Depending on the negative staining method used, between 10^7 and 10^9 particles per milliliter must be present in the original specimen in order to be detected on the grid (Anderson and Doane, 1972b). Because of the high particle-to-infectivity ratio observed with many viruses, this may represent less than 100 $TCID_{50}$ in terms of infectivity (Doane *et al.*, 1967; Valters *et al.*, 1975).

Some of the more fragile viruses may be structurally altered by the negative staining process. Although this may result in structural artifacts, from a diagnostic point of view this may actually be an advantage; for example, it is usually easier to detect a ruptured paramyxovirus with its uncoiled nucleocapsid than an intact virion with the envelope unpenetrated by stain (see Fig. 15A).

B. Materials

1. Negative Stain

Phosphotungstic acid (PTA) serves as an excellent general purpose negative stain. It is used as a 2% aqueous solution, raised to pH 6–7 with a few drops of 1 *N* KOH. The water should be filtered to remove *physically* any bacteria that might be present. It is convenient to store the PTA solution in a small syringe at 4°C and to dispense it by drop from the syringe needle.

2. *Grids*

Copper grids of 200 to 400 mesh can be used, with 300-mesh grids providing optimal support and open area. Although bare Formvar support films have been used successfully for negative staining by some workers (Palmer *et al.*, 1975), we prefer a film of 0.3% Formvar in ethylene dichloride, stabilized by a very thin layer of evaporated carbon.

C. Methods

1. *Direct Application*

Specimens that contain little or no salt can be added directly to the grid and negatively stained without preliminary treatment. A fine-bore Pasteur pipette is used to add a drop of the specimen; a drop of negative stain is then added. Excess fluid is removed by bringing it briefly in contact with a torn edge of filter paper, then allowing it to air-dry (1–2 minutes).

2. *Water Drop Method*

Most fluid specimens contain a high concentration of salt which, if allowed to dry on the specimen grid, will crystallize and obliterate viral particles during EM examination. To circumvent this problem, a simple dialysis method can be used for specimens that contain at least 10^9 virus particles per milliliter (Doane *et al.*, 1969). One microdrop of specimen is placed on a drop of sterile distilled water resting on a waxed surface. A coated grid is touched briefly to the surface of the drop. Negative stain is then added, excess fluid is removed with filter paper, and the grid is air-dried and examined.

3. *Agar Diffusion Method*

This is a modification of the method of Kelen *et al.* (1971). It is useful for salty specimens, especially when the virus concentration is low (Anderson and Doane, 1972b). The limit of detectability by the agar diffusion method is approximately 10^7 virus particles per milliliter of original sample. Cups of disposable microtiter plates are approximately three-fourths filled with 1% aqueous Noble agar, and a 300-mesh coated specimen grid is placed on the solid agar surface of each cup. Plates so prepared can be used immediately, or they can be covered tightly with strips of adhesive sealing tape and stored at 4°C for several weeks. To prepare a specimen for examination, one to two drops are added to a grid and allowed to air-dry (15–30 minutes). A drop of negative stain is then added. After 1 minute the grid is removed with forceps, aid-dried briefly, and examined by EM.

4. Rupture of Cells by Freezing and Thawing

Small pieces of tissue (1 mm^3) are placed in a metal planchet and are frozen and thawed four to six times by alternately touching the planchet to Dry Ice and to the palm of the hand (Doane *et al.*, 1969). A drop of filtered distilled water is then added and the tissue is once more frozen and thawed. The resulting lysed tissue suspension is briefly mixed with a fine-bore Pasteur pipette and a small drop is placed on a coated grid. The specimen is then negatively stained and air-dried.

D. Practical Considerations

Whenever possible, duplicate grids should be prepared of every specimen; one might be prepared by the direct application method, for immediate examination, and the other by the agar diffusion method, to serve as a backup grid. In general, no more than 15 minutes need be spent in examining a grid, unless it is a selected case, e.g., associated with smallpox. Screening is carried out at viewing magnifications of approximately 40,000 times. A single magnification should be selected as a routine, so that the operator can become familiar with the relative size of particles being observed. (On one of the microscopes in our laboratory, a small circle on the viewing screen spans approximately 100 nm at a magnification of 35,000×.)

Virus particles are usually recognized by their ability to trap the negative stain at their outer surface. Thus virus particles usually stand out more clearly than background debris. This enables large viruses, such as myxoviruses, to be detected even at low magnification (Fig. 2). Several areas of the grid should be examined, and with experience, one learns to select moderately dense squares under very low magnification, then proceed to examine those squares at high magnification.

E. Specimens

1. Vesicle Fluid, Smears, or Crusts

Specimens from vesicles produced by herpesviruses or poxviruses provide excellent material for direct examination because of their high content of virus (Fig. 3). The use of the electron microscope in smallpox diagnosis was first indicated, in 1948, by van Rooyen and Scott and by Nagler and Rake; it is probably in the differential diagnosis of chicken pox and smallpox* that the electron microscope is best known in diagnostic virol-

* NOTE: *Extreme* caution should be exercised in handling specimens suspected of containing smallpox (see Section VIII).

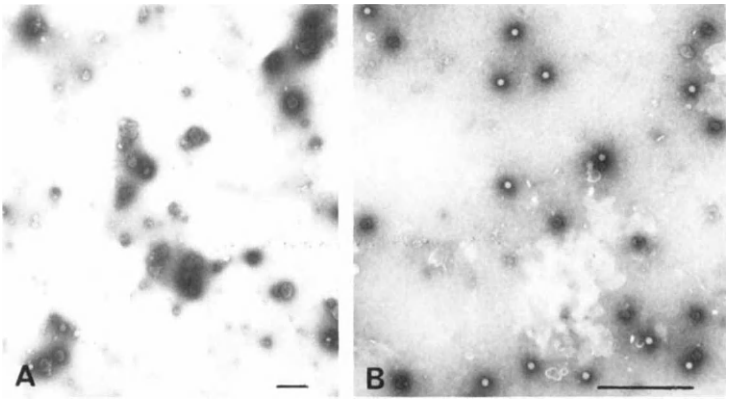

Fig. 2. Low magnification electron micrographs of negatively stained clinical specimens, showing high concentration of virus that may be present in crude specimen. Note dark outline of negative stain around virus paticles. (A) Parainfluenza viruses in nasopharyngeal secretions. (B) Adenoviruses in stool suspension. Scale, 1 μm.

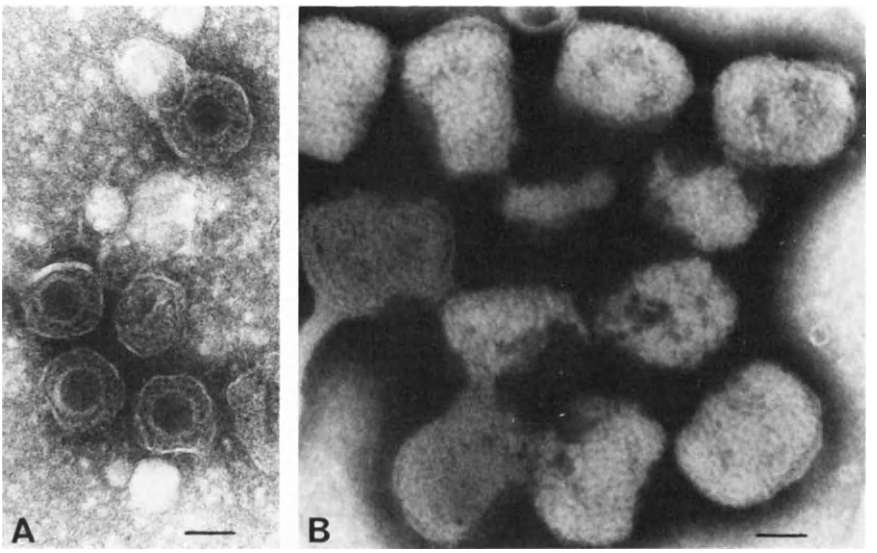

Fig. 3. Negatively stained virus particles from vesicular lesions. (A) Herpes varicella-zoster viruses. (B) Vaccinia viruses. Scale, 100 nm.

ogy. Thus electron microscopy now provides the method of choice in the rapid detection of poxviruses and herpesviruses (Peters *et al.*, 1962; Almeida *et al.*, 1962; Smith and Melnick, 1962; Nagington, 1964; Cruickshank *et al.*, 1966; Macrae *et al.*, 1969; Bland *et al.*, 1970; Gibbs *et al.*, 1970), although for specific identification it is still necessary to compliment morphological detection with virus isolation and identification procedures (Long, 1970).

Coxsackie A16 virus, the etiological agent of "hand, foot and mouth disease" (Robinson *et al.*, 1958; Alsop *et al.*, 1960) can also be detected by direct EM examination of vesicle fluid (P. J. Middleton and M. T. Szymanski, personal communication).

Vesicle fluid is collected in a capillary tube, or into a fine-bore needle attached to a small syringe. If the volume is sufficient, grids are prepared both by direct application and by the agar diffusion method. If the amount of fluid is limited, the agar diffusion method is recommended. Crusts removed from dried vesicles provide an excellent source of viruses (P. J. Middleton and M. T. Szymanski, personal communication). The excised crust is placed, underside facing down, in a drop of 1% unbuffered aqueous ammonium acetate on a glass slide. A smear is made by grinding the crust against the side by means of a scalpel blade. One or two more drops are added to the smear, and a coated grid is touched briefly to the fluid, then negatively stained and examined. Smears can also be made from vesicle fluid, or from scrapings collected from the base of a vesicle with a 25-gauge needle. Dried smears are then resuspended in two to three drops of filtered distilled water or 1% ammonium acetate.

2. *Respiratory Tract Specimens*

Nasopharyngeal secretions collected by the suction method of Auger (1939) have been found suitable for direct EM detection of influenza, parainfluenza, and respiratory syncytial viruses (Fig. 1) (Doane *et al.*, 1967; Joncas *et al.*, 1969). For examination, the aspirated nasopharyngeal secretion is diluted five- to tenfold in balanced salt solution, bacteria are deposited by low-speed centrifugation, and the clarified specimen is prepared for negative staining by the agar diffusion or water drop method. Following similar procedures, herpesvirus particles have been found in tracheobronchial suctions from a child with herpes simplex pneumonia (P. J. Middleton and M. T. Szymanski, personal communication).

