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Proteolytic Processing of Animal Virus Proteins

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Investigations of the proteins synthesized in response to animal viruses have revealed reliance to various extents on the process of proteolytic cleavage for maturation and control of function of the viral proteins. The role of protein cleavage in bacteriophage proteins has been reviewed (*Hershko* and *Fry*, 1975). The major emphasis of this critical review will be on the protein processing of the members of the picornavirus group, where proteases are used for everything from release of the nascent chains from the polysomes to maturation of the virions. An extensive literature has been accumulated in this area which has revealed interesting new insights into the details of these processes. Proteolytic processing also plays a large role in the biosynthesis of the togaviruses and the RNA tumor viruses and each is discussed in turn.

I. The Cleavage Scheme of the Picornaviruses

A. History

There are several reviews dealing with the molecular biology of picornavirus replication and the role of proteolytic cleavages in the formation of the viral proteins (*Baltimore*, 1969; *Rueckert*, 1971; *Sugiyama* et al., 1972; *Shatkin*, 1974; *Hershko* and *Fry*, 1975; *Korant*, 1975; *Butterworth* et al., 1976a; *Rekosh*, 1976).

Studies comparing the cleavage pattern of several picornaviruses show the patterns to be remarkably similar. In general, principles learned in one system seem to apply to the other members of the picornavirus group. Well-studied viruses will be used to illustrate most points. However, where there are interesting differences they will be pointed out.

Two developments facilitated the detailed study of the synthesis and cleavage of picornavirus proteins. The first was the discovery that these viruses inhibited host protein synthesis, so that under the appropriate conditions incubation of infected cells with radioactive amino acids resulted in the specific labeling of only the viral-coded proteins (Fig. 1). The second was the development of high-resolution sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) which provided a straightforward method of separating the polypeptides on the basis of molecular weight.

In 1965 *Summers* et al. published a profile of poliovirus-specific polypeptides obtained from the cytoplasm of infected cells (*Summer* et al., 1965). The pattern contained both capsid and non-capsid polypeptides and there were hints of proteolytic cleavage of some of the larger polypeptides. It was later shown using

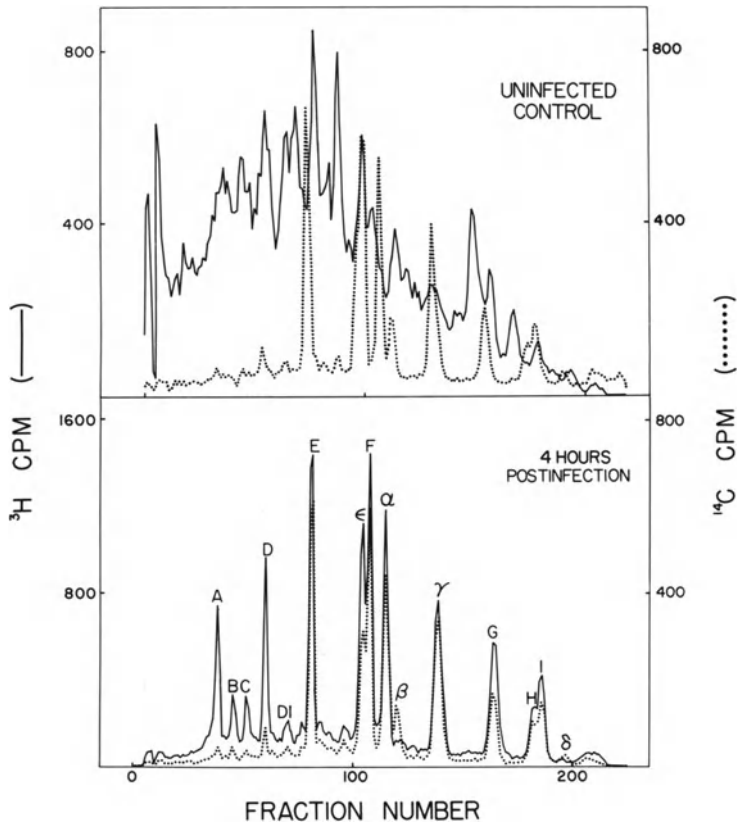


Fig. 1. SDS-PAGE profiles of proteins synthesized in uninfected HeLa cells (top panel) and 4 h after infection with EMC virus (bottom panel) (*Butterworth* et al., 1971). This dramatically illustrates the virus' ability to redirect cellular protein synthesis. Dotted line represents the known stable viral polypeptides which were coelectrophoresed as a marker. Direction of migration is from left to right

pulse-chase experiments (Fig. 2) that some of the large polioviral polypeptides were, in fact, precursor molecules that underwent a series of proteolytic cleavages to generate the smaller polypeptides, and the basic outlines of the precursor-product relationships were established (*Summers* and *Maizel,* 1968; *Maizel* and *Summers*, 1968; *Jacobson* and *Baltimore*, 1968; *Jacobson* et al., 1970; *Summers* et al., 1971).

Subsequent work with encephalomyocarditis (EMC) virus showed it to be more amenable to study than poliovirus. The EMC viral protein profile was simpler, without so many secondary cleavages and there were no apparent overlapping peaks. Typical profiles for the virus-specific proteins of the three most well-studied viruses, EMC virus, human rhinovirus-1A (HRV-1A), and poliovirus are shown in Figure 3. Based on kinetics of synthesis and cleavage, CNBr mapping, and molecular weights, a cleavage scheme was obtained for EMC virus which was analogous to the one determined for poliovirus (Fig. 4) (*Butterworth* et al., 1971).

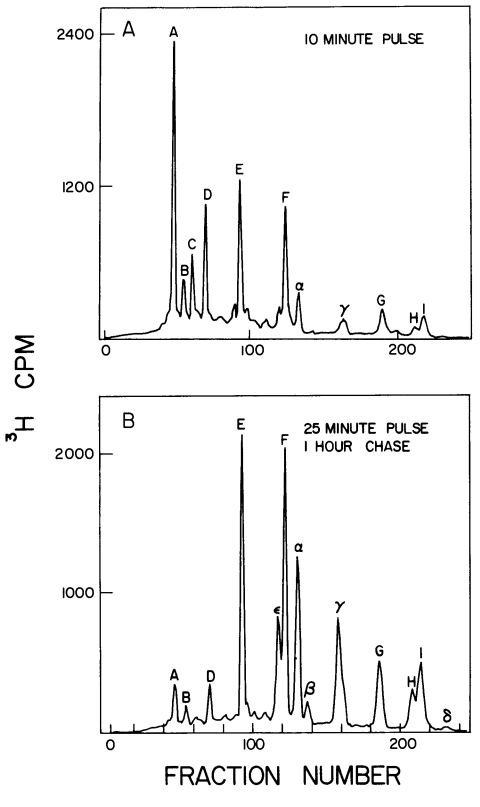


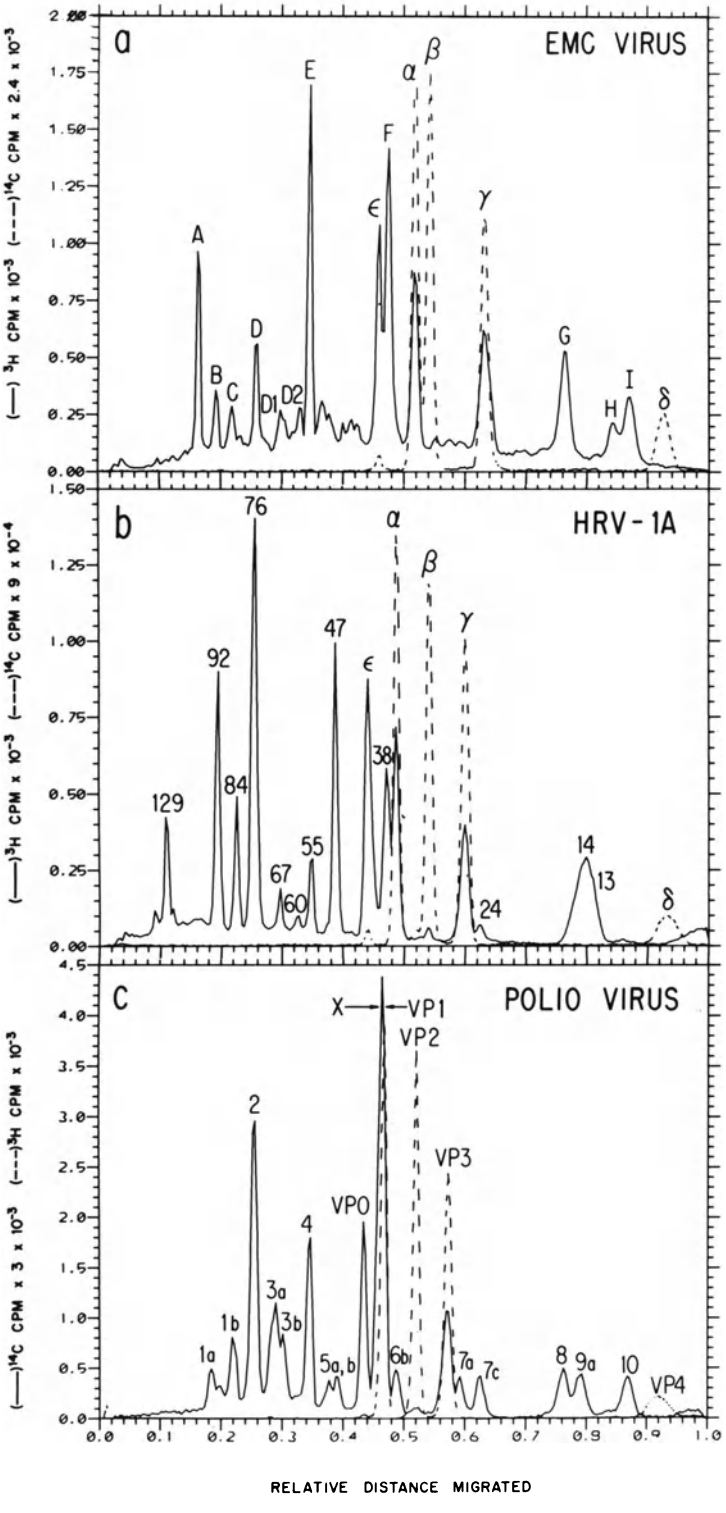
Fig. 2. Flow of radioactivity in a pulse-chase experiment (*Butterworth* et al., 1971). During a brief exposure of EMC virus-infected cells to ^{3}H-amino acids (pulse), label is incorporated primarily into large precursor molecules (panel *A*). If these labeled cells are then incubated in the absence of ^{3}H-amino acids (chase), the pattern changes as precursors cleave to generate the smaller stable products (panel *B*)

Accumulated evidence suggested that the picornaviral mRNA had only a single initiation site (*Jacobson* and *Baltimore*, 1968). This provided the basis for a unique technique to map the polypeptide gene loci on the viral RNA using the drug pactamycin (*Taber* et al., 1971). Newly developed quantitative techniques allowed more detailed studies of the cleavage kinetics and of pactamycin mapping which rely on quantitation for meaningful results (*Butterworth* and *Rueckert*, 1972b).

B. Nomenclature

Unfortunately, a systematic nomenclature has not been agreed to and not only has each virus acquired its own nomenclature but newly discovered peaks have been independently named with new letters and subscripts. The preferred nomenclature and identification of analogous polypeptides for EMC virus, HRV-1A, and poliovirus, are shown in Figures 3 and 4.

Fig. 3a–c. Comparison of the SDS-PAGE profiles of ^{3}H-labeled virus-specific polypeptides of (a) EMC virus, (b) HRV-1A, and (c) Poliovirus (*Butterworth*, 1973). Dashed line represents proteins from the purified ^{14}C-labeled virions, which were coelectrophoresed to identify the virion polypeptides in each pattern


a
EMC VIRUS
A
B
C
D
D1
D2
E
F
ϵ
α
β
γ
G
H
I
δ
(—) ³H CPM x 10⁻³ (---)¹⁴C CPM x 2.4 x 10⁻³
b
HRV-1A
129
92
84
76
67
60
55
47
ϵ
α
β
γ
38
24
14
13
δ
(—)³H CPM x 10⁻³ (---)¹⁴C CPM x 9 x 10⁻⁴
C
POLIO VIRUS
X
VP1
VP2
VP3
VPO
VP4
1a
1b
2
3a
3b
4
5a, b
6b
7a
7c
8
9a
10
(—)¹⁴C CPM x 3 x 10⁻³ (---)³H CPM x 10⁻³
RELATIVE DISTANCE MIGRATED

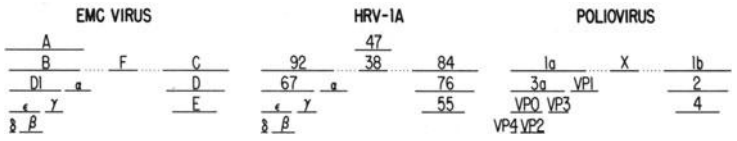


Fig. 4. Common features in the biosynthesis of virus-specific polypeptides of EMC virus, HRV-1A, and poliovirus (*Butterworth,* 1973). Based on molecular weight and position on the genetic map, above portions of the patterns of viral protein biosynthesis were the same for these three viruses. Analogous polypeptides are shown in the same relative positions. Lateral position represents relative location of the corresponding gene locus on the viral RNA (the 5' end of the RNA is to the left). Vertical position represents precursor-product relationships or alternative cleavage forms. Line lengths are proportional to molecular weight

As new viruses are studied it is suggested that the virion polypeptides be named with the prefix VP and the noncapsid polypeptides be numbered according to their apparent molecular weight (in thousands) relative to a standard, as was done for HRV-1A and HRV-2 (*McLean* and *Rueckert*, 1973). This approach was taken for the RNA tumor viruses (*August* et al., 1974). Because of differences in SDS-PAGE techniques and markers chosen there exists an uncomfortably large variation in the reported molecular weights of the virus-specific polypeptides, especially for the larger polypeptides (*Butterworth* and *Korant*, 1974; *Swaney* et al., 1974). As a point of reference the molecular weights of EMC, HRV-1A, and polioviral proteins *relative to each other* have been established (*Butterworth*, 1973).

C. The Pattern of Polypeptide Synthesis and Cleavage

The single-stranded picornaviral RNA genome of molecular weight $2.6–2.8 \times 10^6$ daltons that is found in the virion serves as the mRNA to direct the synthesis of the viral proteins (*Shatkin*, 1974). A detailed comparison of the relative molecular weights and positions on the genetic map of the polypeptides synthesized by the antigenically different viruses, EMC virus, HRV-1A, and poliovirus, revealed that in each case there was a capsid precursor nearest the 5' end of the RNA followed by a stable primary product, then a family of polypeptides analogous to the EMC viral polypeptides C, D, and E (Fig. 4) (*Butterworth*, 1973; *McLean* and *Rueckert*, 1973). This suggests something fundamental about this pattern of biosynthesis because it has remained unchanged while evolutionary pressures have resulted in extensive changes in the antigenicity of the virions and the degree of homology among the RNAs (*Young* et al., 1968; *Dietzschold* et al., 1971; *Yin* et al., 1973).

It is not known if the genes for the three polypeptide families are contiguous on the viral RNA and there are a considerable number of polypeptides which have not been placed in the over-all cleavage scheme. The poliovirus pattern has many more polypeptides of a size below 27000, indicating considerable secondary cleavages that are not seen in EMC virus or HRV-1A. However, tryptic mapping does indicate that polypeptides G, H, and I in the EMC viral pattern may be mixtures of different polypeptides of similar molecular weight (*Dobos* and *Plourde*, 1973).

D. Uniformity of Protein Processing in the Picornaviruses

There have been numerous additional reports of analogous proteolytic processing for members of each of the four major picornavirus subgroups. Several coxsackievirus types, which like poliovirus are members of the enterovirus subgroup, have been studied (*Holland* and *Kiehn*, 1968; *Kiehn* and *Holland*, 1970). There is one report that up to 34 distinct polioviral polypeptides could be resolved, many of which had not been described previously (*Abraham* and *Cooper*, 1975a). However, careful comparison of this pattern with others shows the major peaks to be the same (*Butterworth*, 1973) e.g. p110 = 1a, p90 = 1b, p79 = 2, p29 = 6b. The minor polypeptides present may represent a low level of secondary cleavages or residual synthesis of host proteins. Tryptic mapping of the polypeptides confirms the relationships presented in Figure 4 (*Abraham* and *Cooper*, 1975b).

In general, additional studies characterizing the viral polypeptides of the cardiovirus subgroup, including EMC virus, mouse Elberfeld (ME) virus, and mengovirus, are consistent with the cleavage scheme presented in Figure 2 (*Ginevskaya* et al., 1972; *Dobos* and *Martin*, 1972; *Dobos* and *Plourde*, 1973; *Esteban* and *Kerr*, 1974; *Lucas-Lenard*, 1974; *Paucha* et al., 1974; *Paucha* and *Colter*, 1975).

Members of the rhinovirus subgroup (many of which are responsible for the common cold) show analogous protein processing to the other picornaviruses (*McLean* and *Rueckert*, 1973).

Studies on foot-and-mouth disease virus (FMDV) indicate that the cleavage scheme will be analogous to those of the other picornaviruses (*Vande Woude* and *Ascione*, 1974; *Black*, 1975).

II. The Translation Process

A. Single Initiation Site

The viral RNA is proposed to possess only a single initiation site so that each ribosome completes translation of the entire mRNA portion of the genome with the polypeptide products being separated by proteolytic cleavage (*Rekosh*, 1976). Primary products are released from the growing polypeptide chain by nascent cleavages. Some of these primary products are further processed by posttranslational or secondary cleavages.

Consistent with the concept of a single initiation site are the results of quantitative analysis experiments which showed that the major primary polypeptides and stable products of EMC virus were produced in equimolar amounts (Table 1) (*Butterworth* et al., 1971; *Butterworth* and *Rueckert*, 1972a). Similar values were also obtained for the polypeptides of HRV-1A and poliovirus (*Butterworth*, 1973). However, there are enough variations and minor polypeptides present in less than equimolar amounts so that multiple initiation sites or premature release of ribosomes cannot be completely ruled out.

There are reports that the major polypeptides of mengovirus may be produced in less than equimolar amounts (*Lucas-Lenard*, 1974; *Paucha* et al., 1974; *Paucha*

Table 1. Size and molar ratios of the major EMC viral polypeptides

Polypeptide	Apparent molecular weight	Molar ratio following short pulse	Molar ratio following chase
A	100000	0.80	0.01
B	90000	0.09	0.01
C	84000	0.29	0.01
D	75000	0.29	0.04
E	56000	0.47	0.72
F	38000	1.00	1.00
α	34000	0.20	1.00
G	16000	0.79	1.06
A + B + α		1.09	1.02
F		1.00	1.00
C + D + E		1.05	0.77

Data are from *Butterworth* et al., 1971 and *Butterworth* and *Rueckert*, 1972a. There are several other reports that indicate that the molecular weight of A may be closer to 110000 (*Esteban* and *Kerr*, 1974; *Lucas-Lenard*, 1974; *Paucha* et al., 1974)

and *Colter*, 1975). This could result from premature termination, selective degradation, or preferential loss from the cells (in each case the cells were washed before lysing). The differences observed are small, so it is doubtful that this represents the operation of a major control mechanism in the relative amounts of the polypeptides synthesized. Indeed, this lack of regulation is puzzling, as it appears that the capsid polypeptides should be needed in substantially larger amounts than the other viral polypeptides.

B. Pactamycin Mapping

The single initiation site scheme with each ribosome translating one copy of each gene provided the basis for a unique mapping system in which quantitative changes in the viral protein profile were measured following inhibition of initiation of protein synthesis by pactamycin. During that brief period when the already initiated ribosomes completed translation, those genes nearer the 5' end of the RNA were translated with less frequency than those nearer the 3' end (Fig. 5). The relative amount of each polypeptide formed provided a criterion for ordering the viral polypeptide genes on the RNA (*Taber* et al., 1971; *Summers* and *Maizel*, 1971; *Butterworth* and *Rueckert*, 1972a, *Rekosh*, 1972; *Butterworth*, 1973). The results confirmed previously established relationships and allowed the placement of several unassigned polypeptides in the cleavage scheme. Furthermore, this technique would not have worked if there had not been a single initiation site, supplying further support for the single initiation site hypothesis.

Other means of ordering the genes such as observing the kinetics of appearance of the polypeptides following a short pulse (*Rekosh*, 1972; *Butterworth* and *Rueckert*, 1972b) or following reversal of inhibition of initiation (*Saborio* et al., 1974) have confirmed the relative gene orders in Figure 4.

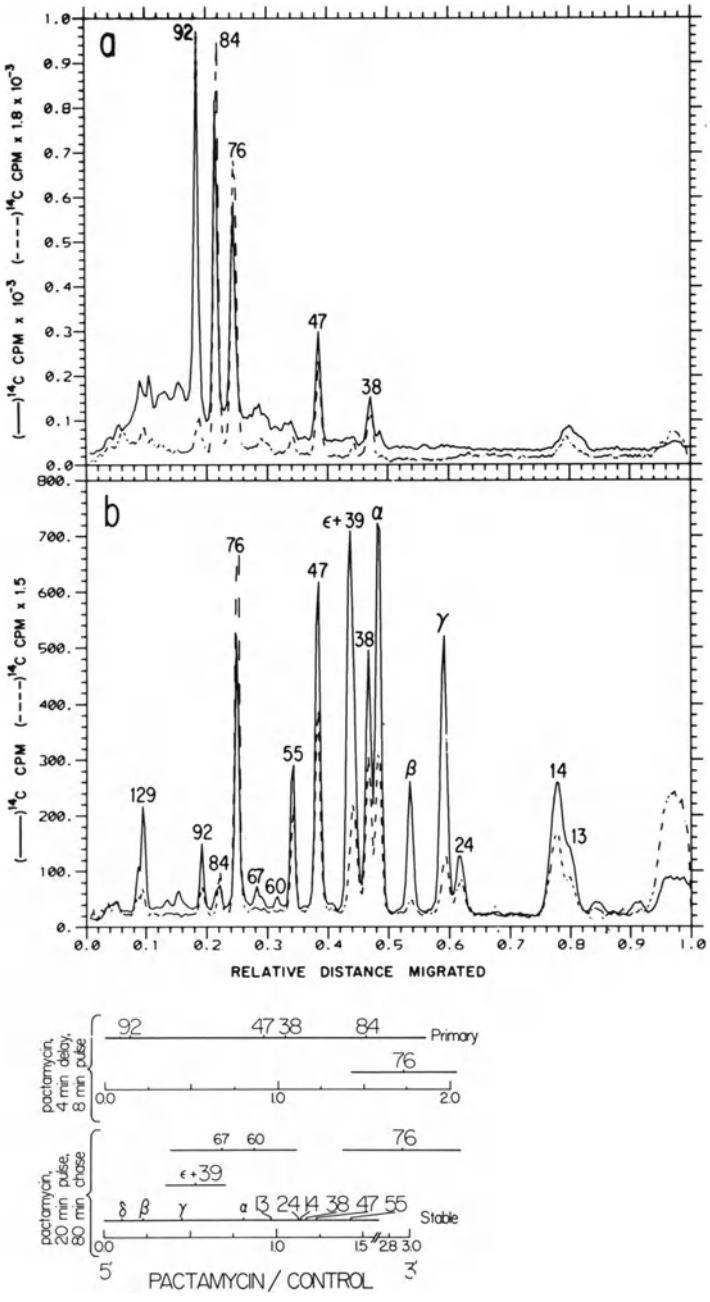


Fig. 5. Effect of pactamycin on the distribution of radioactivity incorporated into virus-specific polypeptides of HRV-1A (*Butterworth,* 1973). Solid line in panel (a), shows normal distribution of label found by pulse labeling HRV-1A-infected HeLa cells. If cells are labeled during that brief period of time following inhibition of initiation of protein synthesis by pactamycin, in which previously initiated ribosomes are running off the RNA, the pattern is changed *(dashed line)*. Those polypeptides translated from nearer the 5' end of the RNA are labeled with less frequency than those translated from nearer the 3' end. Chasing the label into the stable polypeptides allows them also to be ordered [panel (b)]. Pactamycin map derived from these figures is shown below

C. In Vitro Protein Synthesis

Initial work using picornaviral RNA in an in vitro protein synthesizing system showed that EMC viral RNA was far superior to polioviral RNA in stimulating protein synthesis (*Boime* et al., 1971; *Roumiantzeff* et al., 1971; *Egger* and *Shatkin*, 1972; *Boime* and *Leder*, 1972; *Kerr* et al., 1972; *Laskey* et al., 1972; *Kalinina* et al., 1974; *Hunt*, 1976). The in vitro work showed initiation and translation of a substantial part of the genome, but often there was premature termination. Now, various systems have been perfected that synthesize both EMC and polioviral RNA with apparently remarkable fidelity (Fig. 6) (*Esteban* and *Kerr*, 1974; *Lawrence* and *Thach*, 1975; *Villa-Komaroff* et al., 1975). In vivo the largest polypeptides normally observed are the primary products (polypeptides A, F, and C). Products synthesized by the in vitro protein synthesizing systems included the presumed complete translation product (M.W. 250000), cleavage intermediates leading to the primary products, and the primary products themselves. The pro-

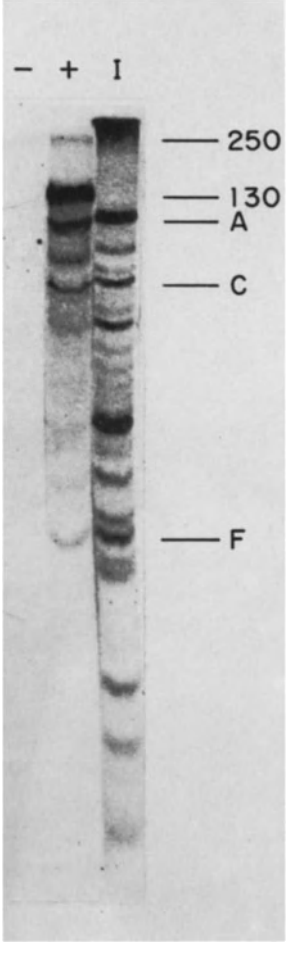


Fig. 6. ^{14}C-polypeptides synthesized in an in vitro protein synthesizing system programmed with EMC viral RNA (*Esteban* and *Kerr*, 1974). SDS-PAGE profiles are from an incubation period of 120 min in the presence (+) or absence (—) of EMC viral RNA. Gel (*I*) shows authentic EMC virus-specific polypeptides labeled in infected cells

duction of primary products in the in vitro protein synthesizing system indicates that those enzymes responsible for performing the nascent cleavages are present and functioning. The extensive secondary and maturation cleavages that occur in vivo are not observed in the in vitro systems.

D. The Initiation Sequence

Met-tRNA$_f^{Met}$ appears to be the unique initiator-RNA in eukaryotic protein synthesis, while Met-tRNA$_M^{Met}$ donates all internal methionyl residues. The fMet residue from (^{35}S)-fMet-tRNA$_f^{Met}$ is incorporated as the amino-terminal peptide into protein coded for by EMC viral RNA in in vitro protein synthesizing systems. Tryptic digests of this product yield only one major ^{35}S-labeled tryptic peptide (*Oberg* and *Shatkin*, 1972) of N-terminal sequence fMet-Ala-Thr (*Smith*, 1973), once again confirming a single initiation site on the picornaviral RNA. Similarly, only one initiation peptide could be identified from in vitro protein synthesizing systems programmed with mengovirus RNA, mouse Elberfeld (ME) viral RNA (*Oberg* and *Shatkin*, 1972; *Oberg* and *Shatkin*, 1974) and polioviral RNA (*Villa-Komaroff* et al., 1975). In contrast, there is a report that two distinct polioviral initiation peptides exist (*Celma* and *Ehrenfeld*, 1975). In fact, careful examination of the tryptic digests of ^{35}S-fMet in vitro labeled EMC viral and mengoviral polypeptides shows small amounts of a second peptide. However, the rigorous characterization of the EMC products shows minor peptides to be overdigestion products and strongly supports the presence of only on initiation peptide (*Smith*, 1973).

The corresponding ^{35}S-fMet initiation peptide could not be located in tryptic digests of EMC virus or mengovirus. Since the capsid polypeptides are translated from the 5' end of the mRNA portion of the genome, this suggests that there is a lead-in peptide sequence, which is removed by a cleavage process in the infected cell (*Oberg* and *Shatkin*, 1972; *Smith*, 1973; *Oberg* and *Shatkin*, 1974). The in vitro protein synthesizing system programmed by poliovirus RNA did produce a protein of the same electrophoretic mobility as polypeptide 1a, which was labeled with ^{35}S-fMet-tRNA$_f^{Met}$, suggesting that 1a does contain the initiation sequence (*Villa-Komaroff* et al., 1975). A major product of the in vitro protein synthesizing system programmed with EMC viral RNA is a polypeptide of apparent molecular weight 130000 that appears to be a precursor of polypeptide A. The species labeled with ^{35}S-fMet-tRNA$_f^{Met}$ had an apparent molecular weight of 130000 indicating that this polypeptide contains the initiation sequence (*Esteban* and *Kerr*, 1974).

III. The Major Viral Polypeptides

A. The Capsid Precursor

Based on size, cleavage rate in pulse-chase experiments, and tryptic mapping, polioviral polypeptide 1a was shown to be the precursor of the capsid proteins VP0, VP1, and VP3 (*Summers* and *Maizel*, 1968; *Jacobson* and *Baltimore*, 1968; *Jacobson* et al., 1970; *Abraham* and *Cooper*, 1975b). The same criterion, with the

addition of cyanogen bromide mapping, were used to establish that the EMC polypeptide A was the capsid precursor (*Butterworth* et al., 1971; *Dobos* and *Plourde*, 1973). The largest nascent EMC viral and polioviral polypeptides observed are the size of the capsid precursor, indicating that the capsid precursor is released by a nascent cleavage very soon after synthesis (*Jacobson* et al., 1970; *Butterworth* and *Rueckert*, 1972b).

Detailed kinetic studies show that the EMC viral polypeptide A is cleaved further only after it is released from the polyribosome (*Butterworth* and *Rueckert*, 1972b). This agrees with evidence that polypeptide A actually begins to assemble and form subviral structures before further cleavage occurs (*McGregor* et al., 1975). In fact, the cleavage of A is probably intimately associated with the assembly process. The cleavage proceeds through the probable intermediates D1 and possibly D2 (3a and 3b in the case of poliovirus) to generate ε, α, and γ (VP0, VP1, and VP3 in the case of poliovirus). At this point EMC has formed the 13s immature promoter $(\varepsilon, \gamma, \alpha)_5$ and poliovirus has formed an entire empty shell consisting of $(\text{VP0, VP3, VP1})_{60}$ (*McGregor* et al., 1975; *Rekosh*, 1976). ε (VP0) is then cleaved to generate the virion polypeptides δ and β (VP2 and VP1). This is termed the maturation cleavage and occurs simultaneously with the addition of the viral RNA to the capsid precursor structures to form the complete virion (*Rueckert*, 1971). This cleavage may be imperative in picornaviral architecture because it occurs in all picornaviruses examined thus far and may play a role such as altering the protein configuration to lock the RNA in the virus structure. There are some reports that the cleavage process is not always complete and small amounts of polypeptide intermediates, ε and D2, are found in the virions (*Rueckert* et al., 1969; *Ziola* and *Scraba*, 1974).

Based on comparative SDS-PAGE studies, the EMC capsid precursor, A, is disproportionately large compared to 92 of HRV-1A and 1a of poliovirus and may go through the intermediate B (*Butterworth*, 1973).

It has been suggested that there can be ambiguity or multiple cleavage sites in the capsid precursor polypeptides, resulting in multiple forms of the polypeptides (*Cooper* et al., 1970; *Vanden Berghe* and *Boeye*, 1972; *Phillips* and *Fennell*, 1973; *Fennell* and *Philips*, 1974). It is difficult to reconcile these observations with other detailed studies which consistently show only single peaks for each of the virion polypeptides (*McGregor* et al., 1975; *Lonberg-Holm* and *Butterworth*, 1976).

A class of defective interfering (DI) poliovirus particles has been identified in which approximately 15% of the length of the normal RNA has been deleted from the capsid coding region of the genome (*Cole* et al., 1971; *Cole* and *Baltimore*, 1973a, 1973b). Even with this defect DI particles can still inhibit cellular macromolecular synthesis, direct the synthesis of viral RNA and proteins, and serve as a mRNA in an in vitro protein synthesizing system (*Cole* and *Baltimore*, 1973a; *Villa-Komaroff* et al., 1975). Of course, no progeny virus is produced. The profile of viral polypeptides produced by DI particles shows a normal complement of noncapsid proteins. In place of the capsid-related polypeptides (1a, VP1, VP2, VP3) there is only synthesis of a new polypeptide termed DI(1)-P, which migrates in the position of 3a and maps with pactamycin in the capsid coding region of the viral RNA (*Cole* and *Baltimore*, 1973a). This protein is rapidly digested and is either the residual fragment of 1a encoded by the DI genome, or may actually be

the capsid intermediate 3a (Fig. 4) which is transiently formed as part of the degradation pathway of the aberrant capsid precursor. These experiments would suggest that the capsid polypeptides are neither responsible for inhibition of cellular RNA and protein synthesis, nor facilitate the synthesis of the viral macromolecules.

B. Polypeptides Analogous to F

EMC viral polypeptide F which is analogous to polioviral polypeptide X and HRV-1A polypeptide 38 is a stable primary product with an apparent M.W. of 38000 that is translated from the center of the genome. Little is known about the function of this polypeptide. However, X has been shown to have an affinity for phospholipid membranes and may play a role in the association of the viral RNA polymerase with cellular membranes (*Butterworth* et al., 1976b). The HRV-1A polypeptide 38 appears to have an alternate cleavage form (polypeptide 47) (*Butterworth*, 1973) which may also be the case for HRV-2 (*McLean* and *Rueckert*, 1973).

Based on molecular weight measurements and peptide mapping it has been suggested that X may be translated independently from the other viral polypeptides (*Abraham* and *Cooper*, 1975a). This is inconsistent with results of pactamycin mapping, molar ratio data, and patterns obtained during inhibition of cleavage; all of which suggest that X is under the same translational controls as the other polypeptides.

C. Polypeptides 1b, 2, and 4 and the Viral Polymerase

The EMC viral polypeptides C, D, and E are analogous to the polioviral polypeptides 1b, 2, and 4 and the HRV-1A polypeptides 84, 76, and 55 (Fig. 4). Relative to the other viral polypeptides C maps nearest the 3′ end of the viral RNA. Polypeptide C cleaves to produce D which in turn cleaves to produce E (*Butterworth* et al., 1971). Kinetic studies show that although C can be translated in the intact form, most of the time the natural cleavages occur in the growing chain, generating D and E as primary products (*Butterworth* and *Rueckert*, 1972b). There are large differences among the picornaviruses in the rates that these proteins are processed. In the profile of EMC viral polypeptides E is always present in large amounts relative to the other polypeptides, whereas in the HRV-1A profile 55 is present in small amounts. In all cases the cleavage analogous to C $\rightarrow$D appears to be rapid (the half-life of C is 10 min (*Butterworth* and *Rueckert*, 1972b)).

The observation that the polymerase functions are on the opposite end of the genetic map relative to the capsid functions (*Cooper*, 1969) suggests that C, D, and E may be components of the viral RNA polymerase. Partial purification of the polioviral RNA polymerase complex did show an enrichment in polypeptide 4 and similar experiments with the EMC viral polymerase showed an enrichment in polypeptides D and E (*Lundquist* et al., 1974; *Traub* et al., 1976). However, these experiments are extremely difficult and it still has not been rigorously shown that these polypeptides are constituents of the polymerase (*Butterworth* et al., 1976).

Inhibition of viral protein synthesis with cycloheximide results in a progessive decrease in the viral RNA polymerase activity, indicating that the enzyme is unstable and must continually be renewed (*Baltimore*, 1969). It is tempting to speculate that one of the polymerase constitutents is an unstable viral precursor polypeptide that loses activity upon cleavage. In fact, changing cleavage rates could be a means of regulating the kinds and amounts of RNA synthesized.

Iodoacetamide inhibits the proteolytic processing of poliovirus polypeptides resulting in the accumulation of 1a, and 2 (*Korant*, 1973) (note: no markers were run and it may actually have been 1b that was accumulated rather than 2). It has been reported that the previous addition of iodoacetamide blocks the decay of the polymerase activity that normally follows the addition of cycloheximide to infected cells (*Korant*, 1975). In addition, the serine protease inhibitor phenylmethane sulfonyl fluoride (PMSF) (*Fahrney* and *Gold*, 1963), which seems to specifically block the $2 \rightarrow 4$ conversion in poliovirus-infected monkey kidney cells, had the ability to prolong the high rate of viral RNA synthesis when compared to untreated infected controls (*Korant*, 1975). These experiments suggest that stabilizing polypeptide 2 also stabilizes the viral polymerase. However, others claim that addition of iodoacetamide or the protease inhibitor TPCK (see below) inhibits the in vivo polymerase activity (*Röder* and *Koschel*, 1974; *Röder* and *Koschel*, 1975). Caution should be used in making conclusions following the use of such reactants as iodoacetamide, PMSF, and TPCK which probably react irreversibly with a variety of cellular proteins. For example, TPCK has been shown to inhibit RNA synthesis in both infected and uninfected cells (*Summers* et al., 1972). The premise of the cleavage pattern controlling RNA synthesis is fascinating but both systems should be explored further using high resolution PAGE and perhaps using zinc as an inhibitor (see below) so that inhibition of cleavage and reversal is more easily controlled.

A mutant of poliovirus, designated P2r, has been selected with an RNA polymerase that exhibits greater stability following inhibition of protein synthesis and less susceptibility to heat inactivation (*Korant*, 1975). Analysis of the viral polypeptide profile of P2r revealed that the $2 \rightarrow 4$ conversion was proceeding much more slowly than with the wild type virus. This more directly implicates the cleavage of polypeptide 2 as playing a role in the activity of the polymerase.

IV. Inhibitors of Cleavage

A. Amino Acid Analogs

Treatment of poliovirus-infected cells with the amino acid analogs p-fluorophenylalanine (FPA), canavanine (arginine analog), azetidine-2-carboxylic acid (proline analog), and ethionine (methionine analog) results in these analogs being incorporated into the polioviral proteins. Use of FPA prevented posttranslational cleavages and causes the accumulation of the primary products (*Jacobson* and *Baltimore*, 1968) while a combination of analogs resulted in the accumulation of larger polypeptides up to 210000 daltons which may represent the complete uncleaved translation product (*Jacobson* et al., 1970). Presumably these analogs

alter the structure and configuration of the protein so that it is no longer recognized by the proteases responsible for protein processing. Other analogs have since been shown to affect the processing of polioviral proteins (*Abraham* and *Cooper*, 1975a).

Similar cleavage inhibition has been demonstrated in cells infected with EMC virus (*Dobos* and *Martin*, 1972), FMDV (*Black*, 1975), and mengovirus (*Collins* and *Roberts*, 1972). In one case the apparent complete translation product of mengovirus was formed along with two intermediates larger than the capsid precursor (*Paucha* et al., 1974). These results are evidence for the single initiation site hypothesis but the proteins thus formed have been permanently altered so that they cannot serve as substrates for studies of the cleavage process.

B. Temperature

It has been reported that shifting LSc poliovirus-infected HeLa cells from 35° to 39° causes the production of low levels of viral polypeptides in the M.W. range of 230000 to 160000 and that shifting back to 35° allows some cleavage of these presumed viral precursor proteins (*Garfinkle* and *Tershak*, 1971). Unfortunately, resolution was not sufficient to specifically identify any of the viral polypeptides. In contrast, others have shown that the thermosensitive defect of the LSc2ab strain of poliovirus was in the assembly of its viral capsid proteins into capsomeres, and saw no effect of elevated temperatures on the cleavage pattern (*Fiszman* et al., 1972). Slight cleavage inhibition of EMC viral proteins in ascites cells at elevated temperatures has been reported (*Dobos* and *Martin*, 1972). Shifting back to the permissive temperature did not result in cleavage of these accumulated polypeptides. Several factors make the interpretation of such experiments difficult, including the observation that elevated temperatures can release lysosomal enzymes which cause the abnormal breakdown of viral macromolecules (*Fiszman* et al., 1972; *Garfinkle* and *Tershak*, 1972).

C. Serine Protease Inhibitors

Tolylsulfonyl-phenylalanyl chloromethyl ketone (TPCK) is an irreversible inhibitor of chymotrypsin. The specificity lies in the phenylalanyl portion of the molecule which has an affinity for the recognition site of chymotrypsin, thus presenting the alkylating agent at the active site (*Shaw*, 1967). Another serine protease inhibitor (*Stroud*, 1974) is L-carbobenzyloxyphenylalanyl chloromethyl ketone (L-ZPCK) which is also recognized by chymotrypsin. An optical isomer of this inhibitor (D-ZPCK) is not recognized and is not inhibitory (*Shaw* and *Ruscica*, 1971). Similarly, tolylsulfoniclysyl chloromethyl ketone (TLCK) is a specific inhibitor of trypsin (*Shaw*, 1967).

It has been reported that polioviral precursor proteins larger than 1a are accumulated in infected monkey kidney cells treated with TPCK and in HeLa cells treated with TLCK (*Korant*, 1972). Others suggest that both TPCK and TLCK allowed the accumulation of large precursors and prevented the appearance of capsid proteins in poliovirus-infected U cells (*Abraham* and *Cooper*, 1975a). A third report showed strong inhibition of polioviral cleavages in infected HeLa

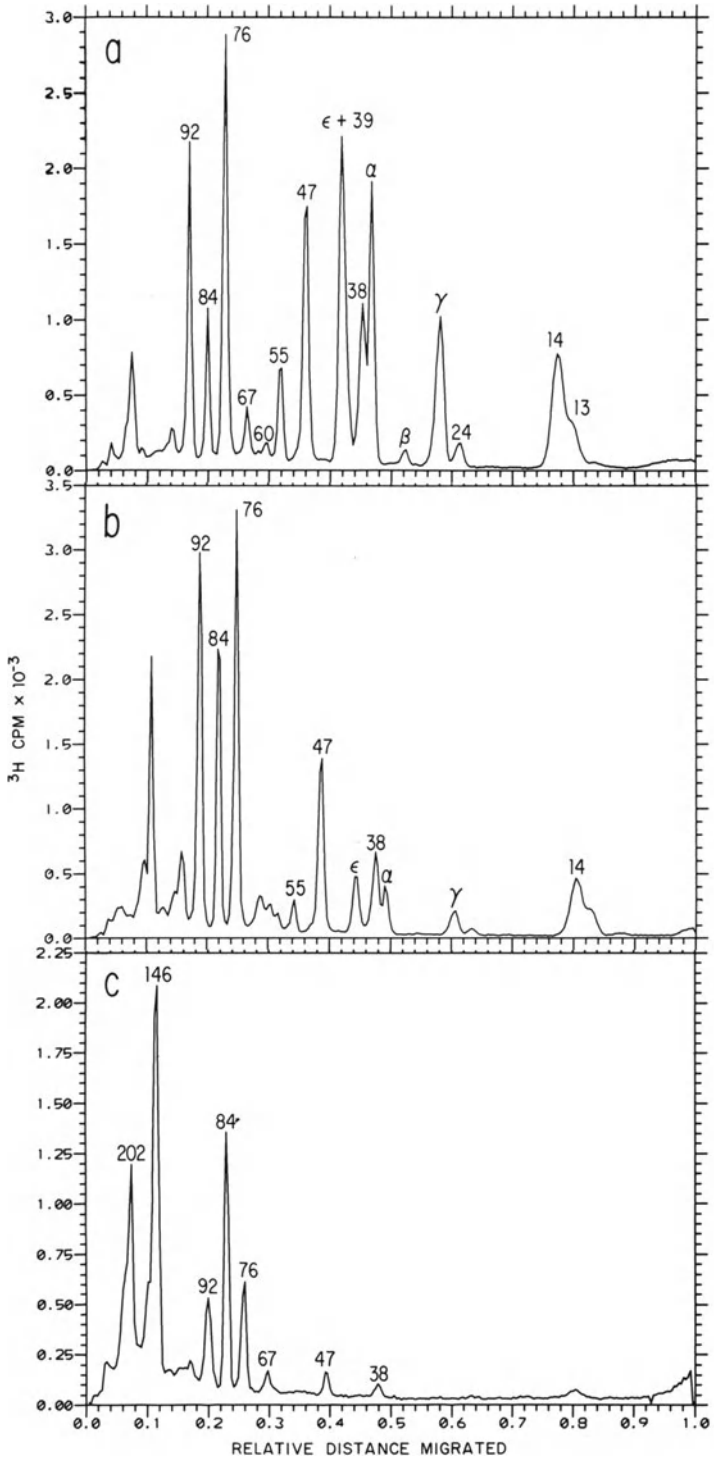
cells treated with TPCK and little inhibition when the cells were treated with TLCK (*Summers* et al., 1972). In this study the pattern of TPCK inhibition revealed that 0.05 mM TPCK, there was an accumulation of the primary products 1a, 1b, and X, while increasing the concentration to 0.1 mM TPCK resulted in the buildup of at least seven distinct protein bands in the size range from 105000 to greater than 210000 daltons. L-ZPCK caused some accumulation of polypeptides larger than 2. The effect of TLCK treatment of poliovirus-infected HeLa cells was only inhibition of the maturation cleavage which generates VP2 (*Summers* et al., 1972). Consistent with this was the observation that neither of the trypsin inhibitors p-amidinophenacyl bromide (APB) or p-guanidino-phenacyl bromide (GPB) (*Schroeder* and *Shaw*, 1971) had much effect on the processing of the large poliovirus precursor proteins (*Summers* et al., 1972). It is not known why various groups report such different susceptibilities to these reagents for the poliovirus system. One suggestion is that the different cell types may be able to employ slightly different enzymes in the cleavage process (*Korant*, 1972).

