Contributors

- M. Bowen
- Tse-Wen Chang
- E. H. Cooper
- Steven J. Fischman
- Michael L. Franklin
- Patrick C. Kung
- Allen L. Pusch
- Randhir S. Sandhu
- Edward V. Savard
- Vincent R. Zurawski, Jr.

Clinical Biochemistry

Contemporary Theories and Techniques

VOLUME 3

Edited by
HERBERT E. SPIEGEL

Department of Clinical Laboratory Research
Hoffmann-La Roche, Inc.
Nutley, New Jersey

1984

ACADEMIC PRESS, INC.
(Harcourt Brace Jovanovich, Publishers)
Orlando San Diego San Francisco New York London
Toronto Montreal Sydney Tokyo São Paulo

ACADEMIC PRESS, INC.
Orlando, Florida 32887

United Kingdom Edition published by
ACADEMIC PRESS, INC. (LONDON) LTD.
24/28 Oval Road, London NW1 7DX

Library of Congress Cataloging in Publication Data

Main entry under title:

Clinical biochemistry.

Includes bibliographies and index.
1. Chemistry, Clinical. 2. Biological chemistry.
I. Spiegel, Herbert E. [DNLM: 1. Chemistry, Clinical.
QY 90 C6406]
RB40.C56 616.07'56 81-14933
ISBN 0-12-657103-1 (v.3)

PRINTED IN THE UNITED STATES OF AMERICA

84 85 86 87 9 8 7 6 5 4 3 2 1

This volume is dedicated to Dr. John J. Burns. His scientific attainments, perception, and skills have commanded my professional and personal admiration for many years.

Contributors

Numbers in parentheses indicate the pages on which the authors' contributions begin.

*M. Bowen** (1), Unit for Cancer Research, University of Leeds, Leeds LS2 9NL, England

Tse-Wen Chang (89), CENTOCOR, Malvern, Pennsylvania 19335

E. H. Cooper (1), Unit for Cancer Research, University of Leeds, Leeds LS2 9NL, England

Steven J. Fischman (215), Mountainside Hospital, Montclair, New Jersey 07042

Michael L. Franklin (153), Advanced Technology Department, Hoffmann-La Roche, Inc., Nutley, New Jersey 07110

Patrick C. Kung (89), CENTOCOR, Malvern, Pennsylvania 19335

Allen L. Pusch (117), Department of Pathology, Christ Hospital, Oak Lawn, Illinois 60453

Randhir S. Sandhu† (215), Mountainside Hospital, Montclair, New Jersey 07042 and Department of Pharmacology, UMDNJ, New Jersey Medical School, Newark, New Jersey 07103

Edward V. Savard (55), Hoffmann-La Roche, Inc., Nutley, New Jersey 07110

Vincent R. Zurawski, Jr. (89), CENTOCOR, Malvern, Pennsylvania 19355

*Present address: Bioscience I Department, Imperial Chemical Industries, Mallesfield, Cheshire Sk10 4T9, England.

†Present address: Mountainside Hospital, Montclair, New Jersey 07042.

Preface

The intent of this volume is to broaden the scope of those laboratorians working in clinical biochemistry. Relevant aspects of serology, microbiology, monoclonal antibody techniques, and instrumentation have been included along with a discussion of hemoglobinopathies and a rather encompassing consideration of tumor markers.

As has been the practice in the previous two volumes of this treatise, encouragement was given for speculation, individual writing style, and presentation of concept rather than detail. All of the chapters were provided by experts in their field anxious to share their talents.

The editor wishes to express his thanks for the kind reception given to the previous volumes. This third volume will complete the present treatise. However, the editorial philosophies which characterize *Clinical Biochemistry: Contemporary Theories and Techniques* will be applied to a new endeavor. As the recently appointed editor-in-chief of *Advances in Clinical Chemistry,* I and a distinguished board of international editors will attempt to survey the leading edge of this burgeoning science.

Herbert E. Spiegel

Acknowledgments

I would like to acknowledge the contributions and professionalism of all those who participated in assembling this volume. My secretary, Mrs. Joan Marks, again has proven to be a valuable and valiant assistant. Finally, to the members of my family, especially my wife Joanne, I express my special thanks for the gifts of patience and concern.