Throat washings or gargles rarely contain sufficient virus to be detected by electron microscopy. Lipman *et al.* (1975) observed Epstein-Barr virions in a single throat washing from a patient with infectious mononucleosis, but only after a 23 × concentration and purification by density gradient centrifugation. Nasal secretions from acute upper respiratory

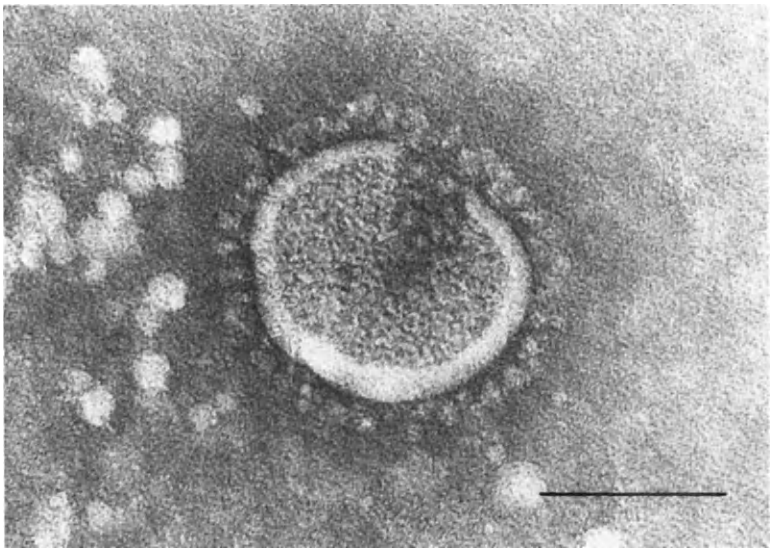

Fig. 4. Coronavirus found in negatively stained nasal secretions. Scale, 100 nm. (Courtesy of M. Szymanski.)

tract infections have been used to demonstrate coronaviruses (Fig. 4) (M. T. Szymanski, personal communication).

3. Cerebrospinal Fluid

Evans and Melnick (1949) reported the EM detection of herpesviruses in cerebrospinal fluid (CSF) from a patient with herpes zoster. In our own laboratory, mumps virus was observed in negatively stained CSF from a patient with suspected mumps encephalitis (Fig. 1) (Doane *et al.*, 1967). Our overall results, however, indicate that CSF rarely contains sufficient quantities of virus to be detected by direct examination. The agar diffusion method is recommended for negative staining of CSF.

4. Stool

A variety of viruses and viruslike particles have been found in negatively stained fecal specimens by electron microscopy (Fig. 5). Included in the rapidly growing list are coronaviruses (Caul *et al.*, 1975), adenoviruses (Anderson and Doane, 1972b; Flewett *et al.*, 1974b) reolike viruses (Doane *et al.*, 1969; Anderson and Doane, 1972b; Flewett *et al.*, 1973, 1974b,c; Middleton *et al.*, 1974), enteroviruses (P. J. Middleton and M. T. Szymanski, personal communication), hepatitis A virus (Feinstone *et al.*, 1973), bacterial viruses (Flewett *et al.*, 1974b), and miscellaneous unclassified viruses (Kapikian *et al.*, 1972b; Paver *et al.*, 1973; Madeley

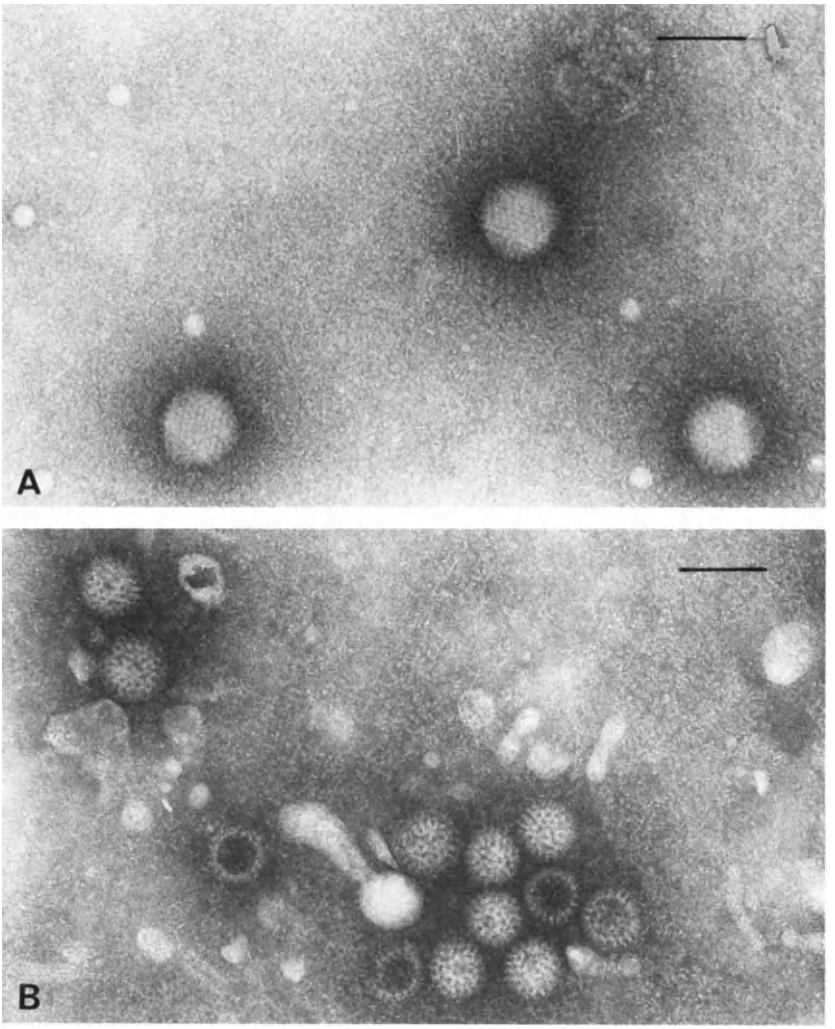

Fig. 5. Viruses detected by EM in negatively stained stool specimens. (A) Adenoviruses with small adeno-associated viruses. (B) Reolike particles from infantile gastroenteritis. Scale, 100 nm. (Courtesy of M. Szymanski.)

and Cosgrove, 1975), as well as "pleomorphic viruslike particles" Mathan *et al.*, 1975). (For a comprehensive review of agents associated with acute gastroenteritis, see Chapter 11, Volume I.)

Ultracentrifuged deposits (UCD) of stool suspensions (clarified of bacteria by light centrifugation) have been used for many years in this laboratory. The UCD is resuspended in two to three drops of water prior

to negative staining. A more satisfactory technique for processing large numbers of specimens, recommended by M. T. Szymanski and P. J. Middleton (personal communication), is to resuspend a small portion of stool in 1% ammonium acetate without any preliminary clarification or concentration. If difficulty is encountered in negative staining the specimen due to, e.g., excess mucus, a smear of the specimen is made on a glass slide. This is allowed to air-dry and is then resuspended in water or ammonium acetate, negatively stained, and examined. Smears of fecal swabs can also be used with considerable success.

5. Urine

It appears that viruses are not readily detected in urine. Efforts to visualize cytomegalovirus (CMV) are generally discouraging. However, Montplaisir *et al.* (1972) found CMV by centrifuging 30 to 50 ml volumes of urine, at 82,000 *g* for 90 minutes, then resuspending the pellet in a few drops of water, followed by negative staining. Papovaviruses and adenoviruses have only rarely been observed in urine (P. J. Middleton and M. T. Szymanski, personal communication).

6. Blood

It would appear that blood is not reliable for direct EM detection of viruses. Hepatitis B antigen has been observed in the serum, but a more

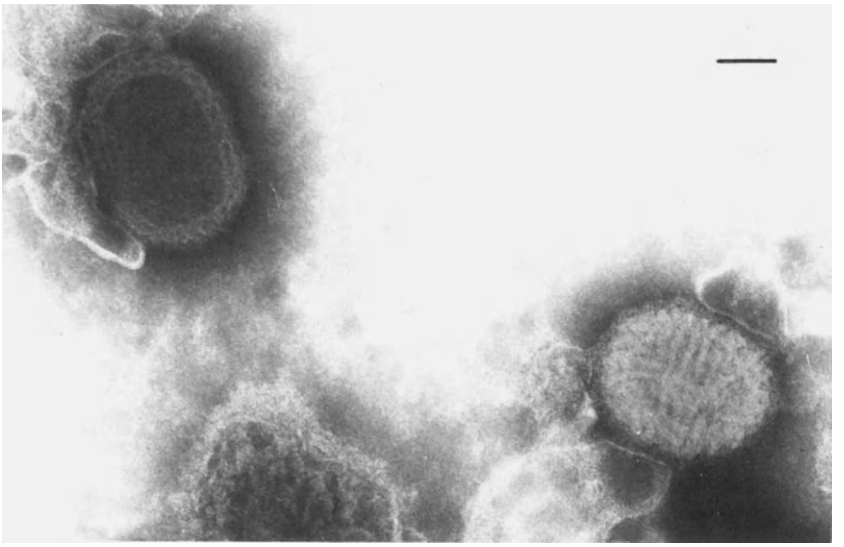

Fig. 6. Molluscum contagiosum viruses from skin biopsy. Note two morphological forms characteristic of negatively stained poxviruses. Scale, 100 nm.

sensitive method for visualizing the components of this antigen is provided by immunoelectron microscopy.

7. Tissues

Skin biopsies can be used to demonstrate molluscum contagiosum (Fig. 6), or virus from contagious pustular dermatitis, and papovaviruses from warts (Macrae *et al.*, 1969; Strauss *et al.*, 1949; Williams *et al.*, 1961). The papovavirus associated with progressive multifocal leukoencephalopathy (PML) has been found by negative staining of brain tissue (Howatson *et al.*, 1965), and Deutsch and Spence (1972) detected hepatitis B virus in liver specimens.

IV. DIRECT EXAMINATION OF CLINICAL SPECIMENS BY THIN SECTIONING

A. Introduction

Although negative staining provides the most rapid method for examining a clinical specimen, the thin sectioning technique is usually more reliable when the clinical specimen is in the form of autopsy or biopsy tissue. Viruses that have been demonstrated, for the first time, as a result of this technique, include the measles-like virus of subacute sclerosing panencephalitis (SSPE) found in brain cell nuclei (Bouteille *et al.*, 1965; Tellez-Nagel and Harter, 1966) (Fig. 7), and the reovirus-like particles of acute gastroenteritis, first revealed in thin sections of duodenal mucosa biopsy tissue (Bishop *et al.*, 1973). Thin sectioning has also been used to demonstrate papovaviruses in brain tissue from progressive multifocal leukoencephalopathy (PML) (Zu Rhein and Chou, 1965; Dolman *et al.*, 1967) and herpesviruses in brain from herpetic encephalitis (Harland *et al.*, 1967).

Useful retrospective EM studies can be done with autopsy material regardless of fixation (Burns *et al.*, 1975), and techniques have been described for processing paraffin-embedded sections to permit visualization of intracellular virus particles (Morecki and Becker, 1968; Blank *et al.*, 1970).