In EMC virus-infected ascites cells there is reportedly little effect of either TPCK or TLCK on protein cleavage (*Dobos* and *Martin*, 1972). TPCK, but not TLCK or the protease inhibitor phenylmethylsulfonyl-fluoride (PMSF), inhibited protein processing in mengovirus-infected L cells (*Lucas-Lenard*, 1974). No inhibition of the cleavage of the FMDV-induced proteins in BHK cells was found by treatment with TLCK (*Black*, 1975). The well-known protease inhibitor diisopropylfluorophosphate (DFP) also inhibits post-translational polioviral protein processing (*Jacobson* et al., 1970). It is premature to conclude that the processing enzymes are serine proteases because the D-isomer of ZPCK also inhibited cleavage in poliovirus-infected HeLa cells and because of other apparently nonspecific effects of these chemicals such as inhibition of RNA and protein synthesis in both infected and uninfected cells (*Summers* et al., 1972).

D. Zinc

Detailed analysis of a sample of a presumed protease inhibitor that apparently had some ability to inhibit rhinovirus protein processing revealed that zinc ion, which was present as a contaminant, was the active species (*J. Kauer* and *B. Korant*, personal communication). It was later shown that 0.1 mM zinc ion was very effective in preventing the multiplication of many rhinoviruses with minimal toxicity to the tissue culture cells (*Korant* et al., 1974). The zinc had only a slight effect on viral RNA and protein synthesis, rather, the mechanism of antiviral action was shown to be the inhibition of the cleavage processing of the viral proteins (Fig. 7). Although neither poliovirus nor EMC virus were nearly so susceptible as HRV-1A, at higher zinc concentrations and in suspension culture, processing of these two viruses was also inhibited (*Butterworth* and *Korant*, 1974).

Fig. 7a–c. Inhibition of cleavages of HRV-1A polypeptides by zinc (*Korant* et al., 1974). Panel (a) shows the normal pattern of HRV-1A polypeptides obtained by labeling with ^{3}H-amino acids for 1 h. Panels (b) and (c) show inhibition of cleavage and accumulation of large precursor polypeptides by labeling in the presence of 0.1 mM $ZnCl_2$ and 0.8 mM $ZnCl_2$, respectively


a
76
92
ε + 39
α
47
84
38
γ
14
55
67
13
60
β
24
b
76
92
84
47
38
ε
α
55
γ
14
c
146
202
84
92
76
67
47
38
^{3}H CPM x 10^{-3}
RELATIVE DISTANCE MIGRATED

It has been proposed that the proteolytic processing of HRV-1A is inhibited by the binding of zinc to the viral polypeptides, particularly the capsid sequences. Consistent with this are the observations that pactamycin mapping shows all of the large precursors accumulated by zinc to contain the capsid sequence, that zinc binds to the HRV-1A virions and prevents their crystal formation, and that zinc-resistant mutants display antigenic alterations in their coat proteins (*Butterworth* and *Korant*, 1974; *Korant* and *Butterworth*, 1976). With EMC virus and poliovirus higher zinc concentrations were necessary to inhibit the cleavage process and there was less evidence that the capsid sequence was so strongly involved.

The utility of zinc may well be as a tool to accumulate the unaltered precursor molecules and to study the cleavage process. Choosing the correct zinc concentrations allows the cleavage process to be frozen at almost any point desired. Further, a simple washing procedure to remove the zinc allows the cleavage process to resume and those precursor proteins which had been accumulated then cleave normally.

V. Source of the Processing Proteases

A. Cellular and Viral Proteases

In the absence of inhibitors the largest polypeptide normally observed in the infected cell is the capsid precursor. Occasionally, early in the infection cycle of coxsackievirus B1 a polypeptide in the molecular weight range of 200000 could be detected (*Kiehn* and *Holland*, 1970). Later in the infection, production of this polypeptide could not be detected, implying that there was an increase with time in the cleavage activities of those enzymes performing the nascent cleavages. Additional experiments suggest that infected cells have acquired new protease activities which may be responsible for the various cleavage reactions (*Korant*, 1972; *Korant*, 1973; *Esteban* and *Kerr*, 1974; *Lawrence* and *Thach*, 1975). Identifying the various processing proteases is complicated by the facts that the substrate is difficult to obtain and that the cell already contains a complement of lysozomal proteases.

It has been reported that high molecular weight polioviral polypeptides accumulated by treatment with TPCK or iodoacetamide could be degraded by crude cell extracts to polypeptides with sizes similar to known viral polypeptides (*Korant*, 1972; *Korant*, 1973). While extracts from both infected and uninfected cells produced products in the size range of the primary products, only extracts from infected cells possessed the ability to cleave the larger molecules to products in the size range of the capsid polypeptides, suggesting that host enzymes were responsible for production of the primary products while viral-specified or induced enzymes were necessary to accomplish the post-translational cleavages. It would be of value to extend these studies using the high-resolution SDS-PAGE techniques that are now available. Other workers doing similar experiments with high-molecular weight EMC viral precursors found degradation from extracts of both infected and uninfected cells and concluded that the cleavages were due to non-specific proteases that were released by the homogenation procedure (*Ginevskaya* et al., 1972).

B. Virion Polypeptide γ

Translation of EMC viral RNA in an in vitro system from infected cells yielded a polypeptide of apparent molecular weight 100000 (polypeptide 100) which CNBr analysis indicated was identical to the capsid precursor, polypeptide A (*Lawrence* and *Thach*, 1975). In addition several smaller polypeptides were produced including one of apparent molecular weight 12500 (Polypeptide 12.5). Translation of EMC viral RNA in uninfected cell systems yielded a polypeptide of apparent molecular weight 112000 (polypeptide 112) but no polypeptide 12.5 was observed. CNBr and kinetics of synthesis studies suggested that there was a protease activity found in infected cells that cleaved 12.5 from the amino terminal end of 112 to generate 100. Using this cleavage reaction as an assay, it was found that the kinetics of appearance of the protease activity was approximately the same as the appearance of the capsid polypeptides. A viral polypeptide of molecular weight 23000 (presumably γ) was found to copurify with the activity, which led the authors to suggest that the virion polypeptide γ was the protease responsible for the cleavage of 112 (*Lawrence* and *Thach*, 1975). It was suggested that this cleavage and perhaps others may be autocatalytic, with the resident γ being responsible for the cleavage as the viral assembly process occurs.

The concept of the dual functionality of this viral polypeptide is interesting. It has been reported that mengo virions have some proteolytic activity, but it appears to be nonspecific and further characterization is yet to be done (*Holland* et al., 1972).

Esteban and *Kerr* also see a polypeptide generated in their protein synthesizing system that appears to be a slightly larger precursor of A (*Esteban* and *Kerr*, 1974), but its relationship to polypeptide 112 has not been studied. The reaction $112 \rightarrow 100 + 12.5$ is inhibited by zinc (*Lawrence* and *Thach*, 1975). Treatment of EMC virus-infected HeLa cells with zinc in vivo causes the production of a polypeptide slightly larger than A that maps in the capsid region (*Butterworth* and *Korant*, 1974), but its relationship to 112 has not been established.

Polypeptides ε, γ, and α are synthesized together and are usually found tenaciously bound together in equimolar amounts (*McGregor* et al., 1975). Since the 23000 dalton polypeptide was found without any other viral polypeptides it would be good to confirm that the polypeptide found is γ and not some other heretofore unrecognized viral polypeptide. A viral-specific polypeptide of M.W. 22000 has been reported (*Medvedkina* et al., 1974). The possibility also exists that the enzyme is a viral-induced host protease, and the host components present in the final preparation should be checked.

VI. Specificity of the Proteases

A. Configuration of the Cleavage Site

Amino acid sequencing of the virion polypeptides is giving some insights as to the specificity of the cleavage site. Table 2 shows the amino- and carboxyl- terminal amino acid sequences of the capsid polypeptides of several picornaviruses. There

Table 2. Amino- and carboxyl- terminal amino acid sequences of the capsid polypeptides of picornaviruses

Polypeptide	RVP2 (β)	VP3 (γ)	VP1 (α)
EMC virus[a]	Asx- ...	Ser-	Gly-
Mengovirus[b]	Asp-Gln-Asn- ... -Leu-Arg-Gln	Ser-Pro-Ile- ... -(Gln)	Gly-Val-Glu- ... -Val-Leu
FMDV A_{12} 119[c]	Asp- -Met- ... (Leu)-Glu	Thr-Thr-Ala- ... -Gln-Ala-Leu	Gly-Ile ... -Ser-Gln
FMDV 0_1 Kaufbeuren[d]	Asx ...	Thr- ...	Gly- ...
FMDV 0_0 Brugge[e]	(Asp)- ...	Thr-Thr-Ser- ...	Gly, Ile, Phe
FMDV C_3 Resende[e]	Asp-Leu ...	Thr-Thr	Gly, Ile, Phe- ...
Poliovirus[f]	Asp- ...	Ser- ...	Gly- ...
BEV VG-5-27[g]	Asx- ...	Ser- ...	Gly- ...

[a] Data for EMC virus are from *D. Omilianowski R. Rueckert* and *D. Rekosh* (*Matheka* and *Bachrach,* 1975).

[b] These sequences have been determined to a greater extent than shown here (*Ziola* and *Scraba,* 1976). The amino terminal of VP4 is blocked. The C-terminal sequence of VP4 is ...-Leu-Leu-Ala.

[c] *Bachrach* et al., 1973; *Matheka* and *Bachrach* 1975. Different means were used to separate the polypeptides in these two reports and it has not been established that the VP1, VP2, and VP3 for which the C-terminal sequences were determined (*Bachrach* et al., 1973) correspond to the VP1, VP2, and VP3 for which the N-terminal sequences were determined (*Matheka* and *Bachrach,* 1975) (*Bachrach,* personal communication). In addition it has been suggested that VP1, VP2 and VP3 in this nomenclature of FMDV may actually correspond to β, γ, and α of mengovirus, respectively (Scraba, personal communication).

[d] *Adam* and *Strohmaier,* 1974.

[e] *Matheka* and *Bachrach,* 1975.

[f] The N-terminal amino acids of the unfractionated virus were determined (*Burrell* and *Cooper,* 1973) and have been assigned in conformity with the other results shown (*Bachrach,* 1976).

[g] Data for bovine enterovirus (BEV) are from *P. Carthew* (*Martheka* and *Bachrach,* 1975). The N-terminal of VP4 is gly

is a striking constancy in the N-terminal amino acids among these antigenically different viruses. Aspartic acid and asparagine residues, found in the N-terminal position of VP2; serine and threonine residues, found in the N-terminal position of VP3; and glycine, found in the N-terminal position of VP1 are all strong breakers of α-helicies and function to initiate β-bends in the folding of protein chains (*Lewis* and *Scheraga*, 1971). It has been proposed that the picornavirus precursors are cleaved in β-bends to the left of a helix-breaker amino acid (*Bachrach*, 1976). There may be something inherently fundamental about this specificity because it has been retained among these widely divergent viruses. However, there are two sequence studies with different strains of FMDV which do not exactly fit the pattern found in Table 2 (*Laporte*, 1969; *Matheka* and *Dietzschold*, 1972).

B. Specificity of the Enzyme

Additional sequencing has been done with mengovirus which indicates that a single viral protease with a specificity for peptide bonds whose carboxyl function is donated by glutamine, may be responsible for the conversion of the capsid precursor to ε, α, γ (*Ziola* and *Scraba*, 1976). Glutamine is also the C-terminal peptide residue in VP1 of FMDV A_{12} (*Bachrach* et al., 1973). Leucine, which is steriochemically slightly similar to glutamine, is the C-terminal peptide residue in VP1 of mengovirus and VP3 of FMDV A_{12}.

VII. The Togaviruses

A. The Virion

indbis virus and Semliki forest virus (SFV) are members of the alpha togaviruses which are also known as group A togaviruses, alphaviruses, and group A arboviruses. These virions contain a nucleocapsid core consisting of one single-stranded RNA genome of molecular weight 4.3×10^6 daltons (*Dobos* and *Faulkner*, 1970; *Simmons* and *Strauss*, 1972a; *Shatkin*, 1974) complexed with a single core protein of molecular weight 30000 daltons (*Strauss* et al., 1968, 1969). Surrounding this core is an envelope membrane which contains at least two virus-coded glycoproteins (*Harrison* et al., 1971; *Schlesinger* et al., 1972).

B. The Cleavage Scheme

Host protein synthesis is sufficiently inhibited by these viruses so that incubation of infected cells with radioactive amino acids under the appropriate conditions reveals a profile of virus-specific proteins. In the case of SFV about 10 virus-specific polypeptides are seen in the molecular weight range of 10000 to 130000 daltons (Fig. 8), some of which have been shown to be uncleaved precursors of the structural virion polypeptides (*Hay* et al., 1968; *Burrell* et al., 1970; *Morser* et al., 1973; *Simons* et al., 1973; *Garoff* et al., 1974; *Morser* and *Burke*, 1974; *Clegg*, 1975; *Kaluza*, 1975; *Kääriäinen* et al., 1975; *Keränen* and *Kääriäinen*, 1975; *Lachmi* et al.,

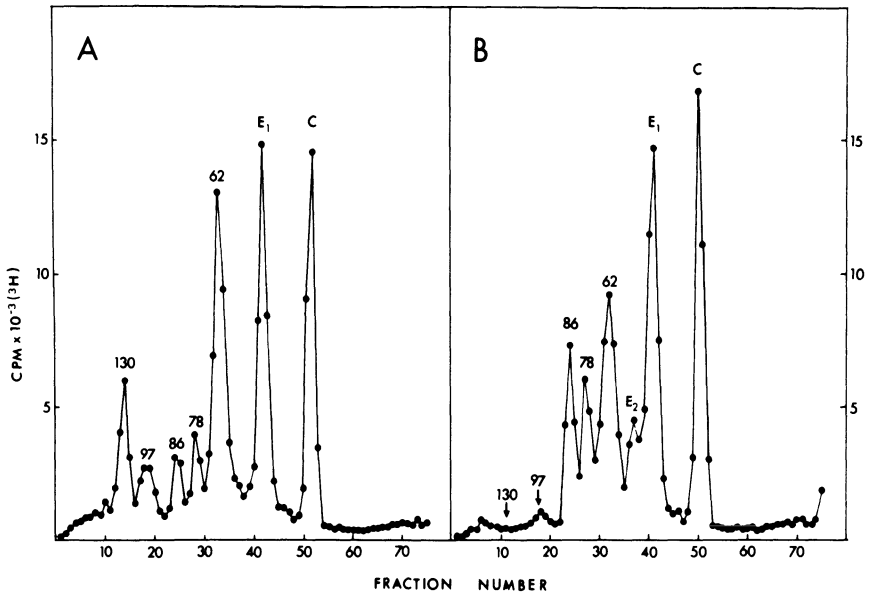


Fig. 8A and B. SDS-PAGE profile of virus-specific polypeptides synthesized in Semliki Forest virus ts-1-infected chick embryo cells (*Keränen* and *Kääriäinen*, 1975). Panel (A): cells were labeled at 39° for 10 min; Panel (B): a batch of the cells was chased for 4 h

1975; *Clegg* and *Kennedy*, 1976; *Wengler* and *Wengler*, 1976). An analogous profile is observed for Sindbis virus (*Strauss* et al., 1969; *Scheele* and *Pfefferkorn*, 1970; *Schlesinger* et al., 1972; *Schlesinger* and *Schlesinger*, 1972; *Pfefferkorn* and *Boyle*, 1972; *Schlesinger* and *Schlesinger*, 1973; *Waite*, 1973; *Snyder* and *Sreevalsan*, 1973; *Snyder* and *Sreevalsan*, 1974; *Jones* et al., 1974).

The patterns of protein processing for Sindbis virus and SFV are shown in Figure 9. Tryptic mapping and pulse-chase experiments indicate that the 130000 dalton Sindbis viral protein (p130) is the precursor of both the core (*L*) and

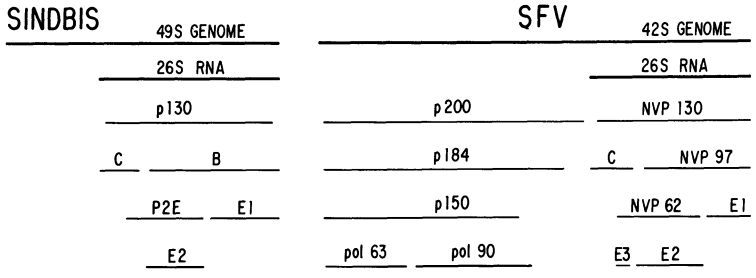


Fig. 9. Protelytic processing of Sindbis virus and Semliki Forest virus proteins. 26S RNA is produced in the infected cell, represents a unique fraction of the viral RNA, and codes for the virion structural proteins. Line lengths are proportional to molecular weights of polypeptides. Lateral position represents relative location of the corresponding gene locus on the viral RNA (the 5' end of the RNA ist to the left). Vertical position represents precursor-product relationships. Detailed explanations and references are given in text

envelope (*E*1 and *E*2) proteins (*Schlesinger* and *Schlesinger*, 1973; *Snyder* and *Sreevalsan*, 1974). The Sindbis virus temperature-sensitive mutants ts-2, ts-5, and ts-13 have a defect in nucleocapsid formation. This defect involves proteolytic processing because at the nonpermissive temperature cells infected with these mutants accumulated p130 (*Strauss* et al., 1969; *Scheele* and *Pfefferkorn*, 1970). In the normal infection process the core polypeptide is probably released by a nascent cleavage before the synthesis of p130 is completed, because little p130 is seen in a normal infection, even with very short pulses. Further, shifting to the permissive temperature did not result in the cleavage of the p130 precursor which had been accumulated at the restrictive temperature for the Sindbis virus nucleocapsid ts mutants (*Scheele* and *Pfefferkorn*, 1970). A polypeptide of 133000 daltons was accumulated at the restrictive temperature in cells infected with the temperature-sensitive mutant ts-11 which is defective in RNA synthesis (*Waite*, 1973). The relationship of this polypeptide to p130 has not been established, but it has been suggested that lack of the normal cleavage which releases the polymerase generates this aberrant 133000 dalton protein (*Waite*, 1973).

Pactamycin mapping has shown that the core protein of Sindbis virus is derived from the amino terminal end of p130, leaving a protein of molecular weight 99000, termed B, which tryptic mapping indicates is the precursor of the envelope glycoproteins (*Strauss* et al., 1969; *Schlesinger* and *Schlesinger*, 1972; *Schlesinger* and *Schlesinger*, 1973). Little B was observed in Sindbis virus-infected chick cells, whereas substantial amounts were observed in Sindbis virus-infected BHK cells, suggesting that host enzymes are responsible for the proteolytic processing of this precursor (*Strauss* et al., 1969). Cleavage of B yields E1 plus PE2 (M.W. 60000). PE2 is then cleaved to form the other envelope protein E2 (*Schlesinger* et al., 1972; *Schlesinger* and *Schlesinger*, 1972). The temperature-sensitive mutant ts-20 accumulates P2E at the nonpermissive temperature (*Jones* et al., 1974). This precursor was found associated with the plasma membrane and it has been suggested that the virus assembles at this site and that the cleavage of PE2 is one of the final events of maturation before the release of the virion (*Jones* et al., 1974).

An analogous cleavage pattern to that of Sindbis virus has been found for Semliki forest virus, including a set of ts mutants that are variously defective in cleavage (*Morser* et al., 1973; *Morser* and *Burke*, 1974; *Keränen* and *Kääriäinen*, 1975; *Lachmi* et al., 1975). A third envelope glycoprotein has been identified in the SFV pattern which corresponds to the other product formed when NVP63 cleaves to form E2 (analogous to the P2E cleavage to form E2 in Sindbis virus) (*Simons* et al., 1973; *Garoff* et al., 1974). Based on kinetics of synthesis the order of the polypeptides within the precursor NVP130 is indicated to be N→C C–E3, E2–E1 (*Clegg*, 1975). A polypeptide of M.W. 86000 was observed which probably contains C and NVP63, in addition to two polypeptides of M.W. 78000 and 86000 which may not be precursors to structural polypeptides (*Morser* and *Burke*, 1974; *Lachmi* et al., 1975). Further work with SFV suggests that two components of the polymerase of molecular weight 63000 and 90000 are produced by proteolytic cleavage of precursor polypeptides which are synthesized from the 5' end of the 42S genome RNA (*Clegg* and *Kennedy*, 1976).

C. Inhibition of Cleavage

The wide variety of ts-mutants described above, which are defective in cleavage, differs from the situation with the picornaviruses where few, if any, such ts-mutants have been found.

There are some indications that TPCK but not TLCK inhibits cleavages in Sindbis virus-infected chick embryo fibroblasts (CEF) (*Pfefferkorn* and *Boyle*, 1972). A slight build-up of large precursors was also seen when SFV-infected BHK or CEF cells were treated with TPCK (*Morser* and *Burke*, 1974). The degree of this inhibition is not nearly as great as is observed with the picornaviruses.

Surprisingly, the amino acid analogs which strongly inhibit post-translational cleavages of picornaviruses do not cause the buildup of viral precursors in Sindbis virus or SFV-infected cells (*Scheele* and *Pfefferkorn*, 1970; *Ranki*, 1972). One report does, however, indicate that here is some cleavage inhibition of SVF proteins with amino acid analogs (*Morser* and *Burke*, 1974).

It was observed that large Sindbis virus proteins would accumulate in chick embryo fibroblasts if the cells were first "aged" for 7 days at 37° (*Snyder* and *Sreevalsan*, 1973). Zinc ion is reported to inhibit some Sindbis virus cleavages (*Snyder*, 1974).

D. In Vitro Protein Synthesis

Sindbis virus-infected cells contain at least two forms of mRNA. Approximately 10% is the 49S genome of molecular weight 4.3×10^6 daltons, while about 90% is 26S RNA of molecular weight 1.6×10^6 daltons (*Simmons* and *Strauss*, 1974b; *Mowshowitz*, 1973). This 26S RNA represents a unique fraction of the viral RNA (*Simmons* and *Strauss*, 1972a, 1972b). Analysis of the products of an in vitro protein synthesizing system programmed with 26S RNA showed that this species of RNA was responsible for specifying the virion structural proteins (*Simmons* and *Strauss*, 1974a). The products formed had the same apparent molecular weight as the 30000 molecular weight core protein (*C*) and the 100000 molecular weight precursor of the envelope protein (*B*) (Fig. 10). The *B* produced was specifically precipitated with anti-Sindbis antibodies (*Simmons* and *Strauss*, 1974a). The *C* produced had the same trypticpeptide fingerprint as the authentic core protein (*Cancedda* and *Schlesinger*, 1974; *Cancedda* et al., 1974a).

The products produced by the in vitro system programmed with 26S RNA from the ts mutant of Sindbis virus mimicked the in vivo pattern of protein processing found by this mutant (*Simmons* and *Strauss*, 1974; *Cancedda* et al., 1974b). At the permissive temperature *C* and *B* were produced; at the restrictive temperature the 130000 molecular weight precursor of *C* and *B* was formed (*Simmons* and *Strauss*, 1974a).

Presumably, the portion of the genome exclusive of that which yields the 26S fragment is responsible for coding for the other virus-specific polypeptides such as the viral RNA polymerase. Translation of the 49S genome in vitro yields several polypeptides in the molecular weight range of 60000 to 180000 daltons; the virion structural polypeptides were not observed (*Simmons* and *Strauss*, 1974a).

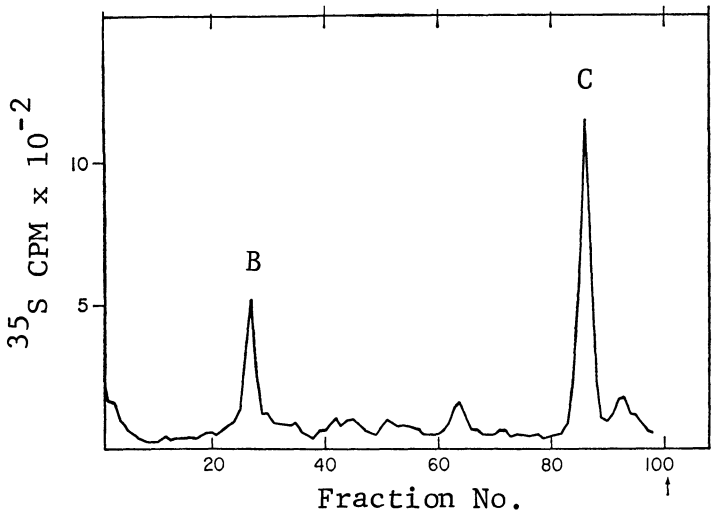


Fig. 10. Proteins synthesized by an in vitro protein synthesizing system programmed with 26S Sindbis virus RNA (*Simmons* and *Strauss'* 1974). Major products synthesized are the core protein (*C*) and the precursor to the envelope glycoproteins (*B*). Background of endogenous polypeptides synthesized in the system has been subtracted

The production of capsid protein and in some cases envelope proteins has been shown in in vitro protein synthesizing systems programmed with SFV 26S RNA or 42S genome RNA (*Clegg* and *Kennedy*, 1974; *Smith* et al., 1974; *Wengler* et al., 1974; *Clegg* and *Kennedy*, 1975). The observation that *C* is produced in these systems indicates that either the protease responsible for this cleavage or some other release mechanism is functioning.

E. Control of Translation

At that time in the infection cycle during which progeny virus is being produced, the predominant polypeptides produced are the virion structural polypeptides or precursors thereof. The production of the sub-genomic 26S RNA to control the relative amounts of the polypeptides produced is not found with the picornaviruses and has not been firmly established, one way or the other, with the RNA tumor viruses.

VIII. The RNA Tumor Viruses

A. The Virion

The RNA tumor viruses, which are also known as leukemiaviruses or retroviruses, include leukemia and sarcoma viruses from mammalian and avian origin (*Tooze*, 1973; *Shatkin*, 1974; *Baltimore*, 1974). The virion structure includes a lipid envelope, which contains at least two major viral glycoproteins, an inner ribonucleo-

protein core, and an RNA-dependent DNA polymerase (*August* et al., 1974; *Bolognesi* et al., 1974; *Strand* and *August*, 1974). The inner proteins among the various avian viruses are very similar as are those of the murine viruses and are known as the group specific (gs) antigens.

The single-stranded RNA genome of the RNA tumor viruses sediments at 60–70S and has an apparent M.W. of 10^7 daltons. However, the genome is segmented and is easily denatured to what appears to be two equivalent 30–40S pieces of M.W. $2–3 \times 10^6$ daltons plus some 4S tRNA and 7S RNA of unknown function (*Shatkin*, 1974; *Duesberg* et al., 1974).

B. The Internal Group-Specific Proteins

Identification of the virus-specific proteins is difficult because the amounts synthesized are small relative to those proteins synthesized by the host and because, unlike the picornaviruses and togaviruses, host protein synthesis is not inhibited by the RNA tumor viruses. Some success has been achieved by using antibodies to the disrupted virion to preferentially precipitate virus specific proteins from the mixture of proteins being synthesized in the cell (*Shanmugam* et al., 1972; *Vogt* and *Eisenman*, 1973; *Naso* et al., 1973; *Halpern* et al., 1974; *Dickson* et al., 1975; *Eisenman* et al., 1975; *Vogt* et al., 1975; *Naso* et al., 1975a; *van Zaane* et al., 1975; *Shapiro* and *Strand*, 1975; *Arcement* et al., 1976; *Arlinghaus* et al., 1976; *Naso* et al., 1976). These studies have revealed that some of the virus-specific proteins are derived from the proteolytic cleavage of precursor molecules (Fig. 11). A summary of the pattern of protein processing for the Avian and Murine RNA tumor viruses is shown in Figure 12.

Evidence is accumulating that the 30–40S pieces each contain substantially the same genetic information (*Duesberg*, 1975). If so, then each virus would have the ability to code for only 200000–300000 daltons of protein. Four major genetic elements have thus far been ascribed to the viral RNA. These have been designated as follows: (1) gag: the gene encoding the internal, virion groupspecific antigens, (2) pol: the gene encoding the viral DNA polymerase, (3) env: the gene encoding the major virion envelope glycoprotein, and (4) onc: the gene encoding the protein responsible for cellular oncogenic transformation (*Baltimore*, 1975). These genes account for the majority of the coding capacity of the 30–40S RNA and are ordered 5' → 3' (gag, pol)-env-onc-poly(A) (*Duesberg* et al., 1976; *Joho* et al., 1976; *Wang* et al., 1976). Based on kinetics of inhibition of protein synthesis with pactamycin, it has been suggested that gag may be nearest the initiation site (*Vogt* et al., 1975).

In the avian myeloblastosis virus (AMV) infected cell system, the major virus-specific polypeptide precipitated following a short labeling period with ^{35}S-methionine was a polypeptide of M.W. 76000 (*Vogt* and *Eisenmann*, 1973; *Eisenmann* et al., 1975; *Vogt* et al., 1975). In vivo pulse-chase experiments and tryptic fingerprinting indicated that Pr76 was cleaved as shown in Figure 12 to generate the four major internal gs antigens. The pactamycin mapping technique was used to order not only the product polypeptides but also the tryptides within the precursor Pr76. The fact that this technique could be employed indicates that

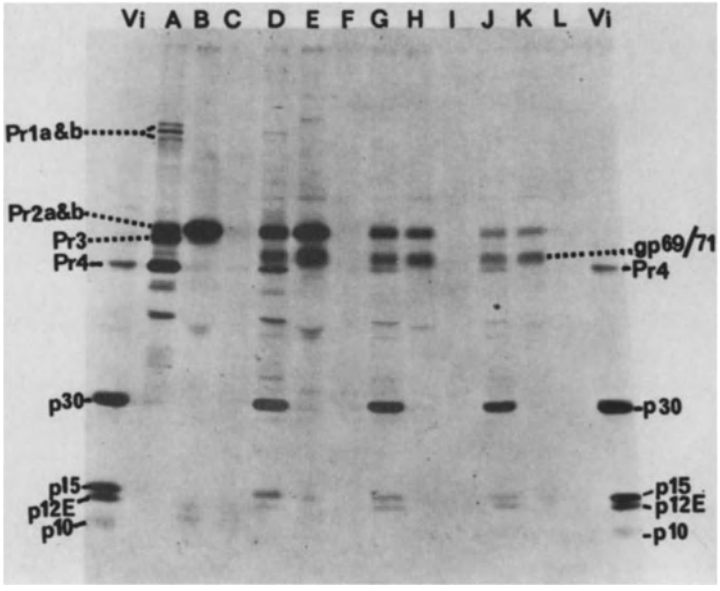


Fig. 11. SDS-PAGE profiles of virus-specific polypeptides synthesized in Rauscher leukemia virus-infected mouse embryo cells and precipitated with antibody to RLV (*Naso* et al., 1976). Colums Vi are ^{14}C-amino acid labeled marker virus. Cells were pulse-labeled for 10 min (*columns A, B, C*) with ^{14}C amino acids and then incubated in excess nonradioactive culture fluid (chased) for 2 h. (*Columns D, E, F*), 4 h. (*Columns G, H, I*), and 6 h (*Columns J, K, L*). *Columns B, E, H,* and *K* were precipitated with antiserum to purified gp69/71. *Columns C, F, I,* and *L* were precipitated with anti-RLV absobed with excess RLV proteins. Direction of electrophoresis was from top to bottom

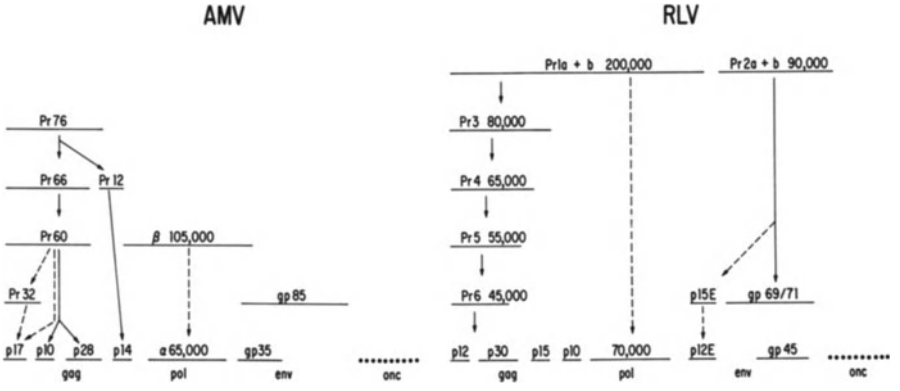


Fig. 12. Proteolytic processing of avian and murine RNA tumor virus proteins. Arrows represent precursor-product relationships. Dashed lines represent tentative relationships. Line lengths are proportional to molecular weight. The gene loci of the major proteins are ordered on the RNA from 5' to 3' as shown (5' end of the RNA is to the left). Avian gs antigens p17, p10, p28, and p14 have been ordered from N to C terminal within the precursor Pr76 and correspond to p19, p12, p27, and p15 in the standard nomenclature (*Vogt* et al., 1975; *August* et al., 1974). RLV polypeptide relationships are those proposed by *Arlinghaus* and coworkers (*Arcement* et al., 1976; *Arlinghaus* et al., 1976). Detailed explanations are given in text

at least this set of polypeptides is being translated in a manner analogous to the picornaviral capsid polypeptides.

An analogous precursor polypeptide and cleavage scheme has also been described for Rauscher murine leukemia virus (RLV) (*van Zaane* et al., 1975; *Shapiro* and *Strand*, 1975; *Arcement* et al., 1976; *Arlinghaus* et al., 1976). In addition, a set of MLV precursors in the molecular weight range of 200000 daltons has been identified which contain the sequence of the p30 gs antigen (*Arcement* et al., 1976). This set of proteins has been designated Pr1 a and b. Earlier work had suggested the existence of a class of very large precursors such as these (*Naso* et al., 1973; *Naso* et al., 1975a). However, those initial estimates of molecular weight were considerably different than those established in the later work (*Arcement* et al., 1976). Results of tryptic mapping, pulse-chase experiments, and identification of those polypeptides precipitated by antibody to purified p30 suggest the cleavage scheme presented in Figure 12 for the gs proteins of RLV (*Arcement* et al., 1976; *Arlinghaus* et al., 1976).

Several groups have reported finding the uncleaved precursor to the internal gs-antigen in preparations of purified virions (*Janjoom* et al., 1975; *Oskarsson* et al., 1975; *Gielkens* et al., 1976). This is reminiscent of finding the uncleaved precursors D2 and ε in picornavirions and suggests that assembly of the virion cores may involve the uncleaved precursor.

C. The DNA Polymerase

The avian reverse transcriptase consists of two subunits designated α and β of molecular weights 65000 and 105000, respectively (*Kacian* et al., 1971; *Green* et al., 1974; *Panet* et al., 1974). It has been suggested that the α subunit may be derived by proteolytic cleavage of the larger β subunit (*Moelling*, 1974).

The murine reverse transcriptase consists of a single subunit of molecular weight 70000 (*Ross* et al., 1971). Antibody to purified polymerase precipitates Pr1 a + b, suggesting that this precursor also carries the polymerase sequence (*Arlinghaus* et al., 1976). Polypeptides in the molecular weight range of 100000 are also seen in the cytoplasmic RLV protein profile, but any precursor relationship to the reverse transcriptase is yet to be established.

D. The Envelope Glycoproteins

The lipid envelope of AMV contains the virus-specific glycoproteins gp85 and gp35. Similarly, the MLV envelope contains the viral antigens gp69/71 and gp45 (*August* et al., 1974). The relationship between the large and small glycoproteins is yet to be determined. Monospecific antiserum to the MLV glycoprotein gp69/71 precipitates a set of proteins in the M.W. range of 85000 to 90000 daltons (*Shapiro* and *Strand*, 1975; *Arcement* et al., 1976; *Arlinghaus* et al., 1976; *Naso* et al., 1976). This set of proteins has been named Pr2a and b. Tryptic mapping indicates that gp69/71, a 17000 dalton protein (designated p15E), and a protein designated p12E are derived from the cleavage of P2a and b (*Arcement* et al., 1976; *Naso* et al., 1976).

Similarly, other workers have reported an MLV-specific polypeptide of M.W. 82000 in extracts of infected cells but have not shown whether it corresponds to Pr3 (*van Zaane* et al., 1975) or Pr2a and b (*Gielkens* et al., 1976).

A 73000 dalton glycoprotein (gp73) detected in mouse mammary tumor virus (MTV)-infected cells was shown by pulse-chase analysis and tryptic mapping to be a precursor to the virion glycoproteins gp49 and gp37.5/33.5 (*Dickson* et al., 1975; *Dickson* et al., 1976).

There is one report that monospecific antiserum to gp85 of avian sarcoma virus B_{77} subgroup C would precipitate a heterogeneous protein fraction with a mean M.W. of 70000 from an extract of pulse-labeled cells, which was suggested to represent an incompletely glycosolated precursor to gp85 (*Halpern* et al., 1974).

E. In Vitro Protein Synthesis

In vitro protein synthesizing systems from *Escherichia coli* have been shown to respond to the RNA genome from several different RNA tumor viruses. The proteins made were in the molecular weight range of 10000–45000 daltons and showed an antigenic relationship to the gs-antigen (*Gielkens* et al., 1972; *Stiegert* et al., 1972; *Twardzik* et al., 1973). There are now several reports that viral RNA or mRNA will function as a mRNA in mammalian in vitro protein synthesizing systems (*Naso* et al., 1975b; *von der Helm* and *Duesberg*, 1975; *Gielkens* et al., 1976; *Kerr* et al., 1976).

The major product synthesized by the Ascites cell-free system in response to Rous sarcoma virus (RSV) RNA is a protein of molecular weight 75000–80000 daltons (*von der Helm* and *Duesberg*, 1975). This protein is precipitated by monospecific antiserum to the gs-antigens and has a similar tryptic peptide composition to the gs-antigen, indicating that it corresponds to the Pr76 precursor of AMV found in vivo (*Vogt* et al., 1975). This confirms that at least this portion of the virion RNA serves as mRNA in the infection process.

There is a report that RLV RNA may direct the in vitro synthesis of viral proteins in the molecular weight range of 50000 to 185000 daltons (*Naso* et al., 1975b). However, more work remains to be done on resolving and sizing the proteins produced. Proteins of M.W. 65000, 76000, and 82000 were synthesized in an in vitro system using endogenous mRNA from RLV infected cells (*Gielkens* et al., 1976). The 65000 and 82000 species probably correspond to authentic precursors found in vivo (*van Zaane* et al., 1975).

Polypeptides of molecular weights 60000, 70000, and 180000 were produced from eukaryotic cell-free systems programmed with Moloney murine leukemia virus (*Kerr* et al., 1976). All three polypeptides were precipitable with antiserum to detergent-disrupted virus. Tryptic mapping indicated that each contained amino acid sequences of the major methionine-containing virion polypeptides.

There are some indications that functional segments of the viral RNA may be formed. Infected cells contain classes of viral mRNA in the range of 35S, 20–22S, and 14S (*Fan* and *Baltimore*, 1973; *Gielkens* et al., 1974; *Shanmugam* et al., 1974). Interestingly, these different size classes of viral mRNA were found to direct the synthesis of distinctly different viral related proteins in vitro (*Gielkens* et al., 1976).

F. Inhibitors of Cleavage

Extracts of AMV infected cells retained a limited ability to cleave the precursor p76 in vitro to yield p28 and p14, indicating that the protease responsible for this cleavage was present and functioning (*Vogt* et al., 1975). The extent of the cleavage was not increased by adding infected or uninfected cell extracts. The in vitro reaction was inhibited by chloroform or detergents, suggesting that intact membranes may be necessary for cleavage.

The protease inhibitors TPCK, TLCK, DFP, iodoacetamide, and zinc had no effect on the in vitro reaction and did not inhibit proteolytic processing in vivo (*Vogt* et al., 1975). However, if the infected cells were exposed to $ZnCl_2$ during pulse labeling the subsequent in vitro cleavage reaction was inhibited. It was also found that copper ion would inhibit the in vitro reaction.

Similarly the in vitro cleavage of the MLV precursor p65 to produce p30 and p15 has also been reported (*van Zaane* et al., 1975). This in vitro cleavage reaction was not affected by PMSF or soybean trypsin inhibitor. In vivo proteolytic processing was not inhibited by TPCK, TLCK, or the amino acid analogs p-fluorophenylalanine or azetidine-2-carboxylic acid. Canavanine did prevent the production of p65 but no corresponding accumulation of larger precursors was observed (*van Zaane* et al., 1975). The addition of these amino acid analogs to the in vitro protein synthesizing system programmed with MLV mRNA did not alter the size of the proteins produced (*Gielkens* et al., 1976).

In contrast to the above reports it has been reported that treatment of RLV infected cells with TPCK but not TLCK resulted in the accumulation of the precursor Pr1a + b (*Arlinghaus* et al., 1976). This is analogous to the cleavage inhibition found in the poliovirus system (*Summers* et al., 1972). Both D-ZPCK and L-ZPCK also inhibited cleavage so that no firm conclusion can be drawn as to the specificity of the processing protease.

A strain of an avian sarcoma virus designated as LA3342 is temperature sensitive in replication and yields noninfectious virus particles of abnormally high density when produced at the nonpermissive temperature. The biochemical nature of the defect appears to be in the proteolytic processing of the precursor protein (*Hunter* et al., 1976). Cleavage of the precursor to the internal structural polypeptides proceeds more slowly at the restrictive temperature and several new antigenically-related proteins appear in the virions which may be the products of the incorrect cleavage of the precursor. Temperature shift-down experiments done in the pressence of cycloheximide suggest that the precursor accumulated at the restrictive temperature may be processed correctly at the permissive temperature (*Hunter* et al., 1976). Similarly, two mutants of MLV have been described which exhibit a temperature-sensitive defect in the cleavage of the precursor polypeptide to the gs antigens (*Stephenson* et al., 1975).

G. Control of Translation

It appears that the picornaviral proteins are translated sequentially in a manditory fashion so that there is no control of the relative amounts of the various proteins

synthesized. Several observations on the production of the RNA tumor virus proteins seem to show an analogous translation to that of the picornaviruses. The major viral mRNA is the 30–40S genome RNA (*von der Helm* and *Duesberg*, 1975) which is similar in size to the 35S picornaviral genome which serves as the mRNA. The 200000 dalton precursors Pr1a and b are approaching a size which would account for translation of a sizable portion of the genome (*Arcement*, 1976).

However, other evidence suggests that controls exist in the production of the RNA tumor virus proteins. There may be some segmenting of the RNA such as is found with the togaviruses because in addition to the 35S mRNA other viral mRNA species of 20–22S and 14S are observed in infected cells (*Fan* and *Baltimore*, 1973; *Gielkens* et al., 1974; *Shanmugam* et al., 1974) and produce distinctly different viral proteins in an in vitro protein synthesizing system (*Gielkens* et al., 1976). Large amounts of the gs antigens are seen relative to the polymerase in the complete virions (*Davis* and *Rueckert*, 1972). In infected cells the gs-protein precursors Pr76 and Pr4 are easily observed, while immunoprecipitation yields little, if any, polymerase (*Eisenman* et al., 1975; *van Zaane* et al., 1975; *Arcement* et al., 1976; *Arlinghaus* et al., 1976). Other immunological studies indicate that the various viral antigens are not always under coordinate control (*Hanafusa* et al., 1974; *Strand* et al., 1974; *Smith* et al., 1976). Large picornaviral precursors (including the presumed complete translation product) are easily produced by treating infected cells with a variety of protease inhibitors. The same inhibitors show less of an effect on the production of the RNA tumor virus proteins (*Vogt* et al., 1975; *van Zaane* et al., 1975; *Arlinghaus* et al., 1976). Of course, these studies with the RNA tumor viruses all suffer because of unknown quantitative differences which may occur in the degree to which the various proteins will precipitate with antibodies to the disrupted virus.