Contents of Previous Volumes

Volume 1

Volume 2

1

Biochemical Monitoring of Cancer

E. H. COOPER AND M. BOWEN

I. INTRODUCTION

Research on the general topic of tumor markers has greatly expanded during the past two decades. Several monographs tracing the history and reviewing the present status of this research have been published during the past few years. They provide a guide to the background of this topic; among them are the books edited by Herberman (1979; Herberman and McIntire, 1979), Sell (1980), Sell and Wahren (1982), Chu (1982), and Colnaghi *et al.* (1982). To this list should be added the excellent overview of the contributions of routine clinical biochemistry of cancer patients built up since 1945 by Bodansky (1975).

Despite the plethora of publications, few of the putative tests for cancer diagnosis or monitoring have been adopted as routine procedures in the

CLINICAL BIOCHEMISTRY
Contemporary Theories and Techniques, Vol. 3

ISBN 0-12-657103-1

practice of clinical medicine. The reasons are complex, in part reflecting the conservatism of medical practice. However, they mainly reflect the view that although the results of many tests are of considerable academic interest, the tests often fail to provide crucial information that influences clinical decision making or are highly correlated with the changes of levels of other substances. In this chapter we will review the advantages and limitations of a number of substances that can be measured to monitor cancer. Before taking too pessimistic a view, however, it must be remembered that the test may yield information on which the clinician may be powerless to act using the treatment modalities currently available. One example would be signaling recurrence when therapy is largely ineffective, as in squamous cell cancer of the lung and other advanced tumors where the force of mortality is such that palliation is all that is left; or warning of a very slow progression of a cancer in an asymptomatic patient after a fundamental treatment has been given, for example, in carcinoma of the prostate. On the more optimistic side, we can see how biochemical measurements can be made that can achieve a significant improvement in the stratification of patients, and, we hope, will aid the interpretation of clinical trials of various modalities of treatment—either alone or in combination. The need for better stratification using clinical, histological, and biochemical parameters has been acknowledged by statisticians (Zellen, 1975) and shown to be highly relevant to the management and prognosis of some malignant disorders. Myelomatosis is a good example (Durie and Salmon, 1975).

Before detailing the performance of the tests available today, we will consider the criteria that the tests need to fulfill. The clinical context in which the tests are being used and the question the test result is intended to resolve can vary immensely. Unfortunately, not all physicians who ask for specific or nonspecific biochemical tests for their cancer patients are fully aware of the limitations of the interpretation of the results—especially the meaning of a "negative test." The hope that tests will eventually be discovered that are really specific and sensitive in identifying all patients with a particular form of cancer has yet to be realized. The growing knowledge that a negative test—carcinoembryonic antigen (CEA) level in primary large-bowel cancer, for example—has a biological advantage for the patient must be set against the climate of opinion that is seeking a very high, level of sensitivity for a given test. Unequivocal "cancer tests," with few exceptions, usually mean advanced cancers with all the biological disadvantage that this state implies.

The general applications of immunological tests have been summarized by Herberman (1982) and are shown in Table I.

TABLE I

Potential Clinical Applications of Immunological Tests for Tumor Markers[a]

1. Detection: screening of populations and high-risk groups
2. Aid in diagnosis of patients with signs or symptoms suggestive of cancer
3. Aid to histopathological evaluation of tumors
4. Aid in staging of cancer patients
5. Localization of tumor and detection of metastases
6. Serial monitoring to determine efficacy of therapy and to detect recurrence of metastases

[a] From Herberman (1982).

II. TUMOR-DERIVED PRODUCTS

A. Oncofetal Antigens

The concept of oncodevelopmental markers, antigens present in embryonic tissues that reappear when the adult tissue undergoes malignant change, is well founded. Carcinoembryonic antigen, discovered by Gold and Freedman in 1965, and α-fetoprotein (AFP) discovered by Abelev *et al.* in 1963, are good examples of oncodevelopmental markers. Indeed, as knowledge has increased, there has been a switch toward the idea that a tumor-associated antigen (TAA) is more realistic and tends to be a less exacting definition than "tumor-specific antigens."