Using standard histological procedures normally employed for electron microscopy, sections of tissue are not generally available until at least 36–48 hours after receipt of the specimen. It has been found that these procedures can be abbreviated considerably, with little loss in ultrastructural detail, to provide sections within 2 to 3 hours (Doane *et al.*, 1974;

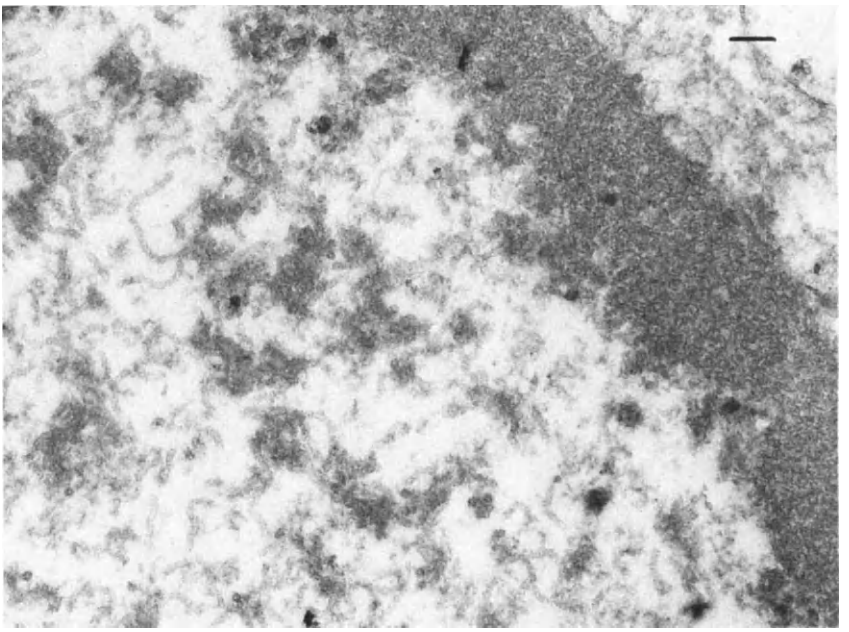

Fig. 7. Thin section (in region of nucleus) of brain biopsy from patient with SSPE. Note margination of nuclear chromatin across upper right corner, and wormlike viral nucleocapsids scattered throughout the nucleus. Scale, 100 nm.

Rowden and Lewis, 1974) (Fig. 8). The rapid embedding method used in this laboratory is described below.

B. Rapid Embedding Method

Tissue specimens not exceeding 1 mm in thickness are collected in cold 2.5% glutaraldehyde in Millonig phosphate buffer. A minimum of 15 minutes is allowed for fixation; however, tissues can safely be stored in glutaraldehyde for several days. Primary fixation is followed by three rinses (1 minute each) in phosphate buffer, and a second fixation of 15 minutes at room temperature in 1% osmium tetroxide in phosphate buffer.

Fixed specimens are dehydrated through acetone as follows: 70% acetone, two changes in 3 minutes; absolute acetone, three changes in 5 minutes. After 10 minutes in a 1:1 mixture of absolute acetone and epoxy embedding plastic (either Spurr's medium, or a mixture of Araldite and Epon), followed by two changes of 100% plastic (5 minutes each), the specimen is placed in fresh plastic in an embedding capsule and heated to 95°C for 60 minutes to achieve polymerization of the plastic. The total processing time is 2 hours.

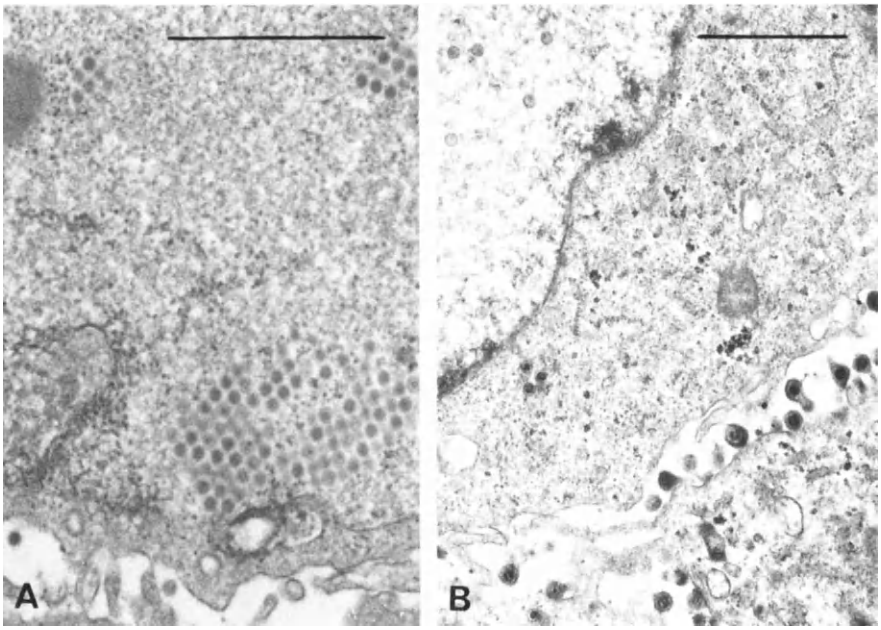

Fig. 8. Thin sections of virus-infected vero cell cultures processed by rapid embedding method. (A) Adenovirus infection. (B) Herpes simplex virus infection. Scale, 1 μm. (From Doane *et al.*, 1974.)

Embedded tissue processed by this method can be trimmed for sectioning within 15 minutes after removal from the oven. Thin sections can be cut with either glass or diamond knives, and are collected on uncoated 300-mesh copper specimen grids prior to staining with lead citrate and uranyl acetate.

V. IDENTIFICATION OF VIRUSES ISOLATED IN TISSUE CULTURES, EGGS, AND ANIMALS

A. Introduction

Undoubtedly the most useful application of the electron microscope in routine diagnostic virology is in the identification of viruses isolated in tissue culture. Because of the impracticability of performing direct EM examination on every clinical specimen submitted for virus studies, most virus laboratories with EM facilities continue to rely on cell cultures for virus isolation, but perform a presumptive morphological identification of

the viral isolate by negatively staining cultures showing a cytopathic effect (CPE) (Doane *et al.*, 1969; Spradbrow and Francis, 1969; Pennington *et al.*, 1975). Viral isolates can readily be identified as to group by electron microscopy; subsequent specific identification can then be performed by standard serological methods.

The amount of virus in a clinical specimen may be well below the limit of EM detectability, making direct examination useless. Following passage in tissue culture, however, the virus concentration can be amplified to yield large quantities of virus that can easily be detected using negative staining techniques. As shown in Table I, some viruses isolated in cell cultures can be identified by electron microscopy as much as 2 days before the appearance of a cytopathic effect (Doane and Anderson, 1972).

EM identification of viruses isolated in organ cultures was used by Almeida and Tyrrell (1967) in their discovery of human coronaviruses. Marsolais *et al.* (1971) reported that this approach permitted a rapid diagnosis of avian coronaviruses isolated in embryonated hen's eggs.

B. Methods

1. Negative Staining

A single tube culture of infected cells provides ample material for EM examination. Although we have found virus in negatively stained infected cultures showing no cytopathic effect, as well as those showing complete cell destruction, in general, it is preferable to work with cultures showing a well-developed CPE (Figs. 9–12). Viruses, such as orthomyxoviruses and paramyxoviruses, can often be detected in the culture medium, simply by withdrawing a few drops and negatively staining by the water drop or agar diffusion methods (Sections III,C,2 and 3). As a routine, however, it is more reliable to negatively stain a sample of the cells, especially if the cytopathic effect has not progressed to involve the entire cell sheet. Furthermore, with viruses that are markedly cell-associated, such as adenoviruses and reoviruses, virus particles remain in the cells long after complete destruction of the culture.

To process the cells for negative staining, the culture medium is withdrawn and held temporarily, and the cells are scraped with a Pasteur pipette into two or three drops of filtered distilled water, thereby lysing the cells. A drop of this lysate is then negatively stained by the water drop method. In the majority of cases this procedure is effective in revealing viral particles if they are present in the cells. If no virus is found in the cell lysate, however, the culture medium is processed both by the water drop and agar diffusion methods.

TABLE I
Earliest Detection of Virus in Cell Culture by CPE and EM

Virus	Inoculum ($TCID_{50}$)	Earliest detection (days)	
		By CPE	By EM
Adenovirus	10	5	3
	100	4	3
Coxsackie B5	10	2	2
	100	2	1
	1000	2	1
Parainfluenza 3	10	4	3
	100	4	2
	1000	4	2
Measles	10	4	3
	100	3	3
	1000	3	2
Herpes simplex	10	3	3
	100	3	3
	1000	2	2

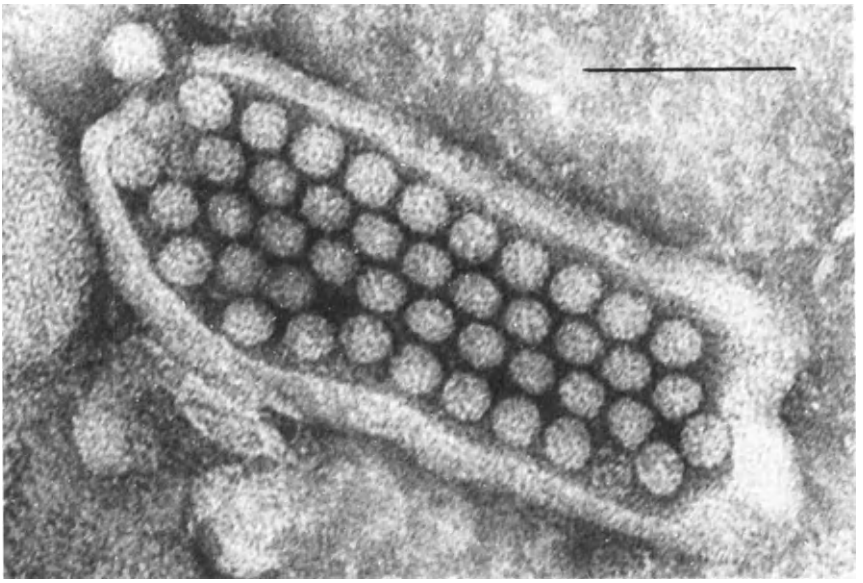

Fig. 9. Enteroviruses in negatively stained cell culture lysate. Note vesicle with membrane around cluster of virus particles. Scale, 100 nm.