H. Nature of the Processing Protease

A nonproducing RSV-transformed hamster cell line (HRSV(BH)) (*Sarma* et al., 1966) has been described which produces Pr76 but does not cleave this precursor to the mature gs-proteins (*Eisenman* et al., 1974). When the virus is rescued by fusion of the HRSV(BH) cells with permissive chick cells, the precursor is synthesized and cleaved as in a normal infection. This implies that the protein responsible for the cleavage is provided by the host cell.

The observation that the N-terminal amino acid residues of the capsid polypeptides of the picornaviruses fall into the class of α-helix breaking amino acids (*Lewis* and *Scheraga*, 1971) suggests that the precursor protein may be cleaved in β-bends to the left of these amino acids (*Bachrach*, 1976) (Table 2). The strongest breaker of the α-helix protein structure is the amino acid residue proline, which is found uniformly as the N-terminal amino acid of the major gs antigens avian p27 and murine p30 (Table 3). However, the analogy and this interpretation for the specificity of the cleavage site breaks down with the avian gs-proteins p12 and p15, which terminate with alanine and leucine, respectively, because both of these residues fall into the α-helix forming class of amino acids.

The only C-terminal amino acid thus far observed for the gs-antigens is leucine,

Table 3. Amino- and carboxyl- terminal amino acid sequences of the gs-polypeptides

Host	Virus	Avian p12	Avian p27 (Murine p30)	Avian p15
Avian	AMV[a]	Ala- ...	Pro-Val-Val- ... -Ala	Leu-Ala-Met- ... -Leu
Avian	PR-RSV[b]	Ala- ...	Pro-	Leu-
Murine	R-MuLV[c]		Pro-Leu-Arg- ... -Leu	
Murine	AKR[d]		Pro-Leu-Arg- ... -Leu	
Murine	WMLV[e]		Pro-Leu-Arg- ... -Leu	
Murine	NZB[f]		Pro-Leu-Arg- ... -Leu	
Rat	M-MSV[g]		Pro-Leu-Arg- ... -Leu	
Feline	FeLV[h]		Pro-Leu-Arg- ... -Leu	
Feline	RD-114[i]		Pro-Leu-Arg- ... -Leu	
Primate	BAB8-K		Pro-Leu-Arg- ... -Leu	

[a] Avian myeloblastosis virus (*Allen* et al., 1970; *Niall* et al., 1970; *Herman* et al., 1975). The protein p19 is cleaved from the amino end of the Pr76 precursor (*Vogt* et al., 1975). The amino terminus of p19 is blocked (*Herman* et al., 1975). This is analogous to VP4 of mengovirus which is derived from the N-terminal end of the capsid precursor (*Paucha* et al., 1974; *Butterworth* and *Rueckert'* 1972a) and has a blocked N-terminus (*Ziola* and *Scraba*, 1976). The amino acid sequences have been determined to a greater extent than shown here for most of these viruses.

[b] Prague strain of Rous sarcoma virus (*Herman* et al., 1975). The amino terminus of p19 was also blocked in this virus.

[c] Rauscher murine leukemia virus (*Oroszlan* et al., 1972).

[d] Virus from AKR mice (*Oroszlan* et al., 1973; *Oroszlan* et al., 1974).

[e] Wild mouse leukemia virus (*Oroszlan* et al., 1974).

[f] Virus from New Zealand black mice (*Oroszlan* et al., 1974).

[g] RaLV pseudotype of murine sarcoma virus (*Oroszlan* et al., 1974).

[h] Thielen strain of feline leukemia virus (*Oroszlan* et al., 1973).

[i] Endogenous cat virus (*Oroszlan* et al., 1973).

[j] Endogenous baboon virus (*Oroszlan* et al., 1975)

with the exception of p27 of AMV which terminates in alanine (Table 3). This suggests that these proteins are being cleaved by an enzyme with a specificity for the hydrophobic side chain of leucine, which also may be similar to the protease responsible for cleavage of the picornaviral capsid polypeptides (Table 2).

IX. Conclusion

The basic outlines of synthesis and proteolytic processing have been reviewed for the picornaviruses, alpha togaviruses, and RNA tumor viruses. Numerous other viruses employ the process of proteolytic cleavage to various extents and at different points in their replication cycles (*Korant*, 1975). Hopefully, the advances discussed here will provide insights and suggest experiments for the study of both other viruses and cellular mechanisms that use this system of proteolytic processing.

It is clear that much remains to be done before the details of these elaborate systems will be understood. Among the questions yet to be answered are:

1. To what extent is proteolytic processing used in the functioning of the cell?

2. What is the location and proportion of the viral genome used as mRNA? Are there small amounts of heretofore unrecognized necessary viral proteins synthesized from different regions of the genome that are not under the same controls as the major primary products?

3. Which host and viral proteases are responsible for the various cleavages observed?

4. What is the nature and specificity of the various cleavage sites?

5. Are the proteolytic cleavages used as control mechanisms to determine events such as the kinds and amounts of RNA synthesized?

6. Did these cleavage mechanisms evolve simply because mammalian cells do not have internal initiation mechanisms or are there special architectural considerations that require cleavage as an obligatory part of building the virion?

7. What is the evolutionary relationship among the many widely divergent viruses that seem to have retained the same basic system of protein cleavage?

8. Is it possible to find or design specific inhibitors of viral proteases that would be of therapeutic value in treating viral diseases?

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Heterophile Antigens and Antibodies in Medicine[1]

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I. Introduction

When an immune serum reacts with cells, tissues, or body fluids of species different from that which supplied the material for immunization, it is generally assumed that these cross-reactions are due to the sharing of antigen(s) or antigenic determinant(s) among various species, as pointed out by *Ehrlich* and *Morgenroth* as early as 1901. The cross-reacting antibodies can be divided into two categories: (1) those reacting only with antigens of closely related species — cross-reacting, species-specific antibodies and (2) those acting on antigens of unrelated species. Probably the first report to describe cross-reacting, species-specific antibodies was that of *Ehrlich* and *Morgenroth* in 1900. They observed that when rabbits were immunized with goat erythrocytes, the resulting antisera lysed sheep erythrocytes and vice versa. *Forssman* antibodies are the best known example of antibodies that act on antigens of unrelated species. In 1907, *Frouin* reported that rabbits im-

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munized with chicken egg yolk formed antibodies that lysed canine erythrocytes. Furthermore, he and *Lisbonne* (1911) clearly showed that the rabbit anti-egg yolk sera could lyse erythrocytes of sheep, horse, and goat, all of which are now known as *Forssman*-positive species. In 1911, *Forssman* reported a similar observation that rabbits immunized with aqueous suspensions of guinea pig, horse, or cat tissues formed antibodies which lysed erythrocytes of sheep, a species apparently unrelated to any of those from which immunizing antigens were derived. *Forssman* named these antibodies heterologous antibodies (*heterologe Antikörper*).

Soon after *Forssman*'s discovery, *Friedberger* and *Schiff* (1913) coined the term heterogenetic antibodies, while *Friedemann* (1917) used the term heterophile antibodies, to denote the *Forssman* antibodies. The terms heterogenetic and heterophile antibodies, however, have come to be used in a collective sense to denote not only *Forssman* antibodies but also all other antibodies which, disregarding phylogenetic order, react with cells or tissues of species apparently unrelated to that of the donor of antigen used for immunization (*Forssman*, 1930).

To distinguish heterophile (*Forssman*) antibodies from cross-reacting, species-specific antibodies, *Forssman* (1911) pointed out that anti-*Forssman* sera, such as anti-guinea pig kidney serum, gave lytic titers with sheep erythrocytes as high as those given by homologous antisera, i.e., antisera to sheep erythrocytes. In contrast, antisera containing cross-reacting, species-specific antibodies usually gave considerably lower titers with cells of heterologous species than the titers of homologous reactions. The degree of reactivity of such antisera with heterologous antigens follows phylogenetic order, i.e., the closer the species, the stronger the cross reaction. We have defined heterophile antibodies, following the original definition of *Forssman*, as antibodies acting upon an antigen that is apparently unrelated to the antigen used for immunization (*Milgrom* et al., 1975). Accordingly, heterophile antigen was defined as an antigen stimulating the formation of, or combining with, heterophile antibodies.

In this review, our discussion will be restricted to heterophile antigen systems well defined by antibodies that are formed by patients under certain clinical conditions. These are: *Paul-Bunnell* (P-B) (*Paul* and *Bunnell*, 1932) and other heterophile antibodies of infectious mononucleosis (IM) *Hanganutziu-Deicher* (H-D) antibodies (*Hanganutziu*, 1924; *Deicher*, 1926), which are related to injections of foreign species sera; heterophile antibodies resulting from allotransplantation (*Rapaport* et al., 1966); and *Forssman* antibodies (*Meyer*, 1922; *Schiff* and *Adelsberger*, 1924). The reader who is interested in antigens shared by human cells and microorganisms is referred to reviews by *Springer* (1971) and by *Lyampert* and *Danilova* (1975), since these subjects will not be covered in this review.

II. Heterophile Antigens and Antibodies in Infectious Mononucleosis (IM)

A. Development of Serodiagnostic Tests for IM

IM has attracted the interest of many immunologists, because patients suffering from this disease exhibit rather unique humoral, as well as cellular, immune

Table 1. Humoral antibody responses in IM

1. Heterophile antibodies
Paul-Bunnell type
Rhesus hemagglutinins
antimurine myeloma cell antibodies
2. Cold-acting antibodies
anti-i antibodies
cold lymphocytotoxins
Donath-Landsteiner antibodies
anti-T cell antibodies
3. Antiviral antibodies
NDVO agglutinins
anti-EBV antibodies
4. Other less frequent antibodies
antimuscle antibodies
antinuclear antibodies
rheumatoid factor

responses. Humoral antibodies of IM patients are summarized in Table 1. Of these antibodies, the (heterophile) P-B antibodies are of the greatest importance, since serodiagnosis of this disease depends mainly on the demonstration of these antibodies in the patients' sera.

In the late 1920's [quoted after *Davidsohn* (1972)], *Paul* was investigating agglutinins against sheep erythrocytes in sera of patients with rheumatic fever. He found that not only sera of some patients with rheumatic fever who had received injections of horse serum but also sera of four IM patients that he included inadvertently contained sheep hemagglutinins with high titers similar to those of sera of the patients injected with horse serum. Together with *Bunnell*, he published this observation in 1932. At the same time, *Davidsohn* was also interested in a sheep hemagglutinin resulting from injections of foreign species sera, which had been described by *Hanganutziu* in 1924 and by *Deicher* in 1926. It was generally believed at that time that any heterophile antibodies (sheep hemagglutinins) found in human pathologic sera were of *Forssman* nature. *Davidsohn* and *Walker* (1935) studied these heterophile antibodies in sera of IM patients and sera of patients who had had injections of foreign species sera. Upon absorption of these sera with boiled guinea pig kidney tissue, the richest source of the *Forssman* antigen, sheep hemagglutinins resulting from injections of foreign species sera were completely removed from the patients' sera, while agglutinins in IM patients' sera were not affected. Thus, the first and most important step toward the "differential" serodiagnosis of IM was established (*Davidsohn*, 1972). Later, *Davidsohn* (1938) added absorption of serum with bovine erythrocytes to absorption with guinea pig kidney and completed the so-called *Davidsohn*'s differential test for IM. Independently, *Bailey* and *Raffel* (1935) and *Stuart* (1935) were also able to differentiate the heterophile antibodies of IM from H-D antibodies on the basis of absorption tests.

Bailey and *Raffel* (1935) and *Stuart* (1935) introduced a new serologic test, namely, lysis of bovine erythrocytes for the demonstration of P-B antibodies.

However, this test was not used until 1951 for diagnostic purposes (*Mason*, 1951). Its value in the serodiagnosis of IM was confirmed by many investigators (*Leyton*, 1952; *Peterson* et al., 1956; *Mikkelsen* et al., 1958; *Ericson*, 1960; *Davidsohn* and *Lee*, 1964). This hemolytic test had obvious advantage over the sheep hemagglutination test, since the target, bovine erythrocytes, has no *Forssman* antigen and, therefore, *Forssman* antibodies need not be absorbed. However, another heterophile antigen, H-D antigen, is present on bovine erythrocytes, and the possible participation of H-D antibodies in bovine hemolysis tests still has to be excluded by absorbing sera with guinea pig kidney, which does not affect P-B antibodies.

In 1936, *Stuart* and his associates pointed out that IM sera also agglutinate or lyse horse erythrocytes. In the same year, *Beer* (1936) compared agglutinating or lytic titers of IM sera against erythrocytes of sheep, ox, goat, and horse and observed that the horse hemagglutinin titer was the highest. This was confirmed later by *Wilkinson* and *Carmichael* (1964). In their extensive studies, *Lee* et al. (1968) found that the serodiagnostic value of horse hemagglutination may be better than that of the sheep hemagglutination test, since the former differentiated IM from non-IM sera more sharply than the latter.

Treatment of sheep erythrocytes with papain (*Wöller*, 1956), plant proteases, receptor-destroying enzyme from *Vibrio cholerae*, and influenza viruses (*Springer* and *Rapaport*, 1957) inactivates P-B antigen selectively, leaving *Forssman* antigen and H-D antigen intact. Such cells could be utilized to remove heterophile antibodies other than P-B antibodies from IM sera. In this way, *Springer* and *Callahan* (1965) established what they called the plant protease test for serodiagnosis of IM. They claimed that any heterophile antibodies left in IM sera after the absorption with protease-treated sheep erythrocytes should be considered as P-B antibodies.

B. Incidence of Paul-Bunnell (P-B) Antibodies

P-B antibodies, defined by *Davidsohn* and *Walker* (1935) and *Davidsohn* (1938) as antibodies agglutinating sheep erythrocytes which are absorbable by bovine erythrocytes but not by guinea pig kidney, have been shown to be present in the sera of almost 100% of IM patients (*Hoagland*, 1952; *Leikowitz*, 1953). According to *Davidsohn's* 37 years of experience (1972), it is extremely rare to find true P-B antibodies in sera of patients suffering from a disease other than IM. The incidence of P-B antibodies in sera of the normal population was shown by *Barrett* (1941) to be less than 2%. *Hoagland's* (1972) data agreed with those of *Barrett*. More recently, *Virtanen* (1962) studied 321 randomly selected sera of blood donors in Finland and found that only three of them contained P-B antibodies. In all these studies, the titers of positive sera from "normal" individuals were very low, suggesting that these few individuals might have suffered from a mild form of IM prior to the tests.

It has been generally agreed that P-B antibodies in IM patients persist for a rather short period of time. According to *Hoagland's* data (1952), many IM patients become seronegative within the first month, and almost all patients within 3 months after the onset of the disease. There have been, however, a few exceptional cases

where P-B antibodies persisted for quite a long time. For example, *Kaufman* (1944) reported that P-B antibodies in a few IM patients persisted for 9 to 12 months. *Springer* and *Callahan* (1965) were also able to show P-B antibodies by means of their plant protease test in some IM patients 3–4 months after recovery from the disease.

The age distribution of P-B antibodies obviously follows that of the incidence of IM itself, since appearance of these antibodies almost always parallels characteristic clinical and hematologic findings of the disease. *Evans* (1972) studied over 3000 IM cases between 1965 and 1967 and found the following age distribution of P-B antibodies: 5–9 years, 4%; 10–14 years, 8%; 15–24 years, 80%; 25–29 years, 3%; and over 30 years, 5%.

An anamnestic response of P-B antibodies in IM patients or return of the disease occur exceptionally if at all. However, *Bender* (1958) and *Hoagland* (1963) found in some IM patients the resurgence of P-B antibodies during upper-respiratory infections several months or even a few years after the initial IM.

C. P-B Antigen-Antibody System

It has been known for some time that the antigen on bovine erythrocytes against which P-B antibodies are directed is by no means a single antigenic determinant. Since *Bailey* and *Raffel* (1935) introduced bovine hemolysin test for determination of P-B antibodies, it has been noted that the bovine hemolysins of IM sera could not be removed completely by absorption with sheep erythrocytes, while sheep hemagglutinins were totally absorbed with bovine erythrocytes (*Leyton*, 1952; *Eyquem* et al., 1955; *Strannegård* and *Lycke*, 1964).

Our own studies on P-B antigen by means of double diffusion in agar gel tests with stroma particles of bovine and sheep erythrocytes clearly showed that the agglutination line with bovine stromata extended as a spur over the line with sheep stromata (*Milgrom* and *Loza*, 1967). This indicated that bovine stromata possess more antigenic sites recognized by IM sera than do sheep stromata. This was further substantiated by the studies performed using crude stroma extracts rather than stroma particles (*Milgrom* et al., 1975b). As seen in Figure 1, separation of the bovine extract lines into two lines, one closer to the antigen well and the other closer to the IM serum well, was often observed. Furthermore, the reaction line

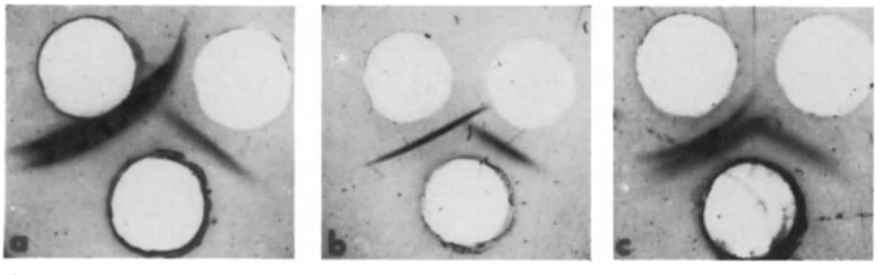

Fig. 1a–c. Upper wells in (a), (b), and (c): Bovine stroma extract (left); Sheep extract (right). Lower wells: IM sera Cw (2), La (b), and Fr (c).
(From *Milgrom* et al., Int. Archs Allergy *48*, 89 (1975))

formed with sheep or horse stroma extracts either merged into a reaction of complete identity or a reaction of partial identity with the bovine line closer to the IM serum well. In a very few instances, a reaction of nonidentity was noted between the sheep line and one of the bovine lines. On the basis of these results, it was concluded that P-B antigen is composed of at least two separate antigens, one which is present only on bovine erythrocytes and another which is shared completely or partially by sheep and equine erythrocytes. In addition, there exists a minor antigen apparently present on sheep erythrocytes but absent from bovine erythrocytes. However, this sheep-specific antigen was recognized by only a few IM sera.

The distribution of P-B antigen in cells and tissues of various mammalian species has been extensively studied in our laboratory for over 10 years. We have attempted to demonstrate the antigen on the surface of dissociated nucleated cells by means of cytolysis tests in agar gel and on cultured cells by mixed agglutination tests. It was found that oxen possess the antigen not only on erythrocytes but also on all other cells thus far examined, including cells of kidney (*Kano* and *Milgrom*, 1964), aortic intima, adrenal and spleen (*Juji* et al., 1971), as well as platelets (*Yoshida* et al., 1974). It should be mentioned that bovine plasma contains very little, if any, P-B antigen as evidenced by its failure to inhibit hemagglutination tests and to disperse agglutinates formed by P-B antibodies and sheep erythrocytes (unpublished data).

Of particular interest was the distribution of the antigen on murine lymphoid cells. It was shown that murine thymocytes and Thy-1 positive lymphoma cells were lysed by P-B antibodies, and that absorption of IM sera with murine thymocytes resulted in the disappearance of P-B antibodies (*Malave* et al., 1973; *Malave* and *Milgrom*, 1973). Significantly, the P-B antigen could not be demonstrated on spleen cells of nude mice (unpublished data). It was, therefore, concluded that the P-B antigen is a marker for murine T lymphocytes. In studying murine bone-marrow cells, it was shown that P-B antigen is also expressed on neutrophilic granulocytes (*Mori* et al., 1976). Reactions of IM sera with rat cells were weaker than those with murine cells, but the distribution of P-B antigen in rat was shown to be quite analogous to the above-described distribution in mice (*Malave* and *Milgrom*, 1973; *Mori* et al., 1976).

Although there has been no solid evidence presented for the presence of P-B antigen on cells of man, *Lowenthal* et al. (1973) showed that some adult lung tissues as well as fetal heart and thymus tissues inhibited the P-B antibody activity of IM sera. In this regard, it is of interest to note that Orientals never develop true P-B antibodies, even those patients who suffer from lymphoproliferative diseases similar to IM (*Kumagai* and *Kawakita*, 1962; *Tan*, 1967). Since many Caucasians, especially American soldiers who served during and after World War II in Asia, suffered from IM and formed P-B antibodies (*Kumagai* and *Kawakita*, 1962), the lack of P-B antibodies in Oriental patients could not be due to absence of the causative agent of IM in that particular geographic region. It is tempting to speculate that P-B antigen may be expressed in the early developmental stages of Orientals, and they are therefore unable to form P-B antibodies.

We have investigated the possibility that P-B antigen may be expressed on lymphoid cells of IM patients. Early experiments using peripheral blood lympho-

cytes of IM patients thus far failed. It appears likely, however, that at the time when specimens from such patients became available, P-B antibodies had reached their peak and combined with the antigen-carrying cells in vivo, so that many of such cells might have been already eliminated from the circulation. More recently, we have had an opportunity to investigate tissues of a patient with IM (*Andres* et al., 1976) who died of heart failure 12 days after the onset of IM when the P-B antibody titer had not yet reached its peak. P-B antibodies were eluted from spleen, liver, and kidney tissues of this patient. Histologic examination of kidney tissue revealed intense infiltration of mononuclear cells, including Downey cells, in interstitial tissues. IgM deposits in the mesangium of the kidney were demonstrated by indirect immunofluorescence staining with anti-IgM conjugate. The IgM deposits could be removed from the tissue sections by heat or acid elution procedures or by incubation with an excess of solubilized P-B antigen preparation. Furthermore, after the elution of the IgM deposits, we were able to reconstitute them by incubation of sections with other IM sera or with the eluate. P-B antibodies were also demonstrated in liver and spleen of this patient. On the basis of these studies, one may conclude that P-B antigen is present in tissues of IM patients in the early stages of the disease, and it forms complexes in vivo with the corresponding antibodies.

The expression of P-B antigen in human malignant lymphoid cells has been studied in our laboratory and by other investigators as well. These studies were initiated with established cultured lymphoid cell lines, many of which harbor *Epstein-Barr* virus (EBV). *Springer* (1972) tested EBV-infected P3J cells by hemagglutination-inhibition tests and failed to demonstrate P-B antigen. We have also tried to absorb P-B antibodies from IM sera with several lymphoid cell lines. The results of our preliminary absorption experiments appeared positive (*Malave* et al., 1972). On the other hand, *Lowenthal* et al. (1973) have shown that two of six fresh Burkitt lymphoma tissues could inhibit P-B antibody activity of IM sera. More recently, we have conducted absorption studies with spleens of patients with lymphomas or leukemias (*Milgrom* et al., 1973). As seen in Table 2, in which one of the protocols of such experiments was presented, extensive absorption of the IM serum with spleens of two patients with Hodgkin's disease removed P-B antibodies almost completely, and absorption with the reticulum-cell sarcoma spleen reduced their titers considerably. Similar absorptions with normal spleen did not affect significantly P-B antibody activity. Table 3 summarizes these absorption studies. Of 59 lymphoma-leukemia spleens examined, 26 (or 44%) reduced P-B antibody titers of IM sera upon extensive absorption, indicating that P-B antigen is expressed in lymphoid tissues of some lymphoma-leukemia patients (*Milgrom* et al., 1975a). It should be stressed, however, that patients suffering from these diseases never form P-B antibodies (*Milgrom* et al., 1975a). It may well be that some mechanism may be operating in these patients to prevent the formation of P-B antibodies or that the P-B antigen may be expressed in a nonimmunogenic form. Whatever the reasons may be, this problem certainly warrants further investigation.

More recently, attempts were made to demonstrate P-B antigen directly on dissociated spleen cells of patients with lymphoma or leukemia (*Kano* et al., 1976). Spleen cells were attached to poly-L-lysine-coated bottoms of wells of U-trays to form a "monolayer." After incubation of such monolayers with IM sera, binding

Table 2. Absorption of IM serum BG with pathologic and normal human spleen cells. Effect on lysis of bovine erythrocytes

Serum dilution 1:	BG serum absorbed with spleen of							Unabsorbed BG serum
	HD[a] ∓ 28	HD ∓ 28	HD ∓ 23–1	RCS[b] ∓ 17	CML[c] ∓ 30	normal man	normal ox	
20	+	++	+++	++	+++	+++	−	+++
40	−	+	+++	++	+++	+++	−	+++
80	−	−	++	++	+++	+++	−	+++
160	−	−	++	+	+++	+++	−	+++
320	−	−	+	−	++	++	−	++
640	−	−	+	−	++	++	−	++
1280	−		+	−	+	++	−	++
2560	−	−	−	−	+	+	−	++
5120	−	−	−	−	−	−	−	−

[a] Hodgkin's disease
[b] Reticulum cell sarcoma
[c] Chronic myelocytic leukemia

From: *Milgrom* et al., Int. Archs Allergy *45*, 633 (1973)

Table 3. Absorption of infectious mononucleosis sera with spleens of lymphomas or leukemias

	Number of spleen specimens which caused		Total
	Significant reduction of Paul-Bunnell antibody titer	No or insignificant reduction of Paul-Bunnell antibody titer	
Hodgkin's disease	11	14	25
Lymphosarcoma	6	5	11
Reticulum cell sarcoma	3	2	5
Unclassified lymphomas	1	3	4
Chronic lymphocytic leukemia	1	4	5
Chronic myelocytic leukemia	3	5	8
Acute myelocytic leukemia	1	0	1
Total	26	33	59
Various other diseases	1	19	20

From: *Milgrom* et al., Transpl. Proc. *VII*, 203 (1975)

of P-B antibodies and, therefore, detection of P-B antigen was achieved by reaction with sheep erythrocytes or trypsinized bovine erythrocytes which served as indicator cells. As shown in Figure 2, in a positive reaction, the indicator cells spread over the monolayer and folding of the edge of the cell sediment was observed. In a negative reaction, indicator cells were sedimented in the middle of the monolayer, forming a button-like sediment. By means of this technique, positive results were

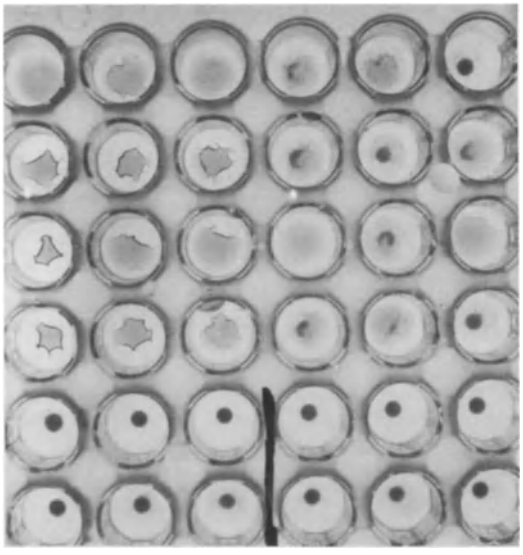

Fig. 2. Mixed agglutination in U tray: Spleen cells of a Hodgkin's patient were tested against four IM sera from the top row to the 4th row and two normal human sera in the 5th and 6th rows at dilutions of 1 : 40 to 1 280 (across from left to right). Trypsinized bovine erythrocytes were used as indicator cells

Table 4. Mixed agglutination with infectious mononucleosis sera and spleen cells of lymphomas or leukemias

	Number of positive spleen specimens at serum dilution of 1:								
	10	20	40	80	160	320	640	1280	Total
Hodgkin's disease	5	2		1	1	3	1		13
Lymphoma	1			1			1	1	4
Chronic myelocytic leukemia	3				1				4
Cancer	1	1							2
Hypersplenism	1								1
Various other diseases	12								12

From: *Kano* et al., Fed. Proc. *35*, 548 (1976) (abstract)

obtained with six of 13 Hodgkin's spleens, three of four lymphoma spleens, and one of four chronic myelocytic leukemia spleens (Table 4). Furthermore, indirect immunofluorescence tests with anti-IgM conjugate revealed that most positive Hodgkin's spleen cells had morphologic characteristics of giant cells (unpublished data).

The antigenic stimulus responsible for formation of P-B antibodies by IM patients still remains to be determined. One would speculate, however, two possibilities. One is that the causative agent of IM may carry P-B antigen which happens to be shared by erythrocytes and/or other cells of ox, sheep, horse, mouse, and other species. Although this possibility was not excluded, it appears to us unlikely, since sera of almost all patients suffering from viral or bacterial infections

thus far examined never contained true P-B antibodies as discussed earlier. Furthermore, there is no evidence for the presence of P-B antigen on EBV, generally considered the causative agent of IM. Significantly, in other diseases associated with EBV, Burkitt lymphoma and nasopharyngeal carcinoma, P-B antibodies have not been convincingly demonstrated. The other, more likely, possibility is that upon viral infection, the patient's own lymphoid cells undergo transformation, and P-B antigen is expressed as a neoantigen which stimulates formation of P-B antibodies by the patient. The prompt production of cytolytic IgM antibodies, such as P-B antibodies by IM patients, might eliminate or at least accelerate elimination of the antigen-carrying cells.

Shope and *Miller* (1973) reported that squirrel monkeys injected repeatedly with autologous lymphoblasts transformed by EBV formed low titers of sheep hemagglutinins which could be absorbed by bovine erythrocytes but not by guinea pig kidney tissues. They speculated that EBV induced P-B-like antigen in the processes of transformation of the monkey lymphocytes. One has, however, to consider the possibilities that the monkeys responded with heterophile antibody formation to infection with the EBV rather than to cells transformed in vitro by this virus. In this regard, it was of interest to note that immunization of man with sheep erythrocytes (*Leikola* and *Aho*, 1969) or with purified P-B antigenic preparations (*Springer* et al., 1972) resulted in formation of antibodies with P-B specificity, even though such immunization with whole sheep erythrocytes also stimulated production of IgG *Forssman* antibodies (*Leikola* and *Aho*, 1969). According to *Springer* (1972), those subjects who had experienced IM 1–5 years prior to the immunization responded to injection of P-B antigen preparations with an apparent anamnestic response.

The remarkable thermostability of P-B antigen of bovine erythrocytes was first noted by *Stuart* et al. (1936). Early studies by *Tomcsik* and *Schwarzweiss* (1947) and *Schwarzweiss* and *Tomcsik* (1948) demonstrated that P-B antigen could be solubilized from bovine erythrocytes by means of a hot 80% ethanol extraction. Their preparation contained glucosamine and glucose, and the P-B antigen in the preparation was thermostable and resistant to proteolytic digestion. *Markowitz* and *Simmonds* (1953), however, obtained by a similar procedure a P-B antigen preparation containing small amounts of carbohydrates, which was sensitive to protease. In 1964, *Strannegård* and *Lycke* succeeded in extraction of P-B antigen from autoclaved and mechanically disrupted bovine erythrocytes or sheep erythrocytes. In their double diffusion in gel tests with the extracts, they observed two separate precipitation lines formed by IM sera with bovine extract. One of these lines merged into a reaction of identity with a single line formed with sheep extract. As mentioned, this observation was fully confirmed by our own studies performed 10 years later (*Milgrom* et al., 1975b). By means of Sepharose 2B fractionation and sucrose density centrifugation, *Springer* and his coworkers (1969, 1972) further purified ethanol extracts of bovine erythrocytes. These investigators suggested that the P-B antigen in their preparation is most likely a glycoprotein but may be a glycolipid or a lipoglycoprotein. More recently, *Fletcher* and *Woolfolk* (1971, 1972) demonstrated that glycoproteins from horse and sheep erythrocytes possessed the P-B antigenic activity. Glycoproteins from horse and sheep erythrocytes were similar in amino acid and carbohydrate composition, while bovine glycoprotein

preparation contained one-third the sialic acid, one-half the total carbohydrate, and one and a half times the nitrogen content of the horse and sheep preparations. It should be pointed out that in all these studies the possible presence of glycolipids in the preparations was never excluded.

The IgM nature of P-B antibodies of the vast majority of IM patients is well established. In 1957, *Fudenberg* and *Kunkel* found P-B antibodies are of high molecular weight. *Lee* et al. (1963) studied the Ig nature of P-B antibodies by DEAE column chromatography, starch-zone electrophoresis, and ultracentrifugation. Results obtained by all these procedures and the 2-mercaptoethanol sensitivity of the antibodies clearly showed IgM nature of P-B antibodies. *Wollheim* and *Williams* (1966) showed that P-B antibodies comprise approximately 5% of the total IgM of IM sera which they studied. By means of immunoelectrophoresis using solubilized P-B antigen preparations, *Stannegård* and *Lycke* (1965) and we (*Milgrom* et al., 1975b) have demonstrated that a precipitation arc was formed with IgM of IM sera. In these studies, we have also found that serum of one patient did contain IgG P-B antibodies in addition to ordinary IgM antibodies, thus confirming previous observations made by *Springer* et al. (1974). Therefore, it may be stated that P-B antibody response in IM shows little or no IgM to IgG conversion, even during convalescence from the disease.

D. Other Antibodies in IM

It has been known for quite some time that a variety of humoral antibodies are formed by IM patients in addition to P-B antibodies (see Table 1). They are heterophile antibodies different from P-B antibodies, antibodies apparently directed against unaltered cell surface antigens of human cells and to viruses.

Sera of IM patients often contain agglutinins to erythrocytes of Rhesus monkeys. *Hoyt* and *Morrison* (1956) and *O'Connell* (1968) have shown elevated titers of Rhesus hemagglutinins in IM sera similar to viral hepatitis sera. Later, *O'Connell* (1970) clearly demonstrated, by means of absorption and inhibition studies, that the Rhesus hemagglutinins in IM sera are distinct from P-B antibodies.

Our recent studies (*Yoshida* et al., 1975) showed that 40% of IM sera lysed murine IgM myeloma cells, MOPC-104, as well as some subpopulation of normal murine B cells. The antibodies responsible for lysis of these cells were different from P-B antibodies. On the basis of absorption studies, three groups of antibodies acting on the myeloma cells were distinguished. Antibodies of the first group appeared infrequently (4%) and could not be absorbed with bovine erythrocytes, murine Thy-1 positive lymphoma cells, or guinea pig kidney. Antibodies of the second group appeared more frequently (13%) and could be absorbed only with the murine lymphoma cells. Antibodies of the third group appeared most frequently (24%) and could be absorbed with any of these three antigens. Antibodies of the third group, therefore, were similar to H-D antibodies.

Of the antibodies to human cell surface antigens found in IM, perhaps anti-i blood group antibodies (*Jenkins* et al., 1965; *Rosenfield* et al., 1965) could be of some clinical importance, since such antibodies are known to be one of the causes of autoimmune hemolytic anemia. However, in most cases of IM, anti-i antibodies

are present only in low titer and are active only at low temperatures (*Rosenfield* et al., 1965). Recently, *Wishart* and *Davey* (1973) reported the presence of *Donath-Landsteiner* cold hemolysins in some IM sera. *Mottironi* and *Terasaki* (1970) found, in IM sera, cold lymphocytotoxins reacting with a majority of human lymphocytes. Similar antibodies were also found in some viral diseases, such as rubella and measles. By means of indirect immunofluorescence tests, *Thomas* (1972) and *Thomas* and *Philips* (1973) found IgM antibodies in 25% of IM sera, which combined with human T cells at low temperature. Whether the cold lymphocytotoxins described by *Mittironi* and *Terasaki* are identical with these anti-T cell antibodies still remains to be determined, since the cold lymphocytotoxins were demonstrated by reactions with peripheral lymphocytes which are rich in T cells. *Holborow* et al. (1973) reported that they found antibodies to smooth muscle in sera of 81% of IM patients. These antibodies were frequently demonstrable in sera of children suspected of IM but having no P-B antibodies. *Carter* (1966) also found rheumatoid factor, antinuclear antibodies and *Wassermann* antibodies in the sera of some IM patients.

In 1946, *Burnet* and *Anderson* reported that human erythrocytes treated by the VIC strain of Newcastle disease virus (NDV) were agglutinated by sera of about 50% of IM cases. Although ample evidence was presented that the agglutinins against NDV-treated human erythrocytes were different from P-B antibodies, the question whether the agglutinins are directed against antigens of the virus itself or modified antigens of NDV-treated erythrocytes was not answered until 1967. By means of double diffusion in agar gel tests, *Barron* et al. (1967) demonstrated that 43 of 65 IM sera formed reaction lines with viral particles of the VIC strain of NDV. Furthermore, when the viral particles and bovine stroma particles were tested simultaneously against IM sera, crossing of lines in a nonidentity reaction was noted. Thus, it was proven that the NDV agglutinins are antibodies to the virion, and it was confirmed that they are distinct from the P-B antibodies.

In 1968, *Henle* and her co-workers presented convincing evidence that EBV may be the causative agent of IM. They observed that one of their technicians working on EBV fell ill with IM. Her pre-disease serum, along with sequential sera obtained during the illness, were examined for P-B antibodies and anti-EBV antibodies. Her pre-disease serum was negative, and the patient formed both types of antibodies during IM. This report has elicited great interest among many investigators and initiated an extensive search for seroepidemiologic evidence linking EBV not only with IM but also with Burkitt lymphoma and nasopharyngeal carcinoma (*Henle* et al., 1969; *Henle* et al., 1970; *Kawamura* et al., 1970).

With regard to the association of EBV and IM, *Henle's* report was immediately followed by several reports which confirmed or supported the original observation (*Niederman* et al., 1968; *Evans* et al., 1968; *Hirshaut* et al., 1969; *Banatvala* and *Grylls*, 1969; *Niederman* et al., 1970; *Sawyer* et al., 1971; *Evans* et al., 1968; *Evans*, 1972). According to the data on over 4000 college students, as summarized by *Evans* in 1972, IM occurred at a yearly rate of 6%. It was most interesting to learn that IM occurred only among those students who did not have anti-EBV antibodies on entering college, and that IM was not found among those students who had such antibodies. These and more recent experiments, which were summarized

by *Henle* et al. in 1974, brought very strong seroepidemiologic evidence that EBV is the causative agent of IM.

More recently, a series of studies to isolate EBV from the patients' material, such as throat washings (*Pereira* et al., 1972; *Miller* et al., 1973; *Niederman* et al., 1976) and peripheral blood (*Rickinson* et al., 1974), have been conducted. According to the results of these studies, EBV was indeed excreted into saliva and oropharynx of IM patients as evidenced by the ability of the throat washings to transform normal lymphocytes in vitro and the appearance of an EBV-associated antigens in the nucleus of the transformed cells. Co-cultivation of peripheral lymphocytes of IM patients with normal lymphocytes of cord blood resulted in transformation of the latter cells into continuous blastoid cell lines, indicating intercellular transfer of EBV. Finally, *Grace* et al. (1969) inoculated seronegative volunteers with EBV, and this was followed by the development of IM. *Kapsenberg* et al. (1970) reported a case of IM which apparently resulted from transfusion of infected blood. All this evidence very strongly supports the association of IM with EBV infection. For further discussion of the role of EBV in IM, the reader is referred to the excellent volume *Infectious Mononucleosis* by *Glade* (1972). It should be stressed again that the anti-EBV antibodies in IM patients are serologically distinct from the P-B antibodies, and that the formation of the latter is virtually always accompanied by clinical manifestation of IM.

III. Hanganutziu-Deicher (H-D) Antigen and Antibodies

In 1924, *Hanganutziu* reported that 12 patients who received therapeutic injections of horse antitoxins formed antibodies that agglutinated sheep erythrocytes at high titers. He claimed also that such sera may give false positive Wassermann tests due to agglutination of sensitized sheep erythrocytes. Independently, *Deicher* (1926) studied sera of 102 patients who were injected with horse or sheep antitoxins and found that sera of 90 patients had high titers of sheep hemagglutinins. Both *Hanganutziu* and *Deicher* observed that the newly discovered heterophile antibodies would agglutinate not only sheep erythrocytes but also erythrocytes of other species: ox, horse, pig, rabbit, and guinea pig. Even in the 1920's, it was obvious that antibodies under study are not of the *Forssman* nature, since they reacted with erythrocytes of *Forssman*-negative species, such as ox and rabbit.

To distinguish the antigen against which this type of heterophile antibodies is directed from the *Forssman* antigen, *Schiff* (1937) coined the term "serum-sickness" antigen, which had been broadly accepted, and the antibodies under discussion were customarily called "serum-sickness" antibodies. However, both *Hanganutziu* (1924) and *Deicher* (1926), as well as *Pirofsky* et al. (1973), pointed out that the appearance of these heterophile antibodies has no direct relationship to any of the clinical symptoms of serum sickness or to the volume of foreign species serum injected. Furthermore, our recent studies (*Kasukawa* et al., 1976) have demonstrated that several patients with various diseases, who had never received injections of foreign species serum, formed the same type of heterophile antibodies. Therefore, we proposed that this type of heterophile antibodies should

be called *Hanganutziu-Deicher* (H-D) antibodies rather than continuing the misnomer of serum-sickness antibodies.

A. Serology of H-D Antibodies

Except for those investigators who tried to find a cause of serum sickness, little interest was at first attached to the discovery of H-D antibodies. According to *Stuart* (1935), what puzzled some investigators was the incubation period required following the injection of foreign species serum before the onset of serum sickness. Prior to the discovery of H-D antibodies, *Taniguchi* (1921) suggested that *Forssman* antibodies preexisting at low titers in the patient's serum are boosted by injections of horse serum, and that this accounts for the incubation period and development of serum sickness. Although both *Hanganutziu* (1924) and *Deicher* (1926) failed to substantiate this hypothesis, *Ramsdell* and *Davidsohn* (1929) and *Davidsohn* (1929) still agreed with the *Taniguchi* view that the sheep hemagglutinins which they demonstrated in sera of patients with serum sickness were of *Forssman* nature and were responsible for the development of serum sickness.

The observation by *Hanganutziu* (1924) and *Deicher* (1926) that the heterophile antibodies which are engendered by injections of horse serum and which combine not only with sheep erythrocytes but also with erythrocytes of several other species was fully confirmed by *Kagan* in 1931. However, further serologic features that distinguish H-D antibodies from *Forssman* antibodies and P-B antibodies had to wait until 1935. *Stuart* et al. (1935) confirmed the above-mentioned observations and performed extensive absorption experiments using erythrocytes of various species. In particular, they found that the sheep agglutinins of IM were hardly influenced by repeated absorptions with rabbit erythrocytes. On the other hand, sheep agglutinins in sera of patients who were injected with horse serum were readily removed by absorption with rabbit erythrocytes. *Schiff* (1937) reasoned that since sera of patients injected with horse serum reacted with erythrocytes of various species, the corresponding antigen should also occur in sera of those species with "positive erythrocytes." Accordingly, he studied serum samples of patients who received rabbit antisera to pneumococcal polysaccharides and found heterophile antibodies serologically indistinguishable from those engendered by injections of horse serum.

The recent development of immunosuppressive therapy utilizing heterologous immune sera to human lymphocytes once again attracted the interest of some investigators in the H-D heterophile system. *Kashiwagi* et al. (1968) found that most patients who received ALS-formed sheep hemagglutinins. *Pirofsky* et al. (1973) noted that the majority of 46 patients who received goat antihuman thymocyte serum globulins formed heterophile antibodies without developing any signs of serum sickness.

Our recent investigations on H-D antibodies (*Kasukawa* et al., 1976) using double diffusion gel precipitation tests revealed that H-D antibodies react not only with bovine serum but also with serum of horse, rabbit, guinea pig, rat, and sheep, indicating wide distribution of their corresponding antigen. Evidence was also presented that H-D antibodies combine with purified fractions of the extract from

bovine erythrocyte stroma which is devoid of P-B antigen. We also showed that H-D antibodies react with tissue fractions resistant to *b*oiling and precipitable by *e*thanol (BE) fractions (*Milgrom* and *Witebsky*, 1962) of serum and organs from ox, rabbit, and guinea pig. Upon incubation of human erythrocytes lacking the H-D antigen with horse serum or bovine serum, such erythrocytes became agglutinable by H-D antibodies. Thus, blood plasma appeared to be the richest source of the H-D antigen which was absorbed by erythrocytes in vivo in a similar fashion as some blood group antigens, such as the J antigen of cattle (*Stromont*, 1949) and the R antigen of sheep (*Rendel* et al., 1954).

B. Nature of H-D Antigens

Tomcsik and *Schwarzweiss* (1948) were the first to isolate and characterize H-D antigen from bovine erythrocyte stromata. They obtained a fraction with H-D antigenic activity by means of extraction in boiling 100% ethanol. Their results clearly indicated that the H-D antigen is thermostable and most likely of lipopolysaccharide nature, which confirmed earlier studies by *Stuart* et al. (1936). Our recent studies, which will be published by *Merrick* et al., showed that the H-D antigen is present in a ganglioside fraction of bovine erythrocyte membrane, and the antigen is therefore most likely a glycolipid.