1. Carcinoembryonic Antigen

The structure, immunology, and assay of CEA have been the subject of many reviews and will not be reviewed again (Fuks *et al.*, 1975; Pritchard and Todd, 1979; Plow and Edgington, 1979). We will consider CEA's use in the more common forms of tumor, emphasizing the prognostic aspects that have come to light as the large clinical trials have run their course of 5–8 years needed to reveal the true statistics.

a. Gastrointestinal Tract: Esophageal Cancer. There have been a few investigations of CEA levels in esophageal cancer. Wahren *et al.* (1979) found a CEA >2.5 ng/ml in 35 out of 59 (59%) patients with tumors of all stages and 7 out of 9 (78%) in metastatic disease. Alexander *et al.* (1978) reported 70% of 41 patients had a CEA >5 ng/ml; CEA levels >10 ng/ml after treatment were associated with a shortened survival.

b. Stomach Cancer. Several studies of primary stomach cancer have repeatedly confirmed the low frequency of raised levels of CEA compared

to that in colorectal cancers of comparable stage (Staab *et al.*, 1982; Rashid *et al.*, 1982), usually being <20% in stages I and II and rarely rising above 50% in advanced disease (Ellis *et al.*, 1978).

Carcinoembryonic antigen measurements have tended to be an impractical test for monitoring the progress of advanced gastric cancer being treated by chemotherapy because the change in levels does not mirror the clinical progress (Ellis *et al.*, 1978). There is an indication, however, that high levels of CEA prior to surgery, especially when combined with a raised level of acute phase proteins such as C-reactive protein or antichymotrypsin, has a high probability of being associated with a tumor too advanced for any form of resection (Rashid *et al.*, 1982).

In Japan, the high prevalence of carcinoma of the stomach and the active campaign to find early cases has provided information on the CEA distribution in stages I and II of the disease, as well as in their advanced stages, the latter being predominant in a European or North American series. Tomoda *et al.* (1981), reporting on 226 Japanese patients with gastric carcinoma, found a CEA >5 ng/ml in 23.9% of those examined, and raised levels were present in 56.3% of patients with unresectable tumors. This is in contrast to the much lower frequency of raised levels reported by Satake *et al.* (1981).

Sequential CEA measurements could give advanced warning of recurrence in stages I and II, but only in about half the patients, the remainder being nonproducers. The general experience of preoperative CEA measurements in carcinoma of the stomach is shown in Table II.

TABLE II

Percentage Increased CEA Levels in Carcinoma of Stomach According to Stage[a,b]

Reference	Number of patients	Origin	Raised CEA (%)			
			Stage I	II	III	IV
Tomoda *et al.* (1981)	226	Japan	8.2	25	23	40
Staab *et al.* (1981)	375	Germany	18.5		23	37
De Mello *et al.* (1983)	100	UK	15		35	52
Stake *et al.* (1981)	22 (early); 70 (advanced)	Japan	0		17	
Ellis *et al.* (1978)	157 (inoperable)	UK	—	—	—	31

[a] From Cooper and Giles (1983).

[b] Defined as >4 or 5 ng/ml by Roche assay or equivalent by other assays.

c. Carcinoma of the Pancreas. The general impression is that CEA measurements can provide little useful information for the diagnosis of pancreatic cancer (Medical Research Council, 1980; Hobbs, 1982; Hunter *et al.*, 1982). Obstructive jaundice will tend to enhance the level of CEA (Kalser *et al.*, 1978; Zamcheck and Martin, 1981). There is considerable overlap in the CEA levels in pancreatic cancer without jaundice and chronic pancreatitis, and the levels in resectable tumors are often normal. Conversely, very high levels in a patient with obstructive jaundice make it highly probable that the lesion is neoplastic. But the high levels cannot distinguish between the origin of the tumor that may be causing the obstruction.

d. Colorectal Cancer. There is now a consensus of opinion that the CEA test lacks the sensitivity and specificity to make it a reliable method for population screening (National Institute of Health, 1981). The Busselton Community Health survey in Australia (Stevens *et al.*, 1975) illustrated the cost ineffectiveness of CEA measurements in asymptomatic subjects and the confusion caused by high levels in heavy smokers. In this study a raised CEA (>5 ng/ml) was found in 44 out of 956 (4.5%) unselected persons over the age of 60 during a 4-year follow up; 6 of these 44 died of CEA-associated cancers, 15 were heavy smokers, 2 had colonic diverticulae, and 1 had a peptic ulcer. On the other hand, 18 (2%) of 912 CEA-negative persons developed CEA-associated tumors during this same time. The 20 persons who were CEA positive at the beginning of the study were reexamined after 4 years: 2 had occult cancers. Carcinoembryonic antigen can provide important prognostic information, and it still remains the most sensitive index for monitoring patients after curative or palliative resection. The value of CEA measurements in the monitoring of chemotherapy for colorectal cancer is more debatable.