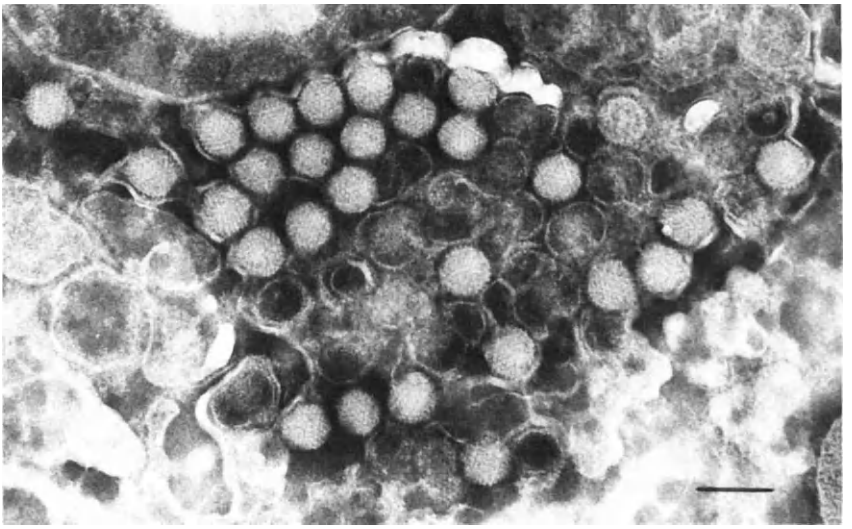

Fig. 10. Adenoviruses in crude cell lysate. Scale, 100 nm.

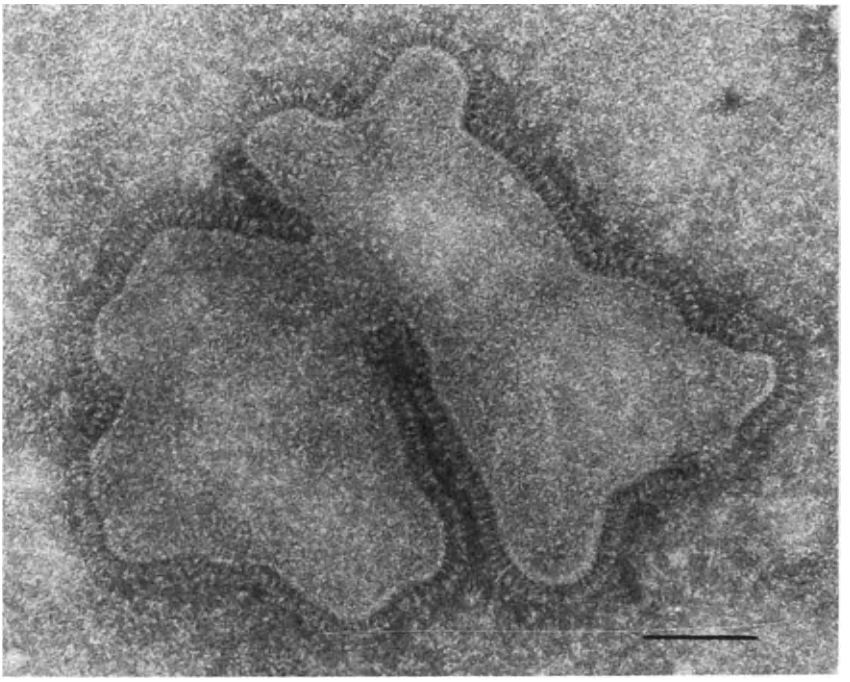

Fig. 11. Respiratory syncytial virus from negatively stained infected culture. Scale, 100 nm. (Courtesy of M. Szymanski.)

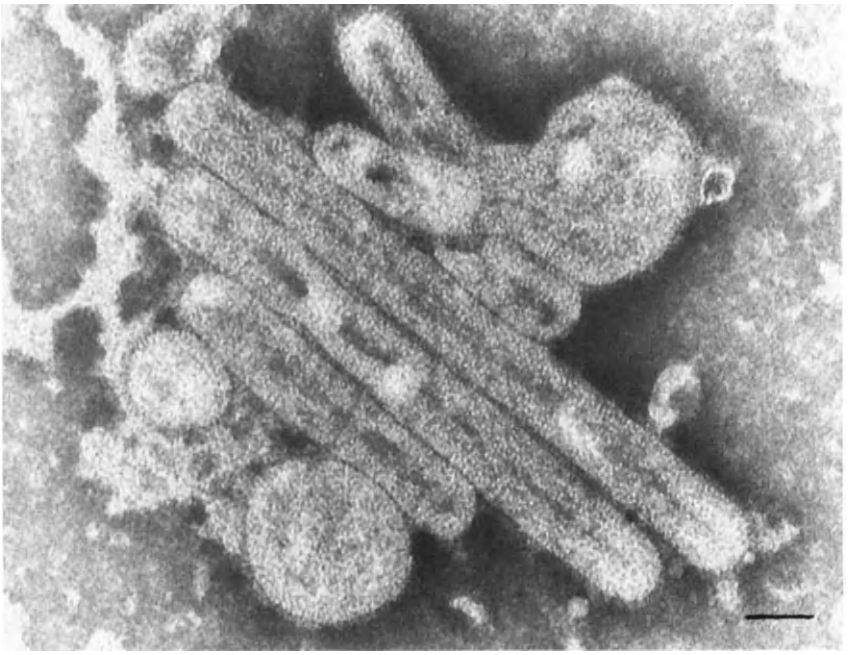

Fig. 12. Influenza viruses from negatively stained cell culture. Scale, 100 nm.

Enveloped viruses such as togaviruses and oncornaviruses are often so severely ruptured by negative staining that they are impossible to recognize by electron microscopy. P. J. Middleton and M. T. Szymanski (personal communication) have overcome this problem by adding a few drops of 2.5% buffered glutaraldehyde to the drained culture for 1 to 2 hours. The cells are then scraped into the fixative and added to a grid for negative staining (Fig. 13).

2. Thin Sectioning

Although negative staining obviously provides the fastest method for identifying viruses isolated in a laboratory host system, thin sectioning may enable the cells or tissues to be examined in a more systematic fashion. This is an important consideration when the viral isolate is a togavirus (Fig. 14) or oncornavirus, which may be difficult to observe in negatively stained preparations, due either to a low particle concentration or to disruption of viral ultrastructure during preparation for electron microscopy. For preparing infected cell cultures for thin sectioning, the following method requires only a single tube culture (Doane *et al.*, 1974). Cells are gently resuspended in the culture medium, transferred to a small

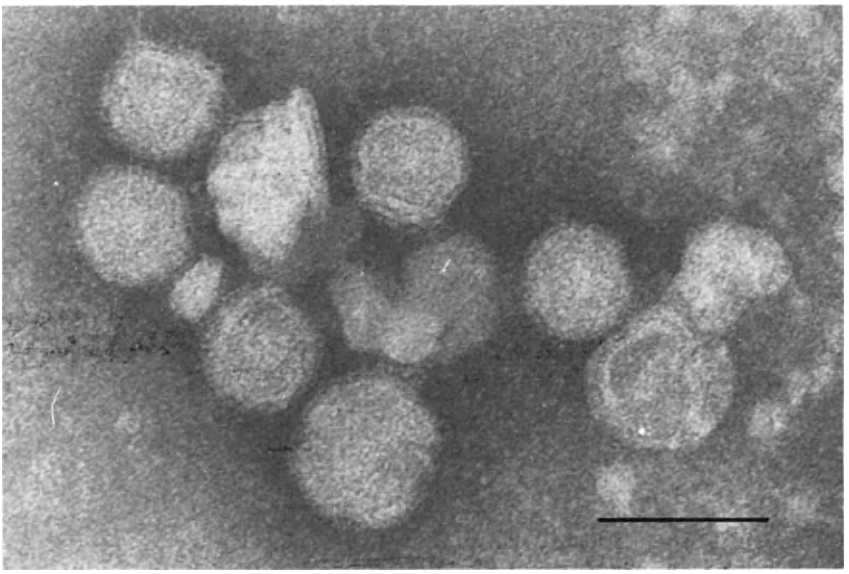

Fig. 13. Rubella viruses from infected cell culture, fixed with glutaraldehyde prior to negative staining. Scale, 100 nm. (Courtesy of M. Szymanski.)

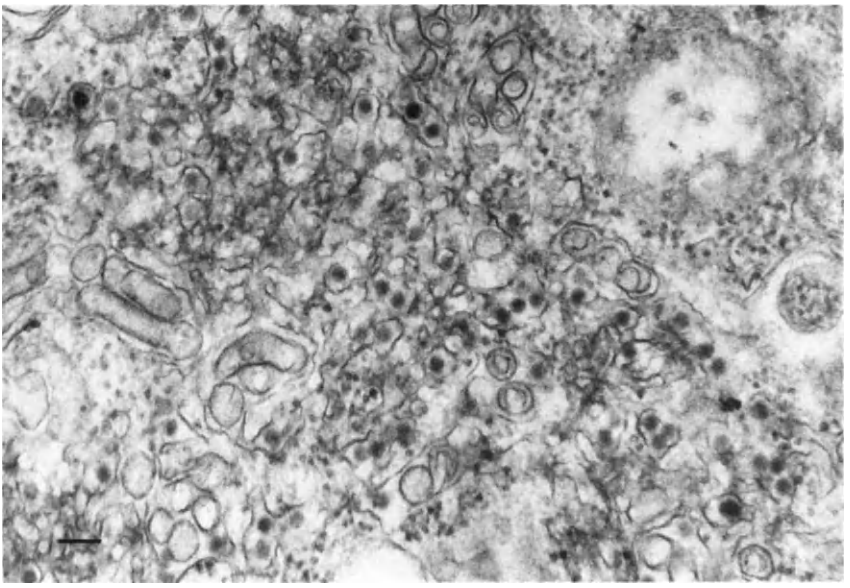

Fig. 14. St. Louis encephalitis virus in thin section of mouse brain. This micrograph was used to confirm the identity of an arbovirus-like agent isolated in Ontario in 1975. Scale, 1 μm. (Courtesy of Drs. L. Spence and H. Artsob.)

conical-tipped centrifuge tube, and pelleted in a clinical centrifuge at 1500 rpm for 3 minutes. The medium is discarded, leaving only two to three drops in the tube to allow for transfer of the cells to a flat waxed surface (e.g., Parafilm). A fine-bore glass tube, 1.3 × 75 mm, is touched to the drop to draw in the cell suspension by capillary action. One end is sealed with Plasticine and the tube is centrifuged in a hematocrit centrifuge for 3 minutes at 12,500 rpm. The cells now form a compact pellet immediately above the Plasticine plug. The tube is scored and broken at a distance of 6 to 7 mm above the cell pellet. The tube is then inverted, and a blunt wire (paper clip) of diameter slightly narrower than the bore of the tube is used to push against the Plasticine, forcing the cell pellet into a vial of fixative. The cell pellet at this and subsequent stages remains tightly packed, and can be transferred easily in the tip of a Pasteur pipette. It can be processed either by standard EM embedding methods or by the rapid embedding method (Section IV,B).