Immunoelectrophoretic studies on the H-D antigen in bovine serum revealed that the antigen is broadly distributed from γ globulin to the albumin region forming a single precipitation line with an H-D antibody-containing serum. When the above-mentioned ganglioside fractions or a thermostable, ethanol-insoluble fraction of bovine serum were tested, they formed precipitation lines only in the albumin region (*Kasukawa* et al., 1976). This latter observation confirmed the early observation by *Deicher* (1926), who found the H-D antigen in the albumin fraction of bovine serum.

IV. Heterophile Antibodies in Allogenic Transplantation

The importance of heterophile antigen-antibody systems in tissue allotransplantation was at first suggested by the discovery of cross reactions between pneumococcal polysaccharides and histocompatibility antigens of A strain mice (*Brent* et al., 1961). *Rapaport* and *Chase* (1964) provided cogent evidence that heat-killed, group A streptococci are able to induce homograft immunity in guinea pigs, which is indistinguishable from that induced by immunization with allogeneic tissues. In view of the fact that most major histocompatibility antigens have protein structure, the above-described cross reactions with bacterial antigens cannot possibly involve major histocompatibility antigens. In fact, *Brent* et al. (1961) stated that the effect of pneumococcal antigens on allograft rejection in mice was not particularly strong. More recently, *Hirata* and *Terasaki* (1970) reported that streptococcal M protein inhibits some human alloantisera with HLA antibodies. The confirmation of this finding indicating cross reactions between proteins of very distant species is eagerly awaited.

A. Heterophile Antibodies in Human Homotransplantation

In collaboration with *F. T. Rapaport*, we (*Rapaport* et al., 1966, 1968) have studied, for heterophile antibodies, sequential serum samples obtained from 49 individuals who received injections of allogeneic leukocyte preparations and skin grafts from the same donors. Of 49 individuals examined, post-treatment sera of 24 individuals showed at least eight-fold increase of the titer of antibodies against erythrocytes of one, two, or all three species studied: sheep, guinea pig, and rat. The peak titers of the heterophile antibodies usually appeared within one week after the skin grafting and were associated with severe graft rejection. The same serum specimens were studied for human blood group antibodies, but no significant increase in antibody titers of any known blood group specificity was observed (*Kuhns* et al., 1966). Similar studies were also performed on renal transplantation sera. Of 22 recipients examined, sera of 10 recipients showed at least an eight-fold increase in rat-hemagglutination titers.

Absorption experiments on selected transplantation sera with multiple hemagglutinins clearly showed that erythrocytes of rat or ox completely removed all antibodies acting on sheep, guinea pig, or rat erythrocytes. Immunochemical studies on the rat hemagglutinins indicated that the heterophile activity of the transplantation sera was associated with both IgG and IgM in some sera and with IgM only in other sera.

By means of antiglobulin consumption tests, *Iwasaki* et al. (1967) demonstrated IgG antibodies to sheep erythrocytes in sera of patients with renal allografts. They found that heterophile antibodies were demonstrable in sera of all 10 patients they studied during the first 4 months after transplantation, and in sera of 13 of 14 patients obtained between 4 months and 2 years after transplantation. Their absorption studies of the heterophile antibodies using "individual" kidney or liver tissue sediments showed that the heterophile antibody activities of the transplantation sera could be abolished by some, but not all, tissue specimens. Furthermore, absorption of transplantation sera with corresponding donor leukocytes resulted in the disappearance of heterophile antibodies.

McDonald (1973a) studied sera of 64 recipients of renal homografts for heterophile antibodies and attempted to assess the significance of these antibodies in graft rejection. According to his data, sensitization of the recipients to heterophile antigen(s) prior to transplantation was associated with rejection in six of eight cases. He also observed that acute rejection episodes were closely associated with a rising titer of heterophile antibodies. On the basis of these studies, he recommended frequent determinations of heterophile antibodies in renal graft recipients as an adjunct to other clinical tests for predicting acute rejection.

B. Serology of Transplantation Heterophile Antibodies

The results described thus far indicated that heterophile antibodies appear as a result of alloimmunization, i.e., transplantation of tissues or injections of allogeneic cells. However, the presence of similar antibodies in some recipients, presumably due to bacterial infections prior to transplantation, tended to confuse

the issue. In this regard, our initial studies (*Rapaport* et al., 1966, 1968) on healthy volunteers who received injections of allogenic leukocytes and subsequent skin grafts from the same donors and who had no immunosuppressive treatment, are of particular importance. The vast majority of their pretreatment sera contained only very minute amounts of heterophile antibodies. Daily inspection of the grafts assured us that none of them had suffered from apparent infections throughout these studies. As mentioned previously, absorption of such transplantation sera with erythrocytes not only of rat, but also ox, removed all antibodies acting on erythrocytes of rat, sheep, and guinea pig. This would mean that rat and bovine erythrocytes possess more antigens recognized by the transplantation sera than other erythrocytes.

Our subsequent studies utilizing agglutination in agar gel (*Milgrom* and *Loza*, 1967) were performed to elucidate the relationship between the antigen of bovine erythrocytes and that of rat erythrocytes. When a heterophile-positive transplantation serum was tested against sonicated stromata of trypsinized bovine erythrocytes and against rat stromata, a reaction of partial identity with a spur extending over the rat stroma line was observed (*Kano* and *Milgrom*, 1970). This would mean that heterophile transplantation antibodies recognized more antigenic sites on bovine than on rat stromata.

More recently, we have conducted similar studies on recipients of renal allografts who had never received injection of any foreign serum such as ALS (*Kano* et al., 1975). Instead of stroma particles, crude extracts prepared from bovine or sheep erythrocyte stromata were used to obtain more analytical data on specificities of the heterophile antibodies (*Milgrom* et al., 1975b). We found that post-transplantation sera of 12 of 32 recipients (37%) contained antibodies to the bovine extract, and five sera of 26 recipients (19%) had antibodies to the sheep extract. These results are in agreement with our previous studies (*Rapaport* et al., 1968) as well as studies of other investigators (*McDonald*, 1973a, b). Furthermore, we noted that transplantation sera from two different recipients frequently gave reactions of nonidentity or partial identity, while two serum samples from the from the same recipient usually gave complete identity reaction.

It was also shown that the antibodies reacting with bovine extract are serologically distinct from those reacting with sheep extract. Clear-cut evidence was also presented that the transplantation heterophile antibodies are different from P-B antibodies of IM.

Some HLA typing sera originating from multiparous women were also shown to contain similar antibodies reacting with bovine stroma extract. However, the antigen(s) recognized by the anti-HLA sera was not related to any HLA specificity. This would indicate that not only transplantation and alloimmunization but also pregnancy may result in the formation of heterophile antibodies. The significance of this observation remains to be explored.

Sequential serum samples of rabbits which received skin and kidney allografts were also studied for heterophile antibodies after skin and/or renal transplantation; three of eight such rabbits formed antibodies combining with bovine stroma extract. Absorption studies of the rabbit transplantation sera showed that donor's, but not recipient's, kidney tissue abolished the heterophile antibody activities in the sera. Thus, these results showed that heterophile antibodies under study are

transplantation alloantibodies and substantiated a similar conclusion reached by both *Iwasaki* et al. (1967) and *McDonald* (1973b) in their studies on human antibodies.

From our studies, expecially those performed by means of double diffusion in gel (*Kano* et al., 1970; *Kano* et al., 1975), it may be concluded that heterophile antibodies resulting from allografts are transplantation antibodies directed against antigens of human or rabbit nucleated cells which are shared by erythrocytes of some foreign species. An individual man or rabbit would possess only some of these antigens, whereas bovine erythrocytes represent most, if not all, of the "repertoire" of these antigens. Therefore, antibodies would be formed as a result of stimulation, with donor cells carrying some of these antigens missing in the recipient. These antibodies would have various specificities, depending on a particular donor-recipient combination with regard to the distribution of the antigens, but most of them would act on bovine erythrocytes.

V. Forssman Antigen and Antibodies

A. Forssman Antigen

What is now called *Forssman* antigen was originally described by *Froin* (1970) and rediscovered by *Forssman* (1911). Early investigations had been devoted mainly to examination of the distribution of the antigen in the animal and plant kingdoms and in bacteria (see *Boyd*, 1966). It is interesting to note that sheep possess the antigen only on erythrocytes and not in other tissues, whereas in guinea pig, this antigen is present in all tissues but erythrocytes. The failure to detect *Forssman* antigen on guinea pig erythrocytes by earlier investigators (*Forssman*, 1911; *Doerr* and *Pick*, 1913; *Buchbinder*, 1935) needs some clarification. *Witebsky*, in 1927, demonstrated the antigen on guinea pig erythrocytes by the complement fixation test. Our studies (*Juji* et al., 1971) using the technique of hemolysis in agar gel, confirmed *Witebsky's* observation. It may well be that the antigen exists on guinea pig erythrocytes in a small quantity and, therefore, is difficult to be demonstrated. In other *Forssman*-positive species, such as dog, cat, and chicken, the antigen is present on both erythrocytes and in other tissues (*Forssman*, 1930). From a practical point of view, it is also important to note the distribution of *Forssman* antigen, P-B antigen, and H-D antigen in sheep, ox, and guinea pig. As already indicated previously, *Forssman* antigen is present on sheep erythrocytes and in guinea pig tissues but not on bovine erythrocytes, while P-B antigen is present on sheep and bovine erythrocytes but not in guinea pig tissues, and H-D antigen appears in all three materials.

The exact location of the *Forssman* antigen in cells and tissues had not been elucidated until the mid-1950's when new serologic techniques became available. *Tanaka* and *Leduc* (1956), using fluorescein-conjugated rabbit anti-*Forssman* globulins, studied various organs of guinea pig, mouse, dog, cat, and chicken. They found that the antigen is present in the form of droplets in the endothelium and adventitial connective tissues in most organs of *Forssman*-positive species. Later, *Spear* (1962a) confirmed these observations by employing a similar technique. He

also studied cultured cells originating from guinea pig kidneys. Here, again, the antigen was found in the cytoplasm of the cells as doplets (*Spear*, 1962b). By means of the mixed agglutination test with rabbit anti-*Forssman* sera and papain-treated sheep erythrocytes as indicator cells, *Hawes* and *Coombs* (1960) demonstrated the *Forssman* antigen on membranes of various cells obtained from guinea pig organs. With the exception of smooth muscles and epithelium of jejunum, cells of all organs studied, such as heart, kidney, liver, spleen, lung, testis, etc., contained the antigen on their surface. Our studies (*Milgrom* et al., 1964) by the mixed agglutination test also demonstrated the antigen on monolayer cell cultures. *Spooner* and *Sell* (1966) demonstrated in vitro toxicity of *Forssman* antibodies for cell cultures originating from *Forssman*-positive animals. More recently, by means of cytolysis in agar gel, we (*Juji* et al., 1971) showed lysis of various nucleated cells of guinea pig, hamster, and mouse induced by *Forssman* antibodies and complement.

The presence of the antigen on vascular endothelium and perivascular connective tissues, as well as the above-mentioned in vitro cytotoxic action of the corresponding antibodies, would account for the well-known toxicity of anti-*Forssman* sera upon their intravenous injection into a guinea pig (*Friedberger*, 1909; *Doerr* and *Pick*, 1913) or the "carotid syndrome" induced by injection of *Forssman* antisera into the common carotid artery of a guinea pig in the direction of the heart (*Forssman*, '920; *Friedberger* and *Schröder*, 1921; *Ingvar*, 1927; *Milgrom* and *Golebiowska*, 1952; *Leibowitz* et al., 1961). *Baumann* and *Witebsky* (1934) described a very interesting reaction on a 2-day old chick embryo exposed to *Forssman* antibodies and complement. The vascular network of the chick embryo shrank and sunk into the yolk, and finally the embryo died when fresh *Forssman* antisera or even fresh normal rabbit serum containing natural *Forssman* antibodies was placed on the embryo.

The physicochemical nature of the *Forssman* antigen obtained from horse organs was first studied by *Doerr* and *Pick* (1913). They showed that the antigen was thermostable and ethanol resistant. Subsequently, *Sordelli* et al. (1918) obtained by ethanol extraction of horse organs a form of the antigen which was active in vitro but nonimmunogenic. They also recognized that the ethanol insoluble residue was fully immunogenic and elicited the formation of *Forssman* antibodies in rabbits. *Landsteiner* and his colleagues (*Landsteiner*, 1921; *Landsteiner* and *Simms*, 1923; *Landsteiner* and *Levene*, 1926) confirmed these observations and coined the term "hapten" which has been much used in immunology ever since. In its original sense, this term identified the ethanol soluble fraction of *Forssman*-positive tissues which was active in vitro but was not immunogenic. They reasoned that the *Forssman* antigen consisted of the hapten determinant, presumably a lipid, and a protein which was bound loosely to the hapten and was necessary for the immunogenicity. To prove this hypothesis, they demonstrated that the hapten could be converted to an immunogen by mixing it in vitro with various proteins foreign to the species of the immunized animal. This procedure of immunization was later used extensively by *Sachs* and his associates (1925 and 1929) and was called combined immunization (Kombinationsimmunisierung).

In 1936, *Brunius* found lipid and hexosamine in the preparation obtained by acid hydrolysis of the *Forssman* hapten of horse origin. *Makita* et al. (1966) isolated the *Forssman* hapten in a water soluble form from horse kidney and

spleen. They recognized the hapten as a glycosphingolipid. The structure of the *Forssman* hapten of horse origin was finally elucidated by *Siddaqui* and *Hakomori* in 1971. The *Forssman* hapten is a ceramide pentasaccharide with the following carbohydrate sequence: acetylgalactosaminyl-α-(1→3) N-acetylgalactosaminyl-β-(1→3) galactosyl-α-(1→4) galactosyl-β-(1→4) glucosyl-(1→1) ceramide.

The *Forssman* hapten with similar structure was isolated from sheep erythrocytes (*Fraser* and *Mallete*, 1974) and goat erythrocytes (*Taketomi* et al., 1975).

B. Forssman Antibodies in Human Sera

In the 1920's and 1930's, many investigators found sheep hemagglutinins in human sera, which they believed to be *Forssman* antibodies (*Schiff* and *Adelsberger*, 1924; *Buchbinder*, 1935). For example, *Meyer* (1922), found two sera with high titers of sheep hemagglutinins which interfered with the Wassermann test. *Kindermann* (1935) claimed that sera of 89% of normal individuals he examined contained low titers of sheep hemagglutinins. According to our present knowledge of heterophile antibodies, i.e., P-B antibodies and H-D antibodies, and blood group A antibodies, we would doubt that all sheep hemagglutinins discovered in the 1920's and 1930's were true *Forssman* antibodies.

Although the differential test for P-B antibodies was established in 1938 by *Davidsohn*, it appeared to be an impossible task to distinguish *Forssman* antibodies from anti-A antibodies because of the close antigenic relationship between the two corresponding antigens. In fact, earlier attempts at this endeavor (*Finland* and *Curnen*, 1938; *Davidsohn*, 1939; *Levine* et al., 1939) led to inconclusive and confusing results. The discovery of the sheep blood groups i, r, and R in 1954 by *Neimann-Sørensen* et al., however, seemed to resolve this confusion. Sheep erythrocytes of group R, but not of i or r, were shown to possess an antigen which was shared by human blood group A erythrocytes (*Rendel* et al., 1954). If this were the case, then one should be able to detect "true" *Forssman* antibodies in human sera by using i or r sheep erythrocytes and to obtain purified *Forssman* antibodies by elution of such erythrocytes following their incubation with human sera containing *Forssman* antibodies. *Flory* (1968) approached this problem by employing a variety of serologic procedures and found that sheep i and r erythrocytes contain not only *Forssman* antigen, but also A antigen, and that guinea pig kidney also contains the A antigen in addition to the *Forssman* antigen. Accordingly, the classic *Forssman* antiserum, rabbit anti-guinea pig kidney serum, contained both anti-A antibody which was inhibited by A_1 secretor saliva and another antibody, presumably *Forssman* antibody, which was not inhibited by A_1 saliva but acted on sheep erythrocytes. By an immunochemical approach using haptenic glycosphingolipids, *Kinsky* et al. (1969) also showed that only 65% of antibodies of rabbit anti-sheep erythrocyte serum were removed by liposomes containing purified *Forssman* hapten indicating that, obviously, such serum has other antibodies beside *Forssman* antibodies.

Our recent studies by hemolysis in agar gel on sera from lymphoma or leukemia patients showed that 26 of 63 sera contained sheep hemolysins of IgM and/or IgG nature (*Milgrom* et al., 1975). The specificity of these sheep hemolysins, however,

still remains to be determined. In 1974, *Joseph* et al. found a patient with *Waldenström* macroglobulinemia whose monoclonal IgM_K agglutinated sheep erythrocytes or lysed them in the presence of complement. The IgM did not react with cells of *Forssman*-negative species. Their subsequent studies showed that the IgM reacted only with purified *Forssman* hapten (globoside 1) but not with other haptenic glycosphingolipids lacking the *Forssman* hapten (*Alving* et al., 1974). The affinity of the IgM for the hapten, however, was much less than that of the IgM fraction of rabbit antiserum to sheep erythrocytes.

It would appear at present that the only reliable way to detect *Forssman* antibodies in human sera is to employ a rather complicated immunochemical procedure, such as complement-dependent glucose release from *Forssman* hapten-containing liposomal (model) membranes described by *Kinsky* (1972). For this test, highly purified *Forssman* hapten-containing glycosphingolipids has to be used along with similar preparations without the hapten as control.

VI. Closing Remarks

Heterophile antigens and antibodies have been known for almost 70 years. Still, it is always quite surprising for an immunologist to find "unexpected" crossreactions between antigens derived from remote species. *Landsteiner* (1946) noted many years ago that such unexpected crossreactions are given by carbohydrates rather than proteins. One is tempted to say that protein antigens behave "with dignity" in following the evolutionary pathway of development. The carbohydrates are those antigens that "play tricks," appearing in an unpredictable way. It should be stressed that the heterophile specificities are of considerable biological and medical importance. The major objective of this treatise was to convey this message.

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Relation of Herpes Simplex Viruses to Human Malignancies

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I. Introduction

We have been asked to provide our evaluation of the available data concerning the role of herpes simplex viruses in human malignancies. Perhaps we can begin by stating a set of criteria which, if fulfilled, would satisfy us that a particular virus was the causative agent of a particular malignancy. These requirements will form the framework around which the topic will be developed. Of course, we do not wish to imply that these criteria are original or that the form in which we have stated them is unique. These criteria include the following points: (1) There should be some epidemiologic or biological association or link between the malignancy and the suspected virus; (2) Purified preparations of the virus should induce tumors in its natural host or a similar species, and the resulting tumors should be similar to naturally occurring ones; (3) Ideally, one might hope to find similar naturally

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occurring malignancies in other animals caused by similar viruses; (4) The virus should transform cells in culture, and transformed cells should induce tumors similar to naturally occurring ones, and conversely, tumor cells cultured in vitro should be similar to transformed cells; (5) It should be possible to prevent the malignancy by preventing infection with the suspected virus. Needless to say, the different criteria carry different weight, and fulfillment of the second or fifth criteria stated above would provide very strong evidence for an etiologic role of the virus.

The first criterion is one of the most important and usually forms the basis for further investigations. An association between a malignancy and a particular virus can be made either by seroepidemiologic investigations or by molecular biological studies. Seroepidemiology should provide evidence for prior infection with the suspected agent and/or its continuous presence in the affected individual. Molecular biology should provide evidence for the presence of the suspected agent associated with the malignancy; it should be possible to detect virus, viral products (e.g., viral antigens, enzymes, or other proteins), or viral nucleic acids. Our belief that the detection of such viral markers in the tumor should be possible stems from the mass of experimental data obtained from studies with experimentally induced tumors and especially from studies with virus transformed cells.

The second criterion cannot be fully satisfied for a virus suspected of causing a human malignancy. However, induction of the disease, or a similar one, by the suspected virus in nonhuman primates would be important support for a causative role in humans.

Finding a similar malignancy in other animals caused by a similar virus would support the hypothesis that the virus induces the human malignancy, but absence of any similar disease in nonhuman species could certainly not be taken as contradicting the suspected etiology.

Studies designed to provide evidence relating to the fourth criterion are important, not so much in defining an etiologic interrelationship of the virus and the malignancy as in determining the mechanisms by which the malignancy might be induced. The in vitro demonstration of oncogenic transformation alone would not weigh very heavily in favor of an etiologic role of the virus in human malignancy, since examples of viruses which can induce transformation of cultured cells, yet play no apparent role in inducing malignancies under natural conditions, are too numerous to mention. If the cells transformed by the suspected virus are similar to cultured tumor cells and induce tumors similar to naturally occurring ones, then, but only then, might transformation be considered a significant indication that the virus could play a role in tumor induction under natural conditions.

The fifth criterion is not only a requirement which must be fulfilled in order to establish beyond doubt that a particular virus causes one or more human malignancies but is also or should be the ultimate goal in the search for the etiologic agents of human malignancies.

In the following sections we will consider each of the criteria in turn and attempt to evaluate the evidence that herpes simplex viruses fulfill the requirements of human cancer viruses. Our primary concern will be with herpes simplex virus type 2 (HSV-2), since the bulk of seroepidemiologic and clinical data concerns this virus as it relates to squamous cell carcinoma of the cervix. However, herpes simplex

virus type 1 (HSV-1) may also be related to squamous cell carcinomas of other sites, and considerable experimental work has been done on HSV-1, including studies which have lead to a somewhat better understanding of the biology of this virus than HSV-2. Studies of HSV-1 will be discussed in this article whenever relevant, on the assumption that from a molecular biological standpoint, the two viruses are similar.

II. Evidence Suggesting a Link Between HSV-2 and Squamous Cell Carcinoma of the Cervix

A. Epidemiologic Investigations

1. Derivation of Hypothesis

A number of attributes have been associated with an increased risk of developing cervical cancer. Included are such factors as age at first coitus, age at first marriage, number of marriages, and number of sex partners. The greatest relative risks are associated with early age at first coitus and multiple sex partners (*Rotkin*, 1973), and these two attributes are interdependent (*Rawls* et al., 1976). Venereal diseases occur with increased frequency among women who have multiple sex partners. On the basis of the data derived from the epidemiologic studies, it has been proposed that venereally transmitted factors are involved in the development of cervical cancer. The available evidence supporting this hypothesis has been recently reviewed by *Kessler* (1976).

The vast majority of herpetic lesions of the genitalia are caused by HSV-2, and there is little doubt that venereal transmission is the usual mode of spread of the virus. The data leading to this conclusion have been reviewed by *Josey* et al. (1972). *Rawls* and *Gardner* (1972), and by *Nahmias* and *Roizman* (1973). Basically, the evidence consists of the following observations: (1) In serologic surveys, antibody patterns associated with past infections by type 2 virus are found only in age groups of persons who have reached sexual maturity; (2) The highest age-specific rates of herpetic lesions of the genitalia are at the ages of greatest sexual activity; (3) The prevalence of genital infections, as determined by virus isolation from genital secretions, correlates with sexual promiscuity; HSV-2 can be isolated with greater frequency from vaginal secretions of prostitutes and women attending venereal disease clinics than from women attending clinics for nonvenereal diseases or cervical cytology clinics. In addition, herpes genitalis is not uncommonly associated with infections by other venereally transmitted agents; (4) The incidence of herpetic infections among sexual consorts of men with genital herpetic lesions has been found to be much greater than the incidence of infection among women exposed to men without herpetic lesions; (5) Finally, venereal transmission of the virus can be demonstrated experimentally in animals.

Infection early in life by HSV-2, with the subsequent development of neoplastic changes of the cervical epithelium, is not an unrealistic hypothesis to be derived from the epidemiologic similarities of the two entities. In fact, the development of anaplastic lesions in women infected by HSV-2 is not an uncommon event (*Naib* et al., 1973; *Nahmias* et al., 1973). However, infection by the virus and the occur-

rence of cervical neoplasia may be covariables and not necessarily etiologically linked. An association would be expected between any venereally transmitted agent and cervical cancer, and indeed, such an association has been reported for several such agents (*Alexander*, 1973). It is also conceivable that intercourse at an early age may predispose to cancer by virtue of the biological events occurring at the squamocolumnar junction during puberty (*Coppleson* et al., 1967). Since women who begin heterosexual activity early in life tend to have multiple sex partners, they are at greater risk of acquiring venereal diseases, and the association between a venereally transmitted agent and cervical neoplasia need not be an etiologic one.

The epidemiologic data data which deal with the interrelation of sexual promiscuity, cervical cancer, and HSV-2 are limited; however, they do suggest that the association between HSV-2 and cervical cancer does not simply represent one of covariability to sexual promiscuity. The frequencies of serologic evidence of syphilis, HSV-2, and the prevalence of trichomoniasis were determined in one case-control study (*Royston* and *Aurelian,* 1970a). While similar frequencies of syphilis and trichomonasis were found among women with cervical cancer and control women, a significantly greater frequency of HSV-2 antibodies was found among the cases than among the controls. Another approach has been to determine the influence of age at first coitus, age at first pregnancy, age at first marriage, number of marriages, number of sex partners, and number of live births on the distribution of HSV-2 antibodies among women with cervical cancer and control women. Controlling for one or all of these factors still left an excess of antibodies to the virus among the women with cancer (*Adam* et al., 1974). Finally, *Schneweis* and coworkers (1975) compared antibody activity to HSV-2 in women with cervical cancer and patients with syphilis; titers of antibody to HSV-2 were greater among the women with cervical cancer than among patients with syphilis.

2. Case-Control Studies (Antibodies to Surface Antigens)

Several types of information can be derived from serologic investigations. The presence of antibodies to a virus implies past infection, and by comparing the occurrence of antibodies among cancer cases and controls, an appreciation of the possible role of the virus in neoplastic development can be derived. An association between a virus and a cancer is also implied if cancer patients have unusually high titers of antibodies to the virus, if antibody titers fluctuate in relation to treatment, progession or recurrence of the cancer, or if antibody titers at the time of diagnosis correlate either positively or negatively with the clinical course of the disease.

Antigens to which neutralizing antibodies are directed are located in the virus envelope and appear on the surface of virus-infected cells (*Nahmias* and *Roizman*, 1973). To assess past infections by HSV-2, neutralizing antibodies to the virus have been assayed in a number of case-control studies. With few exceptions (*Rawls* et al., 1972), antibody activity to HSV-2 has been found with increased frequency among women with cervical cancer when compared to control women. This association between antibodies to HSV-2 and invasive carcinoma of the cervix has been demonstrated in Europe (*Sprecher-Goldberger* et al., 1970; *Skinner* et al., 1971; *Vestergaard* et al., 1972; *Janda* et al., 1973; *Kessler* et al., 1974; *Peltonen*, 1975;

Pasca et al., 1975; *Menczer* et al., 1975; *Christenson* and *Espmark*, 1976), North America (*Rawls* et al., 1969; *Nahmias* et al., 1970a; *Royston* and *Aurelian*, 1970a; *Plummer* and *Masterson*, 1971; *Adam* et al., 1972a; *McDonald* et al., 1974), South America (*Munoz* et al., 1975), Africa (*Adam* et al., 1972b; *Freedman* et al., 1974; *Adelusi* et al., 1975), Asia (*Kao* et al., 1974; *Kawana* et al., 1974), New Zealand (*Rawls* et al., 1972), and the West Indies (*Ory* et al., 1974). Thus, the association between the virus and the malignancy appears to be world wide. However, considerable variation has been found in the strength of the association between antibodies to HSV-2 and cervical cancer in different studies. Within each study, more women with cervical cancer had antibody patterns, thought to represent past infections with type 2 virus, than controls women, but between studies the proportion of cancer cases who were considered type 2 positive varied from 15% to 100%. This variation precludes drawing definitive conclusions regarding the etiologic role of the virus in cervical cancer from these seroepidemiologic studies.

Two major factors appear to contribute to the variations observed in the case-control studies. One factor is the immune response to the antigens of HSV-1 and HSV-2, and the other factor is variations in exposure to the two viruses in different socioeconomic settings. Major antigenic cross-reactivity is displayed by HSV-1 and HSV-2, and the assay systems presently available measure antibodies to cross-reacting antigens as well as type-specific antigens. The accuracy with which these assays detect all past infections by HSV-2 has not been established (*Plummer*, 1973). Evidence suggesting an interdependence of the immune response to HSV-1 and HSV-2 is available; *Smith* et al. (1972), found that the production of specific antibody to HSV-2 was much greater among patients without a prior type 1 infection than among patients who had previously been infected by type 1 virus. Quantitating cytolytic antibodies to cross-reacting and specific antigens by a ^{51}Cr release assay, *McClung* et al. (1976) found that about 80% of antibody activity produced following an initial infection with either HSV-1 or HSV-2 was to the cross-reacting antigen, and 20% of the antibody activity was to the specific antigens. *Skinner* and coworkers (1974) also demonstrated by immunoprecipitation that the major populations of antibodies present after infection with either HSV-1 or HSV-2 was to the crossreacting antigen. Patients infected initially with HSV-1 and then infected with HSV-2 were found to have increased amounts of antibody activity to the cross-reacting antigen, descreased antibody activity to the type 1 specific antigens, and little or no antibody to the type 2 specific antigen (*McClung* et al., 1976). The cross-reacting antigens of the two viruses are not identical, and antibodies developing after infection with HSV-2 appeared to bind more efficiently with HSV-2 than the cross-reacting antibodies present prior to infection. These observations suggest that most infections with HSV-2 occurring in persons previously infected by HSV-1 result in an anamnestic response to the shared cross-reacting antigen of the two viruses.

An increase in antibody which binds more efficiently to cross-reacting antigens would account for some of the observed variability in the results of case-control studies. In the majority of such studies, neutralizing antibodies to HSV-2 have been assayed either by assessing the kinetics of neutralization or by comparing antibody titers to HSV-1 and HSV-2. Greater differences in the occurrence of antibodies to HSV-2 between women with cervical cancer and control women have

Table 1. Titers of antibodies to HSV-1 and HSV-2 from selected case-control studies

Study area	Cases mean titers		Percent positive	Controls mean titers		Percent positive	Reference
	HSV-1	HSV-2	HSV-2	HSV-1	HSV-2	HSV-2	
Israel	1235	86	15	425	22	8	*Menczer* et al., 1975
Hungary	71	51	50	107	32	10	*Pasca* et al., 1976
Yugoslavia							
Moslem	112	42	35	115	33	23	*Kessler* et al., 1974
Others	126	51	40	112	41	25	
Czechoslovakia	59	26	50	46	11	21	*Janda* et al., 1973
Texas, USA							
White	170	89	64	135	56	43	*Adam* et al., 1972
Black	200	151	80	170	102	73	
Uganda[a]	204	155	93	224	144	72	*Rawls* et al., 1973

[a] Cases and controls age 40–59 yrs only included

generally been found when kinetic assays were used than when antibody titers to the two viruses were compared (*Rawls* et al., 1973). Since both antibody binding and antibody concentration are measured in the kinetics test, the assay probably detects past type 2 infection with greater accuracy than assays that measure antibody concentration only.

The potential effect of socioeconomic conditions upon the interpretation of case-control studies can be illustrated by comparing antibody titers to HSV-1 and HSV-2 in different populations. When antibody titers to HSV-1 and HSV-2 have been compared, the differences in antibody concentration to the two viruses has been used to formulate a criterion for positivity (*Plummer*, 1973). The concentration of antibodies to HSV-1 will influence the percentage of women who are considered positive for HSV-2 antibodies. This is illustrated in selected data from several studies (Table 1). For example, the mean geometic titers to HSV-2 were 86 and 51 for women with cervical cancer in Israel and Hungary, respectively. Despite the higher geometic mean titer for Israeli women, only 15% were considered positive for antibodies to HSV-2, while 50% of the Hungarian women were consiedered positive; this is because titers to HSV-1 were higher among Israeli women than among Hungarian women. The data presented in the table were produced in different laboratories, and exactly the same techniques were not necessarily used; thus, there are limitations in their comparative value. However, it would appear that variations in titers to HSV-1 in populations may contribute as much as variations in titers to HSV-2 in determining the percent of women considered to have been infected with type 2 in the past. Although we do not completely know all of the factors which contribute to the levels of antibodies to HSV-1 in individuals, the socioeconomic setting in which the individual lives appears to be one of them (*Black* et al., 1974). Thus, in different socioeconomic settings the percentage of women considered positive for past infections with HSV-2 may differ, while in reality the actual percentage of women infected may be

similar. This is especially true if a fixed criterion of positivity is applied in comparing the levels of antibody activity to HSV-1 and HSV-2.

An influence of relative titers to HSV-1 and HSV-2 on the percentage of population considered positive for past type 2 infections is also suggested by the analysis reported by *Adam* et al. (1974). For this the relative concentrations of antibodies to the two viruses were expressed as a II/I index which was calculated by dividing the $\log_{10}$ titer to HSV-2 by the $\log_{10}$ titer to HSV-1 and multiplying by 100. A II/I index of 85 or greater was found to maximally distinguish patients with clinically evident type 2 infections from patients with clinically evident type 1 infections. When the II/I index of 85 or greater was used as the criterion for past infections by HSV-2, 68%, 80%, and 93% of cervical cancer cases from Houston (Whites), Houston (Blacks), and Uganda were considered positive, respectively. The data were then re-analyzed by abandoning the II/I index of 85, which was derived from patients with clinically apparent lesions, and the II/I index, which maximally distinguished cancer cases from control women, was determined for each study group. This was called the threshold II/I index and was found to be 87, 93 and 89 for white women and black women from Houston and for Ugandan women, respectively. The threshold II/I index values were then used as the criteria for HSV-2 antibodies. The estimated occurrences of past HSV-2 infections did not change for white women from Houston (64%); however, for black women from Houston and Uganda the extimates dropped to 65% and 83%, respectively. As might be expected, the differences in HSV-2 positivity between cases and controls was enhanced in each study group. These observations imply that by comparing the relative titers of antibodies to the two viruses in cancer cases to those in control women within each study group, the estimated occurrence of past type 2 infections among cancer cases becomes more uniform between study groups.

The occurrence of antibodies to HSV-2 among women with preinvasive lesions of the cervix has also been examined. A significant association between antibodies to the virus and the presence of carcinoma in situ or dysplasia was found in some studies (*Aurelian* et al., 1970; *Nahmias* et al., 1970; *Catalano* and *Johnson*, 1971; *Skinner* et al., 1971; *Adam* et al., 1972a; *Heyerdahl*, 1974; *Ory* et al., 1975; *Pacas*, 1975; *Pelton*, 1975), while in other studies the differences in the occurrence of antibodies between women with cervical lesions and control women did not reach statistical significance (*Rawls* et al., 1969; *McDonald* et al., 1974; *Ory* et al., 1974). As with the studies of invasive carcinoma, no definitive conclusions can be drawn because of the problems involved in antibody assays. Despite the limitations of the data, however, we deem the results of the seroepidemiologic studies supportive of the hypothesis that HSV-2 is etiologically related to squamous cell carcinoma of the cervix. This opinion is based on the realization that the serologic assays used are only crude measures of past infections by HSV-2. We suspect that many women with high antibodies titers to HSV-1 do not, upon exposure to type 2 virus, respond sufficiently to be recognized by serologic means. It is quite possible that the occurrence of type 2 infections has been underestimated among women with cervical cancer and overestimated among control women in many of the investigations carried out in the past.

Noteworthy is the occurrence of women who have cervical cancer but do not have antibodies to either HSV-1 or HSV-2. If we assume an etiologic association

between HSV-2 and squamous cell carcinoma of the cervix, we would expect to find evidence of past infections by HSV-2 among all women with cancer. Even though the serologic assays used may not clearly detect all HSV-2 infections, a degree of antibody activity to HSV-1 and HSV-2 would be expected. The qualitative antibody status of 1218 cancer cases has been recorded in the literature, and 25 (2.1%) did not have detectable HSV antibodies. This compared to 76 of 1576 (4.8%) control women. Since the assay methods used were not exquisitely sensitive, the actual fraction of women without evidence of infection may be less. None of 400 only 3 of 477 (0.7%) cancer cases were found to be antibody negative by Dr. S. Subramanian, who used a sensitive antibody-dependent cellular cytotoxicity assay (personal communication). The existence of a small fraction of cancer cases without antibodies does not argue strongly against a carcinogenic role for the virus.

Several groups of investigators have reported a relation between antibodies to HSV-2 and the clinical status of neoplastic lesions of the cervix. *Catalano* and *Johnson* (1971) and *Skinner* et al. (1971) reported a decrease in antibodies to HSV-2 among women who had been treated for carcinoma in situ. An increase in antibody activity assessed by neutralization kinetics was found with greater frequency among women with progressing lesions of cervical dysplasia than among control women (*Sprecher-Goldberger* et al., 1973). *Thiry* et al. (1974) extended these observations and noted high or increasing neutralizing antibodies and decreasing or no complement-dependent cytotoxic antibodies to virus-infected cells among women with preinvasive cervical lesions which were progressing. This was in contrast to treated women and women whose lesions regressed; few showed an increase in neutralizing antibodies, and cytotoxic antibodies increased in the majority of these women. In another study, the long-term survival of women with invasive cancer of the cervix was correlated with neutralizing antibodies and antibodies to the surface of cells infected with HSV-2. Antibodies to the cell surface antigens were quantitated by a mixed hemadsorption assay (*Christenson* and *Espmark*, 1976). A low antibody titer to the surface antigens of infected cells was found predominately among women who subsequently died of their disease or who had recurrence. Titers of neutralizing antibodies to HSV-2 increased 6 to 18 months after therapy and then returned to pretreatment values. The data regarding fluctuating antibody activity could be interpretated as indicating the presence of HSV-2 antigens in the malignant cells. However, the validity of this conclusion awaits more precise definition of the assay techniques and of the antigens used.

3. Case-Control Studies (Antibodies to Other Antigens)

Impressive differences in the occurrence of antibodies to viral-induced antigens other than those involved in neutralization have been reported between women with cervical cancer and control women (*Aurelian* et al., 1973; *Hollinshead* et al., 1973; *Sabin* and *Tarro*, 1973; *Anzai* et al., 1975; *Notter* and *Docherty*, 1976). Certain features of the antigens and antibodies examined are presented in Table 2. An antigen harvested from cells 4 h after infection by HSV-2, called AG-4, has been used to detect antibodies by a complement-fixation test (*Aurelian* et al., 1973). Antibodies to AG-4 were detected in the sera of 35% of patients with cervical

Table 2. Properties of antigens and antibodies in "nonvirion" reactions

Property	Antigen		
	AG-4	HSV-TAA	VP134
Molecular weight	161,000	40–60,000	134,000
Localization in infected cell	Cytoplasm membrane	Membrane	Cytoplasm
Method of detection	Complementfixation	Complementfixation	Radioimmune precipitation
Antibody class	IgM	NT	IgG
Cancer specific:			
Cervix	Yes	No	No[a]
Squamous carcioma	No	Yes	
Present after therapy	No	Yes	Yes[a]

[a] Differences between women with cervical cancer and other women quantitative and not qualitative

dysplasia, 65% of patients with carcinoma in situ and 85% of patients with invasive carcinoma of the cervix. Furthermore, patients with cervical cancer who had been successfully treated did not have detectable antibodies to AG-4; however, with recurrence of the cancer, antibodies to the antigen could be detected. The antibodies were detected in only 0–12% of women in appropriate control groups and were not associated with cancers of anatomic sites other than the cervix.

AG-4 has been characterized as a molecule with a molecular weight of 161,000, and it co-migrates on polyacrylamide gel electrophoresis with a minor virion protein (*Aurelian* and *Strand*, 1976). Antibodies to AG-4 have been identified as IgM, while antibodies to the virus are of the IgG class. Using IgM fractioned from sera which contained antibodies to AG-4, immunofluorescent studies revealed staining in the cytoplasm and membranes of cells infected by HSV-2. IgM antibodies to an early-virus-induced antigen were also found with higher frequency in sera from women with cervical cancer than in sera from control women by *Schneweis* et al. (1975). It has been concluded that AG-4 is localized primarily in the cytoplasm of infected cells and probably also on the cell surface (*Aurelian* et al., 1976). The correlation between the presence of antibodies to AG-4 and the presence of cervical neoplastic lesions along with the reactivity of extracts of tumor tissue with AG-4 positive sera constitutes the main evidence that AG-4 is present in the neoplastic cells (*Aurelian* et al., 1973).

A different antigen prepared from cells of squamous carcinoma of the genital tract was found to react in a complement-fixation test with sera from patients with cervical carcinoma (*Hollinshead* et al., 1972). This antigen was prepared by sonicating the cell membranes and subsequently purifying the solubilized proteins. Using a similar technique, proteins with similar antigenic reactivity and migration

characteristics on polyacrylamide gel electrophoresis were subsequently isolated from cells infected with HSV-1 or HSV-2 (*Hollinshead* et al., 1974). Antibodies to this virus-induced antigen were found among patients with cervical cancer and squamous cell carcinomas of other sites. The titers of antibodies to the induced antigens did not correlate with neutralizing antibodies to HSV-1 or HSV-2 and were detected in patients who had been successfully treated as well as those with active invasive cancers (*Hollinshead* et al., 1973). In a recently published review, the name herpes simplex virus tumor-associated antigen (HSV-TAA) was proposed (*Hollinshead* et al., 1976). The antigenically active region of the polyacrylamide gel upon which the antigen was prepared contained two proteins with apparent molecular weights of about 40–60,000. Of the 141 patients with squamous carcinoma studied, 127 (90%) had detectable antibodies to HSV-TAA. This compared to 3 of 67 (4%) of normal subjects and 4 of 36 (11%) patients with nonsquamous cancer who had detectable antibodies. The presence of antibodies to HSV-TAA appears to correlate with the depressed responsiveness of lymphocytes of patients with squamous cell cancers (*Silverman* et al., 1976). The relatedness of HSV-TAA to virion proteins has yet to be established.

Antibodies to an unstable antigen induced by HSV-1 and HSV-2 were found in patients with a variety of malignancies, including cervical carcinoma (*Sabin* and *Tarro*, 1973). *Sabin* (1974) subsequently reported that he was unable to confirm the observations, althogh a relatedness between the labile antigen and HSV-TAA has been suggested (*Hollinshead* and *Tarro*, 1973).

Using radiolabeled protein synthesized early during the infectious cycle, *Anzari* et al. (1975) examined sera from cancer patients for antibodies to the nonvirion antigens. The assay consisted of reacting the antigen preparation with the serum, precipitating the antigen-antibody complex with goat antihuman IgG, solubilizing the precipitate and electrophoresing it on polyacrylamide gel. The major polypeptide synthesized early has a molecular weight of 134,000 (VP134), and the relative amounts of VP134 precipitated by the sera were calculated. Quantitatively, sera from cervical cancer patients precipitated more VP134 than sera from patients with breast cancer or from control women who did not have cancer (*Melnick* et al., 1976).

Recently, *Notter* and *Docherty* (1976) compared AG-4 and HSV-TAA using the same set of sera. They found antibodies to AG-4 and to HSV-TAA in 78% and 82%, respectively, of sera from cervical cancer patients, while reactions were observed in 13–14% of sera from normal subjects. They confirmed the reaction of HSV-TAA with sera from patients with squamous cell carcinomas of anatomic sites other than the cervix. They also found similar reactivity of these sera with AG-4, which is in contrast to earlier reports which suggested that antibodies to AG-4 were found only among patients with cervical cancer. Sera from patients with nonsquamous malignancies reacted to both antigens with the same frequency as sera from normal subjects. While the numbers studied were small, sera from patients who had been treated for cervical cancer reacted with both HSV-TAA and AG-4 — an observation which confirms the published data on HSV-TAA but which is at variance with the published reports regarding the absence of antibodies to AG-4 among successfully treated patients.

The significance of the high occurrence among cancer cases of antibodies to

HSV-TAA and/or AG-4 antigens is not clear. The antigens could represent "fetal" antigens induced by the virus and the association of antibodies to these antigens, and the neoplastic lesions would not necessarily indicate that the virus was etiologically related to the cancer. Although not proven, it seems unlikely that HSV-TAA or AG-4 represent fetal antigens (*Aurelian* et al., 1976; *Hollinshead* et al., 1976). If the antigens are coded for by viral genes, they may or may not reside in cancer cells. Antibodies to these antigens could simply denote repeated exposure of persons to viral antigens through recurrence and/or reinfection. Even if this were the case, the observed excess of antibodies to these antigens among cancer patients would be supportive of an etiologic role of the virus. Should these antigens be viral coded and present in the cancer cells, the observed distribution of antibodies in cases and controls weighs heavily in favor of a hypothesis in which the virus plays an etiologic role.