e. Prognostic Assessment. The clinical staging of colorectal cancer, whether it be the A, B, and C stage as originally described by Dukes (1932), the B_1 and B_2 modification introduced by Astler and Coller (1954), or the more universal Tumor, Node, Metastasis [TNM] classification of the Union Internationale Contre le Cancer (UICC, 1978), carries strong prognostic implications. Hence any evaluation of the contribution of any laboratory test to refine the allocation of the patients to a particular risk, either time to recurrence or, more often, survival time, must first take clinical staging into account. Several statistical analyses are suitable for this procedure; their general properties and choice are discussed by Peto *et al.* (1977). An essential feature is to decide whether the analyses should use continuous measurements of a substance such as CEA or whether the

data should be converted into discrete measurements by adopting a level to distinguish raised and "normal" values. In recent years, sufficient time has elapsed to allow the prognostic significance of preoperative CEA to be evaluated. The following studies are examples from the literature but are by no means the whole experience available.

Major studies have been reported by Wanebo *et al.* (1978) based on 358 patients treated at the Memorial Hospital, New York. The recurrence rate was higher in Duke's B and C lesions, with a CEA >5 ng/ml. There was a linear inverse correlation between preoperative levels and estimated time to recurrence in patients with Duke's B and C lesions, ranging from 30 months for a CEA of 2 ng/ml to 9.8 months for a CEA of 70 ng/ml. In C lesions the median time to recurrence declined from 28 months in patients with a preoperative CEA <5 ng/ml to 13 months for patients with a CEA >5 ng/ml. Goslin *et al.* (1980) used preoperative CEA to stratify patients after "curative" resection of 134 colorectal cancers (Dukes's stages B and C). In the B lesions preoperative CEA levels were not correlated to risk of recurrence or time to recurrence. In Dukes's C lesions, however, 90% (19 of 21) of the patients presenting with a CEA >5 ng/ml relapsed during a follow up of 36–72 months, whereas only 39% (9 of 23) of Dukes's C lesions with a preoperative CEA <5 ng/ml relapsed in the same time. The Gastrointestinal Tumor Study Group, reviewing their experience of Dukes's B and C tumors of the large bowel since 1975, reported that colon cancer patients with a preoperative CEA >5 ng/ml had a greater probability of recurring than those whose values were lower than 33% versus 18% recurrence with 21 months minimum follow up ($p < .05$). The prognostic significance of preoperative CEA was apparent only in patients with Dukes's C colon tumors. Preoperative CEA was not of prognostic significance in Dukes's B or C rectal cancer (Steel *et al.*, 1982).

In Germany Staab *et al.* (1981), in a study of 563 patients, demonstrated the powerful prognostic effect of TNM stage in the prediction of survival. Dividing preoperative CEA levels into 4, 4.1–10, and >10 ng/ml, they demonstrated that the CEA can provide a further independent prognostic parameter that is additive to TNM stage. Lavin *et al.* (1981), using a comparable multiparametric analysis, examined the interrelationships of preoperative CEA and Dukes's stage in 74 patients who underwent curative resection. The CEA correlated with Dukes's stage and at the same time added significantly to the prediction of recurrence. The highest levels of CEA in Dukes's B and C tumors have been associated with undifferentiated tumors (Arnaud *et al.*, 1980), but there is less agreement about the relationship to tumor size (Mariani *et al.*, 1980).

The evidence from the large statistical analyses that preoperative CEA

levels can aid the assessment of prognosis is strong. Others point out, however, that too much overlap occurs between the outcome of patients showing small or no elevations of CEA prior to surgery (Koch and McPherson, 1979; Blake *et al.*, 1982), although most agree that levels >10 ng/ml carry a high probability of recurrence (Blake *et al.*, 1982). Mariani *et al.* (1980) studied the outcome of patients undergoing a curative resection when the preoperative CEA was normal. In 39 patients 28% had a steadily rising CEA within a year after surgery, compared to 37% of 32 patients whose preoperative levels were raised. This indicates that caution is needed before extrapolating the group statistic of probability of survival to a given time for recurrence for an individual patient, a point reiterated by Blake *et al.* (1982). Persijn and Hart (1981), after a study of 222 colorectal cancers, considered that CEA levels should not be taken to be a contraindication to a treatment intended to be curative and that the preoperative CEA levels are not superior to staging, according to Dukes, as a prognostic reference. In our experience (de Mello *et al.*, 1983), the combination of stage and preoperative levels of C-reactive protein and phosphohexose isomerase were the most powerful terms in the prediction of survival of colorectal cancer: Once they were included in a multivariate model, CEA was no longer significant.