VI. SCREENING CELL CULTURES AND VIRUS POOLS FOR ADVENTITIOUS AGENTS

A. Introduction

Cell cultures are now firmly established as the prime host cell system for the isolation and identification of viruses and for the investigation of virus–cell interactions. Concurrent with their widespread use, however, is the increased recognition that cell cultures are often contaminated with adventitious agents. These may be viruses originating from the host, or they may be viruses or mycoplasma acquired in the laboratory during continuous cultivation. In most instances these contaminants are extremely insidious, causing little or no cytopathic effect; consequently, their presence can often be detected only by electron microscopy.

One of the most notorious groups of contaminants are the simian viruses, which may occur in over 50% of "normal" monkey kidney cell cultures (Hull, 1968; Hsiung, 1968; Anderson and Doane, 1972a). Commonly encountered simian viruses include paramyxoviruses, the papovavirus SV40, foamy virus, and cytomegalovirus. Mixed infections in a single batch of monkey kidney cells are not uncommon (Hsiung and Atoynatan, 1966).

A possible source of contamination is bovine serum, commonly incorporated as a nutrient supplement into most tissue culture media. Fong *et al.* (1975) used electron microscopy techniques to screen 25 lots of bovine serum, and found viruslike particles in 17 lots (68%). Sera were concen-

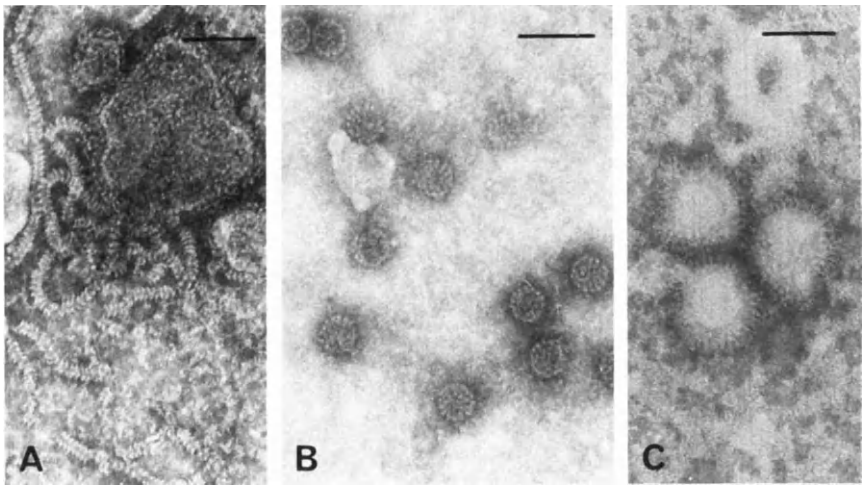

Fig. 15. Negatively stained simian virus contaminants commonly found in monkey kidney cell cultures. (A) Paramyxovirus SV5. (B) Papovavirus SV40. (C) Foamy virus. Scale, 100 nm. (Reproduced by permission of the National Research Council of Canada from the *Can. J. Microbiol.* See Anderson and Doane, 1972a.)

trated 100-fold or more by centrifugation. It was difficult to identify viruses in negatively stained serum; however, when sera were pelleted by ultracentrifugation into Beem capsules, and the pellets were processed for thin sectioning, viruses were found in all 17 lots.

Routine monitoring of cell cultures by negative staining, and preferably also by thin sectioning, serves to keep in check the spread of adventitious agents in stock cultures and also in virus pools prepared in contaminated cultures (Anderson and Doane, 1972a; de Harven, 1973) (Figs. 15–18).

Detection of mycoplasma is often difficult by transmission electron microscopy. Although negative staining provides the most rapid technique, it requires considerable experience to be able to distinguish the pleomorphic microorganisms from normal cellular material (Wolanski and Maramorosch, 1970). Mycoplasma can also be recognized in thin sectioned cell cultures (de Harven, 1973) (Fig. 18). Recent reports suggest that the scanning electron microscope offers a sensitive method for detection of mycoplasma contamination of cell cultures (Brown *et al.*, 1974; Doane and Anderson, 1975) (Fig. 19).

B. Transmission Electron Microscope Methods

Negative staining methods used for screening cell cultures are similar to those described in Section V. For thin sectioning, cells are fixed in 2.5%

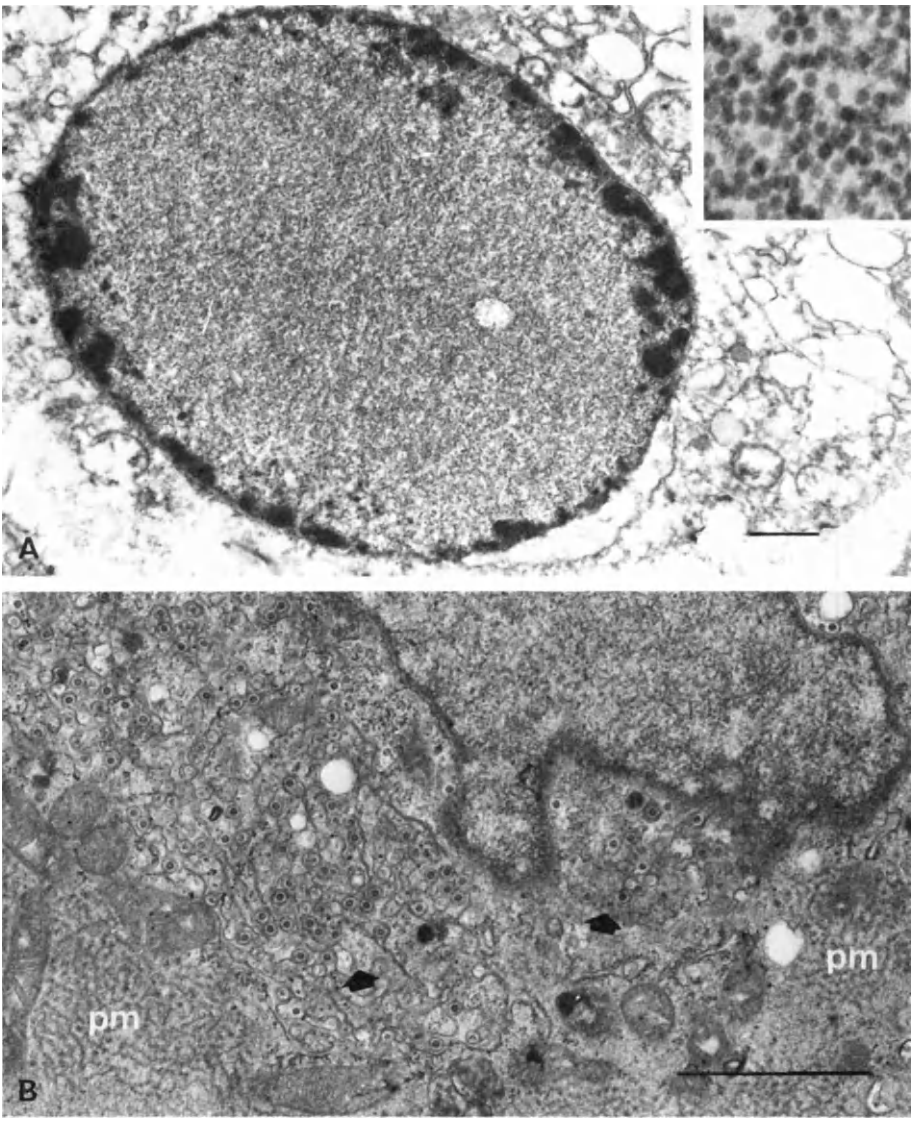

Fig. 16. Thin sections of monkey kidney cell cultures showing simian contaminants. (A) Nucleus packed with papovaviruses. Insert shows higher magnification of nucleus. (B) Mixed contamination of foamy virus (arrows) and paramyxovirus (pm). Scale, 1 μm. (Reproduced by permission of the National Research Council of Canada from the *Can. J. Microbiol.* See Anderson and Doane, 1972a.)

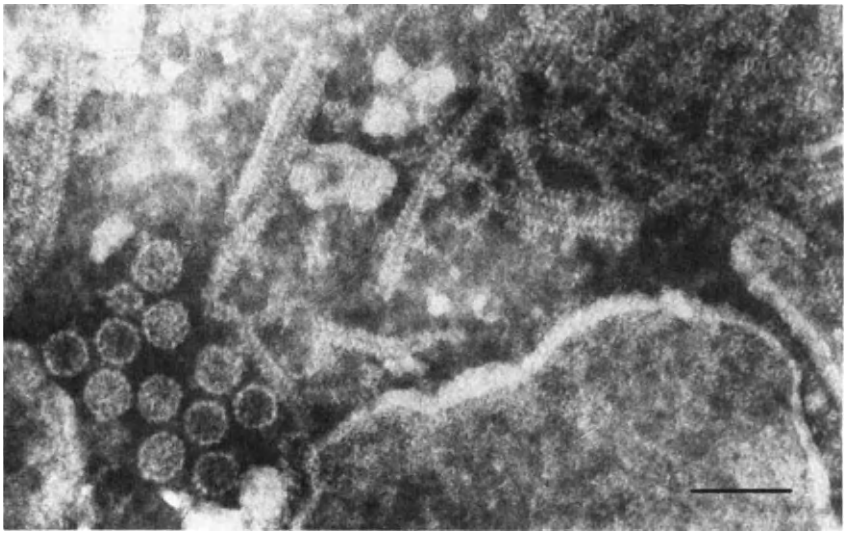

Fig. 17. Negatively stained preparation of crude parainfluenza virus pool prepared in monkey kidney cell cultures that were contaminated with SV40. The spherical papovavirus particles lie in the cell lysate with the "herring bone"-shaped parainfluenza virus nucleocapsids. Scale, 100 nm.

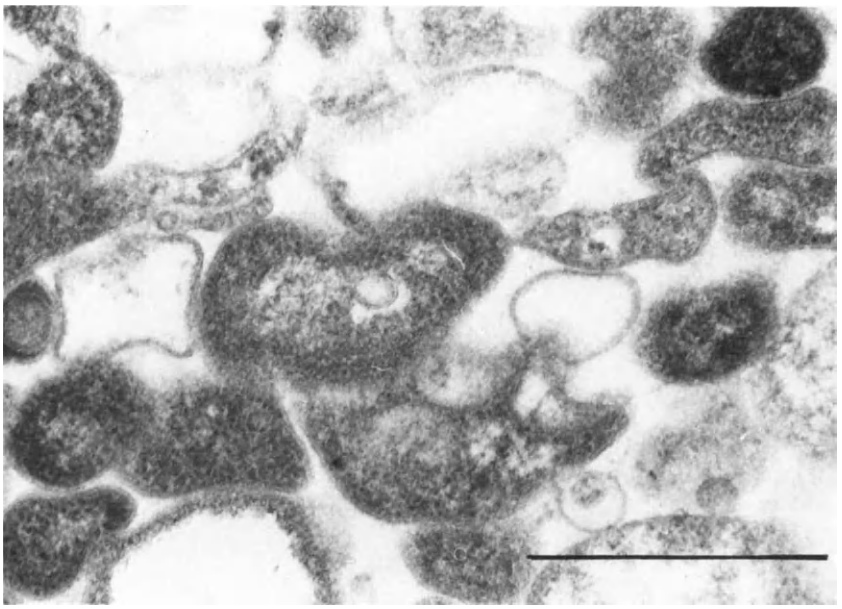

Fig. 18. Thin section of portion of cell culture contaminated with mycoplasma (pleomorphic dense structures). Scale, 1 μm.