B. Molecular Biological Studies

Attempts to detect the presence of intact HSV-1 or HSV-2 specific markers (antigens, enzymes, DNA, or RNA) in human tumors have so far failed to provide convincing evidence for an association between the viruses and any human malignancy.

1. Viral Antigen in Human Tumors

The existence of viral antigens in cervical cancer cells has not been unequivocally established. Using antiserum prepared in rabbits to HSV-2-infected HEP-2 cell debris, *Royston* and *Aurelian* (1970b) searched by indirect immunofluorescence for antigens in neoplastic cells exfoliated from cervical lesions. From 20% to 48% of dyskaryotic cells stained when HSV-2 antiserum was used, while 0–20% of the cells stained when diluent instead of antiserum was used in the reaction; from 10% to 48% of the cells were considered positive for HSV-2 antigens. However, biopsies of neoplastic lesions of the cervix were found not to contain antigens when examined by immunofluorescence using the same reagents (*Aurelian* et al., 1972). *Pasca* and coworkers (1975) were unable to find specific reactions for HSV-2 antigens in biopsies of cervical neoplastic lesions. Using indirect immunofluorescence, *Nahmias* and coworkers (1975) could not demonstrate antigens in biopsy material; however, between 2% and 54% of cells were positive in 25 of 40 specimens examined by an anticomplement immunofluorescence test. A definition of the nature of antigens detected by immunofluorescence and their relatedness to the antigens extracted from cancer and detected by complement fixation (*Hollinshead* and *Tarro*, 1973) will be required before their significance can be appreciated.

2. Viral Nucleic Acids in Human Tumors

The use of molecular probes to detect herpes simplex virus DNA or RNA in human tumors has also provided somewhat equivocal results. *Frenkel* et al. (1972) reported the detection of HSV-2-specific DNA and RNA in a biopsy of a human

cervical carcinoma. Using DNA-DNA hybridization to measure the rate of reassociation between radioactively labelled HSV-2 DNA and tumor cell DNA, *Frenkel* et al. (1972) found that a segment representing 39% of the HSV-2 genome was present in the tumor cells at a concentration of 3.5 copies per diploid cell. Rapidly reassociating tumor cell DNA was enriched in some HSV-2 sequences, which was taken as evidence that part of the HSV-2 DNA was covalently linked to cellular repetitive sequences. Although extremely promising, the results of *Frenkel* et al. were obtained from analysis of a single cervical carcinoma, and controls did not include DNA from normal cervical tissues. Unfortunately, the promise of these studies has not been fulfilled by more extensive studies carried out by other workers. *Zur Hausen* et al. (1974) attempted unsuccessfully to detect HSV-1 or HSV-2 sequences in DNA extracted from biopsies of 10 human cervical carcinomas by hybridization between tumor cell DNA and ^{3}H RNA synthesized in vitro on the viral DNA template (cRNA). The sensitivity of the assay was estimated adequate to detect the equivalent of one HSV genome per diploid cell. Negative results were reported for an additional 46 cervical carcinoma biopsies by *Schulte-Holthausen* (1975), who used assays with sensitivities between 0.2 and 1 HSV genome equivalent per cell. Finally, *Pagano* (1975) used DNA reassociation kinetics with a sensitivity of as little as 0.25 viral genomes per cell but found no evidence for the presence of HSV-2 sequences in three cervical carcinomas. Thus, the bulk of molecular hybridization studies have so far failed to provide evidence for the persistence of HSV-2 sequences in cervical cancers.

III. Tumor Induction by Herpes Simplex Viruses

The classification of herpes simplex viruses as tumor viruses should, strictly peaking, be based on induction of tumors in animals. Unfortunately, this is an area of research with HSV-2 which is as yet rather undeveloped. *Nahmias* et al. (1970b) found that inoculation of newborn hamsters with HSV-1 or HSV-2 resulted in almost 100% mortality at virus doses greater than 10^3 TCD50, a finding that was in agreement with previous studies (*Rapp* and *Falk*, 1964; *Trentin* et al., 1969). At lower doses of live virus, or with UV inactivated virus, the 3-week survival was approximately one-third or three-quarters, respectively. Tumors (undifferentiated sarcomas) were observed 5–28 months after inoculation in a small percentage (2–3%) of animals receiving HSV-2 (live or UV irradiated) but not in animals inoculated with HSV-1. One control animal (out of 101) developed a well-differentiated sarcoma. Attempts to detect HSV-2 specific antibodies in the sera of tumor-bearing animals yielded negative results. Furthermore, neither intact HSV-2 nor HSV-2 antigens could be detected in any of the tumors. Consequently, the role of HSV-2 in the induction of these tumors is uncertain (see also *Munoz*, 1973; *Sever*, 1973; *Wentz* et al., 1975; *Palmer* et al., 1976).

IV. Analogous Malignancies

There is no known malignancy in nonhuman species which is analogous to squamous cell carcinoma of the cervix. A venereally transmitted tumor occurs in

dogs, but the tumor is histologically a lymphosarcoma, and there is no definite evidence of a viral cause (*Orial* and *Hayward*, 1974). It is clear, however, that other members of the herpesvirus group can induce malignancies (reviewed by *Rapp*, 1974). The lymphoreticuloproliferative disease of chickens, known as Marek's disease, is caused by a herpesvirus and can be prevented by vaccination. Herpesvirus-induced lymphomas have also been described in South American monkeys, cottontail rabbits, and guinea pigs. There is good evidence that the Lucké tumor of frogs, an adenocarcinoma, is caused by a herpesvirus. In man, Epstein-Barr virus is associated with both lymphomatous lesions, African Burkitt's lymphoma, and an anaplastic or poorly differentiated carcinoma of the nasopharynx (see *Epstein*, 1976). Unfortunately, there are not enough similarities between these lesions and carcinoma of the cervix to serve as a strong argument for or against the role of HSV-2 in oncogenesis.

V. Transformation by HSV

A. Oncogenic Transformation

Oncogenic transformation of cultured cells is generally considered to be analogous to (or at worst a model system for) the induction of tumors in animals. Accordingly, in vitro transformation of cells affords the possibility of studying processes which are probably similar to those occurring during at least the initial stages of tumor induction. Since cultured cells can exhibit spontaneous transformations or alterations, often leading to oncogenic potential, it is usual to test for the presence of specific viral markers, such as antigens, RNA, or DNA, in virus-transformed cells to distinguish them from spontaneously arising variants. Transformation assays for DNA viruses are usually carried out in nonpermissive cells in which viral replication is blocked but transforming functions are expressed. One of the principle difficulties in studying transformation by the herpes simplex viruses is that no cultured cells have been found which are totally nonpermissive for either HSV-1 or HSV-2. Consequently, in order to avoid viral replication and lysis of infected cells, it has been necessary to eliminate viral infectivity without significantly affecting transforming activity. This has been done in a variety of ways. *Duff* and *Rapp* (1971a, b) used UV-inactivated HSV-2 to induce transformation of primary hamster embryo fibroblasts and obtained foci of morphologically transformed cells from which permanent cell lines could be established. The efficiency of transformation in these early studies appeared to be rather low and was not precisely quantitated. Not more than one or two transformed foci were induced per 5×10^6 cells infected at a multiplicity of infection equivalent to 1 PFU (pre-irradiation titer)/cell. Of 15 HSV-2 isolates tested, seven were found to have detectable transforming potential (*Rapp* and *Duff*, 1973); however, in view of the low numbers of foci detected, these differences may be quantitative rather than qualitative.

Transformation of hamster embryo fibroblasts by UV-irradiated HSV-1 has also been obtained with two out of 12 HSV-1 strains (*Rapp* and *Duff*, 1973; *Duff* and *Rapp*, 1973). In contrast to HSV-2-transformed cells which were predominatly

fibroblastic and induced fibrosarcomas in hamsters (*Duff* and *Rapp*, 1971a, b; *Duff* et al., 1974), HSV-1-transformed cells had a predominantly epithelioid morphology. Two transformed lines (one for each of the transforming HSV-1 strains) were tested for oncogenicity in newborn hamsters, and one was found to be oncogenic. The tumors induced by HSV-1-transformed cells, which appeared in 47% of treated hamsters, were similar to adenocarcinomas. Sera from these animals had a low level of neutralizing antibodies against HSV-1 and HSV-2. Most of the studies on the oncogenic properties of herpes simplex virus transformed cells were carried out with one line, transformed by HSV-2, and designated 333-8-9. It was found that inoculation of these cells into newborn hamsters resulted in induction of tumors in 30–40% of surviving animals within 10–16 weeks. Later studies (*Rapp* and *Duff*, 1973) resulted in the isolation of more highly oncogenic lines. With these, tumor induction was essentially 100%, and the latent period only 2–4 weeks. In addition, some transformed lines were established which did not induce tumors following injection into newborn hamsters. *Rapp* and *Duff* (1973) classified herpes simplex virus transformed cells into three groups: highly oncogenic, moderately oncogenic, and nononcogenic, but no cellular characteristics are yet known which might explain these differences in oncogenicity. One observation of interest was that in vivo passage of transformed cells significantly increased their oncogenic potential, a finding which has frequently been noted in other systems. Infectious virus could not be isolated from either transformed cells or the induced tumors, consistent with the method used to obtain transformation. However, transformed cells (333-8-9 line) contained viral antigens as indicated by indirect immunofluorescence with antisera prepared against HSV-1 or HSV-2 (*Duff* and *Rapp*, 1971), Diffuse cytoplasmic staining was observed in 1–10% of transformed cells, while with normal hamster serum or serum against SV40 T antigen, no immunofluorescence was detected. Finally, HSV neutralizing antibodies to HSV-1 and HSV-2 were present in sera from tumor-bearing hamsters (*Duff* and *Rapp*, 1971; *Rapp* and *Duff*, 1973).

A second method of inactivating herpes simplex virus infectivity has also permitted the demonstration of transforming activity and yielded results similar to those obtained with UV inactivation (*Rapp* et al., 1973; *Rapp* and *Li*, 1975; *Li* et al., 1975). It was found that inactivation with visible light of neutral red treated HSV-1 or HSV-2 resulted in virus preparations with transforming capability. As was the case with UV inactivation, the resulting transformed cell lines were oncogenic.

RNA-DNA hybridization studies detected the presence of virus-specific RNA in 333-8-0 cells which hybridized to both HSV-1 and HSV-2 DNA. Transformed-cell RNA competed with only 10–13% of RNA from infected cells, indicating that transcription of the HSV-2 DNA in the transformed cells was limited to a relatively small portion of the viral genome (*Collard* et al., 1973). Analysis by DNA-reassociation kinetics of several HSV-2 transformed hamster cell lines, including the 333-8-9 line, revealed that viral DNA sequences were present in all of the lines in amounts varying from 8% to 32% of the viral genome at levels of one to three copies per cell (*Frenkel* et al., 1976). Two parallel passages of the same transformed cell line differed in their content of viral DNA, and, of particular interest, one hamster tumor as well as a tumor-drived cell line, contained a smaller portion of

the viral genome than the serially passaged parental line. *Frenkel* et al. pointed to the need for highly sensitive probes for the detection of such small amounts of viral DNA in transformed or tumor cells. Thus, the failure to detect HSV-2 sequences in hamster cells transformed by HSV-2 (*Davis* and *Kingsbury*, 1976) could be due simply to insufficient sensitivity of the methods used, and the same may be true for failure to detect HSV-2 sequences in human tumors (*Zur Hausen* et al., 1974; *Schulte-Holthausen*, 1975; *Pagano*, 1975).

Additional evidence for the presence of functional HSV-2 viral products comes from studies on the growth of HSV-2 *ts* mutants in HSV-2-transformed cells (*Kimura* et al., 1974; *Benyesh-Melnick* et al., 1974). Two temperature sensitive (*ts*) mutants of eight tested showed enhanced growth at the nonpermissive temperature in hamster cells transformed by HSV-2 as compared to normal hamster embryo fibroblasts. These two mutants are capable of synthesizing DNA, and the *ts* functions appear to be located in the same region of the genetic map. This suggests that the transformed lines contain or express only a specific part of the HSV-2 genome.

Morphologic transformation of a continuous line of hamster cells was observed by *Kutinova* et al. (1973) following infection of the cells with UV-inactivated HSV-2. The resulting transformed line expressed viral antigens and was more highly oncogenic than the parental line. Sera from tumor-bearing animals contained neutralizing antibodies against HSV-2. Recently, *Boyd* and *Orme* (1975) succeeded in transforming a nononcogenic 3T3-like line of Balb/c mouse cells with UV-inactivated HSV-2. One HSV-2-transformed fibroblastic line was isolated; these cells were oncogenic in Balb/c mice, inducing fibrosarcomas in 100% of animals injected with 10^6 cells. Indirect immunofluorescence with HSV-2 antiserum suggested the presence of virus-specific antigens in the cytoplasm of the transformed cells, while sera from tumor-bearing mice contained neutralizing antibodies. Attempts to rescue virus from the transformed cells or to detect HSV particles by electron microscopy were unsuccessful.

Transformation of human embryonic lung cells with infectious HSV-2 has also been reported (*Darai* and *Munk*, 1973; *Munk* and *Darai*, 1973). Cells were infected and incubated for 8 days at 42°C, a temperature which is nonpermissive for viral replication but at which some infected cells apparently survive. The transformed cells formed syncytia, appeared to contain viral antigens in their cytoplasm, and were resistant to superinfection with HSV-2.

The use of *ts* HSV mutants to transform cells at temperatures nonpermissive for lytic replication has been reported by several investigators. *Macnab* (1974) obtained transformation of rat embryo cells with five *ts* mutants of HSV-1 at a frequency of 1–2 foci/10^6 cells. The transformed cells expressed viral antigens both in the cytoplasm and on the cell surface and some transformed lines were tumorigenic in rats (*Macnab*, 1975). One line of transformed cells was capable of complementing the *ts* defect in three *ts* mutants from different complementation groups, suggesting expression of at least three HSV functions in the transformed cells. Seven other *ts* mutants were not complemented (*Macnab* and *Timbury*, 1976). Transformation of both hamster and human cells by HSV-2 *ts* mutants has been reported by *Takahashi* and *Yamanishi* (1974). Three out of 12 *ts* mutants tested were able to transform hamster embryo cells, and one *ts* mutant could transform human embryonic lung cells. As has been repeatedly observed by previous in-

vestigators, the transformed cells stained by indirect immunofluorescence had cytoplasmic fluorescence in 5–10% of the cells, indicating the presence of viral antigens. Two lines were also found to contain HSV-2-specific RNA by RNA-DNA hybridization analysis (*Bacchetti*, unpublished). Two transformed hamster lines were tested for oncogenicity and were found to induce tumors in the majority of animals within 10–16 weeks; metastases in the lungs were frequently observed. Similar results were reported by *Kimura* et al. (1975), who used UV-inactivated HSV-2 or a *ts* mutant to transform hamster cells. Oncogenicity could be demonstrated by *Kimura* et al. (1975) only after the transformed cells had been cultured through 33 passages, and with increasing passage the latent period was reduced and the frequency of tumor induction increased.

Duff and *Rapp* (1975) observed that C type virus was released from HSV-2-transformed 3T3 cells after several passages in culture, and *Hampar* et al. (1976) found that infection of Balb/C mouse cells with ultraviolet-irradiated HSV-1 and 2 resulted in activation of an endogenous type C virus. While the significance of the observations is not clear, one obvious and intriguing possibility is that induction of C type virus synthesis is a step in induction of oncogenic transformation, as suggested by *Huebner* and *Todaro* (1969).

A somewhat different approach to studying transformation by HSV has been adopted by *Wilkie* et al. (1974). It had previously been shown by means of the calcium technique (*Graham* and *van der Eb*, 1973) that exposure of mammalian cells to intact DNA resulted in production of infectious virus (*Graham* et al., 1973). However, by mechanically shearing HSV-1 DNA, infectivity could be eliminated, and thus it became possible to induce transformation of primary rat embryo cells (*Wilkie* et al., 1974). Alternatively, cells could be transformed at the nonpermissive temperature with DNA extracted from *ts* mutants of HSV-1. Cells transformed by HSV-1 DNA appeared to be similar to those transformed with *ts* mutants of HSV-1 by *Macnab* (1974). Studies on oncogenicity of lines transformed by HSV-1 DNA have not yet been reported. This type of approach is potentially very useful, since in combination with restriction enzyme technology it should permit the determination of the minimum size of transforming DNA fragments and the location of the transforming genes in the viral genome as has been done for SV40 (*Abrahams* et al., 1975) and human adenoviruses (*Graham* et al., 1974a, b). Identification and purification of restriction enzyme fragments containing transforming activity would in turn permit the synthesis of more refined probes for detecting the presence of HSV-transforming genes in human malignancies.

B. Biochemical Transformation

Up to this point we have discussed only herpes simplex virus induced morphologic transformations of the type which usually lead to the establishment of oncogenic cell lines. However, a second type of transformation induced by these viruses has also been extensively studied, and although this may not be directly relevant to the question of oncogenicity, it may shed some light on some of the processes involved in oncogenic transformation. HSV-1 and HSV-2 code for several enzymes (deoxypyrimidine kinase, DNA polymerase, deoxyribonuclease) (*McAuslan*, 1974),

electrophoretically and/or biochemically distinguishable from their cellular counterparts and easily detectable in productively infected cells (*Kit* and *Dubbs*, 1963; *Hay* et al., 1971; *Weissbach* et al., 1973; *Kit* et al., 1974). Among these enzymes, the deoxypyrimidine kinase (which for convenience we will refer to as TK) has been most extensively studied. The enzyme phosphorylates both TdR and CdR and is necessary for virus replication in serum-starved cells but not in actively growing cells (*Jamieson* et al., 1974). The kinases coded for by HSV-1 and HSV-2 can be distinguished from each other and from the TKs found in the cytoplasma and mitochondria of cells on the basis of thermal stability, electrophoretic mobility, and serologic properties (*Munyon* et al., 1971; *Lowry* et al., 1971; *Thouless*, 1972; *Kit* et al., 1975).

Munyon et al. (1974) infected TK^- mouse or human cells with UV-inactivated HSV-1 or HSV-2 and by selection in HAT (Hypoxanthine, Aminopterin, Thymidine) medium (*Littlefield*, 1964) isolated clones of cells carrying the viral TK. Similar results were obtained by *Hughes* and *Munyon* (1975) using *ts* mutants of HSV-1 unable to replicate at 39° C. The transformed cells contained at least two viral-specific antigens in the perinuclear cytoplasmic region (*Chada* and *Munyon*, 1975), one of which might be the TK itself, since it was absent in a TK^- revertant line. Transfer of the HSV-2 TK to TK^- human cells has also been achieved by infecting the cells with sheared HSV-2 DNA (*Bacchetti* and *Graham*).

Since in all of the above studies the parental TK^- cells are oncogenic, it was not possible to relate the conversion of TK^- cells to cells carrying the viral TK with changes in oncogenicity. It seems unlikely that the acquisition of the viral TK by mammalian cells would affect their oncogenic potential, although co-transfer of other viral genes could have such an effect. However, the processes involved in the converion of TK^- cells to TK^+ cells by infection with HSV-1 or HSV-2 may be similar to those which result in oncogenic transformation, and the TK transformation system is an interesting and valuable approach to study these processes.

VI. Prevention

If cervical carcinoma is caused by HSV-2, then, as stated previously in the introduction, it should be possible to prevent the malignancy by preventing or modifying HSV-2 infections. At present, the link between HSV-2 and cervical carcinoma is still too tenuous to make any such attempt at prevention worthwhile in relation only to this particular malignancy. However, herpetic infections are a sufficiently serious medical problem to warrant investigations into the development of effective means of preventing or treating the disease independent of any possible link with human malignancy. Two basic preventive measures, i.e., prevention of exposure and wide-spread vaccination, may be difficult to effectively develop. Prevention by reducing exposure would presumably involve radical changes in sexual mores, hygiene, and public education and is unlikely to be totally effective. Effective vaccines are not presently available, and our understanding of immunity to HSV-1 or HSV-2 is not sufficient to predict the probability of success of developing such a vaccine. Clearly, reinfection by HSV-1 and HSV-2 is possible (*Blank* and *Haiver*, 1973) and appears to occur in natural settings (*Nahmias* and *Roizman*, 1973). In

addition, the data derived from studies on transformation of mammalian cells by herpes simplex viruses and other viruses suggest that inactivation of viral functions required for lytic replication may render the virus capable of inducing transformation of permissive cells, and the inactivated viruses may be theoretically oncogenic, or more oncogenic, for its normal host than viruses not inactivated. Thus, vaccines prepared from viruses capable of inducing oncogenic transformation should consist of nucleic acid-free virion components, or of attenuated strains of virus which are defective in transforming functions (*Seth* et al., 1974). Unfortunately, the biology of herpesviruses is still not sufficiently advanced to permit the preparation of either type of vaccine.

VII. Conclusions

At the beginning of this discussion we listed five criteria, the fulfilment of which would convince us that an etiologic relationship exists between a malignancy and a particular virus. It would appear that none of these criteria are completely satisfied by HSV-2 in relation to cervical carcinoma. The first evidence, and still the bulk of presently available evidence for an association between cervical carcinoma and HSV-2, comes from epidemiologic and seroepidemiologic studies which are extremely suggestive but insufficient to fully satisfy the first criterion. Molecular biological data are now needed to provide further support or refutation of a putative link. The most convincing molecular biological evidence for a link between HSV-2 and cervical carcinoma would be the detection of virus specific RNA or DNA sequences in tumor cells, an approach which so far has yielded almost exclusively negative results. It would be premature to be overly discouraged by the failure of molecular-hybridization studies to detect the presence of HSV-2 sequences in cervical cancer cells, since in other systems it has been demonstrated that oncogenic transformation of mammalian cells can be induced by small segments of tumor virus genomes — indeed as little as $1–2 \times 10^6$ Daltons of DNA may be sufficient (*Graham* et al., 1974a, b; *Abrahams* et al., 1975). If the same absolute amount of the HSV-2 genome were sufficient to induce oncogenic transformation, then the sensitivity of molecular probes would have to be capable of detecting as little as 1–2% of the virus genome before negative results become meaningful. The detection of neoantigens — possibly virus specific in cervical carcinomas cells — suggests that these cells should contain viral DNA sequences. However, the total molecular weight of the putative viral antigens reported to date is about 260,000, which would represent only about 2–3% of the viral genome. This is well below the limits of detection of molecular-hybridization studies carried out to date. The need is, therefore, for more sensitive probes, and a possible approach to this end has been worked out in other systems. Through the use of restriction enzymes and transformation of cultured cells with DNA fragments, it should be possible to determine the size and location of the transforming genes of HSV-2 as has been done for the human adenoviruses (*Graham* et al., 1974a, b). Purified preparations of these genes can then be used to prepare highly specific probes for homologous viral sequences in tumor cells. All the technology needed for this approach is currently available or rapidly being developed; transformation

of cultured cells by herpes simplex virus DNA has been demonstrated (*Wilkie* et al., 1974; *Bacchetti* and *Graham*, unpublished), and the genome structure and restriction enzyme-cleavage patterns of the virus DNA are currently under investigation (e.g., see Cold Spring Harbor Symp. Quant. Biol. *39*, 1974; *Skare* et al., 1975; *Hayward* et al., 1975a, b; *Steinhart* et al., 1975).

Little information is available at the present time regarding the second and third criteria. Attempts have been made to induce tumors in rodents with HSV, but the results have not been very helpful. Studies with primates are currently in progress (*Palmer* et al., 1976) and may provide more useful data. There appears to be no naturally occurring malignancy in other animals which significantly resembles cervical carcinoma, although it is relevant that several members of the herpesvirus group can induce malignancies. (Indeed, of all the DNA viruses, the herpesvirus group is the only one containing members known to cause malignant disease in their natural host.)

Herpes simplex viruses do, in part, fulfill the requirements of our fourth criterion in that it is now well established that HSV-1 and HSV-2 can oncogenically transform cells in culture. However, as we indicated previously, we do not feel that the mere demonstration of transforming activity carries much weight in deciding whether or not a virus can induce malignant disease under natural conditions. Rather, the importance of such studies lies in the contributions they are likely to make toward an understanding of the mechanisms underlying the induction of malignancy.

All the evidence required to establish an etiologic relationship between a virus and a neoplasm in man has not yet been obtained. From animal studies, a model has been constructed in which the induction of neoplasia is compared to oncogenic transformation of cultured cells resulting from integration of viral genetic information into the cell genome. Viral-coded neoantigens are present in the transformed cells, and inoculation of the transformed cells or virus into the appropriate host leads to tumor formation. An association has been established between EB virus and African Burkitt's lymphoma and nasopharyngeal carcinoma. In this association, the characteristics of the model have been demonstrated, and yet there is reluctance to accept the virus as etiologic. Short of preventing tumor formation by vaccination or inducing tumor regression by antiviral therapy, little additional observational data could be acquired which would add fundamentally to our appreciation of the etiologic role of the virus (*Epstein*, 1976). Since EB virus probably comes closer than any other virus to fulfilling all the criteria needed to establish it as a human cancer virus, it is instructive to end this discussion with a comparison between EBV and HSV-2.

Studies of the relation between HSV-2 and carcinoma of the cervix are not as advanced as with EB virus. However, sufficient information is available to indicate obvious differences in the two systems. Unlike the association of EB virus with lymphoma or nasopharyngeal carcinoma, the association of HSV-2 with cervical cancer has no peculiar geographic or racial distribution. Epidemiologically, HSV-2 is a suitable candidate for cervical cancer, while the unusual distribution of EB-virus-associated tumor is poorly understood. The prediction from the model that viral DNA and proteins should be present in cancer cells has been readily fulfilled in neoplasms associated with EB virus, but HSV-2 DNA and proteins

have not been easily and reproducibly demonstrated in cervical cancer cells. A lymphomatous disease develops in marmosets inoculated with EB virus, but invasive cervical cancer has not yet been produced in animals with HSV-2. EB virus has a narrow host range; transformation occurs primarily in B lymphocytes, and infectious virus can be recovered from lymphoblastoid cells, while HSV-2 has a wide host range, and infectious virus cannot be rescued from transformed cells. The association between the two viruses and the different malignancies appears to be fundamentally different. We feel that it is not possible at this time to conclude that HSV-2 is etiologically related to cervical cancer; especially since the role of EB virus in the genesis of Burkitt's lymphoma and nasopharyngeal carcinoma still remains in doubt. As additional data become available, it should be possible to accurately evaluate the meaning of the intriguing inter-relations between these members of the herpesvirus group and human malignancies.

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Enzymology of Carbohydrate Transport in Bacteria

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Abbreviations: APG_0 = 2-Nitro-4-acidophenyl-1-thio-β-D-galactopyranoside; APG_2 = 2′-N-(2-Nitro-4-acidophenyl) aminoethyl-1-thio-β-D-galactopyranoside; ATP = Adenosine triphosphate; CCCP = Carbonylcyanide m-chlorophenyl hydrazone; DCCD = N, N′-dicyclohexylcarbodiimide; DDA = Dibenzyl dimethyl ammonium; DEAE = Dicthylaminoethyl; DG_0 = 1-(N-dansyl) amino-β-D-galactopyranoside; DG_2 = 2′-(N-dansyl) aminoethyl-1-thio-β-D-galactopyranoside; DG_5 = 5′-(N-dansyl) aminopentyl-1-thio-β-D-galactopyranoside; DG_6 = 6′-(N-dansyl) aminohexyl-1-thio-β-D-galactopyranoside; D-LDH = D-lactate dehydrogenase; DTT = Dithiothreitol; FAD = Flavin adenine dimeleotide; IPTG = Isopropyl-1-thio-β-D-galactopyranoside; HPr = Heat stable phosphocarrierprotein of the PTS; NEM = N-ethyl maleimide; ONPG = O-Nitrophenyl-β-D-galactopyranoside; PEP = Phosphoenol pyruvate; PG = Phosphatidyl glycerol; PTS = Phosphotransferase system; SDS = Sodiumdodecylsulfate; TDG = β-D-thiodigalactoside; TMG = β-D-thiomethylgalactoside.

I. Introduction

The structure of biological membranes is still one of the major problems of modern biochemistry which remain to be solved.

One important physiologic feature of biological membranes is their capability to regulate and determine the flow of metabolites through this permeability barrier. More detailed knowledge of biochemical processes such as solute transport or electron transport, both of which occur at the level of the membrane, will certainly lead to a better understanding of membrane function and structure itself.

Transport studies in whole living cells such as bacteria are not capable of elucidating molecular mechanisms of solute transport, because single cells are still very complex systems consisting of several thousand components. More detailed molecular mechnisms, therefore, can be investigated only in simple systems composed of a limited number of purified components. In recent years many papers dealing with the genetics and biochemistry of bacterial solute transport have appeared. The reader is referred to numerous reviews in this field (*Cirillo*, 1961; *Pardee*, 1968; *Kaback*, 1970a, b, 1972; *Oxender*, 1972; *Halpern*, 1974; *Boos*, 1974, 1975; *Hamilton*, 1975; *Simoni* and *Postma*, 1975; *Konings*, 1976;

Konings and *Boonstra*, 1976; *Maloney* et al., 1975). The genetic and kinetic data available show the biochemist that even very complex transport systems consist of a limited number of components which can be isolated and characterized.

Great progress has been made by developing subcellular systems such as *Kaback's* bacterial membrane vesicles (*Kaback*, 1972, 1974) which have greatly improved our understanding of biological transport processes and are still a powerful tool for penetrating further into the complicated mechanisms of energy coupling during active transport processes (*Konings*, 1976; *Konings* and *Boonstra*, 1976). In this review I try to summarize the information which has been obtained from studies with isolated bacterial transport proteins.

Quite often the isolated transport proteins are inactivated during purification or extraction procedures. Also enzymic assays for translocation of a substrate only work with intact membraneous compartments. Therefore, I will also cover substrate binding studies with membrane fragments and membrane vesicles and work with specially designed substrate analogs which helped to characterize the substrate binding sites.

Many pioneering studies with isolated bacterial transport proteins have been performed with components involved in the transport of carbohydrates. I therefore restrict myself to isolated proteins which are involved in carbohydrate transport. Other transport proteins, for instance proteins involved in amino acid transport, ion transport, and phosphate and sulfate transport, have been treated in recent reviews listed above.

II. Active Transport of Carbohydrates in Bacteria

There are two basically different mechanisms of carbohydrate transport in bacteria (*Simoni* and *Postma*, 1975): (1) indirect coupling and (2) solute modification. In the former, the solute is transported actively across the membrane without being changed. This mechanism requires a carrier molecule in the membrane which changes its affinity during the translocation process: carrier outside high affinity for the substrate, carrier inside low affinity. The affinity change of the carrier during the transport process requires energy which is derived from cellular energy via an indirect coupling mechanism such as production of an ion or proton gradient or a membrance potential. In the latter, the solute is also transported actively across the membrane, but during the transport process the substrate is chemically modified, for instance, the sugar ist phosphorylated. This mechanism has been called group translocation (*Roseman*, 1969, 1972; *Postma* and *Roseman*, 1976). However more felicitous designation is vectorial phosphorylation (*Kaback*, 1970a, b), which means that the transport process is achieved by a vectorial chemical reaction whereby the solute is phosphorylated into the cell.

Other bacterial transport systems besides that are known to operate via solute modification. For example, adenine is glycosylated to adenosine monophosphate in a vectorial reaction catalyzed by a membrane-bound enzyme (*Hochstadt-Ozer* and *Stadtman*, 1971; *Hochstadt-Ozer*, 1972). A second example is that of fatty acids, which are converted to acyl-Co A during the transport process in which the acyl-Co A synthetase in *E.coli* serves as catalyst (*Klein* et al., 1971).

III. Active Sugar Transpoort via Indirect Coupling

A. The Lactose Transport System of E. coli

1. Isolation of the Lactose Carrier

Interest in bacterial transport was initiated by the pioneering work of *Rickenberg* et al. (1956) which presented genetic and physiological evidence for a lactose transport system in *E. coli*. This system consists of a carrier which is able to equilibrate lactose into a cell, a catalytic step demonstrating Michaelis Menten kinetics (*Kennedy*, 1970; *Kepes*, 1971). Under physiologic conditions, the transport system is able to concentrate lactose or galactosides against a concentration gradient. If uncouplers of oxidative phosphorylation are added, the system is poisoned, but the carrier function per se can now be studied.

The isolation of the lactose carrier molecule was achieved by the ingeneous work of *Fox* and *Kennedy* (1965), who labeled the lactose carrier selectively by protecting a reactive Sulfhydryl group of the carrier against N-ethyl maleimide (NEM) with the substrate analog β-D-thiodigalactoside (TDG). Upon removal of the TDG this reactive Sulfhydryl group was labeled with radioactive NEM, so that lactose carrier (M-protein) could then be extracted with detergents. The protein's molecular weight in SDS was shown to be about 30000, and its solubility behaviour in detergents was compatible with an integrated membrane protein. Furthermore, the amount of lactose carrier war estimated to be about 3% of the total membrane protein (0.3% of total cell protein). The assay procedure of the M-protein (labeling with NEM), however, led to biologically inactive material, the function of which could not be studied further (*Jones* and *Kennedy*, 1969).

2. The Galactoside Binding Site of the Lactose Carrier of E. coli

In a later contribution *Kennedy* et al. (1974) demonstrated the direct binding of galactosides to membrane fragments. Further information about the lactose binding site was obtained from studies of isolated membrane vesicles which contained the lactose carrier, which showed that the vesicles bound the fluorescent lactose analogs 2′-(N-dansyl) aminoethyl-1-thio-β-D-galactopyranoside (DG_2) (*Schuldiner* et al., 1975a, b, c) and 6′-(N-dansyl) aminohexyl-1-thio-β-D-galactopyranoside (DG_6) (*Reeves* et al., 1973). This binding required energized membrane vesicles or lactose efflux from the vesicles and resulted in an increased intensity of fluorescence for the bound DG_2 or DG_6 molecules. Recently these studies of binding have been repeated, but this time using tritium labeled DG_6. Flow dialysis experiments confirmed the observation that the binding of DG_6 required energized membrane vesicles (*Schuldiner* et al., 1976). *Kennedy* et al. (1974), however, observed the direct binding of galactosides to membrane fragments obtained by sonication of *E. coli* cells and subsequent differential centrifugation. In these fragments, which probably do not exhibit proper orientation as do the membrane vesicles, the high affinity binding site may have been exposed during the disruption procedure so that binding of a galactoside is observed, but no energizing of the membrane.

Further information about the galactoside binding site could be obtained by using the photo affinity label 2′-N-(2-nitro-4-acidophenyl) aminoethyl-thio-β-D-galactopyranoside (APG_2), which reacts covalently with the lactose carrier upon illumination. This compound specifically inactivates the lactose binding site of the lactose carrier by forming a covalent linkage. Lactose is a competitive inhibitor of the inactivation. Thus, energized membrance vesicles are again necessary in order that inactivation may be observed. (*Rudnick* et al., 1975a, b). The analogs DG_2, DG_6 and APG_2 are not accumulated by the membrane vesicles (*Walsh*, 1976).

3. Substrate Speicificity of the Lactose Carrier

A large variety of galactosides are recognized by the lactose carrier (Table 1).

Table 1. Substrates of the lactose carrier of *E. coli*

Substrate	K_M [μM]	Special properties	References
TMG	510		*Barnes* and *Kaback*, 1970
Lactose	90	Vesicle studies	
Lactose	900	Whole cells ML 308–325	*Kennedy*, 1970
Lactose	70	Strain W 2244	
TMG	500	Whole cells	
ONPG	1000	Whole cells	
β-D-thiodi-galactoside (TDG)	67		*Kennedy* et al., 1974
DG_2	32	Fluorescent substrates not transported	*Reeves* et al., 1973 *Schuldiner*, 1975a, b, c
DG_0	550		
DG_6	6.6		
APG_2	30–40	Photo affinity labels not transported	*Rudnick*, 1975b
APG_0	75	Actively transported	*Rudnick*, 1975a
p-nitrophenyl α-D-galactoside	7		*Kennedy* et al., 1974
Phenyl-αD-galactoside	70		
Melibiose	500		
Methyl-α-D-galactoside	2000		

Carter et al. (1968) observed that the galactosides could be divided into two classes:

Class I: Lactose, O-nitrophenyl-β-D-galactopyranoside (ONPG), thiomethyl-β-D-galactopyranoside (TMG), isopropyl-1-thio-β-D-galactopyranoside (IPTG) did not protect the M-protein from inactivation with NEM.

Class II: TDG, melibiose p-nitrophenyl-α-D-galactoside protected the M-protein from inactivation with NEM. These findings suggest that the lactose carrier may possess two distinct binding sites with different affinities, one of which contains a sulfhydryl group in close proximity to the binding site.

4. Reconstitution of the Lactose Transport System

Recently *Müller* et al. (1976) succeeded in restoring the lactose transport activity of a mutant defective in the β-D-galactoside transport system by using a solubilized preparation of the M-protein prepared from wild-type membrane vesicles. These reconstituted membrane vesicles accumulated lactose in the presence of electron donors such as D-lactate or ascorbate and phenazine methosulfate. However, lactose uptake was inhibited by the uncoupler carbonylcyanide m-chlorophenyl hydrozone (CCCP) and by the substrate analog TDG.

5. Interaction of Membrane Phospholipids with the Lactose Carrier

The lactose carrier protein behaves like a typically integrated membrane protein (*Singer* and *Nicolson*, 1972; *Singer*, 1974). It is soluble only in detergents or certain combinations of aprotic solvents (*Müller* et al., 1976). When such a carrier protein is embedded into the phospholipid bilayer of the cytoplasmic membrane, one expects the action of the carrier molecule to be influenced by the lipid environment. Indeed, *Fox* (1969) observed that integration of the M-protein into the membrane after induction of the *E. coli* lac operon required phospholipid biosynthesis, since a fatty acid auxotroph of *E. coli* with defective biosynthesis of oleate was no longer able to integrate the functional lactose carrier into the membrane if grown in the absence of oleate. When supplied with oleate, the mutants produced functional M-protein upon induction. These experiments indicate that lipid biosynthesis is necessary to integrate biologically active lactose carrier protein into the cato-plasmic membrane.

The more detailed studies by *Overath* et al. (1971, 1976) indicate that the structure of the acyl part of the phospholipid has an influence on the functioning of the lactose carrier. (For a review of genetic modification of membrane lipids consult *Silbert* (1975). In these studies a fatty acid auxotroph of *E. coli* grown in a medium containing a defined mixture of oleate and elaidate showed a marked transition point in the activation energy for galactoside transport. The transition point temperatures in the Arhenius plots were correlated to the phase transitions of the corresponding *E. coli* membrane phospholipids. Shifting the culture from a medium containing elaidate to one with oleate shortly before induction of the lac operon did not result in a transition point of the Arhenius plot characteristic of pure oleate but rather a transition point characteristic of the randomized phospholipids of the membrane. Thus, these experiments show very clearly that phospholipid biosynthesis is necessary during integration of a transport protein into the membrane. The phospholipids as well as the carrier molecules can diffuse laterally in the lipid bilayer. There is apparently no preference for newly synthesized lipid in close proximity to the carrier molecule.

6. Enzymology in the Energy Coupling of the Lactose Transport System in E. coli

a) The Membrane-Bound D-*Lactate Dehydrogenase*

Around 1970 there was still great confusion about the energy coupling mechanism of the lactose transport system. *Scarborough* et al. (1968) suggested that adenosine

triphosphate (ATP) alone was involved in the lactose accumulation process. However, *Pavlasova* and *Harold* (1969) had evidence that in anaerobically grown cells of *E. coli* TMG transport could be blocked by uncouplers of oxidative phosphorylation without significantly altering ATP levels. Even more confusion was produced by the suggestion of *Kundig* et al. (1966) that components of the phosphoenol pyruvate-(PEP-) dependent phosphotransferase system might be involved in galactoside transport of *E. coli*.

Then *Barnes* and *Kaback* (1970) showed very clearly that D-lactate is able to serve as an energy source during active galactoside transport in *E. coli* membrane vesicles. In their experiment, the vesicles oxidized D-lactate to pyruvate, which required the existance of a membrane-bound D-lactate dehydrogenase (D-LDH). This membrane-bound D-LDH of *E. coli* has since been solubilized and purified to homogeneity (*Kohn* and *Kaback*, 1973). *Futai* (1974) the enzyme has a molecular weight of 75000 daltons and contains 1 mole of FAD per mole of enzyme. In the presence of the purified D-LDH, vesicles derived from a mutant strain defective in D-LDH could be reconstituted (*Short* et al., 1974) by the following procedure. The enzyme preparation in 0.1% triton X100 0.6 M guanidine-HCl was diluted about 20-fold into a suspension of D-LDH membrane vesicles. In contrast to wild-type membrane vesicles which are insensitive to antibody against D-LDH, the D-LDH-reconstituted vesicles are inhibited by D-LDH antiserum. This suggests that in native wild-type vesicles D-LDH is bound at the inner surface of the membrane vesicles, whereas in reconstituted vesicles the enzyme is bound at the outer surface. It could be shown that the mutant strain defective in D-LDH which was used in the reconstitution studies still contained inactive D-LDH protein that reacted with D-LDH antibody (*Short* et al., 1975). This may be the reason why D-LDH is inserted at the wrong side of the membrane after reconstitution.

b) Other Protein Compounds Involved in the Energy Coupling of the Lactose Transport System

An interesting model, which integrated the lactose carrier into the electron transport chain, was proposed by *Kaback* and *Barnes* (1971). In this model they postulated that the lactose carrier performed its cycle of conformational changes via a redox process occurring at the level of the carrier.

This model has been called into question since *Hirata* et al. (1973–1974) showed that active lactose transport can be driven by an electrical potential across the vesicular membrane. This potential can be generated by the efflux of K^+ ions from preloaded vesicles with valinomycin or by the generation of a pH gradient which can be monitored by the uptake of a lipophilic organic cation such as the dibenzyl dimethyl ammonium (DDA) ion (*Altendorf* et al., 1975; *Schuldiner* and *Kaback*, 1975). Experimental evidence is still accumulating that the lactose pump of *E. coli* is indeed a proton symport, as was reported earlier by *West* and *Mitchell* (1973). As a logical consequence of the validity of the *Mitchell* hypothesis about energy coupling of bacterial sugar transport, the involvement of the membrane-bound ATPase in the energy coupling of proline transport in *E. coli* could be demonstrated. Membrane vesicles prepared from an ATPase mutant (DL 54) originally isolated by *Simoni* and *Shallenberger* (1972) did not accumulate proline

during D-lactate oxidation. However, the defect could be restored by treating the vesicles with N, N′-dicyclohexylcarbodiimide (DCCD), which inhibits the ATPase complex and reduces the permeability of the DL 54 membrane vesicles to protons, which is higher than in the wild-type strain. Thus, a proton gradient sufficient to drive the proline pump can be generated by D-lactate oxidation (*Altendorf* et al., 1974). DCCD was also shown to react with a protein of the integral membrane-bound part of the *E. coli* ATPase (*Altendorf* and *Zitzmann*, 1975).

Involvement of the membrane-bound ATPase in TMG uptake has been described in detail by *Schairer* et al. (1976), who observed that potassiumcyanide-poisoned cells are still able to accumulate TMG, although with half the efficiency of the unpoisoned cells. Various ATPase mutants (for reviews of mutants defective in oxidative phosphorylation see *Cox* and *Gibson* (1974) and *Simoni* and *Postma*, (1975)) accumulate TMG at a very low rate compared to the wild-type strain. The defects in the different ATPase mutants have been assigned to the subunits of the ATPase; its soluble part has been purified to homogeneity and its active complex can be reconstituted from isolated subunits (*Vogel* and *Steinhart*, 1976). The chemiosmotic hypothesis of *Mitchell* (1973), which is discussed in detail in many reviews (*Harold*, 1972; *Boos*, 1974, 1975; *Simoni* and *Postma*, 1975; *Konings*, 1976), now seems to explain many features of the energy coupling mechanism in the lactose transport system of *E. coli*.

7. Conclusions About the Lactose Carrier Protein of E. coli

The lactose carrier protein belongs to the lac operon, one of the best-characterized regions of the *E. coli* chromosome (*Kennedy*, 1970). The protein has been partially purified after reaction with labeled NEM or after induction in the presence of labeled amino acids. According to its solubility behavior in detergents or aprotic solvent it belongs to the integrated membrane proteins. Kinetic studies and binding experiments using a large variety of galactosides have revealed the existence of two binding sites at the lactose carrier, one high-affinity and one low-affinity site. Studies of membrane vesicles using substrate analogs with membrane probe properties (fluorescent substrates) have shown that the high affinity site is only then accessible when the membranes are in the energized state. This state can be achieved by several methods: (1) Oxydation of D-lactate or other suitable electron donors, (2) generation of membrane potentials, and (3) efflux of lactose.