Finlay and McArdle (1982) have demonstrated that CAT scanning is a more effective method of identifying patients with occult liver metastases at the time of resection of colorectal cancer than is an elevated CEA. In 35 patients undergoing "curative resection" the preoperative CEA was raised in 9, whereas the occult liver metastases at the time of surgery were detected in 11. The raised CEA was of no prognostic significance, but only 9% of patients with occult hepatic metastases survived 30 months. In contrast, 88% of patients in whom occult metastases were not detected survived more than 30 months.

f. Monitoring Patients with Colorectal Cancer. One of the certainties that emerged with time was that CEA measurements could provide early warning of recurrence of colorectal cancer. This was evident by the mid 1970s (Mach *et al.*, 1974; Zamcheck, 1975; Neville and Cooper, 1976), and recent studies have reinforced this point of view. The lead time provided by this test, ahead of the discovery of recurrence by virtue of its symptoms or clinical signs, is usually 3–5 months and sometimes much longer. In other patients the evidence from a rising CEA and the discovery are coincidental, or the tumor may reappear without any rise in CEA. Clearly, the lead time will depend on the frequency of the observations, and they depend on the clinicians' policy and attitudes toward treatment

(which are by no means uniform) for what is correct for a given patient after a curative or palliative resection. The issue is far from settled. Persuasive arguments in favor of adjuvant chemotherapy, chemotherapy for advanced disease, second-look surgery, or no intervention until symptoms develop all have their opinion leaders and followers. The laboratory staff may well find such differences within an institution confusing. Here, we can only put forward the use of CEA for monitoring, as envisaged and practiced by different clinicians, to show the varying degree of involvement of the patients in the monitoring schemes.

Reviewing the literature, Evans *et al.* (1978) considered that an elevated CEA appears in about one-third of recurrent or metastatic cancers of the large bowel, with a lead time of 3 months or more. Moertel and colleagues (1978) from the Mayo Clinic have pointed out that this lead time is gained at not inconsiderable financial burden to the patient or the state; they also considered that the information gained by the test was of little profit to the patient.

Those who favor second-look surgery have adopted a policy of close monitoring of patients, with repeated CEA measurements after resection. Minton *et al.* (1978) advocated taking a CEA two standard deviations above the postoperative low as an indication of recurrence and reported that 11 out of 14 (78%) patients had resectable lesions at second look. The rate of change of CEA is important: The rapidly rising levels are more likely to be associated with inoperable lesions than are the slowly rising levels (Staab *et al.*, 1978; Wood *et al.*, 1980; Attiyeh and Stearns, 1981). Minton's experience is exceptional; more typical of the general findings at second-look surgery initiated on the evidence of a rising CEA are the results from the Memorial Hospital (New York) in which 33 out of 37 laparotomies demonstrated tumor. In 16 cases the disease was resectable, and in 17 it was unresectable; the mean CEA level was 16.7 ng/ml in the resectable group and 56 ng/ml in the unresectable group (Attiyeh and Stearns, 1981). Other surgeons feel the information of recurrence usually comes at too late a stage to have any therapeutic advantage. A conservative estimate suggests that it is more likely to be only in 5–7% of patients with rising CEAs that further resection will be beneficial (Beart *et al.*, 1981).

Unfortunately, despite the lead time given by CEA compared to other tests such as serum enzymes and other nonspecific indicators of cancer, it is still insensitive to small tumor burdens as shown by certain primary tumors of Dukes's A or B stage and by monitoring small masses of tumor known to have been left behind either locally or in the liver after palliative resection (Lawton *et al.*, 1980); it often fails to give warning of an occult hepatic metastasis (Findlay and McArdle, 1982).