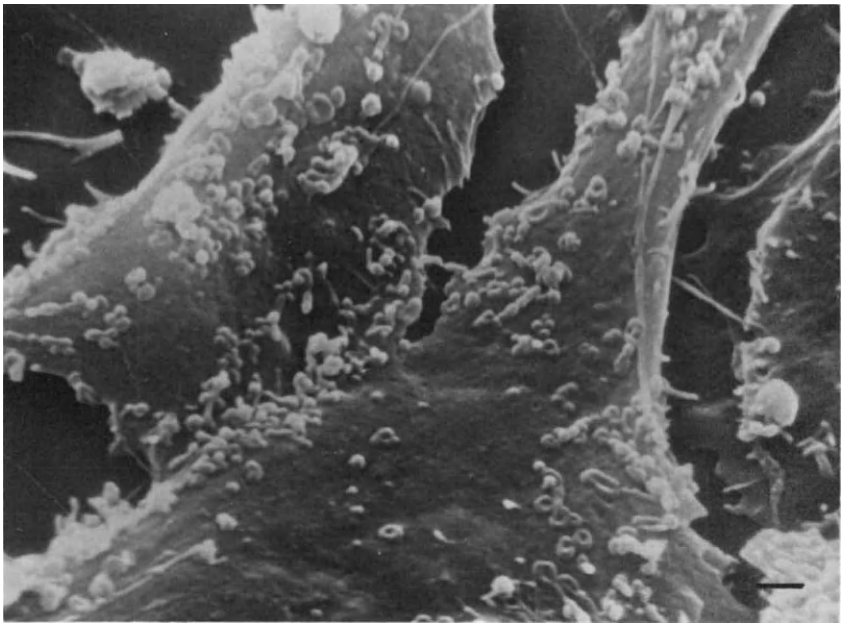

Fig. 19. Scanning electron micrograph of cell culture contaminated with mycoplasma. The pleomorphic contaminant can be seen on the cell surface in several forms, including rings, spheres, and chains. Scale, 1 μm.

glutaraldehyde in phosphate buffer, followed by 1% osmium tetroxide in phosphate buffer, dehydrated either with acetone or alcohol, and embedded in epoxy resin, either by standard EM histological procedures or by the rapid embedding method (Section IV,B).

C. Scanning Electron Microscope Methods

Cultures to be checked by scanning electron microscopy (SEM) for mycoplasma contamination are grown on glass cover slips, preferably for several days, to allow the mycoplasma (if present) to become well established. They are then processed by the following method: cultures are fixed at 4°C in 2.5% glutaraldehyde in 0.1 *M* cacodylate buffer (pH 7.2) for at least 18 hours. They are then rinsed in three changes of 0.1 *M* cacodylate buffer, and immersed in 1% osmium tetroxide in 0.1 *M* cacodylate buffer (pH 7.2) for 30 minutes at room temperature. Fixed cultures are then rinsed in cacodylate buffer, passed through 5-minute changes of 50, 70, and 95% alcohol, three changes of absolute alcohol, then quickly transferred to a critical point dryer, where they are dried by passing

through the critical point of CO_2. In preparation for SEM examination, dried cover slips are mounted on SEM stubs by means of silver paint, and coated with gold-palladium.

VII. IMMUNOELECTRON MICROSCOPY

A. Introduction

Immunoelectron microscopy (IEM) is the direct visualization by electron microscopy of an antigen–antibody complex, and was first reported in 1941 by Anderson and Stanley and by von Ardenne *et al.* Its usefulness in the differentiation of viral antigens owes much to the work of Almeida and her colleagues who recognized the diagnostic potential of the technique as a means of serotyping viruses directly on the EM grid (Almeida *et al.*, 1963; Almeida and Waterson, 1969).

IEM has proved invaluable in the detection and identification of elusive viruses such as rubella virus (Best *et al.*, 1967), coronaviruses (Kapikian *et al.*, 1973), and rhinoviruses (Kapikian *et al.*, 1972a), and of viruses that are difficult or impossible to culture, such as wart viruses (Almeida and Goffe, 1965), hepatitis virus (Bayer *et al.*, 1968; Feinstone *et al.*, 1973), and the reovirus-like particles associated with gastroenteritis (Flewett *et al.*, 1974a). In the serotyping of viruses within a major virus group, IEM has been successfully applied to the differentiation of papovaviruses (Gardner *et al.*, 1971; Penney *et al.*, 1972; Penney and Narayan, 1973), enteroviruses (Anderson and Doane, 1973), myxoviruses and paramyxoviruses (Kelen and McLeod, 1974), and adenoviruses (Luton, 1973; Vassall and Ray, 1974; Edwards *et al.*, 1975). For a more detailed survey of the several applications of IEM, the reader is directed to reviews by Almeida and Waterson (1969), and Doane (1974).

B. Materials

1. Viruses in Clinical Specimens

Viruses in clinical specimens can be detected by IEM by mixing with homologous antibody—in standard typing serum or in the patient's own convalescent serum (Fig. 20). This approach has been valuable in demonstrating viruses that are present in specimens at too low a concentration to be detected by direct EM examination (Paver *et al.*, 1973). No preliminary clarification or concentration of the specimen is needed for IEM if the virus is present at detectable levels. In general, however, a clearer

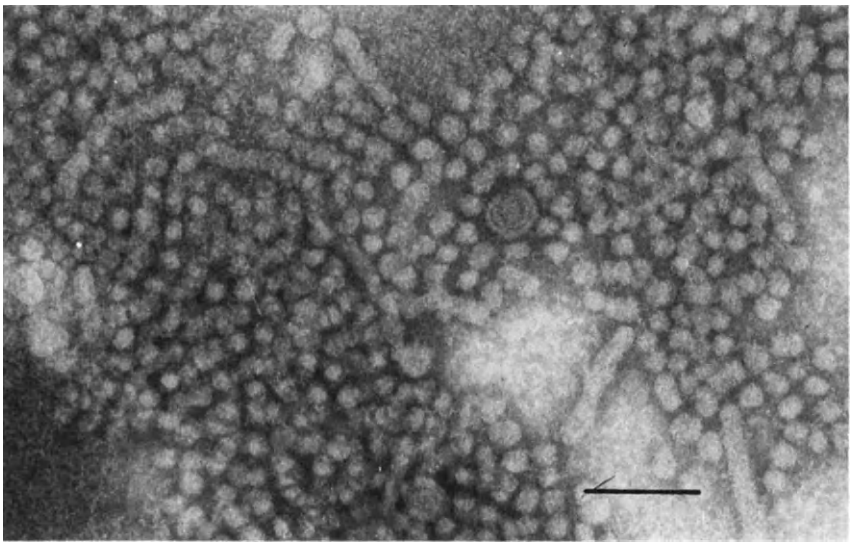

Fig. 20. Preparation of serum from chronic hepatitis B antigen carrier, incubated for 1 hour with rabbit anti-HB_s serum prior to negative staining. Note various morphological forms of antigen. Scale, 100 nm. (Courtesy of M. Fauvel.)

preparation is obtained if a suspension of the specimen is clarified of bacteria and debris by centrifugation for 30 minutes at approximately 8000 *g* prior to IEM and negative staining.

Greater sensitivity is obtained by more time-consuming methods such as ultracentrifugation or density gradient centrifugation (Flewett *et al.*, 1974b).

Devine and Lee (1975) detected poliovirus particles in stool at a concentration of 10^4/ml when they combined IEM with virus concentration by the polyelectrolyte PE60. This compared with a limit of EM detection of 10^6 when PE60 was used alone.

2. *Viruses in Cell Cultures*

Crude infected cell lysates can be used in identifying viral isolates by IEM; no concentration or purification of virus is necessary (Fig. 21). Sensitivity tests by Valters *et al.* (1975) on IEM typing of adenovirus showed that as few as 16 to 32 $TCID_{50}$ per 0.5 ml could be detected. Anderson and Doane (1973) were able to serotype as few as $10^{3.5}$ $TCID_{50}$ per milliliter of poliovirus from infected culture lysate, representing a detection sensitivity of approximately 100-fold over that required for EM detection in the absence of antibody.