The lactose transport system has proven very helpful in describing the influence of the membrane phospholipids on transport processes in general. The lipid composition of the cytoplasmic membrane can be easily altered in *E. coli* cells by constructing mutants with defective biosynthesis of fatty acids and phospholipids. The modified phospholipids give rise to altered transport properties which can be correlated to typical changes of the physical properties of the phospholipids by a variety of different physical measurements (*Overath* et al., 1976). Thus, a bacterial transport system has greatly improved our general understanding of the role of the membrane lipid during membrane-bound biochemical processes.

Knowledge of the structure of the lactose carrier molecule is rather limited. Its purification and preparation in substantial quantity still challenges the biochemist. Also the technology of handling the hydrophobic membrane proteins

needs to be further developed. Indeed, this field of research is still very empirical and needs more methods of general applicability. Consequently, many important questions about the structure of the lactose carrier protein have not yet been answered. The amino acid composition is not known; so no data about the ratio of polar and apolar amino acids are available. Is the lactose carrier a protein molecule which penetrates through the membrane or is it localized preferentially on one side of the cytoplasmic membrane?

Such key questions about structure and topology of bacterial transport proteins have been partially solved for other transport systems isolated from highly specialized organelles of higher organisms, for instance the $(Na^+ K^+)$ ATPase of the plasma membrane of eukaryotic cells or the (Ca^{2+}) ATPase of the sarcoplasmic reticulum. According to *Guidotti* (1976) these proteins appear to have several common features one of which is that, they are transmembrane (bilayer spanning) proteins composed of several subunits. In the case of the mammalian systems the transport protein contains carbohydrates, a property which has not yet been reported for bacterial substrate translocating proteins.

The detailed structural analysis of these membrane transport proteins is usually simplified by the fact that highly specialized membranes contain only a very few protein species which can be separated rather easily. For instance, the (Ca^{2+}) ATPase of the sarcoplasmic reticulum accounts for 70% of the membrane protein (*Meissner*, 1975). In comparison, the *E. coli* lactose carrier protein is only 3% of the *E. coli* membrane proteins in the complex mixture and its purification is much more difficult.

B. Activite Transport of Other Sugars in E. coli via Indirect Coupling

Membrane vesicles of *E. coli* are able to accumulate several other sugars after energizing the vesicles with electron donors, for instance galactose, arabinose, glucuronate, and hexosephosphate (compare Table 3 of the review of *Konings*, 1976; *Dietz*, 1976). These findings show that the cytoplasmic membrane of *E. coli* contains a large number of sugar carriers which are energized in a manner similar or identical to that of the lactose transport system.

No further isolation of these carbohydrate carrier proteins has been attempted. For the transport of other sugars in a wide variety of microorganisms energized by the lactose type energy coupling system compare Table 3 of the review of *Konings* (1976).

C. Sugar Transport Mediated by the Periplasmic Binding Proteins

1. The Binding Proteins for Monosaccharides

According to *Heppel* (1969) an osmotic shock procedure applied to *E. coli* cells released, in addition to other components, proteins which bind carbohydrates. The release of these binding proteins from the *E. coli* cells resulted in a decreased transport activity of the sugar, which is bound by the released protein. A large variety of such binding proteins has been isolated and purified in recent years (see the reviews of *Boos*, 1974, 1975). Restoration of transport with these binding

proteins is reported in several cases; however, such experiments often were not reproducible, and the results should treated with caution.

The most extensively investigated sugar binding protein is probably the galactose binding protein of *E. coli*. Its involvement in galactose transport has been shown by *Boos* (1972), *Silhavy* et al. (1974), and *Boos* (1975) by using a mutant strain defective in this binding protein. Other sugar binding proteins of *E. coli* which were obtained in homogeneous form are the arabinose binding protein and the ribose binding protein, the properties of which have been the subject of reviews by *Oxender* (1972) and *Boos* (1974, 1975). Recently the three-dimensional structure of the L-arabinose binding protein has been published (*Phillips* et al., 1976) with up to 3.5 Å resolution.

2. The Maltose Transport System of E. coli

The maltose binding protein has been purified to homogeneity by *Kellermann* and *Szmelcman* (1974). It is a monomeric protein of 40 000 daltons similar to the other sugar binding proteins and is coded for by the *mal*E gene of *E. coli*. The maltose transport of the *E. coli* cell can be affected by a mutation in the gene *lam*B, the structural gene for an outer membrane protein which acts as a receptor for bacteriophage λ. Mutations in *lam*B lead to an increase of the K_M for maltose uptake by a factor of 100–500 and block the uptake of larger maltose derivatives such as maltotriose. *Szmelcman* et al. (1976) suggest that the λ receptor protein facilitates the diffusion of maltose and maltodextrins through the outer membrane. Other phage receptors in the outer membrane of *E. coli* are known to be involved in the transport of iron citrate, iron enterochelin complexes, and ferrichrome (*Hantke* and *Braun*, 1975).

3. Involvement of the Periplasmic Carbohydrate Binding Proteins in the Translocation Step

The substrate binding proteins released by osmotic shock from the periplasmic space of Gram-negative bacteria show high affinity for the corresponding substrate ($K_{Diss} \sim 10^{-6}$–10^{-7} M). These proteins are typically soluble proteins with no detectable lipophilia, although lipophilia often is not a stringent requirement of membrane proteins (*Guidotti*, 1972). The binding proteins alone are probably not the translocating principle, since galactose transport mutants have been described which are no longer able to transport galactose but still possess intact galactose binding protein. So far, three genetic loci for the β-methyl-galactoside transport system have been found in *E. coli* — *mgl*A, B and C, where gene *mgl*B codes for the galactose binding protein. Using two-dimensional electrophoresis, *Silhavy* et al. (1974) were able to detect two membrane proteins which were co-induced with the galactose binding protein by adding the inducer D-fucose. These proteins, called MTA α and MTA γ, showed respective molecular weights of $\sim$ 80 000 and $\sim$ 50 000 in SDS gels. So far, a physical interaction between the membrane components and the galactose binding protein or free galactose has not been detected.

Recently, genetic evidence for such interaction between a substrate binding protein and a presumably membrane-bound component has been presented for the

histidine transport system of *Salmonella typhimurium*. This amino acid transport system contains a histidine binding protein (J protein) which is isolated from the periplasmic space by an osmotic shock procedure similar to that used to isolate the galactose binding protein.

A histidine transport mutant has been isolated (*Ames* and *Spudich*, 1976) which was defective in the J protein. Although this mutant J protein had different electrophoretic mobility when compared to the wild-type protein, it still bound histidine with the same efficiency. The transport defect of this mutant can be corrected by introducing a mutation in another genetic locus, linked to his J. The gene product of the his P locus has not yet been identified among the cell proteins (*Ames* and *Nikaido*, 1976). This double mutant showed histidine transport even more efficient than that observed in the wild-type strain. Such experiments provide strong genetic evidence that the J protein interacts with P protein.

It is hoped that the genetic evidence for the P protein will be strengthened by the isolation and localization of this protein. Physicochemical measurements with the purified J and P proteins should show the interaction between them. Since the P protein is also necessary for growth of the cells in arginine, it is likely to be a common membrane component which is required by several other substrate binding proteins. Perhaps a complex of membrane components that bind the protein conducts the translocation step in transport processes which are mediated by substrate binding proteins. In addition to the transport of the carbohydrate maltose, galactose, and ribose, which is catalyzed by the corresponding periplasmic binding proteins, these components also serve as signal receptors for the chemotactic response of *E. coli* (*Adler*, 1975).

4. Energy Coupling During Sugar Transport

This subject is still at the stage where the energy coupling process is characterized and described by the isolation of mutants defective in the various energy transduction systems. Therefore no data on experiments with purified energy coupling components are available yet (*Boos*, 1974, 1975; *Simoni* and *Postma*, 1975). Studies with whole cells using mutants defective in electron transport and ATPase have been performed. The conclusions obtained from these experiments again show that energy coupling can be achieved if one of the energy transduction systems is still intact in the mutant. The involvement of the membrane-bound ATPase seems to be more pronounced than in the energy coupling of the lactose system (*Berger* and *Heppel*, 1974; *Parnes* and *Boos*, 1973).

Unfortunately, studies that show binding protein mediating transport in membrane vesicles are not possible due to the loss of binding proteins during vesicle preparation (*Parnes* and *Boos*, 1973).

IV. Active Sugar Transport by Solute Modification: the Bacterial PEP-Dependent Phosphotransferase System (PTS)

As pointed out in the Introduction, there is another mechanism of carbohydrate transport in bacteria which is called vectorial phosphorylation. The most charac-

teristic feature of this transport system is the fact that the sugar occurring in the growth medium is phosphorylated during passage through the membrane and appears as a phosphorylated derivative in the cytoplasm. This phosphorylation of the sugar is catalyzed by the PEP-dependent phosphotransferase system (PTS) which was discovered in *E. coli* by *Kundig* and co-workers (*Kundig* et al., 1964). Several reviews of PTS have appeared in recent years (*Roseman*, 1969, 1972; *Postma* and *Roseman*, 1976), and genetic and biochemical evidence for its function in sugar transport has been discussed (*Egan* and *Morse*, 1965; *Lin*, 1970; *Kaback*, 1970a, b).

The following reactions occur during vectorial phosphorylation of carbohydrates (Fig. 1):

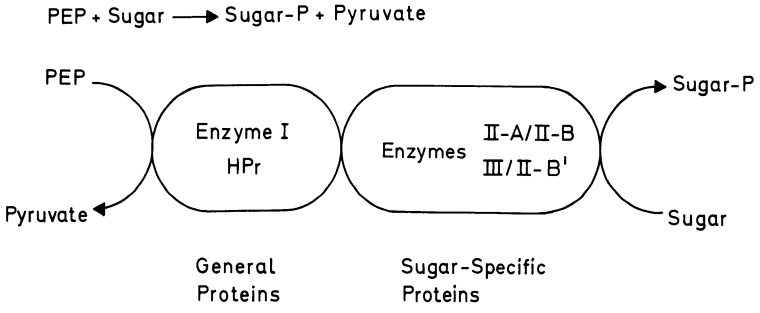


Fig. 1. Reaction scheme of the PEP-dependent phosphotransferase system (*Postma* and *Roseman*, 1976)

The PTS proteins, enzyme I and HPr, are synthesized constitutively by the bacterial cells. They serve as phospho-donors for several sugars. A genetic analysis of these components has been presented using *E. coli* and *Salmonella* (*Epstein* et al., 1970; *Cordaro* and *Roseman*, 1972).

Enzyme II-B is sugar specific and membrane bound. A family of Enzyme II-B exists which is recognized by the enzyme II-A or the sugar-specific Factor III components. Enzyme II-B is the component responsible for the translocation of the sugar.

A. The Constitutive Soluble Proteins of PTS

Compared to the sugar transport systems which work via an indirect coupling mechanism, the phosphotransferase system is unique. It is the only bacterial transport system where the energy-coupling components as well as the membrane-bound components have been characterized genetically and virtually all of the components have been isolated and partially characterized biochemically. As indicated in the reaction scheme, PTS consists of several interacting proteins which have been characterized and isolated from different microorganisms, mainly from *E. coli*, *S. typhimurium*, and the Gram-positive organism, *Staphylococcus aureus*. The nomenclature for these proteins has been introduced by *Roseman* and is discussed in a review (*Roseman*, 1972).

1. Enzyme I

a) Properties of Enzyme I

This protein catalyzes the phosphorylation of the low molecular weight component HPr. It has been partially purified in several laboratories from *S. aureus*, *E. coli*, and *S. typhimurium*. The preparation of an apparently homogeneous protein from *Salmonella* has been reported (*Roseman*, 1972), the enzyme is sensitive to oxygen and SH reagents, and a partial purification is possible only in the presence of DTT or mercaptoethanol. Extensive attempts in our laboratory to obtain this protein in pure form from *S. aureus* did not result in a homogeneous preparation (*Stein*, 1977). However, we were able to demonstrate that Enzyme I could be isolated as an intermediate product of phosphorylation. Incubation with ^{32}P-labeled PEP of very high specific activity (*Lauppe* et al., 1972) yielded phospho-Enzyme I having a molecular weight estimated at 90000 daltons on a Sephadex column. By using SDS gel electrophoresis, a single ^{32}P-labeled band was obtained with a molecular weight of around 80000; from this it was concluded that Enzyme I probably consists of a single, rather large polypeptide chain. For the phosphorylation of Enzyme I the presence of Mg^{2+} ions was necessary. In the presence of EDTA only very minor amounts of phospho-Enzyme I could be detected, whereas the transfer of the phospho-group to HPr did not require any metal ions (*Stein* et al., 1974).

b) The Phosphorus Protein Bond of Enzyme I

The phosphorus protein linkage in HPr of *E. coli* and in Factor III of *S. aureus* was derived from kinetic data on the hydrolysis rates of the phospho-proteins and from the isolation of phosphohistidine after alkaline hydrolysis (*Anderson* et al., 1971; *Hays* et al., 1973). These techniques were also applied to Phosphoenzyme I. Correct kinetic data on Phosphoenzyme I decay were, however, very difficult to obtain, since Enzyme I denatured during the hydrolysis experiments, resulting in a steadily decreasing rate of hydrolysis (*Stein*, 1977). Alkaline hydrolysis permitted the isolation of ^{32}P-labeled 3-P-histidine, which was identified by coelectrophoresis with chemically synthesized 3-phospho-histidine (*Hengstenberg* et al., 1976).

Although Enzyme I has not yet been obtained in pure form from *S. aureus*, the experiments mentioned above resulted in a more detailed description of the first reaction in the phosphotransferase system:

$$\text{PEP} + \text{Enzyme I} \overset{Mg^{2+}}{\rightleftharpoons} \text{P-Enzyme I} + \text{Pyruvate}.$$

It should be mentioned that evidence is accumulating that the soluble components of the phosphotransferase system, expecially Enzyme I, are involved in the regulation of protein synthesis of catabolic enzymes (*Peterkofsky* and *Gazdar*, 1975; *Jones-Mortimer* and *Kornberg*, 1974; *Saier* et al., 1970; *Roseman*, 1972; *Postma* and *Roseman*, 1976).

2. The Phosphocarrier Protein HPr

a) Purification and Properties

This protein, which is heat stable, was purified to homogeneity from several microorganisms. During the purification of *E. coli* HPr it was noted that heating resulted in the occurrence of several HPr species which were produced by the loss of amide residues (*Anderson* et al., 1971). Although the phosphotransferase systems of different bacterial species are rather similar in terms of molecular weight of the components (*E. coli* HPr 9500, *S. aureus* HPr 8600) (*Simoni* et al., 1973), the proteins are not interchangeable in the two systems. This behavior can be deduced from the difference in amino acid composition of the two HPr molecules. Whereas *S. aureus* HPr contains one histidine residue, one phenylalanine residue, and three tyrosine residues, HPr from *E. coli* or *S. typhimurium* contains two histidines, four phenylalanines, and no tyrosine residues (*Roseman*, 1972; *Hengstenberg* et al., 1976).

Recently homogeneous HPr was isolated from *B. subtilis* and shown to have an amino acid composition and molecular weight almost identical with HPr derived from *S. aureus* (*Marquet* et al., 1976). A third species of HPr which contains 2 moles of cysteine has been isolated from mycoplasma (*Jaffor Ullah* and *Cirillo,* 1976).

b) The Phosphorus-Protein Bond of HPr

Phospho-HPr can be prepared using Enzyme I and excess PEP as phospho-donor, (*Schrecker* et al., 1975). The rate of hydrolysis of phospho-HPr is quite pH dependent: at alkaline pH, P-HPr is very stable; at low pH, P-HPr decays rather fast, which was compatible with a protein the imidazole residue of which was phosphorylated at the N-1-residue. In addition after alkaline hydrolysis 1-phospho histidine was isolated from both P-HPr of *E. coli* and P-HPr of *S. aureus* (*Anderson* et al., 1971); *Hengstenberg* et al., 1976).

Recently, however, the conclusion that the phospho-group is bound at N-1 of the histidine residue in intact HPr has been questioned. The experiments forming the basis for this interpretation were rather indirect. As a rule, kinetic methods are not sufficient to establish a defined chemical intermediate. Alkaline hydrolysis of a protein is a rather drastic method, for the possibility can never be excluded that the phospho-group simply migrated from an unidentified residue to the histidine where it was able to survive the drastic conditions of alkaline hydrolysis.

Since HPr is a small, stable protein molecule which is quite easy to isolate, we were able to develop a large-scale purification procedure to obtain ample amounts of the protein from *S. aureus*. The yield from 1 kg of cells wet weight was about 150 mg of pure protein (*Beyreuther* et al., 1977). This protein gave a ^{1}H NMR spectrum, the aromatic region of which was easy to interpret (Fig. 2). The line at very low field is the C2 proton resonance of the only histidine residue of the molecule. The signals at higher field between 6.5 and 7.5 ppm a caused by the aromatic protons of tyrosine which can be clearly separated from each other by titration methods (*Maurer* et al., 1977). The spectrum of enzymatically phosphory-

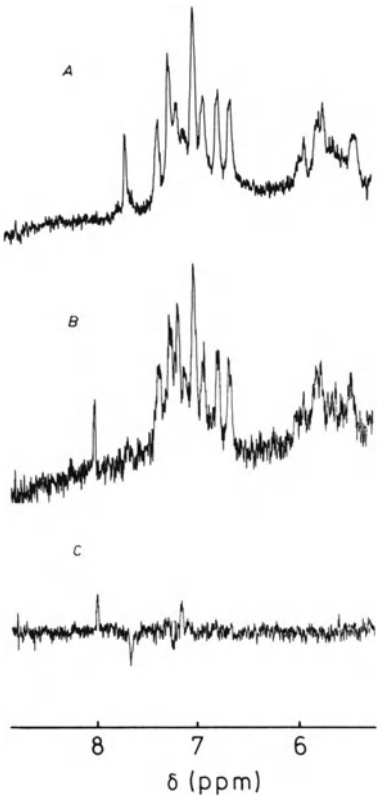


Fig. 2. Aromatic region of the 270 MHz ^{1}H NMR spectrum of HPr and phospho-HPr at pH 9.3. *A* Spectrum of HPr (40 mg Protein/ml), *B* Spectrum of phospho-HPr, *C* Difference spectrum *B–A*

lated HPr showed a very characteristic shift of the C2 proton signal to lower field at pH 9.3 which had already indicated that the phosphoryl group must be attached to the imidazole ring of the only histidine residue of *staphylococcal* HPr. Comparison of the C2 and the C4 proton resonances with the corresponding resonances of the chemically synthesized phosphohistidine isomers allowed the obvious interpretation that also in native phospho-HPr the phospho-group was attached to the N1 of the only imidazole residue of the protein (*Schrecker* et al., 1975; *Gassner* et al., 1977).

Another type of phospho-HPr has been prepared chemically through reaction with phosphoamidate. The resulting mixture contained about 50% phospho-HPr as judged from its electrophoretic mobility which was identical with the electrophoretic mobility of phospho-HPr prepared with PEP and Enzyme I. This species of P-HPr, containing one phospho-group per molecule of HPr, was separated by ion-exchange chromatography from residual HPr. It was no longer active in the phosphotransferase assay. The NMR spectrum of this compound indicated that the phospho-group was linked at the N-3 of the imidazole residue of the protein, and no considerable denaturation had occurred during the chemical phosphorylation of HPr (*Gassner* et al., 1977).

Thus, the NMR studies on the two types of phospho-HPr have demonstrated very clearly that in the intact and biologically active phosphoprotein the phospho-

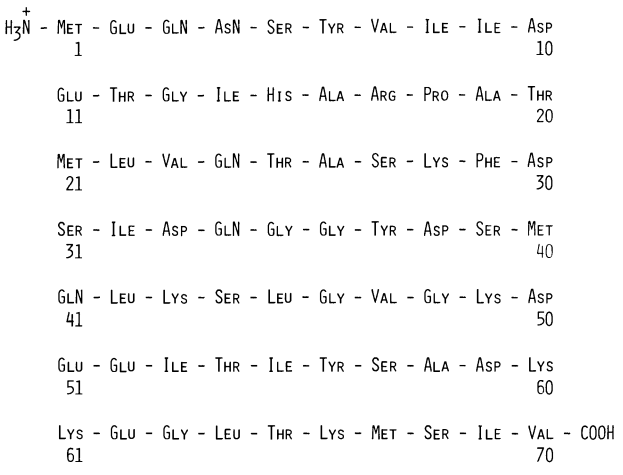


Fig. 3. Amino acid sequence of HPr from *S. aureus*. The molecular weight of HPr calculated from the amino acid sequence is 7685 daltons

group is bound to the N-1 of the imidazole residue. So the high energy bond ($\sim$ 10 kcal/Mol) of P-HPr really is of the P-N type.

c) Primary Structure of HPr

The large amount of HPr prepared from *S. aureus* was very convenient in order to establish the primary structure of this protein, which was accomplished by *Beyreuther* and his co-workers (1977) (Fig. 3). The sequence of HPr from *E. coli* is at the time of publication almost complete in the laboratory of *Roseman* (*Postma* and *Roseman*, 1976). It is hoped that the comparison of the two primary structures will enable the determination of some common features of the two phosphocarrier proteins.

B. The Soluble Sugar-Specific Phosphocarrier Proteins

1. Properties of Factor III ^{lac} of S. aureus

In contrast to one of the constitutive glucose phosphotransferase systems of *E. coli* which contains two membrane-bound components, one of which seems to be sugar specific, *S. aureus* contains a sugar-specific soluble component (*Simoni* et al., 1968; *Hengstenberg*, et al., 1969). The two proteins have been purified from *S. aureus* and are designated Factor III^{lac} and Factor III^{mtl} (*Simoni* et al., 1968). Factor III^{lac}, which is part of the *staphylococcal* lactose operon, has been purified to homogeneity in several laboratories (*Hays* et al., 1973; *Schrecker* and *Hengstenberg*, 1971). It is a protein of molecular weight 33000 — determined by several methods — and the protein is dissociable into three subunits. Earlier experiments based only on SDS gels resulted in four subunits (*Schrecker* and *Hengstenberg*, 1971). This discrepancy can now be explained. According to *Fish* (1975), SDS-protein complexes of approximate molecular weight 7000–10000 no longer separa-

te on SDS gels according to molecular weight. Whereas protein SDS complexes of large polypeptides appear as rodlike molecules, the SDS complexes of small polypeptides behave like ellipsoids or spheres, which usually results in somewhat low molecular weight. Recent studies with cross-linking reagents also indicated three subunits (*Rached*, 1976). Moreover, automated Edman degradation studies showed that the three subunits are identical as far as the sequence was established (*Hays* et al., 1973).

Factor III can be loaded with 3 moles of phosphorus, which means that each subunit carries one phosphogroup. Hydrolysis studies with ^{32}P-labeled Factor III as well as alkaline degradation of P-factor III^{lac} have further demonstrated that Factor III contains the phosphogroup bound to the N-3 position of an imidazole residue (*Hays* et al., 1973; *Hengstenberg* et al., 1976). P-factor III is the phosphodonor of the actual translocation reaction which is catalyzed by the Enzyme II-B′component.

2. Significance of the Subunit Structure of Factor III^{lac} for its Mechanism of Action

Experiments have been performed with Factor III^{lac} to find an explanation for its subunit structure (*Schrecker* and *Hengstenberg*, 1976). Factor III was bound covalently to Sepharose 4B by the method of *Cuatrecasas* and *Parikh* (1972). The thus immobilized Factor III could then be phosphorylated with PEP, HPr and Enzyme I and serve as phosphodonor in the galactoside-specific PTS of *S. aureus*. Matrix-bound phospho-factor III^{lac} dissociated readily under the following conditions: pH higher than 9; presence of the non-ionic detergent Triton X100 which does not dissociate matrix-bound unphosphorylated Factor III^{lac}. (Dissociation could be easily followed by the appearance of ^{32}P-labeled Factor III in the supernatant of the Sepharose-bound material.) The addition of soluble Factor III to the matrix-bound material leads to subunit exchange. The phosphorylated subunits exhibit lipophilic behavior as visualized by electrophoresis in the presence of Triton X100.

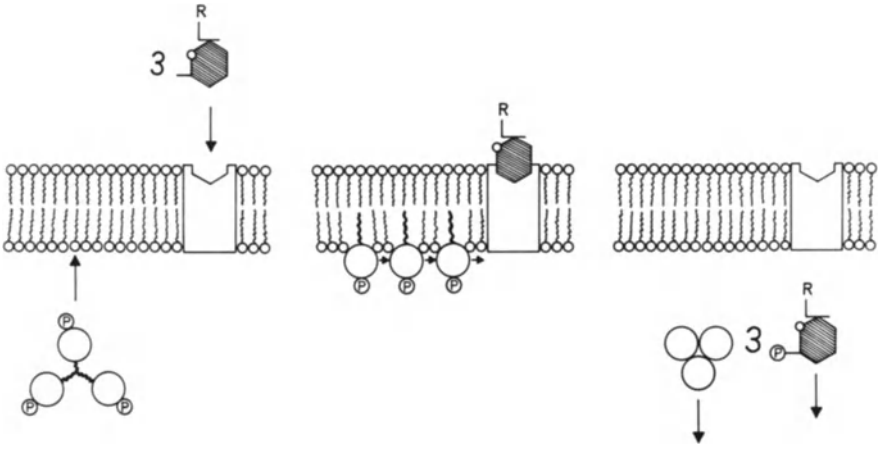


Fig. 4. Model of the function of the soluble sugar-specific phosphocarrier protein Factor III^{lac}

The above experiments suggest that P-Factor III^{lac} may occur in two forms: (1) the trimeric phosphoprotein, a soluble hydrophilic protein, and (2) the monomer, hydrophobic protein which is generated from the trimer on contact with mild nonionic detergents or, perhaps, membrane lipids.

The monomer may be able to penetrate the lipid phase of the membrane in order to transfer its phosphogroup to the sugar via the membrane-bound Enzyme II-B′. A working model of the action of Factor III^{lac} is shown in Fig. 4.

Dissociation of P-factor III^{lac} prior to the phosphorylation of the galactoside by the membrane-bound Enzyme II may explain the higher affinity of P-Factor III^{lac} for Enzyme II^{lac} observed by *Hays* and *Sussman* (1976) during kinetic measurement of PEP-dependent phosphorylation of TMG in the presence of Triton X100.

3. Factor III of the Glucose High Affinity System of E. coli

More recently a high affinity glucose PTS of *E. coli* has been isolated which contains a soluble Factor III–type component. This Factor III is constitutively synthesized by the *E. coli* cells. It has a molecular weight of about 20000 daltons and consists of three or four subunits. The gene locus in *S. typhimurium* is closely linked to the PTS operon.

In addition to the phospho transfer activity, this Factor III also possesses phosphatase activity specific for hexose-6-phosphates.

The phosphoryl linkage in this protein has been intensively studied. In contrast to Factor III^{lac} of *S. aureus*, this protein contains an acyl phosphate residue, as demonstrated by the formation of a protein-bound hydroxamic acid derivative (*Kundig*, 1976).

C. The Membrane-Bound Sugar-Specific Enzyme II-B

1. Enzyme II-B′ Specific for Galactosides in S. aureus

a) Purification and Properties

Enzyme II-B′ of *S. aureus* has been purified to apparent homogeneity after solubilization with Triton X100 (*Korte* and *Hengstenberg*, 1971). Among several ionic and non-ionic detergents used, Triton X100 gave the best results of solubilization. Even the enzymic activity of the solubilized preparation could be partially retained. (The solubilization process of the membrane proteins by detergents is the subject of a very comprehensive review (*Helenius* and *Simons*, 1975).) Moreover the solubilized preparation could be purified further. *Roseman* and co-workers, however, questioned the soluble character of our Enzyme II preparation (*Simoni* et al., 1973). They were puzzled by the fact that "soluble" Enzyme II gave a very high apparent molecular weight of about 10^6 daltons when analyzed on molecular sieve columns (*Hengstenberg*, 1970), whereas the S value obtained after centrifugation in a sucrose gradient containing Triton X100 is about 4.5 S, this is almost identical with the sedimentation velocity of serum albumine.

There are several possible explainations for this behavior. The membrane-bound Enzyme II^{lac} may bind large amounts of the detergent Triton X100, or the

solubilized Enzyme II may consist of a complex of common perhaps identical, protein subunits. Certain properties of the solubilized preparation indicate that solubilization has been achieved. For example in the presence of Triton X100, the Enzyme II^{lac} is able to penetrate 5% acrylamide gels, its activity has been demonstrated in the gel after slicing it. Also membrane components can be separated from each other on DEAE cellulose in the presence of Triton X100 (*Korte* and *Hengstenberg*, 1971). Almost all phospholipid can be removed from the membrane protein after centrifugation through a sucrose gradient which does not contain Triton X100. This indicates that the phospholipids no longer bind to the membrane proteins; they have been exchanged by the detergent (*Lengsfeld* et al., 1973).

After solubilization the protein was chromatographed on DEAE cellulose in the presence of Triton X100, and appeared as a single protein band on an SDS gel. Some minor band could also be detected on the SDS gel. Material which had been obtained from electrophoresis in the presence of Triton gave two bands after SDS gel electrophoresis, one of which had the same molecular weight as the band obtained after SDS electrophoresis of material isolated from the DEAE cellulose column. We concluded from these data that we had purified the Enzyme II-B'^{lac} of *S. aureus* almost to homogeneity. However, we are aware of the fact that we demonstrated homogeneity by the use of SDS gels which separate only according to molecular weight. It is known from the literature than many proteins involved in bacterial transport have molecular weights around 30000–40000 (*Boos*, 1974). Therefore it may still be possible that the SDS band contains several membrane-bound proteins. Other more specific methods of purifying this protein, such as affinity chromatography using a Sepharose column with a covalently attached galactoside ligand (*Cuatrecasas* and *Anfinsen*, 1971), have so far been unsuccessful (*Hengstenberg* and *Schrecker*, 1974). The molecular weight of the polypeptide chain of the Enzyme II^{lac} was estimated to be 35000. We did not find genetic or biochemical evidence for another membrane-bound component which was necessary for the phosphorylation of galactosides.

b) Lipid Dependence of the Staphylococcal Enzyme II-B'^{lac}

A preparation of Enzyme II^{lac} essentially free of lipid has been obtained by sucrose gradient centrifugation of solubilized Enzyme II containing membranes. Whereas triton and the phospholipids stayed at the top of the gradient, the protein penetrated the gradient, formed aggregates, and was finally found on the bottom of the gradient as a precipitate. Though containing only trace amounts of phospholipids, the precipitate showed membranelike structure in the electron microscope (*Lengsfeld* et al., 1973). The precipitate exhibited only very low phosphotransferase activity in the presence of other purified soluble PTS components, but this activity could be increased by adding Triton X100. These results are interpreted in the following way: Enzyme II-B'^{lac} of *S. aureus* needs a lipophilic environment to function properly. In the intact membrane, this environment consists of the surrounding phospholipid molecules. The lipophilic environment can also be created by replacing the membrane phospholipids with detergents which apparently are able to mimic the function of a phospholipid. Further

evidence for the role of phospholipids in the transport process was obtained by *Mindich* (1971) who used a mutant strain of *S. aureus* which was an auxotroph for glycerol, an essential compound for phospholipid biosynthesis. Moreover, this strain had a heat-sensitive repressor, so that induction of the *staphylococcal* lactose operon could be achieved simply by a temperature shift. In the absence of glycerol the induced cells showed reduced accumulation of galactosides. However, the ability to phosphorylate galactosides in the crude extract was normal. This means that Enzyme II^{lac} was integrated into the membrane in the glycerol auxotroph, resulting in wild-type phosphotransferase activity. Transport, however, was considerably reduced in this strain, a feature which may have been caused by a wrong orientation of the vectorial component Enzyme II^{lac} in the membrane.

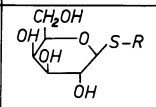
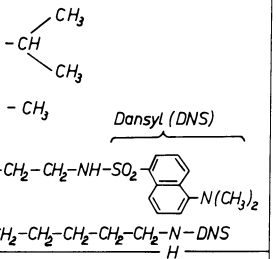
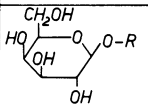
In the literature, membrane proteins have been described which show a very specific requirement for their biological activity, such as the Enzyme II-A/II-B complex specific for glucose transport and phosphorylation (*Kundig* and *Roseman*, 1971). (This complex will be discussed later in this review.) Other examples, like the C_{55} Isoprenoid alcohol phosphokinase from *S. aureus* membranes, not need a specific lipid for biological activity (*Sandermann* and *Strominger*, 1971).

c) Sugar Binding Site of Enzyme II-B'^{lac} of S. aureus

S. aureus transport a large variety of β-D-galactosides via PTS, some representative examples are listed in Table 2. To be phosphorylated, the substrates have to possess the β-D-galactopyranoside ring. The bridge atom can be oxygen or sulfur; the aglycon can be an aliphatic residue, an aromatic resude, or another carbohydrate. Even very bulky and nonphysiologic derivatives, such as DG_2 or 5′-(N-dansyl) aminopentyl-1-thio-β-D-galactopyranoside (DG_5), are phosphorylated and transported by the *staphylococcal* cells.

The substrates DG_2 and DG_5, being dansyl derivatives, are highly fluorescent. As mentioned earlier in this review, these substrates were used by *Kaback* and coworkers in binding studies of membrane vesicles of *E. coli*. They observed that

Table 2. Galactosides phosphorylated and transported by the staphylococcal PTS

	Glycon	Aglycon R
Thiogalactoside: Isopropyl-β-D-thiogalactoside ITPG Thiomethyl-β-D-galactoside TMG 2-(N-Dansyl)-aminoethyl-β-D-thiogalactoside DG_2 DG_5		
O-galactoside: O-nitrophenyl-β-D-galactoside ONPG Lactose Allolactose		 Glucose (β-1-4) Glucose (β-1-6)

binding of the fluorescent galactosides to membrane vesicles occurred only if the vesicles were energized with D-lactate or another suitable electron donor.

In contrast to the findings of *Kaback* and co-workers using *E. coli*, *staphylococcal* membrane fragments bind DG_2 or DG_5 without prior energization. The binding has been demonstrated using lactose as a competitor for the galactoside binding site of Enzyme II^{lac}. When DG_2 is removed from the binding site of Enzyme II^{lac} containing staphylococcal membrane framents, the fluorescence intensity is depressed. This implies that DG_2 bound to Enzyme II^{lac} is in a more lipophilic environment than the unbound DG_2.

Table 3. Affinity of some β-D-galactosides to the lactose-specific PTS

Substrate	K_i of ONPG hydrolysis in whole cells [μM]	K_M of PTS assay [Factor III^{lac}] $=\infty$	K_M of transport [μM]	K_D [μM]
Lactose		0.7[a]	60[a]	0.25[a]
			5[c]	0.6[b]
TMG		600[a]	1 200[a]	
IPTG	130[b]	5[a]	100[a]	70[b]
DG_2	9[b]			10[b]
ONPG	900[b]			

[a] *Postma* and *Roseman*, 1976; [b] *Hengstenberg* et al., 1976; [c] *Egan* and *Morse*, 1966

The measurement of fluorescence depression upon removal of DG_2 can be used to determine the dissociation constant of other nonfluorescent substrates. In Table 3 the dissociation constants of some β-D-galactosides are listed. They have been measured by using the fluorescence depression effect after adding the corresponding galactoside. The values are compared with K_i values obtained from the inhibition of whole cell ONPG hydrolysis, with K_M values of the PTS assay, and K_M values of transport. The significant differences in these values may be caused by the use of different bacterial strains. Removal of the substrate from its binding site can also be achieved by different mechanism. If one adds soluble PTS components (Enzyme I, HPr, and Factor III) to the Enzyme II^{lac}-DG_2-complex, the fluorescence decreases until all the DG_2 is phosphorylated. This experiment implies that DG_2 is removed from its lipophilic binding site upon PEP-dependent phosphorylation. Phosphorylation is, therefore, a mechanism to lower or abolish the affinity of the membrane-bound substrate which is an essential step in releasing the substrate into the cell interior after vectorial phosphorylation (*Hengstenberg* et al., 1976). Recent studies by *Hays* et al. (1975) have shown that galactose substituted at C6 with a tosyl residue is a competitive inhibitor of TMG uptake and phosphorylation in *S. aureus*. They concluded from their experiments that the tosyl galactoside behaves like a transition state analog where the tosyl residue has structural analogy to the phosphoimidazole residue of Factor III^{lac}, and thus prevents phospho-Factor III from reaching its correct position to transfer the phosphate to the sugar.

2. The Membrane Component Enzyme II-B′ of E.coli Specific for Glucose

a) Purification and Properties

The Enzyme II-B′ component in the glucose high-affinity system of *E.coli* has been isolated by *Kundig* and co-workers. It was solubilized from purified membranes by using chaotropic reagents ($NaClO_4$) and detergent. The protein was solubilized by 1% sarcosyl, it was precipitated with ammonium sulfate, and was further fractionated by DEAE cellulose chromatography.

b) Lipid Requirement of Enzyme II-B′^{glc}

The protein exhibited a lipid specificity which is less pronounced than the lipid dependence of Enzyme II-B discussed later in this article. Whereas PG gave maximal rates of phosphotransferase activity, cardiolipin activated only 30%, sarcosyl only 10%, and triton X100 was inactive. Though the data on the lipid dependence of Enzyme II-B′ are not yet complete, quite considerable differences exist between the lipid requirement of the Enzyme II-B′ and that of the Enzyme II-B systems. The Enzyme II-B′ system of *E.coli* may resemble in its properties the Enzyme II-B′ isolated from *S.aureus* more. The glucose-specific Factor III of the Enzyme II-B′ system of *E.coli* shows features which are similar to the *S.aureus* lactose phosphotransferase system, though considerable differences exist in the type of high energy phospho-protein bond of the two Factor III components.

3. The Enzyme II-A/II-B Complex of E.coli

a) Purification and Properties

E.coli possesses a high affinity glucose transport system of the type Factor III Enzyme II-B′. In *S.aureus* the lactose transport system and probably the mannitol transport system belong to the Factor III Enzyme II-B′ type also.

Quite different biochemical properties have been reported for the low affinity II-A/II-B system of *E.coli* which is specific for glucose. The isolation and reconstitution of the system has been described in detail (*Kundig* and *Roseman*, 1971). The components of the II-A/II-B system were solubilized with urea butanol followed by fractionation with ammonium sulfate, ion exchange chromatography, and isoelectric focusing.

b) Lipid Requirement of the Enzyme II-A/II-B Complex

Restoration of an active enzyme complex capable of transferring the phosphogroup from P-HPr to the sugar required Enzyme II-A and II-B, Mg^{++}, and a specific phospholipid (phosphatidyl glycerol). In order to achieve proper functioning, the complex had to be reconstituted in a specific order. Only mixing II-B, Mg^{++}, PG, and Enzyme II-A in this sequence gave the active Enzyme II complex. By using ^{32}P-labeled PEP Enzyme I and HPr, the transfer of the phosphoryl group to Enzyme II-A could be demonstrated. The P-transfer to Enzyme II-A also

occurred without Enzyme II-B being present in the reaction mixture. The phosphoryl linkage in P-Enzyme II-A has not yet been clearly established but preliminary experiments indicate that the phosphogroup is bound to an acyl group of the protein (*Kundig*, 1976).

D. Phosphotransferase Systems Specific to Other Carbohydrates in E. coli and S. aureus

In *E. coli* and *S. typhimurium* sugars of the D-manno and D-gluco configuration, β-D-glucosides, fructose, and several hexitols (*Lengler*, 1975a, 1975b) are transported via PTS. As already mentioned earlier in this review, the sugars not taken up via PTS in Gram-negative bacteria are the disaccharides lactose, melibiose, maltose, and the sugars galactose, pentose, and hexose-6-phosphate, which are taken up by other transport systems where energy coupling occurs probably via a proton motive force–type mechanism. In gram-positive organisms such as *S. aureus*, the disaccharides lactose and sucrose in addition to the monosaccharides are transported by PTS, while maltose is taken up by a different mechanism (*Button* et al., 1973).

E. Occurrence of PTS in Other Microorganisms

Widespread in microorganisms, the PTS is generally present in anaerobic organisms and facultative aerobes, but not in obligate aerobes (*Romano* et al., 1970). It also seems to be absent from yeast and fungi. One report claimed the discovery of a PTS in the vertebrate intestine, but this report has never been confirmed or extended (*Weiser* and *Isselbacher*, 1970).

V. Conclusions

Enzymology of carbohydrate transport in bacteria is a rapidly expanding branch of modern biochemistry which has become the successor of microbial genetics and kinetics of transport which dominated the field for many years. Genetics and kinetics were research tools which showed the great variety and complexity of the bacterial transport systems. However, since the contribution of these two disciplines to knowledge of the structure and function of bacterial membranes was limited, many laboratories became interested in the biochemistry of transport processes in bacteria, with the aim of describing the transport mechanism in molecular detail.

The biochemical approach has been most successful with sugar transport systems which are mediated by vectorial phosphorylation. All components involved in the transport process were isolated in pure form if possible. The reactions which couple the cellular energy of PEP with the actual transport process are known in detail (Fig. 5). Some of the sugar-specific proteins have been isolated and purified, and substrate binding studies have been performed. Still unknown however, is the primary or tertiary structure of a sugar translocator. This task will

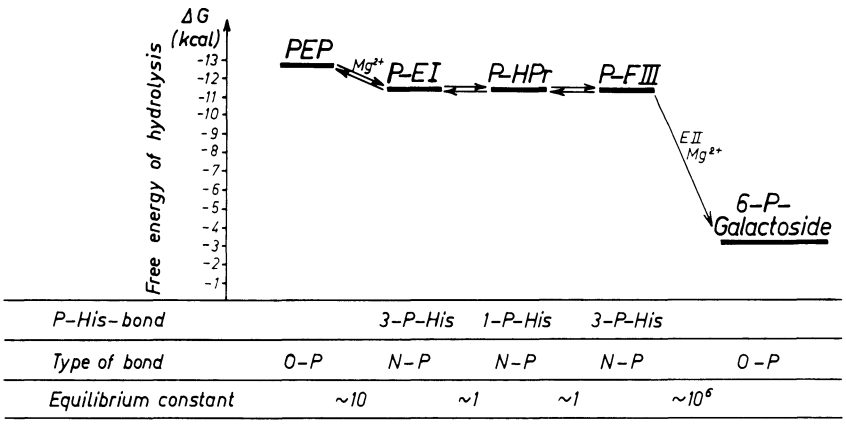


Fig. 5. Bioenergetics of the lactose-specific PTS of *S. aureus*

probably require many more years' research due to the complex composition of the bacterial membranes.

In other bacterial sugar transport systems that are probably driven by a proton motive force–type mechanism, only the soluble sugar-binding proteins are well characterized. No structural details are known about the membrane-bound carriers, probably due to the same difficulties mentioned above.

The process of energy coupling is still governed by the evaluation of concepts such as the chemiosmotic theory or the respiratory chain model. Evidence is accumulating that the chemiosmotic theory may be the proper way to describe the energy coupling process (*Ramos* et al., 1976).

More advanced than the study of the biochemistry of transport in bacterial systems are the studies of some transport systems of highly specialized organelles of higher organisms, such as mitochondria or, for instance, the calcium pump of the sarcoplasmic reticulum. Some of these organelles have been successfully reconstituted using purified phospholipids and isolated pure protein components (*Meissner* and *Fleischer*, 1974). The rapid progress in studies of these organelles, especially the sarcoplasmic reticulum, was possible because of the simple composition of their membrane protein. Moreover, reconstitution experiments are a powerful method for describing membrane protein-lipid interaction.

The broad knowledge about the variety and specificity of bacterial transport process which has been accumulated by genetic and kinetic techniques is now being rapidly keepened and augmented by biochemical methods. The wealth of information already accumulated may be very important in helping to defeat bacterial infections which still pose a serious problem in medicine, especially in surgery. For example, we know that the sugar transport systems of bacteria are often very specific for the glycon part of the glycoside molecule, whereas the aglyconic part of the molecule can be varied in a broad range (compare galactoside transport in *S. aureus*). This suggests that naturally occurring bacterial transport systems could be used to transport antibacterial compounds, specifically when these compounds (e.g. antibiotics) could be applicated for instance as glycosidic derivatives. Such chemically modified antibiotics would be extensively accumulated in the interior

of the bacterial cell by the suitable transport system. Thus, the target organism would be sensitive to much lower concentrations of antibiotic modified in this way.