g. Response to Chemotherapy. Several authors have reported that serial CEA measurements are of value in determining response of colorectal cancer to radiotherapy (Sugarbaker *et al.*, 1976). Ravry *et al.* (1974) found 13 out of 20 patients with progression of advanced gastrointestinal (GI) cancer during chemotherapy showed a rising CEA. In 14 patients with stable disease, 57% showed no change in CEA levels, and 6 out of 8 (75%) showed a fall of CEA associated with clinical improvement. Al-Sarraf *et al.* (1979) found that CEA levels were a useful index of response in 49 patients with colorectal cancer being treated with chemotherapy. Lawton *et al.* (1980) found it to be useful in monitoring adjuvant therapy. Despite the varying reports on the usefulness of CEA measurements, it has now become a routine test in many institutions concerned with the treatment of colorectal cancer. The interpretation of borderline results has to be tempered with clinical experience.

h. Breast Cancer. The levels of CEA tend to be raised infrequently in early stages of breast cancer. In an analysis of 1085 patients, Myers *et al.* (1978) found an elevated level in 20% of patients with breast cancer. The other studies have confirmed that a primary breast cancer limited to the breast or involving local lymph nodes tends to produce an infrequent and modest rise of CEA, and often this is present only if a 2.5 ng/ml cut-off is used (Searle *et al.*, 1974; Steward *et al.*, 1974; Cowen *et al.*, 1978). Other investigators consider the frequency to be higher. Wang *et al.* (1975) reported 69% >2.5 ng/ml, Tormey *et al.* (1975) found 74% >5 ng/ml, and Wahren *et al.* (1978) found 70% >2.5 ng/ml. But as a rule the preoperative CEA level has no relation to prognosis (de Jong-Bakker *et al.*, 1981).

In metastatic breast cancer the situation is clearer. Waalkes and Tormey (1978) recorded 79% positivity with bone metastases, 82% with liver metastases, and 52% with skin metastases. Chu and Nemoto (1973) observed 93% positivity with liver metastases, 74% with bone metastases, 83% with pulmonary metastases, and 41% with soft-tissue metastases. Myers *et al.* (1979) observed 259 patients with metastatic breast cancer and obtained an overall frequency of 4-ng/ml levels in 189 of them (73%). An actuarial survival analysis showed a significant difference in the survival for each site except soft-tissue metastases when the population was subdivided according to whether the CEA was >4 ng/ml or <4 ng/ml, as shown in Table III. They also concluded that a postoperative CEA >4 ng/ml taken within 3 months of surgery for stages I–III disease enhances the risk of recurrence. The use of CEA as a monitor for chemotherapy and hormone therapy in disseminated breast cancer has been explored in many centers. A study of patients by Lamerz *et al.* (1980) has shown that,

TABLE III

Effect of CEA Levels on Survival: Median Survival (in weeks) from Date of CEA Estimation[a,b]

Subgroup	CEA ≥ 4.0 ng/ml	CEA < 4.0 ng/ml	Significance
Age ≥50	70	104	$p = .003$
Age <50	72	96	n.s.
Bone metastases	76	>104	$p = .008$
Soft-tissue metastases	>104	>104	n.s.
Visceral metastases	50	>104	$p = .055$
All patients	70	>104	$p = .002$

[a] From Myers *et al.* (1979).
[b] n.s., No significance.

following mastectomy in 1462 women without metastases, 91% had normal CEA levels (<3 ng/ml, 98% <5 ng/ml), whereas in 633 patients with metastases the CEA remained elevated in 54.3%. Lamerz and colleagues showed that the serial changes of the CEA level can be helpful in monitoring the subsequent changes in the evolution of the disease. de Jong-Bakker *et al.* (1981) found elevations of CEA in 35% of patients who developed metastases. This gave an average lead time of 200 days prior to clinical evidence. Several other authors support the view that CEA can help monitor the progress of established metastatic disease (Steward *et al.*, 1974; Waalkes and Tormey, 1978; Lamerz *et al.*, 1980).

Far less confidence has been expressed in CEA as a marker for the follow up of patients after treatment of nonmetastatic primary tumors. Undoubtedly, in about one-third of patients, a rising CEA can occur 2–6 months ahead of the clinical or radiological diagnosis of metastases. Random transient elevations may occur, however, that can make the interpretation difficult (Chu and Nemoto, 1973; Steward *et al.*, 1974; Coombes *et al.*, 1980; Lamerz *et al.*, 1980).