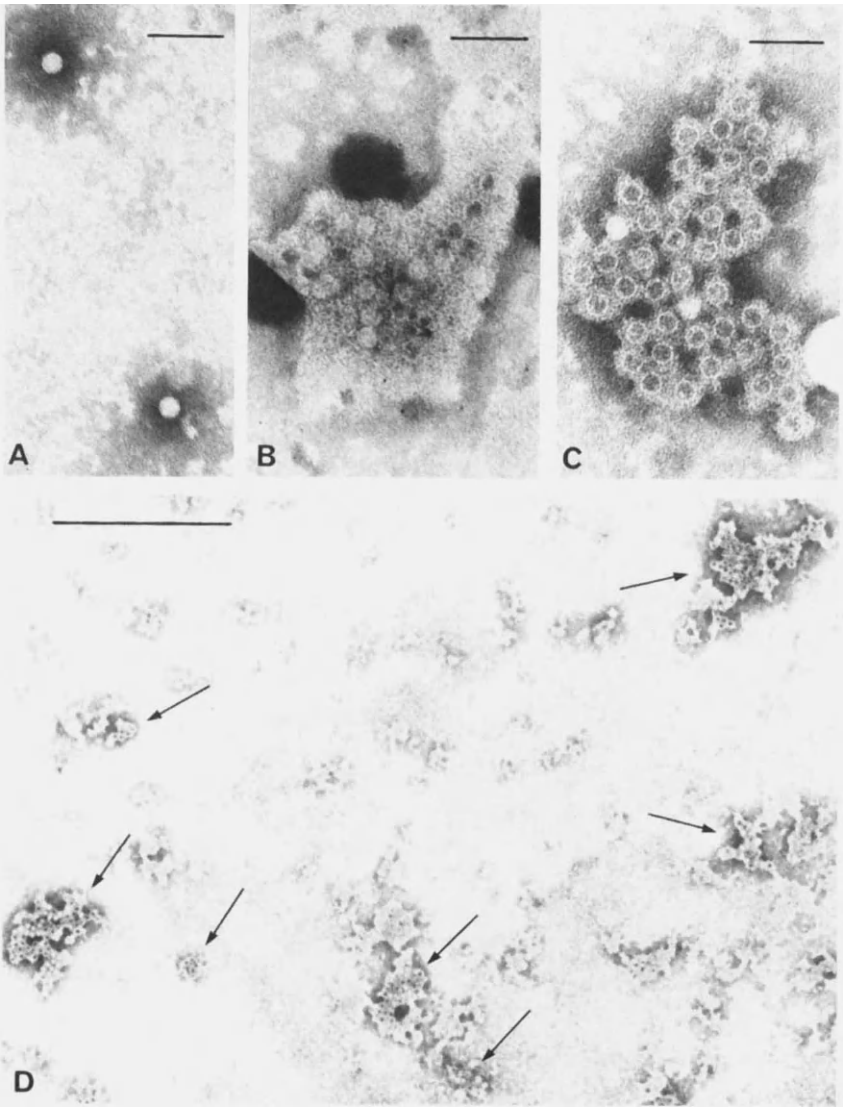

Fig. 21. Serotyping of enteroviruses by immunoelectron microscopy. (A) Two negatively stained coxsackie viruses in crude culture harvest exposed to heterologous antiserum in SIA typing test. (B) Echo 9 virus preparation mixed with $\frac{1}{50}$ dilution of homologous antiserum. (C) Echo 9 virus preparation mixed with a higher dilution of homologous antiserum. (D) Low magnification of coxsackieviruses in presence of homologous antiserum to show abundance of immune complexes (arrows) obtained by SIA typing test. Scale (A), (B), and (C) 100 nm; (D) 1 μm. [Reproduced by permission of the National Research Council of Canada from Anderson and Doane, *Can. J. Microbiol.* **19**, 585–589 (1973).]

3. *Antisera*

Whether working with standard viral antisera or patients' convalescent sera, unfractionated serum is satisfactory for most IEM tests in diagnostic virology. Where problems are encountered with cloudy serum, or where fine resolution of the immune complex is required, the serum should be clarified by centrifugation at 40,000 rpm for 1 hour (Almeida and Waterson, 1969). Even greater definition can be achieved by working with the globulin fraction (Lafferty and Oertelis, 1963; Bayer and Mannweiler, 1963; Mandel, 1971). Almeida and Waterson (1969) recommend working with heat-inactivated sera to avoid complications arising from the presence of complement.

In serotyping enteroviruses and adenoviruses, antisera have been used either individually or in pools, with similar results (Anderson and Doane, 1973; Luton, 1973; Vassall and Ray, 1974). The concentration of antibody used, relative to the concentration of virus, may affect the specificity of the test. Enteroviruses can be distinctly differentiated by IEM when typing sera are used diluted (generally at $\frac{1}{50}$ or greater) (Anderson and Doane, 1973) but cross-reactions occur when sera are used undiluted, or at very low dilutions (Doane, 1974). Similar problems of cross-reactivity with myxoviruses have been reported by Kelen and McLeod (1974).

Antiserum concentration also affects the appearance of the immune complex. When there is little antibody present in relation to viral antigen, aggregates are small, consisting of only a few clearly outlined virus particles. As the concentration of antibody increases, aggregates become larger and more numerous, the interconnecting antibody layers become thicker, and the surface details of the virus particles become more obscure (Fig. 21). At high concentrations of antibody, individual virus particles are surrounded by a halo of antibody, and the incidence of aggregates is greatly reduced.

C. Methods

1. *Direct Method*

The most commonly used method for serotyping by IEM is the direct mixing of virus and serum, recommended by Almeida and Waterson (1969). The viral antigen is reacted at 37°C for 1 hour with an equal volume of antiserum, then left overnight at 4°C. The following morning the mixture is spun at 10,000 rpm for $\frac{1}{2}$ hour to sediment immune complexes. For smaller viruses, e.g., picornaviruses, centrifugation of at least 15,000 rpm is recommended, whereas vaccinia–antibody complexes will sediment using a clinical-type bench centrifuge. The resultant pellet is usually

resuspended in a small volume of distilled water prior to negative staining and examination in the electron microscope. Some workers have shortened the entire procedure to 1 hour incubation at 37°C (Lutton, 1973; Vassal and Ray, 1974; Doane, 1974).

2. *Indirect Method*

Edwards *et al.* (1975) and Valters *et al.* (1975) described a more sensitive indirect IEM method, whereby the virus–antibody complexes were further aggregated by anti-immune globulin sera (anti-IgG). When applied to serotyping of adenoviruses, it was from 4 to over 32 times more sensitive than the direct method, indicating its value not only in differentiation of viruses, but also as a sensitive method for revealing new viruses (e.g., when used with patients' serum).

The indirect method, as used by Valters *et al.* (1975) for detecting adenovirus type 7 in throat swab specimens (and which identified 19 out of 25 adenovirus-positive specimens), is as follows: ½ ml of diluted adenovirus antiserum was mixed with an equal volume of throat swab fluid and incubated for 1 hour at 37°C and then for 3 hours at 6°C. The mixture was centrifuged at 12,000 *g* for 30 minutes, and the pellet was suspended in 0.5 ml of phosphate-buffered saline. An equal volume of anti-IgG serum, diluted to the optimal concentration (as determined by a chess board titration of adenovirus type 7 antiserum and anti-IgG) was added, and this mixture was incubated at 6°C for 16 to 20 hours. The mixture was then centrifuged at 12,000 *g* for 30 minutes, the pellet was suspended in 0.1 ml of distilled water, negatively stained, and examined by EM.

3. *Agar Gel Diffusion Filtration (ADF) Method*

Kelen *et al.* (1971; Kelen and McLeod, 1974) have described a practical microtechnique for demonstrating hepatitis B antigen–antibody complexes and for serotyping myxoviruses and paramyxoviruses. Virus and antiserum are incubated together at 37°C for ½ hour. A microdrop of the mixture is then deposited on the surface of a standard microscope slide that has previously been covered with 5 ml of 0.8% agar. Immediately thereafter, a Formvar-carbon coated grid is placed upside down and left floating on top of the drop. When diffusion of the fluid phase into the agar is complete (a matter of a few minutes), the grid is removed for negative staining and EM examination. Specimens prepared by the ADF method are both partially purified and concentrated, the agar acting as a molecular sieve and effectively removing interfering salts, macromolecules, and tissue debris of 15 nm in diameter, while retaining the virus particles on the surface.

4. Serum-in-Agar (SIA) Method

A further modification of the ADF method has been developed by Anderson and Doane (1973) for serotyping of enteroviruses by IEM. In this method, the antisera are incorporated in the agar itself.

Dilutions of single or pooled typing sera are added to a cooled (56°C) molten solution of 1% aqueous agar, which is then pipetted into the cups of disposable microtiter plates. Once the agar has solidified at room temperature, Formvar-carbon coated grids are placed on the agar surface. Viral specimens to be typed by IEM are added to the grids in volumes of 1 to 2 microdrops, and allowed to air-dry (approximately 30 minutes). Preparations are then negatively stained and examined by electron microscopy.

The SIA method is rapid and simple to perform, and requires only small quantities of virus and serum. Although the antisera are contained within the agar itself, homologous antibodies apparently diffuse rapidly into the viral specimen, resulting in the formation of immune complexes. The sensitivity and specificity of this method for enterovirus typing has been found to be comparable to that obtained using the direct method. From a practical point of view, however, the SIA method is more useful in that cups can be stored at 4°C for several months prior to use, and cups containing different types of antisera can be color-coded for reference.

VIII. SAFETY PRECAUTIONS

All too often, little attention is paid to safety precautions that should be taken while examining pathogenic viruses. A report on "Classification of Etiologic Agents on the Basis of Hazard," compiled by the United States Department of Health, Education and Welfare (1974), lists the following viruses as Class 4 agents, viz., "that require the most stringent conditions for their containment because they are extremely hazardous to laboratory personnel or may cause serious epidemic disease":

Alastrim, smallpox, monkey pox, and whitepox, when used for transmission or animal inoculation experiments

Hemorrhagic fever agents, including Crimean hemorrhagic fever (Congo), Junin, and Machupo viruses, and others as yet undefined

Herpesvirus simiae (Monkey B virus)

Lassa virus

Marburg virus

Tick-borne encephalitis virus complex, including Russian spring-

summer encephalitis, Kyasanur forest disease, Omsk hemorrhagic fever, and Central European encephalitis viruses

Venezuelan equine encephalitis virus, epidemic strains, when used for transmission or animal inoculation experiments

Yellow fever virus—wild, when used for transmission or animal inoculation experiments

A similar report on "Laboratory Use of Dangerous Pathogens" has been published by the Department of Health and Social Security of Great Britain (1975). Microscopists working with any of these pathogens are advised to avail themselves of these reports.

Maximum precautions should be taken when preparing specimen grids for electron microscopy. The production of aerosols, through operations such as grinding or sonication, should be avoided, and all stages of specimen preparation should be carried out in a safety cabinet. Potentially hazardous specimens, such as those from suspected smallpox, should be irradiated by ultraviolet light prior to EM examination. There is a paucity of information in the published literature concerning optimum conditions necessary for inactivation of viral infectivity. Personnel working with these specimens should be vaccinated against smallpox at least every 3 years. In the event of smallpox virus being detected in specimens, all associated personnel should immediately be revaccinated.

Because of the highly infectious nature of hepatitis B antigen, serum suspected of containing this agent should be treated with β-propiolactone prior to EM examination. The method recommended by L. Spence and M. Fauvel (personal communication) is as follows: to 1 ml of serum add 0.1 ml of 3% saline solution of β-propiolactone. Incubate at 37°C for 4 hours. Add 1 drop of 5 *N* NaOH to bring the pH to neutrality.

Forceps used to handle specimen grids should be flamed thoroughly after use, cooled, dipped in alcohol, and flamed again. Grids to be discarded should be placed in specially marked containers and autoclaved immediately, care being taken to ensure that the steam can penetrate the container.