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The Eosinophil and its Role in Immunity to Helminth Infection

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I. Introduction

For many years, an association has been noted between helminth infection and increased levels of eosinophils in the peripheral blood. In human disease, this association is particularly marked in patients with schistosomiasis, filariasis, ascariasis, trichinosis, and visceral larva migrans; but most helminth infections in which there is a tissue stage of the parasite usually elicit at least some degree of eosinophilia. In addition, the common condition of tropical pulmonary eosinophilia is now believed to be associated with cryptic filarial infections (*Neva* et al.,

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1975). High levels of blood eosinophils are also seen in livestock infected with helminths such as *Fasciola hepatica, Dictyocaulus viviparus* and *Oesophagostomum radiatum;* while in experimental animals, infection with *Trichinella spiralis* remains one of the most reliable methods of inducing a peripheral blood eosinophilia.

In spite of the striking and consistent nature of this relationship between eosinophilia and helminth infection, it has proved difficult to attribute a *functional* role to the eosinophil in such infections. Quite recently, however, several functional properties have been described which may have a part to play in limiting the deleterious effects of helminth infection. These include: a preferential capacity for phagocytosing immune complexes; a role in the modulation or dampening down of anaphylactic hypersensitivity reactions of Coombs Type I (*Coombs* and *Gell*, 1975); and the ability to mediate antibody-dependent damage to helminths, especially the larval stages.

In this review, the basic structure and biochemistry of the eosinophil will first be summarized, with particular emphasis on those aspects in which the eosinophil differs from other cell types, especially the neutrophil. It is in these differences that unique functional attributes may be expected to lie. Next, the way in which large numbers of eosinophils can accumulate in the tissues will be briefly discussed. This again is of importance in considering a functional role for the eosinophil, since it must act locally at the site of parasite invasion or migration. Finally, the functional properties of the eosinophil will then be considered in more detail, with particular reference to helminth infection. For more extensive discussion of structure and kinetics, the reviews by *Zucker-Franklin* (1974), *Kay* (1974) and *Cline* (1975) are recommended.

II. Eosinophil Structure

A. Morphologic Observations

The stage at which the eosinophil precursors differentiate from neutrophil precursors, and the factors which govern such differentiation, are poorly understood (*Zucker-Franklin*, 1974), although the occurrence of hereditary enzyme defects specific for each granulocyte type (*Presentey* and *Szapiro*, 1969; *Lehrer* and *Cline*, 1969) and the finding that neutropenic patients can have normal or raised eosinophil levels (*Connell*, 1969) suggest that differentiation occurs at an extremely early stage. The first distinguishable cell of the eosinophil series, the eosinophilic promyelocyte, is found in the bone marrow in extremely small numbers and is characterized by the presence of about ten small eosinophilic granules (*Cline*, 1975). Subsequent differentiation through the myelocyte, metamyelocyte, and band forms proceeds in a fashion comparable to that of the neutrophil series, with peripheral condensation of nuclear chromatin, loss of nucleoli, and diminution of the cytoplasmic reticulum. Nuclear lobation in eosinophils is less marked than in neutrophils, and mature cells generally have two or three lobes. The Golgi apparatus and the mitochondria are more prominent in mature eosinophils than in neutrophils.

The most striking difference between the neutrophil and the eosinophil,

however, is in the morphology of their granules. These highly acidophilic organelles, which may contain up to half the total protein content of the cell (*Archer* and *Hirsch*, 1963a), consist of polyhedral or biconvex particles containing a crystalloid electron-dense core. This is surrounded by an amorphous matrix, which is in turn bounded by a membrane. The core has the characteristics of a cubic lattice with repeating units at intervals of 30–40 A in different species (*Miller* et al., 1966). Crystalloid-containing granules are not present during the earliest stages of differentiation, when the characteristic structure is a spherical, homogeneously dense granule slightly larger than that found in neutrophil precursors (*Bainton* and *Farquhar*, 1970). As differentiation proceeds, both immature and crystalloid-containing granules are found, with forms intermediate between the two types; and it is usually suggested that, during differentiation and division, immature granules are continually being produced and are continually transforming into the mature, crystalloid-containing forms (*Hudson*, 1966). In the circulating eosinophil, the granule population is usually restricted to the crystalloid-containing forms, although a second granule type has recently been identified in human late eosinophils (*Parmley* and *Spicer*, 1974); this granule is round or oblong in shape and is smaller than the homogeneous granule of the immature cell.

In normal individuals, the number of eosinophils in the peripheral blood is usually less than 0.25 to 0.55 $\times 10^9$/l (*Felarca* and *Lowell*, 1967; *Tai* and *Spry*, 1976). Under abnormal conditions, including helminth infections, this figure can increase by 10- to 100-fold. Under such conditions, eosinophil morphology is frequently abnormal. The cells may be vacuolated (*Connell*, 1968; *Saran*, 1973; *Spry* and *Tai*, 1976); there may be a reversal of the relative densities of the matrix and core of the granules (*Zucker-Franklin*, 1974; *Tai* and *Spry*, 1976); and the cells may be relatively degranulated (*Zucker-Franklin*, 1971; *Tai* and *Spry*, 1976). These morphologic alterations may reflect functional changes occurring in these cells (*Spry* and *Tai*, 1976).

Morphologically, therefore, the main distinguishing feature between the neutrophil and the eosinophil is the presence in the latter of an unusual and characteristic granule. Although differences between eosinophils in different individuals have been described, it is not at present possible to state on morphologic grounds whether or not there exist separate subpopulations of eosinophils. This problem — of some importance in considering functional properties of eosinophils — will be discussed in later sections.

B. Histochemical and Biochemical Observations

Since the major morphologic feature of the eosinophil is the crystalloid-containing granule, much attention has been paid to the composition of this organelle. Its acidophilic nature is attributable to its high content of an arginine-rich basic protein (*Vercauteren*, 1950–51). This "major basic protein" has been isolated from human and guinea pig eosinophils (*Gleich* et al., 1973, 1976); in man it has a molecular weight of about 9000 daltons and shows a marked tendency to form disulphide-linked aggregates. Using peroxidase-labeled antisera, *D. Lewis* et al.

(1976a) have shown by electron microscopy that this protein is localized to the core of the granule; none is found in the granule matrix or in the surrounding cytoplasm. The protein lacks peroxidase activity, and its functions are poorly understood. *Gleich* et al. (1974) have suggested that it has no unique functions other than those that would be expected of a basic protein with free sulphydryl groups: namely the ability to react with and alter the function of free or cell-bound acidic molecules, and the ability to activate enzymes which depend on free sulphydryl groups.

Other characteristic features of the granule are its high content of zinc, associated with the noncrystalloid matrix (*Pihl* et al., 1967), and its abundant peroxidase activity. In the mature granule, peroxidase is restricted to the matrix rather than the crystalline core; in immature cells, it is also found in the rough endoplasmic reticulum, the cisternae of the Golgi apparatus, and the immature granules (*Bainton* and *Farquhar*, 1970). It is suggested that synthesis of enzymes and packaging into granules usually occurs in immature stages in the bone marrow, and that mature, circulating eosinophils are not actively synthesizing enzymes. In some situations, however, immature enzyme-synthesizing eosinophils may be found in extramedullary sites (*Bogitsh*, 1971). Eosinophil peroxidase differs histochemically and antigenically from neutrophil myeloperoxidase (*Rytömaa*, 1960; *Salmon* et al., 1970), and genetic disorders exist in which there is deficiency of either eosinophil or neutrophil peroxidase, but not both (*Presentey*, 1969; *Salmon* et al., 1970). The relative lack of efficiency of eosinophil peroxidase in bacteriostasis will be described in Section IV. B.

In contrast to peroxidase, eosinophil acid phosphatase is not associated with the crystalloid-containing granule (*Wetzel* et al., 1967). Instead, it appears to be localized in the homogeneous spherical granule characteristic of late eosinophils (*Parmley* and *Spicer*, 1974). Eosinophil granules have lower acid phosphatase activity than neutrophil granules (*Archer* and *Hirsch*, 1963a), while alkaline phosphatase activity is low (*Archer* and *Hirsch*, 1963a) or absent (*Wetzel* et al., 1967) in different species. Such alkaline phosphatase as has been reported to be present in human eosinophils is not distinguishable from neutrophil alkaline phosphatase (*Cao* et al., 1973); other workers fail to find alkaline phosphatase in human eosinophils (*West* et al., 1975), and the possibility of neutrophil contamination of eosinophil preparations must be considered. Although eosinophils do not contain lysozyme, plasminogen (*Riddle* and *Barnhart*, 1965) and kininase (*Melmon* and *Cline*, 1968) have both been reported to be present. In addition, eosinophils contain a collagenase as active as that of neutrophils (*Bassett* et al., 1976), while their β-glucuronidase and acid β-glycerophosphatase content is approximately twice as great (*West* et al., 1975).

Two enzymes of potential importance in modulating type I reactions are present in much higher concentrations in eosinophils than in other cell types. Phospholipase D, previously recognized in mammals only in rat brain (*Saito* and *Kanfer*, 1975), has now been isolated from human eosinophils (*Kater* et al., 1976). Arylsulfatase, which is localized in the matrix of the granule (*Bainton* and *Farquhar*, 1970), is also present to a much greater extent in eosinophils than in other circulating leukocytes (*Tanaka* et al., 1962; *Wasserman* et al., 1975a). Possible functional roles for these enzymes are described in Section IV. C.

Although little work has been done on eosinophil metabolism — primarily because of difficulties in obtaining sufficiently large numbers of pure cells — such work as there is suggests that the eosinophil resembles the neutrophil in many respects. The relatively large and well-developed mitochondria suggest an aerobic metabolism for energy production, but biochemical evidence points to a mainly anaerobic system. Of the glucose utilized by the cell, 90% is converted to lactic acid, while only 10% is oxidized to CO_2 or used for glycogen, lipid, or amino acid synthesis (*Stjernholm* et al., 1969). Hexose monophosphate shunt activity is greater in eosinophils than in neutrophils, both at rest and after phagocytosis (*Baehner* and *Johnston*, 1971; *Mickenberg* et al., 1972). Some caution is necessary in evaluating these findings, since the eosinophils used in such studies have come from patients with eosinophilia. Such eosinophils may be in a "stimulated" or otherwise functionally abnormal state.

In summary, although most of the differences between eosinophils and neutrophils are quantitative variations on the granulocyte theme, a few qualitative differences have also been described. It is possible that further qualitative differences in enzyme distribution remain to be discovered and that such differences may be important in attributing unique functional properties to the eosinophil. Recent improvements in techniques for obtaining pure preparations of eosinophils, from normal as well as from eosinophilic patients (*Sher* and *Glover*, 1976), may help in the detection of subtle differences.

C. Receptors

In spite of the finding that eosinophils show a marked capacity for phagocytosing immune complexes (Sect. IVB) and for mediating antibody-dependent damage to helminth larvae (Sect. IVD), relatively little work has been done on the presence of cell surface receptors for immunoglobulins or complement components. *Rabellino* and *Metcalf* (1975) examined cells from mouse eosinophil colonies grown in vitro for the presence of IgG and C3 receptors and compared them with cells from macrophage and neutrophil colonies. In contrast to the macrophage and neutrophil preparations, eosinophils showed no detectable C3 receptors. By the seventh day of culture, however, 50–60% of eosinophils showed receptors for a heterologous (rabbit) IgG, as detected by a rosetting technique. Such cells showed the phenomenon of cap formation after incubation in the presence of indicator cells for 30 to 120 min at 37°C. In contrast, *Gupta* et al. (1976) have reported that human eosinophils have no "high affinity" Fc receptors detectable in a rosette test, although a proportion of such cells do bind aggregated IgG. Human eosinophils do, however, have receptors for C3b, C3d and C4, which are detectable by rosetting techniques.

One possible reason for the discrepancy in these findings concerning Fc receptors may be a species specificity with respect to the IgG-eosinophil combination tested. *Butterworth* et al. (1976a) have shown that eosinophils from the peritoneal cavities of normal guinea pigs have receptors capable of reacting in a rosetting test with IgG from guinea pig and pig, but not from rabbit. Both IgG_1 and IgG_2 were reactive in the guinea pig. Subsequently, *Fuenmayer* (1976, personal

communication) has found that human eosinophils react better with pig than with rabbit antibody, and that the difference in distribution of receptors on eosinophils from atopic compared with normal patients is more marked with pig antibody.

The recent findings of *Tai* and *Spry* (1976) and *Spry* and *Tai* (1976) must be viewed in this light. These workers reported that only 3–10% of eosinophils from normal human subjects formed rosettes with red cells sensitized with rabbit antibody, whereas eosinophils from patients with eosinophilia associated with various conditions, including Löffler's cardiomyopathy, showed more rosettes, 89% being the highest recorded. The rosetting reaction was blocked by heat-aggregated normal human serum and by aggregated myeloma proteins of subclasses IgG_1 and IgG_3, but not IgG_2 or IgG_4. When IgG from other species was used to sensitize the red cells, however, *normal* eosinophils showed a higher proportion of rosettes, ranging from 33% with rat antibody to 62% with pig antibody. The porcine sera used in these experiments and in those described by *Butterworth* et al. (1976a) may be unusual, in that other porcine sera have failed to induce the formation of human eosinophil Fc rosettes (*Spry*, 1977, personal communication).

Two points should be emphasized from these various findings: first, that the eosinophil Fc receptor shows a strong specificity with respect to IgG from different species; and secondly, that the proportion of eosinophils bearing detectable Fc receptors increases in at least some eosinophilic conditions. This does not necessarily mean that there are two populations of eosinophils, one with Fc receptors and one without; it could equally well imply that only those cells with the highest density receptors are detected in the rosette test and that the mean density increases in patients with eosinophilia. Analogous alterations in Fc binding properties may be seen after "stimulation" of other cell types, including macrophages and T-lymphocytes. A suggestion that this might not be the explanation comes from the work of *Ottesen* (1976, personal communication), who has preliminary evidence to suggest that Fc and C3 receptors may be on different cells.

Further evidence concerning C3 receptors has come from the work of *Tai* and *Spry* (1976), who found that 30–43% of normal human eosinophils formed C3 rosettes, and of *Sher* and *Glover* (1976), who found that 11–38% of patients with helminth-induced eosinophilia formed C3 rosettes. The finding mentioned above, namely that eosinophils from bone marrow cultures fail to form C3 rosettes (*Rabellino* and *Metcalf*, 1975) may therefore reflect either a species difference or an unusual feature of cells from bone marrow colonies.

A final point concerning eosinophil receptors is that *Hübscher* (1975a) has reported that some patients with ragweed antigen hypersensitivity have IgE demonstrable on a proportion of their eosinophils and that these eosinophils will bind ragweed antigen. Similarly, *Fujita* et al. (1975) have reported that eosinophils recovered from the nasal secretions of patients with ragweed allergy have bound and possibly phagocytosed IgE-ragweed antigen complexes. More extensive studies are required, however, before the existence of IgE receptors on eosinophils can be regarded as proven. *Wilson* and *Heller* (1976) have demonstrated an antibody cytophilic for eosinophils in chickens, but the class of this antibody is not known.

In summary, receptors both for immunoglobulins and for complement components have been detected on eosinophils. The potential importance of such

receptors, both in the phagocytosis of immune complexes (Sect. IV B) and in the mediation of antibody-dependent damage to helminths (Sect. IV D), is self-evident. More detailed studies of the distribution of such receptors in different eosinophil subpopulations and in different categories of eosinophilia would therefore be extremely desirable.

D. Antigens

A useful inroad into the study of eosinophil function has been the recent demonstration that they bear cell-specific antigens, together with the associated production of antisera specific for these antigens. *Mahmoud* et al. (1973), by immunizing rabbits with mouse eosinophils and absorbing the sera obtained with macrophages, lymphocytes, and neutrophils, produced an anti-mouse eosinophil serum (AES) with a remarkable degree of specificity, as judged by a cytotoxicity assay in vitro and by reduction of circulating eosinophil counts in vivo. Later, these workers produced anti-human eosinophil sera (*Mahmoud* et al., 1974a, 1974b). *Gleich* et al. (1975) produced an antiserum with specificity in vivo and in a cytotoxicity assay in vitro for guinea pig eosinophils; they showed, however, that although neutrophils were not directly affected by this serum, such cells would absorb out the anti-eosinophil activity. They therefore suggested that the antigen was present on both cell types, but in quantitatively greater amounts on the eosinophil. The cellular localization of the antigen recognized is not known.

Administration of AES to mice which had been rendered eosinophilic by primary or repeated intravenous inoculations of *Schistosoma mansoni* eggs led to a marked reduction in mature eosinophils, both in the circulation and in the granulomatous reactions around the deposited eggs (*Mahmoud* et al., 1975b). In contrast, eosinophil precursors in the bone marrow, up to the metamyelocyte stage, increased in numbers. The finding that more mature stages in the bone marrow, as well as in the blood, were also reduced, suggested that the increase in immature stages was not attributable to inaccessibility of these cells to the antiserum, but rather that they had not yet acquired the specific antigen recognized by the AES.

Other components within the eosinophil also show a degree of cell or species specificity. For example, the major basic protein is antigenically distinguishable in the guinea pig and man (*D. Lewis* et al., 1976b), while eosinophil peroxidase can be distinguished from neutrophil peroxidase on the basis of antigenic properties (*Salmon* et al., 1970).

E. Conclusions

These findings have been summarized very briefly to make the point that the eosinophil differs from the neutrophil not only in its morphology but also in its composition, its distribution of enzymes and receptors, and its antigenicity. Although many of these differences are only quantitative, the picture is beginning to emerge that the eosinophil represents a quite distinct entity from the neutrophil. Further differences in physiology are considered in the next section, and the possi-

ble evolutionary significance of such differences, in functional terms, is considered in Section IV.

III. Eosinophil Kinetics

A. Introduction

In considering eosinophil kinetics, it is important to remember that the eosinophil is primarily a tissue cell. *Rytömaa* (1960), for instance, has estimated that for every circulating eosinophil there are about 300–500 in the tissues. Particularly in helminth infections, as will be seen in Section IV, eosinophils exert their function at the stage when the parasites are migrating through or lodged in the tissues, rather than on blood-stream or intestinal stages. In this section, therefore, the mechanisms whereby large numbers of eosinophils can localize at the site of a tissue parasite will be discussed. This will involve consideration of three topics: first, the dynamics of eosinophil turnover, under both normal and abnormal conditions (Sect. III B); secondly, the mechanisms which elicit an increase in peripheral blood eosinophil counts (Sect. III C); and thirdly, the mechanisms by which these eosinophils leave the blood and localize at the site of the parasitic lesion (Sect. III D). These three problems have not yet been fully worked out, but a considerable body of information now exists on the ways in which eosinophils *may* selectively accumulate around an invading parasite.

B. Eosinophil Dynamics in Normal and Abnormal Situations

Many of the studies that have been carried out on the dynamics of eosinophil turnover have made use of situations in which the peripheral blood eosinophil levels are abnormally high. Such studies cannot be used to derive information on the turnover in normal individuals; they do, however, provide a certain amount of data for comparisons with other cell types. These studies will be considered later in this section. First, some attention must be given to the relatively few studies that have been carried out on normal individuals.

Foot (1965) used a technique of autoradiography after continuous infusion of tritiated thymidine to determine turnover times in normal rats. He found that there was a delay of 2.5–3 days between synthesis of DNA by the myelocyte and emergence of mature eosinophils in the blood. Eosinophils subsequently left the blood with a half-life of 8–12 h and entered the tissues, especially the gastrointestinal tract; here they persisted with a half-life of 22 h. In a later study, *Spry* (1971 a, 1971 b) showed that in normal rats the cycle time of dividing bone marrow precursors was 30 h; this shortened to 9 h in rats rendered eosinophilic with *T. spiralis*, and in such animals the precursors underwent five or six extra divisions. The emergence time of eosinophils from the bone marrow of normal rats was 41 hours; again this was shortened, to 18 h, in *Trichinella*-infected animals, possibly due to the action of an eosinophil-releasing factor. *Spry* suggested that the eosinophils, after leaving the bone marrow, underwent a further maturation

step in the spleen and that they subsequently left the circulation with a half-life of 6.7 h. A similar study has very recently been carried out on three normal human subjects by *Parwaresch* et al. (1976), who concluded that peripheral blood eosinophils were composed of *two* populations derived from different precursors. One population, derived from a slowly dividing precursor, emerged rapidly from the bone marrow and had a long blood half-life. The second population, derived from a rapidly dividing precursor, emerged from the bone marrow later and had a shorter half-life. The mean half-life of all eosinophils in the circulation was 8 h. This interesting observation requires more extensive study, since it provides the first indication of different eosinophil populations derived from different precursors.

Turning now to experiments on eosinophilic individuals, several workers have determined blood transit times on ^{51}chromium-labeled eosinophils reinfused into the donor. Such studies must be viewed with caution, because of the unknown effects of cell preparation and of isotope-labeling on subsequent viability or sequestration. *Herion* et al. (1970) showed in two patients an initial removal of eosinophils with a subsequent reappearance over the second 24 h, whereas *Carper* and *Hoffman* (1966) found that Pelger-Huet eosinophils in the dog were rapidly removed from the blood with a circulating half-life of only 30 min. In a more extensive study, *Dale* et al. (1976) have compared the kinetics of labeled eosinophils from eosinophilic subjects with labeled neutrophils from normal individuals. Neutrophils progressively left the circulation with a blood half-life of 12 h. Eosinophils, in contrast, consistently left the circulation over the first 3 h, subsequently reappearing over the next 12 h, and finally disappearing exponentially with a blood half-life of 44 h. In two patients with splenomegaly, there was an initial increase in surface counts over the spleen; however, three patients with normal-sized spleens and one who had been splenectomized still showed the initial transient drop in counts, even though they showed no increase in surface counts over the spleen. There was no evidence, therefore, for a splenic localization during the initial disappearance phase.

A rather different approach has been followed by *Bass* (1975) in his interesting studies on the eosinopenic response to acute inflammatory stimuli of mice already rendered eosinophilic by infection with *T. spiralis*. In mice with pneumococcal abscesses, *Escherichia coli* pyelonephritis, Coxsackie viral pancreatitis, or lesions induced by subcutaneous injection of turpentine, there was a marked suppression of *Trichinella*-induced eosinophilia. This was associated with a rapid accumulation of eosinophils at the periphery of the inflammatory lesion, in contrast to neutrophils, which localized more centrally. In addition, there was an inhibition of egress of mature eosinophils from the bone marrow; and only with prolonged inflammation was there an inhibition of bone marrow eosinopoiesis. Among other points, these studies emphasize that the eosinophils are only transiently in the circulation and that changes in peripheral blood eosinophil counts may bear no relationship either to eosinopoiesis or to eosinophil numbers at specific sites. This is a particular example of a more general point, namely, that peripheral blood eosinophil levels fluctuate rapidly and markedly on a diurnal basis, presumably as the cells leave the circulation or marginate on endothelia. Part of this diurnal fluctuation may be attributable to adrenal corticosteroid levels, possibly acting

through a central mechanism (*Speirs* and *Meyer*, 1949; *Szczeklik* and *Podolec*, 1976) and may be induced by other factors, including stress. Other, more long-term fluctuations may be associated with sex hormone levels or with deposition and perhaps death of eosinophils in the uterus (*Bjersing* and *Borglin*, 1974; *Ross* and *Klebanoff*, 1966).

The main point of emphasis in this section, then, is that the eosinophil is a highly labile cell in terms of both maturation and blood transit time, showing marked variability in its tendency to enter, leave, and perhaps re-enter the circulation under different conditions. Although the blood represents a convenient organ for sampling eosinophils, no prediction can be made from peripheral eosinophil counts as to the localization of eosinophils at any one particular site. This is a point of importance in considering tissue responses to migrating larval helminths and will be discussed in more detail in Sections III C and IV D.

C. Mechanisms of Induction of Blood Eosinophilia

The approach described in the last section provides data about the changes in eosinophil turnover that occur in conditions of peripheral blood eosinophilia but gives no clue as to the mechanisms whereby such an eosinophilia might be induced. Such mechanisms have mainly been studied in various helminth infections; but the administration of particulate antigenic material and of antigen-antibody complexes has also yielded some useful information.

Of the different helminth systems, the most extensively studied has been *T. spiralis* in the rat. Although it has been known for many years that *T. spiralis* infection induces a profound eosinophilia, both in man and in experimental animals (*Gould*, 1970), the mechanism of the eosinophilia was very poorly understood until the recent experiments by *Beeson* and his colleagues. *Basten* et al. (1970) showed that a prolonged but variable eosinophilia could be induced by oral administration of live muscle-stage larvae. Intravenous injection of live larvae elicited a pronounced and more clearly defined eosinophilia. The same effect could be achieved by intra-aortic injection but not by subcutaneous, intramuscular, or intraperitoneal injection. Homogenates of larvae, even when injected intravenously, failed to elicit an eosinophilia. The efficiency of intravenous injection of live larvae in inducing an eosinophilia has recently been confirmed in the baboon (*Sturrock* et al., 1977).

Next, *Basten* and *Beeson* (1970) showed that neonatal thymectomy, repeated administration of antilymphocyte serum, chronic thoracic duct drainage, or combinations of these treatments abolished the ability of rats to mount an eosinophilic response to intravenously injected larvae. Irradiated rats mounted an eosinophilic response only if they were reconstituted with lymphocytes as well as bone marrow cells. If the reconstituting lymphocytes were derived from an animal which had already been exposed to *T. spiralis* larvae, then an augmented "secondary" type of eosinophilia was seen in the recipient animals after challenge. The stimulus to an ongoing eosinophilia could be adoptively transferred with thoracic duct or peripheral blood lymphocytes but not with lymph or plasma; and

eosinophilia was still observed if the transferred cells were restricted to a cell-tight diffusion chamber.

The conclusions drawn from these experiments were that eosinophilia depended on the release of a diffusible factor from thymus-derived lymphocytes in animals which had received intact *T. spiralis* larvae intravenously. Further confirmation of the T-dependence of the eosinophilia came from the work of *Walls* et al. (1971) in mice, who showed that T-depleted mice failed to mount an eosinophilic response to injection of *T. spiralis* larvae, although they produced a normal neutrophil response to *E. coli*-induced pyelonephritis. The eosinophilic response in T-depleted animals could be restored by implantation of a thymus graft.

The importance of both the route of administration and the physical state of the injected larvae was then studied in more detail by *Walls* and *Beeson* (1972). Eosinophilia was observed only under conditions in which there were local tissue reactions to larval material; intact larvae injected intravenously, and larval extracts administered in Freund's complete adjuvant, both elicited an eosinophilia, whereas intravenous injection of larval extracts or of homogenized larvae gave no eosinophilia. However, intravenous administration of larval extract would prime an animal to give a "secondary" type of eosinophilia to a subsequent injection of intact larvae. Equally, homogenates and extracts which failed to elicit an eosinophilia in normal animals did so in animals which had previously been primed with intact larvae. The requirement of a local tissue response was further suggested by the work of *Despommier* et al. (1974), who found that newborn larvae, which pass through the lungs without dying, failed to elicit an eosinophilia.

Before coming to possible interpretations of these studies, some comments may be made about the induction of eosinophilia in other helminth infections. In mice infected with *S. mansoni*, two waves of eosinophilia occur: the first, of low intensity, during the prepatent period, and a second and higher peak during the immediate postpatent period (*Colley* et al., 1973). Thereafter, eosinophilia fluctuates erratically. In mice depleted of T cells by different techniques, the second wave of eosinophilia is markedly reduced, while the effect on the first wave is variable (*Fine* et al., 1973). In *Trichostrongylus colubriformis* infection of the guinea pig, a rather complicated pattern of changes in eosinophil counts takes place after T-depletion (*Rothwell* and *Love*, 1975). The elevation of peripheral blood eosinophil counts is reduced, although not markedly, while bone marrow and intestinal eosinophil counts remain unchanged. However, after reconstitution with immune mesenteric lymph node cells as well as bone marrow cells, *intestinal* eosinophil counts after infection increase dramatically. A similar, although less dramatic, local increase is seen after passive administration of immune serum to T-depleted and infected animals, consistent with the local generation of an eosinophil chemotactic factor. These findings emphasize the distinction that must be drawn between blood and tissue eosinophilia. This will be considered in more detail in the next section.

The interpretation of these various findings must remain somewhat uncertain. Clearly, the involvement of a T-lymphocyte response is a necessary requirement for eosinophilia in the *T. spiralis* system. One way in which this might act is by release of a diffusible stimulator of eosinopoiesis, as suggested by the original experiments of *Basten* and *Beeson* (1970). Diffusible mediators of this type have

now been described in several situations. For instance, *McGarry* and *Miller* (1974) and *Miller* and *McGarry* (1976) have described an eosinopoietic factor released by immune spleen cells after challenge with specific antigen (tetanus toxoid). In their experiments, immune or normal spleen cells were separated from normal bone marrow cells by a cell-impermeable filter and were implanted in cell-tight chambers in normal mice. Challenge of these mice with antigen led to an increase in eosinopoietic activity in the bone marrow cells adjacent to the immune spleen cells, when compared with parallel chambers in the same animal containing bone marrow cells adjacent to normal spleen cells. A similar activity has now been demonstrated with spleen cells from *S. mansoni*-infected mice after challenge with schistosome egg antigens (*Miller* et al., 1976). Using a different system, namely stimulation of bone marrow colonies in vitro, *Ruscetti* et al. (1976) have demonstrated an eosinophil colony stimulating activity in supernatants from antigen-stimulated mouse lymphocyte cultures. Both of these approaches indicate that lymphocyte products *may* affect eosinophil stem cell activity.

In view of the requirement for antigen localization observed in the *T. spiralis* system, however, it is unlikely that a simple T-lymphocyte response to antigen is the *only* requirement for the induction of eosinophilia. In the *T. spiralis* model, such antigens are present in extracts and homogenates of larvae, which prime for a secondary type of eosinophilia in response to intact larvae but do not in themselves induce an eosinophilia (*Walls* and *Beeson*, 1972). It must therefore be postulated that the lodging of particulate material, especially in the lungs, must also be a necessary condition, although it is difficult to see how this would operate. One possibility is that the *local* formation of large amounts of immune complexes may lead to the generation and release of T-dependent eosinopoietic substances, whereas a more generalized and diffuse immune complex formation may be ineffective. In this context, several observations are relevant. First, as will be seen in the next section, one of the eosinophil chemotactic factors depends for its activity on the interaction between a T-lymphocyte product and immune complexes (*Cohen* and *Ward*, 1971). It is possible, though not proven, that this chemotactic factor also has eosinopoietic activity. Secondly, there is no necessity for the eliciting antigen to be helminthic in origin; simple protein antigens, providing they are in the right form, can elicit an eosinophilia. For example, *Schriber* and *Zucker-Franklin* (1975) have demonstrated that latex particles coated with human gamma globulin elicit an eosinophilia after intravenous injection into the pulmonary bed of rats, whereas uncoated particles do not. Eosinophilia is associated with the development of a delayed hypersensitivity reaction around the injected particles and of a positive lymphocyte transformation response to human γ-globulin. Finally, in addition to the extensive work on the role of complexes in eliciting a tissue eosinophilia, described in the next section, several studies have shown that administration of immune complexes, usually by the intraperitoneal route, can also induce a blood eosinophilia. In the guinea pig, complexes containing IgG_1 are most efficient in this respect (*Litt*, 1968; *Parish* and *Coombs*, 1968; *Parish*, 1972).

In summary, although the exact mechanism is not yet certain, it is clear that blood eosinophilia depends both on a T-lymphocyte response to the introduced antigen, possibly involving the release of a lymphocyte mediator, and on the

structural nature and distribution of that antigen; this in turn may reflect a requirement for local immune complex formation. The nature of the diffusible eosinopoietic mediators involved and their relationship to the chemotactic factors described in the next section is not yet known.

D. Localization of Eosinophils in Tissues

1. Studies in vitro: Chemotactic Factors

Failure to draw a sharp distinction between peripheral blood and tissue eosinophilia has in the past led to some confusion. The important point to make is that peripheral blood eosinophilia reflects an increase in eosinopoiesis or an alteration in eosinophil dynamics, whereas tissue eosinophilia may reflect *both* an increase in eosinopoiesis *and* a local chemotactic effect. It has already been emphasized that the eosinophil is principally a tissue cell; and, particularly in helminth infections, it is likely that it will play its functional role, if any, in localized lesions within the tissues. In this section, therefore, attention will be paid to mechanisms whereby eosinophils may be attracted to and localized at one particular site within the tissues. Much of the work in this area has involved the study of chemotactic factors in vitro, and these will be considered first. Any attempt to extrapolate these observations in vitro to events that occur in vivo must necessarily be hypothetical; some direct evidence concerning the nature of eosinophil localization in vivo does exist, however, and will be considered in the next section.

A great variety of agents have now been reported to have a more or less selective chemotactic effect on eosinophils in vitro. One of the most extensively studied has been a low molecular weight polypeptide released after antigenic challenge of sensitized guinea pig lung or human lung and referred to as eosinophil chemotactic factor of anaphylaxis (ECF-A) (*Kay* et al., 1971; *Kay* et al., 1973; *Kay* and *Austen*, 1971). This substance has recently been shown to be one of two tetrapeptides of composition Ala-Gly-Ser-Glu or Val-Gly-Ser-Glu (*Goetzl* and *Austen*, 1975), and synthetic analogues have been shown to possess chemotactic activity (*Goetzl* and *Austen*, 1976). ECF-A exists as a preformed mediator and can be isolated from the granules of rat peritoneal mast cells (*Wasserman* et al., 1974). After interaction with low concentrations of ECF-A, eosinophils enter a state of "functional deactivation", in which they no longer respond to chemotactic concentrations of ECF-A or of C5a, discussed below (*Wasserman* et al., 1975b). This process may serve to localize eosinophils once they have been attracted to the site of ECF-A release. ECF-A is not restricted to the mast cell; it is also found in leukemic basophils (*R. Lewis* et al., 1975) and in neutrophils. Release from the latter may be induced by treatment with the calcium ionophore A23187 (*Czarnetski* et al., 1976) or by phagocytosis, especially of complement-coated particles (*König* et al., 1976). Neutrophil-derived ECF-A may be important in localizing eosinophils to the sites of acute inflammatory reactions.

In addition, products of complement activation, including C567 and C5a, are chemotactic for eosinophils as well as neutrophils (*Ward,* 1969; *Ward*, 1971; *Kay*, 1970; *Lachmann* et al., 1970; *Kay* et al., 1973). These substances show cross-deactivation with ECF-A (*Wasserman* et al., 1975b). Antigen-antibody complexes,

as well as leading to complement activation and release of C567 and C5a, may also be more directly involved in eosinophil chemotaxis. *Cohen* and *Ward* (1971) described a precursor substance, ECF-P, which was released from sensitized lymphocytes cultured in the presence of antigen. This substance, after interaction with homologous antigen-antibody complexes, gave rise to an active eosinophil chemotactic factor. Later experiments (*Torisu* et al., 1973) indicated that the precursor substance contained an antigen fragment. Subsequent interaction with complexes led to the removal of this fragment and the generation of the active factor.

A spontaneous eosinophil chemotactic activity (SECA) has been reported to be present in higher concentrations in the sera of patients with eosinophilia than in normal patients (*Robinson* and *Miller*, 1975); its nature is unknown. A substance associated with delayed tissue eosinophilia after induction of anaphylactic reactions in the skin with DNP-*Ascaris* extracts has been shown to have eosinophil chemotactic activity in vitro and can be dissociated from two other substances with macrophage chemotactic activity (*Hirashima* et al., 1976). Histamine itself, as well as certain histamine metabolites, are chemotactic in vitro (*Turnbull* and *Kay*, 1976), although there is a possibility that the tissue eosinophilia induced by histamine in vivo is due to acidity rather than any more specific properties (*Litt*, 1976).

Finally, a lymphocyte mediator has been described which enhances the migration of eosinophils out of agarose droplets in vitro. This mediator, referred to as eosinophil stimulation promoter (ESP), was first observed in the supernatants of mouse lymphocytes stimulated with phytohemagglutinin or with specific antigen (*Colley*, 1973). In the mouse, it is produced by T and not by B cells (*Greene* and *Colley*, 1976); its production is dependent on *de novo* protein synthesis (*Greene* and *Colley*, 1974) and requires only a brief exposure to antigen (*Colley*, 1976a). Secretion of ESP can also be demonstrated by isolated schistosome egg granulomas cultured in vitro (*James* and *Colley*, 1975). As well as having migration-enhancing properties, it has also recently been reported to be chemotactic for eosinophils (*Colley*, 1976b, personal communication). It is not yet known if ESP "activates" eosinophils to show an enhanced functional capacity, in a comparable fashion to the enhancement of macrophage activity by migration inhibitory factor (*Stubbs* et al., 1973).

In summary, it may be seen that several immunologic reactions lead to the production of factors which are likely to cause eosinophil accumulation in vivo. These include: anaphylactic hypersensitivity reactions of Coombs Type I (ECF-A, histamine), immune complex formation ($C\overline{567}$, C5a, ECF-P), and delayed hypersensitivity reactions (ESP, ECF-P). Their possible role in vivo will be described in the next section.

2. Studies in vivo

There is as yet no direct evidence that the chemotactic factors described above are actually responsible for eosinophil accumulation in vivo. Several lines of evidence now exist, however, which strongly suggest that at least some of these factors may be involved.

Many workers, for instance, have now found that local immune complex formation or deposition leads to eosinophil accumulation. This effect can be observed in the peritoneal cavity (*Litt*, 1961; *Speirs* and *Osada*, 1962), in the lymph nodes draining the site of injection (*Cohen* et al., 1963; *Cohen* et al., 1964), and at the site of injection in the skin (*Cohen* and *Sapp*, 1965). Eosinophil accumulation can be induced not only by the injection of artificially formed complexes (*Litt*, 1961; *Cohen* and *Sapp*, 1965) but also by injection of aggregated γ-globulin (*Cohen* et al., 1964) or of antigen alone into repeatedly immunized animals (*Litt*, 1963; *Turner* et al., 1968). *Walls* et al. (1974) have shown that after intraperitoneal injection of *T. spiralis* larvae, close interactions form between eosinophils and macrophages. In these interactions, the eosinophil appears to bind to immune complexes on the surface of the macrophage; the effect can be ablated by trypsinizing the macrophage and can be mimicked by incubating normal macrophages in the presence of antigen-antibody complexes, followed by addition of eosinophils.

Chemotaxis in such situations may be attributable to the release of modified complement components. In some circumstances, when the antibody within the complex is reaginic, mast cell factors may also be involved. Thus *Litt* (1961) and *Parish* (1972) have found that complexes containing the guinea pig skin-sensitizing IgG_1 antibody are more efficient in inducing eosinophilia than those containing IgG_2.

Local eosinophilia can also be directly induced by type I anaphylactic hypersensitivity reactions. Following the original observation by *Samter* et al. (1953) that a factor from anaphylactically shocked lungs would elicit an eosinophilia in guinea pigs, *Eidinger* et al. (1962), using a skin-window technique, demonstrated an accumulation of eosinophils at the site of antigenic challenge in ragweed-sensitive humans. This finding was confirmed by *Fowler* and *Lowell* (1966), while *Hirashima* and *Hayashi* (1976) and *Hirashima* et al. (1976) have recently reported that both early (6-h) and late (24-h) eosinophil responses can be demonstrated at the site of cutaneous anaphylaxis reactions induced by DNP-*Ascaris* extracts in sensitized guinea-pigs. Extracts of these skin lesions had chemotactic effects on eosinophils. In the case of the early reactions, the factor responsible was a heat-stable material of molecular weight less than 1400, while the factor isolated from late lesions had a high molecular weight and was heat labile. The latter could be separated from two factors, also present, which were chemotactic for macrophages. In no case has the chemotactic activity been accurately identified, in terms of the in vitro mediators described in the previous section; and a recent report by *Litt* (1976) indicates that much of the early work on the chemotactic effect of histamine in vivo may have been an artefact attributable to the acidity of this molecule.

Finally, eosinophils can also be observed at the site of prolonged delayed-hypersensivity reactions. As well as being seen in naturally induced chronic granulomatous reactions, such as those that form around *S. mansoni* or *Capillaria hepatica* eggs (*Mahmoud* et al., 1975b; *Solomon* and *Soulsby*, 1973), local eosinophilia can also be observed in artificial chronic granulomas (*Steele* and *Rack*, 1965). Although such granulomas can be shown in some cases to be producing mediators which affect eosinophils (*James* and *Colley*, 1975), the nature of the eosinophilotactic activity is not accurately known.

In summary, a variety of mediators have been identified in vitro which could attract and by functional deactivation localize eosinophils at the sites of various immunologic reactions. These reactions include immune complex formation with complement activation, anaphylactic hypersensitivity reactions, and delayed hypersensitivity reactions. However, although such reactions in vivo are associated with a local accumulation of eosinophils, it cannot yet be said with certainty which, if any, of the various mediators are responsible.

IV. Eosinophil Function

A. Introduction

The preceding sections have emphasized that the eosinophil differs structurally, biochemically, and physiologically from other leukocytes, including the neutrophil. The main differences include the morphology and enzyme content of the cell; its kinetics under normal and abnormal conditions, especially helminth infections; its response to chemotactic stimuli; and its localization in a variety of pathologic lesions. It now remains to be asked: can these differences be associated with any functional properties which are characteristic of the eosinophil in contrast to other cell types?

Three such properties have now been described. The most closely related to helminth infection is the ability to mediate antibody-dependent damage to helminth larvae or eggs in vitro (Sect. IV D 1), which may be associated with immunity to helminth infection in vivo (Sect. IV D 2 and 3). In addition to this, however, two characteristics of helminth infection are the formation of immune complexes and the development of Type I anaphylactic hypersensitivity reactions. The preferential phagocytosis of immune complexes by eosinophils and the role of eosinophils in modulating Type I reactions will therefore be described first, though not solely in the immediate context of helminth infection.

B. Preferential Phagocytosis of Immune Complexes

The findings described in Sections III B and III C, namely that eosinophilia can be induced with antigen-antibody complexes and that products derived from antigen-antibody interactions are chemotactic for eosinophils in vitro and in vivo, have already suggested that the eosinophil bears some special relationship to immune complex formation. In this section a further aspect of this relationship, namely preferential phagocytosis of immune complexes, will be discussed.

It is now generally agreed that eosinophils can phagocytose a variety of particles, including bacteria (*Cline* et al., 1968; *Cohen* and *Sapp*, 1969; *Cotran* and *Litt*, 1969; *Douglas* and *Spicer*, 1971; *Baehner* and *Johnston*, 1971), other microorganisms (*Zucker-Franklin* et al., 1966; *Lehrer*, 1971; *Ishikawa* et al., 1972), and nonliving material (*Archer* and *Hirsch*, 1963b; *Zucker-Franklin* and *Hirsch*, 1964; *Roberts*, 1966; *Kostage* et al., 1967; *Cline* et al., 1968; *Komiyama* and *Spicer*, 1975). It is usually suggested, however, that they phagocytose such particles less

well than do neutrophils (*Cline* et al., 1968; *Mickenberg* et al., 1972); and that even after phagocytosis their bactericidal or fungicidal properties are less well developed (*Baehner* and *Johnston*, 1971; *Mickenberg* et al., 1972; *Bujak* and *Root*, 1974). Although oxidation of 1-^{14}C-glucose is greater in eosinophils than in neutrophils, both at rest and after phagocytosis, and although ^{14}C-formate oxidation and hydrogen peroxide generation are also greater (*Baehner* and *Johnston*, 1971; *Mickenberg* et al., 1972), the peroxidase-H_2O_2-halide system which is at least partially responsible for antimicrobial activity in neutrophils (*Klebanoff*, 1967) appears to be inactive in eosinophils (*Bujak* and *Root*, 1974). In this respect then, the eosinophil appears to behave like a rather inefficient neutrophil.

In contrast to this, the phagocytosis of immune complexes by eosinophils is regarded as being particularly efficient, both in vivo and in vitro. This statement must still be regarded with some caution, since in some studies the complexes have been particulate, whereas the original material has been soluble; while in others, in spite of an increased uptake by eosinophils of opsonized materials, there have been no attempts to test for a similar increase in uptake by neutrophils.