ACKNOWLEDGMENTS

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REFERENCES

Almeida, J. D., and Goffe, A. P. (1965). *Lancet* **2,** 1205–1207.
Almeida, J. D., and Tyrrell, D. A. J. (1967). *J. Gen. Virol.* **1,** 175–178.
Almeida, J. D., and Waterson, A. P. (1969). *Adv. Virus Res.* **15,** 307–338.
Almeida, J. D., Howatson, A. F., and Williams, M. G. (1962). *Virology* **16,** 353–354.
Almeida, J. D., Cinader, B., and Howatson, A. F. (1963). *J. Exp. Med.* **118,** 327–340.
Alsop, J., Flewett, T. H., and Foster, J. R. (1960). *Br. Med. J.* **2,** 1708–1711.
Anderson, N., and Doane, F. W. (1972a). *Can. J. Microbiol.* **18,** 299–304.
Anderson, N., and Doane, F. W. (1972b). *Appl. Microbiol.* **24,** 495–496.
Anderson, N., and Doane, F. W. (1973). *Can. J. Microbiol.* **19,** 585–589.
Anderson, T. F., and Stanley, W. M. (1941). *J. Biol. Chem.* **139,** 339–344.
Auger, W. J. (1939). *J. Pediatr.* **15,** 640–645.
Banatvala, J. E., Chrystie, I. L., and Flower, A. J. E. (1975). *Lancet* **2,** 79–80.
Bayer, M. E., and Mannweiler, E. (1963). *Arch. Gesamte Virusforsch.* **13,** 541–547.
Bayer, M. E., Blumberg, B. S., and Werner, B. (1968). *Nature (London)* **218,** 1057–1059.
Best, J. M., Banatvala, J. E., Almeida, J. D., and Waterson, A. P. (1967). *Lancet* **2,** 237–239.
Bishop, R. F., Davidson, G. P., Holmes, I. H., and Ruck, B. J. (1973). *Lancet* **2,** 1281–1283.
Blank, H., Davis, C., and Collins, C. (1970). *Br. J. Dermatol.* **83,** 69–80.
Bouteille, M., Fontaine, C., Vedrenne, C., and Delarue, J. (1965). *Rev. Neurol.* **118,** 454–458.
Brown, S., Teplitz, M., and Revel, J. P. (1974). *Proc. Natl. Acad. Sci. U.S.A.* **71,** 464–468.
Burns, W. A., Zimmerman, H. J., Hammond, J., Howatson, A., Katz, A., and White, J. (1975). *Hum. Pathol.* **6,** 467–478.
Caul, E. O., Paver, W. K., and Clarke, S. K. R. (1975). *Lancet* **1,** 1192.
Cruickshank, J. G., Bedson, H. S., and Watson, D. H. (1966). *Lancet* **2,** 527–530.
de Harven, E. (1973). *In* "Contamination in Tissue Culture" (J. Fogh, ed.), pp. 205–231. Academic Press, New York.
Department of Health and Social Security of Great Britain. (1975). "Laboratory Use of Dangerous Pathogens." H. M. Stationery Office, London.
Deutsch, G. F., and Spence, L. (1972). *Lancet* **1,** 447.
Devine, R. D., and Lee, V. P. (1975). *Can. J. Public Health* **66,** 43.
Doane, F. W. (1974). *In* "Viral Immunodiagnosis" (E. Kurstak and R. Morisset, eds.), pp. 237–255. Academic Press, New York.
Doane, F. W., and Anderson, N. (1972). *Can. J. Public Health* **63,** 83.
Doane, F. W., and Anderson, N. (1975). *Proc. Microsc. Soc. Can.* **2,** 58–59.
Doane, F. W., Anderson, N., Chatiyanonda, K., Bannatyne, R. M., and McLean, D. M. (1967). *Lancet* **2,** 751–753.

Doane, F. W., Anderson, N., Zbitnew, A., and Rhodes, A. J. (1969). *Can. Med. Assoc. J.* **100,** 1043–1049.

Doane, F. W., Anderson, N., Chao, J., and Noonan, A. (1974). *Appl. Microbiol.* **27,** 407–410.

Dolman, C. L., Furesz, J., and MacKay, B. (1967). *Can. Med. Assoc. J.* **97,** 8–12.

Edwards, E. A., Valters, W. A., Boehm, L. G., and Rosenbaum, M. J. (1975). *J. Immunol. Methods* **8,** 159–167.

Evans, A. S., and Melnick, J. L. (1949). *Proc. Soc. Exp. Biol. Med.* **71,** 283–286.

Feinstone, S. M., Kapikian, A. Z., and Purcell, R. H. (1973). *Science* **182,** 1026–1028.

Flewett, T. H., Bryden, A. S., and Davies, H. (1973). *Lancet* **2,** 1497.

Flewett, T. H., Bryden, A. S., Davies, H., Wood, G. N., Bridger, J. C., and Derrick, J. M. (1974a). *Lancet* **2,** 61–63.

Flewett, T. H., Bryden, A. S., and Davies, H. (1974b). *J. Clin. Pathol.* **27,** 603–608.

Flewett, T. H., Davies, H., Bryden, A. S., and Robertson, M. J. (1974c). *J. Clin. Pathol.* **27,** 608–614.

Fong, C. K. Y., Gross, P. A., Hsiung, G. D., and Swack, N. S. (1975). *J. Clin. Microbiol.* **1,** 219–224.

Gardner, S. D., Field, A. M., Coleman, D. V., and Hulme, B. (1971). *Lancet* **1,** 1253–1257.

Gibbs, E. P. J., Johnson, R. H., and Voyle, C. A. (1970). *J. Comp. Pathol.* **80,** 455–464.

Harland, W. A., Adams, J. H., and McSeveney, D. (1967). *Lancet* **2,** 581–582.

Howatson, A. F., Nagai, M., and Zu Rhein, G. M. (1965). *Can Med. Assoc. J.* **93,** 379–386.

Hsiung, G. D. (1968). *Bacteriol. Rev.* **32,** 185–205.

Hsiung, G. D., and Atoynatan, T. (1966). *Am. J. Epidemiol.* **83,** 38–47.

Hull, R. N. (1968). *Virol. Monogr.* **2,** 1–66.

Joncas, J. H., Berthiaume, L., Williams, R., Beaudry, P., and Pavilanis, V. (1969). *Lancet* **1,** 956–959.

Kapikian, A. Z., Almeida, J. D., and Stott, E. J. (1972a). *J. Virol.* **10,** 142–146.

Kapikian, A. Z., Wyatt, R. G., Dolin, R., Thornhill, T. S., Kalica, A. R., and Chanock, R. M. (1972b). *J. Virol.* **10,** 1075–1081.

Kapikian, A. Z., James, H. D., Kelly, S. J., and Vaughn, A. L. (1973). *Infect. Immun.* **7,** 111–116.

Kelen, A. E., and McLeod, D. A. (1974). *In* "Viral Immunodiagnosis" (E. Kurstak and R. Morisset, eds.), pp. 257–275. Academic Press, New York.

Kelen, A. E., Hathaway, A. E., and McLeod, D. A. (1971). *Can. J. Microbiol.* **17,** 993–1000.

Lafferty, K. J., and Oertelis, S. (1963). *Virology* **21,** 91–99.

Lipman, M., Andrews, L., Niederman, J., and Miller, G. (1975). *J. Infect. Dis.* **132,** 520–523.

Long, G. W. (1970). *Appl. Microbiol.* **20,** 497–504.

Luton, P. (1973). *J. Clin. Pathol.* **26,** 914–917.

McFerran, J. B., Clarke, J. K., and Curran, W. L. (1971). *Res. Vet. Sci.* **12,** 253–257.

Macrae, A. D., Field, A. M., McDonald, J. R., Meurisse, E. V., and Porter, A. A. (1969). *Lancet* **2,** 313–316.

Madeley, C. R., and Cosgrove, B. P. (1975). *Lancet* **2,** 451–452.

Mandel, B. (1971). *In* "Methods in Virology" (K. Maramorosch and H. Koprowski, eds.), Vol. 5, pp. 375–397. Academic Press, New York.

Marsolais, G., Berthiaume, L., DiFranco, E., and Marois, P. (1971). *Can. J. Comp. Med.* **35,** 285–288.

Mathan, M., Mathan, V. I., Swaminathan, S. P., Yesudoss, S., and Baker, S. J. (1975). *Lancet* **1,** 1068–1069.

Middleton, P. J., Szymanski, M. T., Abbott, G. D., Bortolussi, R., and Hamilton, J. R. (1974). *Lancet* **1,** 1241–1244.

Montplaisir, S., Belloncik, S., Leduc, N. P., Onji, P. A., Martineau, B., and Kurstak, E. (1972). *J. Infect. Dis.* **125,** 533–538.

Morecki, R., and Becker, N. H. (1968). *Arch. Pathol.* **86,** 292–296.

Nagington, J. (1964). *Br. Med. J.* **2,** 1499–1500.

Nagler, F. P. O., and Rake, G. (1948). *J. Bacteriol.* **55,** 45–51.

Palmer, E. L., Martin, M. L., and Gary, G. W. (1975). *Virology* **65,** 260–265.

Paver, W. K., Ashley, C. R., Caul, E. O., and Clarke, S. K. R. (1973). *Lancet* **1,** 237–240.

Pavilanis, V., Joncas, J. H., Skvorc, R., Berthiaume, L., Blanc, M. F., Martineau, B., and Podoski, M. O. (1971). *Union Med. Can.* **511,** 2195–2202.

Penney, J. B., and Narayan, O. (1973). *Infect. Immun.* **8,** 299–300.

Penney, J. B., Weiner, L. P., Herndon, R. M., Narayan, O., and Johnson, R. T. (1972). *Science* **178,** 60–62.

Pennington, T. H., Follett, E. A. C., and Timbury, M. C. (1975). *Lancet* **2,** 182.

Peters, D., Nielsen, G., and Bayer, M. E. (1962). *Dtsch. Med. Wochenschr.* **87,** 2240.

Robinson, C. R., Doane, F. W., and Rhodes, A. J. (1958). *Can. Med. Assoc. J.* **79,** 615–621.

Rowden, G., and Lewis, M. G. (1974). *J. Clin. Pathol.* **27,** 505–510.

Smith, K. O., and Melnick, J. L. (1962). *Science* **137,** 543–544.

Spradbrow, P. B., and Francis, J. (1969). *Vet. Rec.* **84,** 244–246.

Strauss, M. J., Shaw, E. W., Bunting, H., and Melnick, J. L. (1949). *Proc. Soc. Exp. Biol. Med.* **72,** 46–50.

Tellez-Nagel, I., and Harter, D. H. (1966). *Science* **154,** 899–901.

U.S. Department of Health, Education and Welfare. (1974). "Classification of Etiologic Agents on the Basis of Hazard." USDHEW, Washington, D.C.

Valters, W. A., Boehm, L. G., Edwards, E. A., and Rosenbaum, M. J. (1975). *J. Clin. Microbiol.* **1,** 472–475.

van Rooyen, C. E., and Scott, G. D. (1948). *Can. J. Public Health* **39,** 467–477.

Vassall, J. H., and Ray, C. G. (1974). *Appl. Microbiol.* **28,** 623–627.

von Ardenne, M., Freidrich-Freska, H., and Schram, G. (1941). *Arch. Gesamte Virusforsch.* **2,** 80–86.

Williams, M. G., Howatson, A. F., and Almeida, J. D. (1961). *Nature (London)* **189,** 895–897.

Wolanski, B., and Maramorosch, K. (1970). *Virology* **42,** 319–327.

Zu Rhein, G. M., and Chou, S.-M. (1965). *Science* **148,** 1477–1479.

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