Sabesin (1963) demonstrated by electron microscopy the uptake of ferritin-antiferritin complexes by eosinophils after intraperitoneal injection of ferritin into sensitized guinea pigs. In a comparable study, *Litt* (1964) injected fluorescein-labeled bovine serum albumin (BSA) and rhodamine-labeled anti-BSA intraperitoneally into guinea pigs. Eosinophils showed only a yellow fluorescence, indicating the uptake of both materials simultaneously as immune complexes. *Archer* and *Hirsch* (1963b) reported that horse eosinophils would readily phagocytose antigen-antibody preicpitates; antibody-coated, but not normal, red cells were also taken up, while yeast cell walls were phagocytosed better in the presence of antibody. Similar findings have been reported by *Ishikawa* et al. (1972), who showed that eosinophils would phagocytose *Candida albicans* spores only when these were opsonized. The opsonic activity of the sera tested correlated with anti-*Candida* IgG immunofluorescence titers, and IgG was detected on the surface of phagocytosed spores, but the activity was partially abolished by heating at 56°C for 30 min, suggesting that the reaction was partially complement dependent. In a later study (*Ishikawa* et al., 1974), they showed that human eosinophils would also take up soluble immune complexes. When prepared in antigen excess, complexes containing IgE were taken up to a greater extent than complexes containing IgG or IgM. In antibody excess, however, IgG complexes were preferentially taken up. After injection into rats, complexes containing homologous antibody were ingested to a greater extent that those containing heterologous (rabbit or human) antibody; this may be related to the apparent species specificity of the eosinophil Fc receptor (*Butterworth* et al., 1976a; *Tai* and *Spry*, 1976).

Phagocytosis in these circumstances is followed by degranulation of the eosinophil, with an initial fusion of granules with the phagocytic vacuoles (*Archer* and *Hirsch*, 1963b), followed by discharge of lysosomal enzymes into the vacuole (*Cline* et al., 1968) and sometimes into the surrounding medium. Similar degranulation may occur after phagocytosis or attempted phagocytosis in vivo (*Skinnider* and *Ghadially*, 1974; *Tai* and *Spry*, 1976). This may contribute to the formation of Charcot-Leyden crystals (*Welsh*, 1959; *El-Hashimi*, 1971); and it has been suggested that release of toxic eosinophil granule contents may be involved

in the pathogenesis of heart lesions in Löffler's cardiomyopathy (*Zucker-Franklin*, 1971; *Spry* and *Tai*, 1976).

Phagocytosis of immune complexes represents a general property of eosinophils, rather than one which is specifically associated with helminth infection. It should be noted, however, that helminth infections may be a frequent source of immune complexes, representing as they do a continued antigenic stimulus. For example, circulating immune complexes have now been demonstrated in schistosomiasis, both in man and in experimental animals (*Phillips* and *Draper*, 1975; *Houba* et al., 1976), while local formation of immune complexes may play a part in the pathogenesis of the egg granuloma (*Houba*, *Sturrock* and *Butterworth*, unpublished observations). The potential role of eosinophils in phagocytosing immune complexes is therefore likely to be of particular importance in helminth infection.

C. Modulation of Anaphylactic Hypersensitivity Reactions

It has already been pointed out (Sect. III D) that eosinophils tend to localize at the site of Type I anaphylactic hypersensitivity reactions. The question may now be asked of whether they play any functional role in such lesions. Although no definite answer can yet be given, eosinophils have the potential at any rate for modulating or dampening down such reactions in several different ways: first, by inactivation of slow-reacting substance of anaphylaxis (SRS-A); secondly, by inactivating or preventing the release of histamine; thirdly, by inactivating platelet-activating factor (PAF); and finally, by modifying histamine replenishment after Type I reactions have occurred. Most of the evidence for these activities is based on the properties of eosinophils or extracted eosinophil enzymes in vitro, and a note of caution must be sounded: namely, in the hypersensitivity reaction in vivo, it is not known whether the micro-environment around the eosinophil, in terms of effector molecule concentration and the possible presence of enzyme inhibitors, is sufficient to allow the putative mechanism to act.

SRS-A is an acidic, sulfur-containing mediator of low molecular weight which is released during Type I hypersensitivity reactions in vivo and in vitro (*Orange* et al., 1973; *Orange* et al., 1974; *Stechschulte* et al., 1973) and which causes smooth muscle contraction, increased vascular permeability and decreased pulmonary compliance. It is inactivated by limpet arylsulfatase (*Orange* et al., 1974), and it has already been noted that the eosinophil has a high content of arylsulfatase (Sect. II B). *Wasserman* et al., (1975a) have shown that the arylsulfatase extracted from purified human eosinophils in vitro has the capacity to inactivate SRS-A and have suggested that this enzyme may play a part in limiting Type I reactions. Release of arylsulfatase can be induced not only by phagocytosis but also by high concentrations of ECF-A and is therefore likely to occur at the site of Type I reactions (*Goetzl* et al., 1975).

The possibility that eosinophils might inactivate histamine in anaphylactic reactions was first suggested by *Archer* (1963). Two ways in which eosinophils may prevent histamine-mediated effects are: first, by the action of the high content of eosinphil histaminase (*Zeiger* et al., 1976); and secondly, by the action of the eosinophil-derived inhibitor (EDI) of histamine release recently described

by *Hübscher* (1975a, b). This inhibitor is released from eosinophil-rich fractions of peripheral blood from atopic patients by sonication or by interaction with ragweed antigen-IgE complexes, with ragweed antigen alone, or with anti-IgE (suggesting that IgE is already present on the eosinophils). EDI has been found to exert its inhibitory effect by increasing the levels of cyclic AMP in target leukocytes and is composed of a mixture of acidic glycolipids physicochemically and biologically indistinguishable from prostaglandins E_1 and E_2. Indomethacin, an inhibitor of prostaglandin synthesis, blocks release of EDI. These findings strongly suggest that EDI is a mixture of prostaglandins.

Interaction of IgE-sensitized basophils with specific antigen leads to the release of a soluble mediator, platelet-activating factor (PAF), which induces release of amines from normal platelets and may therefore be involved in the pathogenesis of Type I hypersensitivity reactions (*Barbaro* and *Zvaifler*, 1966; *Siraganian* and *Osler*, 1969; *Benveniste* et al., 1972; *Benveniste*, 1974). This factor may be destroyed by cabbage phospholipase D, but not by phospholipases A or B or by mild hydrolysis (*Kater* et al., 1975). It has already been mentioned that phospholipase D is preferentially present in human eosinophils; and this enzyme has recently been shown to destroy PAF activity (*Kater* et al., 1976). It has therefore been suggested that a further way in which eosinophils may affect Type I reactions is by neutralization of PAF.

Finally, *Jones* and *Kay* (1976) have shown that eosinophils play a role in controlling the replenishment of histamine in the skin after cutaneous anaphylaxis. In guinea pigs treated with an antieosinophil serum, the replenishment of skin histamine following passive cutaneous anaphylaxis is accelerated. They have suggested that eosinophils in such reactions act by partially preventing re-accumulation of histamine and have postulated that this effect might serve to control those Type I reactions in which there is a continuous antigenic stimulus.

Eosinophils can therefore probably act by dampening down Type I reactions at several stages: by preventing mediator release, by inactivating mediators once they have been released, and by preventing their reaccumulation. No specific relationship of this process to helminth infections, as opposed to Type I reactions in general, is suggested. It should be borne in mind, however, that there is a threefold association between helminth infection, eosinophilia, *and* IgE levels (*Johansson* et al., 1968; *Hogarth-Scott* et al., 1969; *Rosenberg* et al., 1970; *Rosenberg* et al., 1971; *Spitz* et al., *Jarrett*, 1973; *Radermecker* et al., 1974; *World Health Organization*, 1974; *Neva* et al., 1975), and that formation of IgE to simple protein antigens may be potentiated by helminth infection (*Jarrett*, 1973). Specific IgE levels may be increased during helminth infection, and a variety of Type I reactions can be demonstrated in vivo, both by skin testing and during the natural course of the disease. Examples of such reactions include the systemic manifestations of *T. spiralis* infection in man; asthma during migration through the lung of *Ascaris lumbricoides, Toxocara canis* or *T. cati*; swimmer's itch, and sometimes asthma in schistosome infections; systemic anaphylaxis in *Dracunculus medinensis* infection and hydatid disease; and local edematous reactions to migrating *Loa loa* (*Zvaifler*, 1976). In view of these findings, it might be suggested that the eosinophilia is in part a secondary adaptive response on the part of the host to control the deleterious effects of these IgE-mediated reactions.

D. Damage to Helminths

1. Schistosoma mansoni: Studies in vitro

Most of the evidence for a role for the eosinophil as an effector cell in helminth immunity stems from studies on *Schistosoma mansoni*. This evidence will be discussed in two parts: first, experiments in vitro which have shown that an antibody-dependent eosinophil-mediated mechanism can damage schistosome larvae; and secondly, experiments which strongly suggest that this eosinophil-mediated mechanism may be important in immunity in vivo.

Several ways in which the larval stages (schistosomula) of *S. mansoni* can be damaged in vitro have now been described. The first was the "lethal" effect of IgG from immune rhesus monkeys, acting in concert with high concentrations of a heat-labile material, presumably complement, present in normal serum (*Clegg* and *Smithers*, 1972). At one stage it was believed that this activity might account for immunity to reinfection in vivo; but later work showed that, although rats could be immunized to produce high levels of lethal antibody by injection of partially purified adult worm antigens in Freund's complete adjuvant, these rats were not immune to infection (*Sher* et al., 1974a). This indicated that lethal antibody, at any rate on its own, was insufficient to account for immunity.

Later workers therefore investigated cell-mediated effector mechanisms of various types. *Dean* et al. demonstrated a complement-dependent IgG antibody in immune rats (1974) and guinea pigs (1975) which, in the presence of neutrophils but not of eosinophils or macrophages, would rapidly damage schistosomula in vitro. This damage was associated with discharge of neutrophil enzymes onto the surface of the organism. The antibody activity, however, was not distinguishable from the lethal antibody described above, and it is possible that the effect of neutrophils may simply have been to accelerate and enhance the damage already initiated by antibody and complement.

More recently, two macrophage-dependent reactions capable of damaging schistosomula have also been demonstrated in rats. The first, described by *Perez* (1974), depends on a heat-stable IgG antibody, which is cytophilic for normal rat macrophages and which confers on such macrophages the ability to adhere to and kill schistosomula. The time course of development and decline of this activity in rats exposed to a primary infection parallels that of immunity to reinfection, and the activity can be separated from lethal antibody on QAE Sephadex. A second activity, described by *Capron* et al. (1975), is heat labile and is removed by passage through IgE-specific immunoadsorbent columns. Normal macrophages, when incubated in the presence of this material for 3–14 h and then added to schistosomula without further washing, adhere to and damage the organisms. The possibility that IgE-antigen complexes may be involved has not yet been excluded. Both of these mechanisms, although extremely interesting in their own right, must be viewed with a certain amount of caution in the context of natural immunity to *S. mansoni*, since the rat is a highly abnormal host.

Finally, the eosinophil has been implicated in an antibody-dependent, complement-independent reaction which induces release of ^{51}chromium from labeled schistosomula. *Butterworth* et al. (1974, 1976b) have adapted the ^{51}chromium release technique, currently in widespread use for assaying cytotoxicity to single

cell suspensions of mammalian or avian targets (*Cerottini* and *Brunner*, 1974), to provide a short-term assay for estimating damage to schistosomula. These organisms take up and retain large amounts of the isotope and will release up to 65% of the incorporated isotope under conditions of extreme damage. Release of isotope correlates well with damage to the parasite, as judged by phase contrast microscopy (*Glauert* and *Butterworth*, 1977).

Using this technique, it has been shown that normal human and baboon peripheral blood leukocytes can damage schistosomula in the presence of heat-inactivated sera from infected but not from normal individuals (*Butterworth* et al., 1974; *Butterworth* et al., 1976b). The activity in sera from infected individuals is detectable at much higher dilutions than is generally the case for the complement-dependent antibodies. It is associated with an IgG fraction off DEAE-cellulose and can be removed on IgG-specific immunoadsorbents (*Butterworth* et al., 1977b). The subclasses of IgG involved have not yet been identified. The activity is opsonic but not cytophilic and can be removed by absorption of sera with whole schistosomula. Such absorbed sera have lost their capacity to bind to adult worm membranes, as judged by immunofluorescence studies on worm sections (*Butterworth* et al., 1977b). In baboons exposed to a primary infection, the development of such antibodies is a necessary but not sufficient prerequisite for immunity (*Butterworth* et al., 1977c, in preparation). Infected baboons which fail to develop cell-dependent cytotoxic antibodies show no resistance to reinfection, but not all baboons which develop antibodies are immune. Possible reasons for this finding are discussed below. In humans, the levels of cytotoxic antibody correlate with the intensity of infection (*Sher* et al., 1977), while preliminary results on treated patients have indicated that cell-dependent antibody levels are lower in patients who become reinfected within 3 years of treatment than in those who do not (*Butterworth*, *McMahon*, *Kimani*, and *Sturrock*, unpublished results). Correlative studies of this type thus suggest that the antibody activity concerned may be relevant to immunity in both man and the baboon.

The nature of the effector cell mediating this complement-independent reaction has been studied in several ways. *Butterworth* et al. (1975) have found that the effector cells in normal human peripheral blood are associated with a polymorphonuclear-rich fraction after density centrifugation. Furthermore, the effector activity of mixed, unpurified peripheral blood leukocytes can be ablated by pretreatment with an antieosinophil serum and complement (*Butterworth* et al., 1975), whereas antineutrophil and antibasophil sera have little or no effect (*Butterworth* et al., 1975; *Butterworth* and *Mahmoud*, unpublished results).

These findings strongly suggest that the eosinophil is a major cell type present in normal human peripheral blood that is capable of mediating antibody-dependent damage to schistosomula. This has been confirmed in further experiments (*Butterworth* et al., 1977a), in which it has been found that preparations containing up to 98% eosinophils can also mediate damage. In contrast, mononuclear cells neither induce detectable chromium release nor enhance the damage initiated by purified eosinophils. The role of neutrophils is not yet clear.

The interaction between purified eosinophils and schistosomula has been studied by phase contrast and electron microscopy (*Glauert* and *Butterworth*, 1977, and unpublished observations). The reaction is initially characterized by intimate

contact between the eosinophil and the schistosomulum, a feature which is not seen in control preparations without antibody. This is followed by eosinophil degranulation, and by the appearance of an electron-dense deposit, probably granule contents, on the surface of the parasite. Subsequently, there is evidence of extensive fine structural damage in the surface layers (*Glauert* et al., in preparation), while analysis by phase contrast microscopy shows a good correlation between the proportion of damaged schistosomula and the degree of ^{51}chromium release.

Comparable results have been obtained by *Mackenzie* et al. (1976, personal communication) using rat eosinophils, with the additional findings that there is an initial reversal of the relative densities of the core and matrix of the granules and that the granules discharge their contents into vacuoles within the eosinophil before these vacuoles finally discharge onto the surface. The discharged material contains peroxidase.

The mechanism of this antibody-dependent damage to schistosomula has been further studied with the aid of metabolic inhibitors (*David* et al., 1977), using either purified eosinophils or unpurified leukocytes as effector cells. Agents which inhibit oxidative phosphorylation, such as antimycin A and potassium cyanide, fail even at high concentrations to inhibit leukocyte-mediated release of isotope from antibody-coated schistosomula. Similarly, no inhibition is seen with inhibitors of protein synthesis, including puromycin and cycloheximide, nor with colchicine, an inhibitor of microtubule function, nor with mitomycin C, an inhibitor of DNA and mRNA synthesis. Indomethacin, an inhibitor of prostaglandin synthesis, also fails to inhibit damage; this is of some interest, in view of the finding that the eosinophil-derived inhibitor of histamine release is a prostaglandin (*Hübscher*, 1975a, b). Finally, prednisolone, an active inhibitor of antibody-dependent lymphocyte-mediated cytotoxicity, has no effect in the eosinophil-mediated system, even at high concentrations.

In contrast, cytochalasin B produces a marked inhibition of the isotope release induced both by unpurified leukocytes and by purified eosinophils (*David* et al., 1977). This agent, as well as acting on microfilament function (*Wessells* et al., 1971), affects other aspects of cell metabolism including hexose transport (*Cohn* et al., 1972). However, the finding that cytochalasin A, which also affects microfilament function but which fails to act on hexose transport in lymphocytes (*Bubbers* and *Henney*, 1975), does inhibit eosinophil-mediated damage strongly suggests that the effect of the cytochalasins is indeed on microfilament function. An example of such inhibition is shown in Table 1. The inhibitory effect is reversible by washing, and the agent must be present during the very early stages of the eosinophil-schistosomulum interaction — within the first 5 min, in the case of cytochalasin A. This suggests that its effect is on the binding of the eosinophil through its Fc receptor and on the subsequent spreading and interdigitation of the eosinophil over the surface of the schistosomulum.

Although inhibitors of oxidative phosphorylation have no effect on leukocyte-mediated damage, agents which inhibit glycolysis, including sodium fluoride, iodoacetate, and 2-deoxyglucose, are markedly inhibitory (*David* et al., 1977). Iodoacetate and sodium fluoride are relatively nonspecific in their action, and both can inhibit enzymes other than those involved in glycolysis. But the finding that the inhibition induced by 2-deoxyglucose is reversed by addition of excess glucose

Table 1. Comparison of effects of cytochalasins A and B on antibody-dependent eosinophil-mediated damage to schistosomula

Concentration (μg/ml)	% Isotope release			
	Ab +		Ab −	
	CA	CB	CA	CB
10	17	18	16	15
3.2	15	25	13	14
1	16	39	12	12
0.32	34	39	13	15
0	45		19	

Cytochalasin A (*CA*) and cytochalasin B (*CB*) were added at the final concentrations shown to mixtures of peripheral blood leukocytes and antibody-coated (*Ab* +) or control (*Ab* −) schistosomula. Release of ^{51}chromium from the labeled schistosomula was measured after 7 h of incubation. Both agents caused significant inhibition ($P < 0.05$) of antibody-dependent cell-mediated isotope release at concentrations down to 0.32 μg/ml. (*T. Nutman* and *A.E. Butterworth:* for methods, see *David* et al., 1977)

and that other sugars have no effect strongly suggests an effect on hexokinase (*Hochster*, 1963).

As in other antibody-dependent cytotoxic reactions (*Garovoy* et al., 1975), eosinophil-mediated damage is inhibited by agents which raise cyclic AMP levels. Aminophylline, which inhibits the catabolism of cyclic AMP by phosphodiesterase, and isoproterenol (isoprenaline), which activates adenylate cyclase, act synergistically at low concentrations to inhibit eosinophil-induced release of isotope from antibody-coated schistosomula. Finally, as in antibody-dependent lymphocyte-mediated cytotoxicity (*Trinchieri* and *De Marchi*, 1976), eosinophil-mediated damage is inhibited by relatively nonspecific esterase inhibitors such as tosyl-lysyl-chloromethylketone (TLCK). The specificity of the esterase involved and its distribution within the cell are not yet known.

In summary (Table 2), it would appear both from electron-microscopic and from metabolic studies that cytotoxicity depends on: 1) a close attachment of the eosinophil to the schistosomulum surface; 2) glycolysis-dependent degranulation of the eosinophil, with release of granule contents onto the surface; and 3) presentation of a TLCK-inhibitable enzyme, possibly lysosomal in origin, to the surface. This enzyme then initiates damage. Although the nature of the enzyme responsible for initiating damage is unknown, the finding that lymphocytic K cells *fail* to damage antibody-coated schistosomula suggests that the enzyme involved is preferentially or selectively concentrated in the eosinophil. In this case, it might be speculated that the schistosomulum and possibly other helminth larvae present to the host unusual substrates in their surfaces and that one selective advantage of the eosinophil to its host is the capacity to deal with such substrates.

Another interesting point concerning eosinophil-mediated damage is that eosinophils from different individuals differ markedly in their cytotoxic activity. *Butterworth* et al. (1977 a) have found that unpurified cells from patients with high levels of eosinophilia associated with schistosome infection do not show the greater

cytotoxic activity, when compared with unpurified normal leukocytes, which would be predicted if all eosinophils were equally active. Although it has not yet proved possible to prepare eosinophils from normal blood of sufficient purity and concentration to make direct comparisons with eosinophils from eosinophilic blood, the finding of "relative inactivity" of some eosinophil preparations is very consistent. The reasons for this relative inactivity are not yet clear, but several alternative explanations may be offered. First, cytotoxicity induced by eosinophils may be enhanced by another cell type present in normal peripheral blood, and this cell may be present in limiting numbers. Mixed-cell experiments (*Butterworth* et al., 1977a; *Butterworth* and *Franks*, unpublished observations) have failed so far to reveal any such enhancement, but a role for the neutrophil cannot yet be excluded. Secondly, eosinophils in patients with schistosomiasis may have their Fc receptors blocked by circulating immune complexes. Although the findings of *Tai* and *Spry* (1976), namely that the proportion of eosinophils bearing detectable Fc receptors increases in some eosinophilic conditions, must be borne in mind in this respect, the possibility of blockade by immune complexes remains strong. Cytotoxicity by unpurified cells can be artificially inhibited by addition of exogenous complexes (*Butterworth* et al., 1977b), and circulating complexes can be demonstrated in patients and experimental animals at various stages during *S. mansoni* infection (*Phillips* and *Draper*, 1975; *Houba* et al., 1976). A third possibility is that eosinophils in patients with schistosome-associated eosinophilia may be immature or functionally abnormal, and some evidence for eosinophil abnormalities in various eosinophilic conditions has already been discussed (Sect. II A). Alternatively, there may be different eosinophil subpopulations with different functional capacities. Clarification of this point must await the identification of the relevant damaging enzyme and analysis of its distribution in different eosinophil populations.

A summary of the various findings concerning eosinophil-mediated damage to schistosomula is given in Table 2. The relevance of this in vitro mechanism to immunity in vivo will be considered in the next section. One point, however, may be made at this stage: namely that, from the results presented above, immunity may depend not solely on antibody levels, but rather on the number of *active* eosinophils *locally* available at the site of cercarial penetration. If this is the case, then antibody alone may be insufficient for immunity. There may also be a need for the presence of large numbers of unblocked eosinophils in the circulation and for a mechanism for attracting them to the site of penetration, of one of the types described in Section III D. This may explain some of the unusual features of immunity to schistosomiasis in different hosts, including its dependence on complement and/or mast cells (Sect. IV D 2); its elicitation by living adult worms, which may induce an eosinophilia, rather than by soluble antigenic extracts; the failure of some serum pools to transfer immunity; enhancement of immunity by the presence of eggs; loss of immunity after successful treatment; and other aspects which will be considered in the next section.

A final point that may be made about eosinophil function in vitro is that, as well as damaging antibody-coated schistosomula, eosinophils have also recently been implicated in the destruction of schistosome eggs. Using a technique involving morphologic observation of damage to eggs which have been cultivated in vitro in the presence of various cell types, *James* and *Colley* (1976, and personal com-

Table 2. Characteristics of antibody-dependent eosinophil-mediated damage to *S. mansoni* schistosomula in vitro

Antibody involved:	Associated with IgG fraction of sera from infected humans and baboons
	Opsonic, complement independent, noncytophilic
	Absorption with schistosomula results in loss of activity and loss of reactivity with adult worm membranes
	Partial correlation with immunity in baboons, and possibly in man
Effector cell involved:	Associated with polymorph-rich fraction of human peripheral blood
	Activity removed by pretreatment with antieosinophil serum and complement
	Purified eosinophils show marked activity
	Mononuclear-enriched preparations neither initiate damage nor enhance eosinophil-mediated damage
	Some eosinophil preparations are relatively inactive. Possible explanations:
	Blockade by immune complexes
	Functional immaturity or abnormality
	Eosinophil subpopulations
Mechanism of damage:	Early tight attachment to antibody-coated schistosomulum
	Degranulation onto surface of schistosomulum
	Not dependent on DNA or mRNA synthesis, *de novo* protein synthesis, oxidative phosphorylation or microtubule function
	Inhibitable by cytochalasins, glycolysis inhibitors, and esterase inhibitors
	Postulated mechanism:
	Tight attachment via Fc receptor
	Glycolysis-dependent degranulation
	Presentation of surface-damaging lysosomal enzyme

munication) have shown that eosinophils but not macrophages, lymphocytes, or neutrophils can damage isolated *S. mansoni* eggs over a 24-h period. The reaction is temperature dependent and requires the presence of antigenically intact eggs. This suggests that the reaction is antigen specific, a suggestion which has been confirmed by the finding that only eosinophils from schistosome-infected mice are active. Normal eosinophils or eosinophils from mice infected with *T. spiralis* are inactive. The activity of eosinophils from schistosome-infected animals can be abolished by treatment with trypsin. Such trypsinized eosinophils regain activity after incubation in serum from infected humans or mice, whereas serum from *Trichinella*-infected animals fails to restore activity. This suggests that specificity depends on a cytophilic antibody present in the serum of infected individuals, in contrast to the non-cytophilic nature of the activity responsible for eosinophil-dependent damage to schistosomula.

2. Schistosoma mansoni: Studies in vivo

The findings described above, namely that eosinophils can damage antibody-coated schistosomula in vitro, reflect an interesting aspect of eosinophil behavior but do not in themselves imply that eosinophils are necessarily involved in immunity to schistosomiasis in vivo. Recent work from several laboratories, however, strongly implicates an eosinophil-mediated reaction of this type as a major component of the host's protective response against secondary schistosome infection.

In such infections, the worms derived from a primary cercarial exposure rapidly protect themselves from the host's response, probably by acquiring a coating of host material which masks the antigenic determinants on the worm's surface (*Smithers* et al., 1969; *McLaren* et al., 1975). In spite of this, some host species do acquire the capacity to reject the migrating larvae of a second challenge infection. This phenomenon, of acquired resistance to reinfection in the face of a continued primary infection, has been referred to as "concomitant immunity" (*Smithers* and *Terry*, 1969). Although there is an early period after primary exposure of rats when such immunity may be transferred by lymphoid cells rather than by serum (*Phillips* et al., 1975), in most situations it has been serum that has been effective in transferring immunity (*Perez*, 1974; *Sher* et al., 1975, 1976b; *Phillips* et al., 1975). The serum activity is associated with an IgG_1 fraction in the mouse (*Sher* et al., 1976b), and in the rat it depends for its effect on the presence in the recipient of a radiosensitive cell (*Perez*, 1974). These findings so far suggest that some combination of antibody and a nonspecific effector cell is required. The question may now be posed whether this nonspecific effector cell is an eosinophil.

The implication of concomitant immunity is that the challenge worms of a secondary infection are only susceptible to immune attack during the early stages of larval migration. Most damage probably occurs before the schistosomula reach the lungs, although some recent experiments (*Sher*, 1976, personal communication) suggest that there may also be a phase of susceptibility during migration between the lung and the liver. This means that, for any effector mechanism to be active in protection, the various components of that mechanism must be able to localize at the site of larval migration. All experiments in vivo and in vitro must therefore relate not only to the efficacy of a particular mechanism but also to the accessibility of that mechanism to the schistosomulum. Inaccessibility may severely limit eosinophil-mediated immunity; conversely, other immune responses which enhance eosinophil access (of the types described in Sect. III) may enhance immunity. The role of the eosinophil must now be viewed in this rather more general context in vivo.

Early histologic observations on inflammatory lesions in the skin and lungs of immune animals (*Vogel* and *Minning*, 1953; *Lin* and *Sadun*, 1959; *von Lichtenberg* and *Ritchie*, 1961) emphasized the polymorphonuclear nature of the infiltrate without special consideration of the eosinophil. The first suggestion that eosinophils might be involved in immunity in vivo came from the histopathologic observations of *Hsü* et al. on rhesus monkeys immunized with irradiated cercariae of *S. japonicum*. In a single immune animal, large numbers of degenerating schistosomula were found in the dermis and epidermis at the site of cercarial challenge (*Hsü* et al., 1971). Schistosomular degeneration was associated with an intense infiltrate composed mainly of eosinophils. This infiltrate reached its peak three days after challenge, and eosinophils were seen in close contact with degenerating schistosomula. In a control animal, the infiltrate was much milder and was composed mainly of neutrophils; few signs of damage to the schistosomula were observed. The attrition rates of schistosomula in the skins of the two animals, however, were not quantitated. Later (*Hsü*, 1973; *Hsü* and *Hsü*, 1974; *Hsü* et al., 1974; *Hsü* et al., 1975), the eosinophil infiltrate was shown to be associated with a mononuclear cell infiltration and an Arthus-like reaction. On the basis of these

histologic findings, these workers proposed: 1) that the eosinophil was responsible for destruction of schistosomula in the skin; and 2) that eosinophil localization resulted from local immune complex formation with release of ECF-C and from a local delayed hypersensitivity reaction with formation of ESP (*Hsü* et al., 1975). More recently (*Hsü* and *Hsü*, 1976) they have demonstrated the local deposition of IgE at cercarial challenge sites and have suggested that ECF-A may be also involved in eosinophil localization. All these experiments, however, are based on histologic observations, and direct evidence for the participation of any of these factors is lacking.

More recently, a quantitative study of the inflammatory reactions in the skins of immune and normal mice has been carried out by *von Lichtenberg* et al. (1976). The most striking difference between immune and normal animals is a tenfold increase in the percentage of eosinophils in the reactions to the schistosomula in immune animals. This difference is already present 6 h after challenge and remains marked until the latest time studied (48 h). More schistosomula are surrounded by an inflammatory reaction in the immune animals; and in such reactions, more cell contacts with the schistosomula are observed. These differences can be observed after injection of schistosomula as well as after percutaneous challenge with live cercariae, implying that the response is not directed solely against residual cercarial components. However, no quantitative difference can so far be established between nonimmune and immune mice with regard to the frequency of damaged schistosomula in the skin, partly because of sampling problems and partly because of the difficulty in reliably recognizing subtle damage. In the lungs of these animals, in contrast to previous findings with the macaque (*von Lichtenberg* and *Ritchie*, 1961), only minor reactions to migrating schistosomula are observed, but fewer organisms are seen in the immune lungs. This implies that most of the damage has occurred at the skin stage and that the schistosomula are protected by the time they reach the lung.

The formation of eosinophil-rich lesions in the skin has been found to depend on the release of vasoactive amines from cellular components, probably mast cells (*Sher* et al., 1976a, personal communication). Administration of the serotonin-depleting drug reserpine before challenge prevents the formation of lesions in both immune and normal animals; this effect can be reversed by simultaneous administration of a monoamine oxidase inhibitor. At the same time, administration of reserpine ablates immunity, as measured by the lung recovery technique of *Sher* et al. (1974b), and enhances the recovery of organisms from normal animals. These findings suggest that chemotaxis of eosinophils by mast cell mediators is a necessary component of immunity and again emphasize the multifactorial nature of immunity in vivo.

In order to quantitate schistosomulum damage in more detail, *von Lichtenberg* et al. (1977) then went on to develop a technique whereby schistosomula prepared in vitro are injected intravenously. The advantage of this technique is that the schistosomula arrive in the lungs immediately and simultaneously; the degree of damage and the host response to the organisms can then be sequentially quantitated. In normal mice, a small proportion of the injected organisms elicit focal reactions composed mainly of neutrophils. By the sixth day, these reactions have converted into "residual killing foci" containing no parasites or parasite remnants.

In contrast, a much higher proportion of organisms injected into immune mice elicit focal inflammatory reactions. These reactions are mainly eosinophilic in nature and convert within 24 h into residual killing foci. The association between the histologic changes and death of the schistosomula has been confirmed by the demonstration of a decrease in viable organisms recoverable from the lung on the sixth day. More recent work (*Sher* and *von Lichtenberg*, 1976, personal communication) has shown that the ability to mount an eosinophilic killing reaction can be transferred from immune to normal animals by injection of very small amounts of serum and is again dependent on the release of vasoactive amines. Serum has to be transferred either 24 h before or 3 h after intravenous injection of organisms; transfer at 24 h after injection is ineffective. Congenitally athymic (*nu/nu*) mice fail to develop killing foci and do not become immune: transfer of serum from infected *nu/nu* mice to normal mice fails to transfer the ability to mount a killing reaction. On the other hand, transfer of immune serum into *nu/nu* mice *is* associated with the subsequent development of eosinophilic killing reactions after challenge, implying that in this case the accumulation of eosinophils is not a T-dependent process. Irradiation of either actively or passively immunized mice 5 days before challenge results in a loss of the ability to produce eosinophilic killing reactions, whereas irradiation 1 or 2 days before challenge has no effect. This may be related to the finding (Sect. III A) that eosinophils pass into a nondividing reserve pool in the bone marrow before emerging into the peripheral blood. Schistosomula which have been cultured in vitro for 2 days before injection fail to elicit killing reactions even in immune animals. All of these findings support the conclusion that, in this particular model of a host-parasite interaction, damage to the parasite depends on: 1) interaction of a T-dependent antibody with the unprotected parasite; and 2) a vasoamine-dependent localization of mature eosinophils around the organism, leading to its destruction.

Mahmoud et al. (1975c) in an important paper used a different approach to determining the requirements for eosinophils in immunity to *S. mansoni* in mice. Following the earlier observations described above — that antibody-dependent effector cell activity in vitro can be ablated by pretreatment with an antieosinophil serum (AES) and complement (*Butterworth* et al., 1975, Sect. IV D 1) — they went on to test the effect of AES on immunity to reinfection in vivo. Mice rendered immune by primary infection were treated on the day before challenge and on the first, third, fifth, and seventh days after challenge, either with a monospecific AES (*Mahmoud* et al., 1973) or with antineutrophil, antilymphocyte, antimacrophage, or normal sera. Immunity was assessed both by the rapid lung recovery technique (*Sher* et al., 1974b) and by the more conventional technique of recovery of adult worms. AES induced a marked reduction in circulating eosinophil counts and completely abolished immunity; the other sera, although inducing a reduction in other cell types, had no effect on immunity. AES, in addition to eliminating actively acquired immunity, also abolished the resistance conferred by passive transfer of immune serum. These results implied that an antibody- and eosinophil-dependent reaction was necessary for the expression of immunity and suggested that the effect was manifest during the first few days after challenge, at the time when the AES was being administered.

In addition to abolishing immunity to reinfection, treatment with AES also

reduced the size and eosinophil content of the granulomas formed after intravenous injection of schistosome eggs (*Mahmoud* et al., 1975b). Eight days after a primary intravenous injection of eggs, eosinophils constituted 25–50% of the granulomas in mice treated with normal serum, but were undetectable in mice treated with AES. In AES-treated animals the granulomas were less than one-third the size of control granulomas. Similarly, in mice presensitized by intraperitoneal injection of eggs, the size and eosinophil content of granulomas elicited by a subsequent intravenous injection of eggs were markedly reduced. The effect of such treatment on the host may be complex. A reduction in size of the granuloma is usually associated with a reduction in severity of the disease (*Warren* et al., 1974; *Mahmoud* et al., 1975a): but, on the other hand, the ability of eosinophils to destroy eggs (Sect. IV D 1) will be lost. In this context, *Bogitsh* (1971) has noted invasion of egg shells in early hepatic granulomas by eosinophils, with discharge of eosinophil contents onto the inner surface of the egg shell.

A final indication of a role for eosinophils in immunity has come from the work of *Knopf* and *Cioli* (1976, and personal communication). Various preparations of adult worm antigens were administered to rats by various routes, including mesenteric vein injection. The development of eosinophilia, of antibodies reactive in a passive hemagglutination assay, and of resistance to infection were then followed. Only those procedures which resulted in the development of both antibodies *and* eosinophilia, namely mesenteric vein injection of glutaraldehyde-fixed worms, induced resistance to challenge infection, again suggesting that the eosinophil was a necessary and limiting component in the expression of resistance.

In summary, the relevance to immunity of in vitro observations on eosinophil-mediated damage to schistosomula is now supported by a number of findings in vivo. These findings have also emphasized that it is not sufficient simply to have eosinophils and antibody present within a host for that host to be immune. Mechanisms must also be available for localizing eosinophils to the site of the migrating schistosomulum during its "unprotected" phase. The various components necessary for immunity are therefore likely to be numerous and complicated, and it is hardly surprising that attempts at active immunization with poorly characterized antigenic extracts have failed. More detailed analysis of the exact components of an adequate immune response is required before it will be possible to decide how to stimulate these components by immunization.

3. Other Helminths

Although most of the direct evidence for an effector role for the eosinophil in immunity has stemmed from the work on *S. mansoni* described in the previous two sections, a number of studies have been carried out in other helminth infections. Many of these studies provide only equivocal evidence, but they will be briefly summarized here in order to make a few general points.

Two prerequisites for an effector role for the eosinophil are that the cell must be able to reach and adhere to the parasite and that the parasite's surface must be susceptible to damage by the cell's effector mechanisms. In many helminth infections, adult worms of a primary infection continue to survive for long periods of time. In such situations, it must be assumed that these conditions (as

well as the conditions necessary for other immunological effector mechanisms) are not met. In other words, the helminth has evolved a way of evading the host's response. Sometimes, however, the challenge organisms of a secondary infection are destroyed; and in these situations, it must be assumed either that the larval stages are passing through sites within the host where effector mechanisms can operate or that they are intrinsically more susceptible to damage. We have already seen how these ideas are important in schistosomiasis, in which the eosinophil in immune animals acts on the migrating schistosomula soon after penetration of the skin, but not on adult worms. We now have to consider the situation with other helminths.

In trematodes and cestodes, in contrast to adult nematodes, the tegument is a fragile and frequently absorptive surface, which may be assumed to be susceptible to antibody-dependent eosinophil-mediated damage. Evasion of such damage should therefore depend on a privileged location of the worm or on masking of antigenic determinants. In *Opisthorchis sinensis* infection of man, for example, the larvae migrate directly from the intestine up the biliary tract, where they elicit an inflammatory reaction in and hyperplasia of the overlying epithelium. Inflammatory cells fail to come directly into contact with the worm, however, and there is no evidence for an effective acquired immunity. In contrast, in *Fasciola hepatica* infection, the larvae migrate through the intestinal wall, across the peritoneal cavity, and through the liver parenchyma before reaching the presumably protected site in the bile ducts. In fascioliasis, although there is no evidence for immunity in sheep (*Sinclair*, 1975), both cattle and rats develop an acquired resistance to reinfection (*Doyle*, 1971; *Armour* and *Dargie*, 1974). In rats, this operates on the migrating larval stage, and is transferable both with immune lymphoid cells and with serum (*Armour* and *Dargie*, 1974). In such animals, there are no data concerning the role of eosinophils in immunity, although extensive infiltration of eosinophils is frequently observed, especially in the larval penetration tracts. It is sometimes suggested that they also act as repair cells, or that they induce the migration of the flukes to the bile ducts (*Smithers*, 1976).

In lumen-dwelling intestinal cestodes, such as *Diphyllobothrium latum* infection of man, there is no evidence for an effective acquired immunity. In contrast, cestodes which have a tissue stage in the mammalian host may elicit an acquired resistance to reinfection. For instance, *Hymenolepis nana* infection of mice is associated with development of resistance to reinfection against the invading oncosphere, and it has been suggested that these are damaged by eosinophil enzymes (*Gemmell*, 1976). There is no evidence for effective eosinophil responses against the cysticercus stages of *Taenia solium* or *Taenia saginata*, however, nor against the hydatid stage of *Echinococcus granulosus*.

In nematode infections, the adult worm is bounded by a tough cuticle, and effective immune responses frequently depend on the interaction of a number of immunologic components, with eosinophils playing a limited role (*Ogilvie* and *Worms*, 1976). There is no evidence, for example, that eosinophils are causally involved in the expulsion of *Nippostrongylus braziliensis* worms from the intestines of immune rats (*Ogilvie* and *Worms*, 1976), and *Mackenzie* et al. (1976, personal communication) have found that eosinophils attach to but fail to damage antibody-coated adult worms. In *T. colubriformis* infections of guinea pigs, however,

both mast cells and eosinophils infiltrate the intestine, more markedly during the accelerated rejection that occurs in reinfected animals, and a causal association between these cells and the rejection process has been suggested, although not established (*Rothwell* and *Dineen*, 1972; *Rothwell*, 1975). In *T. spiralis* infection of mice, rejection of adult worms is associated with the manifestations of both cell-mediated and acute inflammatory reactions, but there is no evidence that the eosinophil plays any major part in this process (*Larsh*, 1970). In other situations, however, adult worms may be affected by eosinophils; for instance, *Worms* and *McLaren* (1976, personal communication) have found that up to 75% of the cells adhering to adult *Litomosoides carinii* after transplantation into cotton rats are eosinophils. Not all worms are damaged, but in those that are the course of events is an initial attachment of eosinophils to the middle of the worm, followed by progressive encapsulation and death of the worm. Macrophages and fibroblasts are not detected in the lesion until the latest stages, after the worm is grossly damaged.

Nematode larvae may be more susceptible to eosinophil-mediated damage than adult worms. In *T. spiralis* infection of mice, for example, *Mahmoud* (1976, personal communication) has shown that repeated treatment with AES over a two-week period has no effect on the adult worm burden. In contrast, the number of muscle-stage larvae is doubled in AES-treated animals in comparison with controls treated with normal serum, this increase being apparent both three and four weeks after infection. The implication of these findings is that eosinophils are inactive against helminths within the gut but are able to approach and destroy organisms which are migrating through tissues. In this context, *Mackenzie* et al. (1976, personal communication) have shown that eosinophils are able to attach in vitro to antibody-coated *Trichinella* organisms, both adults and larvae, although in neither case have they observed damage to the organism under the conditions of the assay.

Suggestive evidence for the involvement of eosinophils in immunity against larval nematodes has also been obtained with a variety of other organisms. For example, *Jones* and *Rubin* (1974) found that mice immunized against *Nematospiroides dubius* by the oral route rapidly expelled a challenge dose of larvae, before their establishment in the tunica muscularis of the stomach; in this process, eosinophils appeared to play little part. In contrast, mice immunized by the subcutaneous route allowed the establishment of larvae within the tunica muscularis. Rejection in these animals was associated with an intense encapsulation of larvae by eosinophils. This reaction was greater than in unimmunized animals and was associated with marked damage to the encapsulated worms.

Similarly, during reinfection of calves with *Oesophagostomum radiatum* (*Elek* and *Drurie*, 1966), a biphasic pattern of rejection of both third- and fourth-stage larvae was associated with eosinophil infiltration around the larvae, and their subsequent death and discharge from gut tissue. In contrast, the reaction to third-stage larvae during a primary infection was minimal (*Elek* and *Drurie*, 1967) and was not associated with death of larvae, while the reaction to fourth-stage larvae was of lesser degree.

In *Wuchereria bancrofti* infection, *Higashi* and *Chowdhury* (1970) reported that eosinophils adhere to infective larvae in vitro in the presence of IgG from immune serum. Enhancement of adherence was seen after addition of fresh normal

serum, and the reaction could be abolished by addition of chelating agents. However, no damage to the larvae was observed over a 24-h period. Adhesion of eosinophils has also been reported to occur with guinea-worm larvae removed from the synovial spaces of patients with arthritis induced by *Dracunculus medinensis* (*Reddy* et al., 1969). Although the synovial fluid contained only 10% eosinophils, 30% of the cells attached to the larvae were eosinophils. Both live and dead larvae were recovered from the joints. In an earlier study, *Soulsby* (1961) reported that after injection of third-stage *Ascaris* larvae intraperitoneally into immune rabbits, eosinophils attached to and destroyed these larvae. This reaction was not seen in normal animals.

Finally, some suggestion of eosinophil involvement in immunity comes from work on *Dictyocaulus viviparus* infection of cattle. *Jarrett* and *Sharp* (1963) reported that during primary immunization of calves with irradiated larvae some organisms reached the lungs and evoked a slight eosinophilic infiltrate. Subsequent challenge with a high dose of infective larvae was associated with an intense eosinophilic infiltrate around dead and dying larvae and with temporary plugging of bronchioles by an eosinophilic exudate.

Although the findings described above cannot in any way be regarded as conclusive evidence, it seems likely that eosinophils are rather generally involved as effector cells in immunity to many helminths in addition to the schistosomes; but also that this role is mainly, if not exclusively, limited to tissue-stage parasites and to those organisms, usually the larval stages, the teguments of which are susceptible to damage by eosinophil components. The eosinophil can be viewed as one isolated component of a continually evolving series of host-parasite relationships; on the one hand, the host evolves specific mechanisms for damaging parasite surfaces, while on the other, the parasite evolves mechanisms for escaping from such damage. The outcome of the interaction between the parasite and the host's responses cannot be predicted with certainty in any host-parasite combination, and it would be unwise to assume that any one particular immune mechanism, including eosinophil-mediated damage, is solely responsible for immunity in any situation.

V. Conclusions

The emphasis in this review has been twofold: first, in distinguishing between the structure and physiology of the eosinophil and that of other cell types; and secondly, in showing how these distinguishing features may be related to functional properties, especially in helminth infections. Although much more work remains to be done, we can now propose that the unusual kinetics of the eosinophil will lead to its accumulation in large numbers in the vicinity of tissue parasites, particularly when these parasites have already elicited other types of immune response and that the unusual structure and enzyme content of the eosinophil then results in functional effects on the parasite or on parasite-associated processes. These effects include phagocytosis of immune complexes, modulation of local Type I reactions, and antibody-dependent destruction of the parasites themselves. It remains to be seen whether these beneficial effects of the eosinophil

response can be artifically modified or enhanced in any useful way in controlling the pathologic manifestations of tissue helminth disease.

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