i. Lung Cancer. The important aspects of CEA measurements in lung cancer during the past few years have been in establishing their role as predictors of survival. A large survey of 682 patients was reported by Vincent *et al.* in 1979. In 118 patients treated by surgical resection, only 2 with CEAs above 10 ng/ml survived more than 24 months. Twenty-seven out of 33 patients (82%) who survived more than 24 months had a CEA <5 ng/ml, the lower the CEA the longer the median survival. Histological type influenced the initial CEA. The median level in 199 cases of adenocarcinoma was 9.5 ng/ml, and in 483 other forms it was 2.2 ng/ml. The distribution of levels according to histological type is shown in Table IV. Support for the view that a preoperative that raised CEA would be a sign

TABLE IV

Initial CEA Value in Patients and Blood Donors[a]

	Number of patients	>2.5 (%)	Median CEA value
Adenocarcinoma	199	68	9.5
Squamous	194	40	2.0
Small	130	49	2.3
Large	94	51	2.6
Other	65	46	2.2
Total	682	52	3.0
Healthy blood donors	487	2.7	0.5

[a] From Vincent *et al.* (1979).

that a lung cancer has already metastasized and it would be unlikely that surgery alone would benefit the patient comes from the reports of Dent and McCulloch (1979) and Concannon *et al.* (1978). However, a normal CEA level has little prognostic significance in individual patients undergoing tumor resection; 50% of this group were dead within 2 years in Stokes's series (Stokes *et al.*, 1980).

A second valuable use for CEA is the monitoring therapy in those patients for whom the test is an appropriate marker (Vincent, 1975; Ford *et al.*, 1979). The recent interest in intensive chemotherapy for small-cell carcinoma has shown that CEA is a valuable indicator of tumor response (Goslin *et al.*, 1981), and a level >10 ng/ml was frequently associated with extrathoracic spread (Waalkes *et al.*, 1980).

Carcinoembryonic antigen plays no part in the diagnosis of lung cancer, because of the low frequency of strongly elevated levels in early-stage cancers and the overlap of elevated CEA found in heavy smokers and obstructive lung disease.

j. CEA in Pleural Effusions. The CEA concentration in the blood and pleural fluid probably is important in the diagnosis of cancer in a patient with pleural effusion (Vladutiu *et al.*, 1979; Milano *et al.*, 1980b; Martinez-Vea *et al.*, 1981; Asseo and Tracopoulos, 1982). Very high CEA levels in the pleural fluid (>20 ng/ml) are pathognomonic of disseminated malignancy and tend to make other investigations redundant. It must be remembered that a low CEA does not preclude cancer, and it is often difficult to make a diagnosis using biochemical tests in patients not producing CEA. For this reason other clinical investigations (pleural biopsy, bronchoscopy, and modern imaging techniques) are preferred to biochemical analysis of the fluid as a means of diagnosis.

k. Other Cancers. Elevations in CEA may occur in a variety of other forms of cancer, mainly of endodermal origin. Generally the frequency is too low to be of value for routine use as a monitor, as in head and neck cancer and bladder cancer, or it tends to indicate advanced disease, as in pancreatic cancer (Zamcheck, 1976; Wood and Moossa, 1977). The use of CEA in gynecological cancer has created some interest. The highest levels are seen in endocervical cancer and lower levels in endometrial cancer; the highest levels are encountered in advanced disease (Seppälä, 1982). In ovarian cancer, modest increases of CEA can occur (Rutanen, 1978), but they contribute little to monitoring the disease (Meerwaldt *et al.*, 1983). Recent experience from Norway (Kjorstad and Orjasaester, 1982) indicates CEA is a useful prognostic agent in early cervical cancer, and a rise >5 ng/ml occurred in 65% of patients who developed recurrence after stage IB disease. Urinary CEA has been investigated as a method of monitoring recurrence of bladder cancer and the differential diagnosis of hematuria (Hall *et al.*, 1973; Fraser *et al.*, 1975; Guinan *et al.*, 1975). Although urinary CEA increases with increasing size of a bladder tumor, most authors, with few exceptions (Zimmerman *et al.*, 1980), find it to be an impractical test because of the production of CEA or CEA-like material from the urothelium during infection. Considerable differences in the CEA present in the urine occur in patients with carcinoma *in situ* and can be released from the urothelium after exposure to intravesical chemotherapy, but these differences do not have any immediate relationship to the natural history of the disease (Yu *et al.*, 1982).

2. α-Fetoprotein

α-Fetoprotein (AFP) is a serum protein normally present in low concentrations in adults (5–10 ng/ml) but occurs in high concentrations in fetal serum up to 10 ng/ml at 12 weeks gestation; the level of concentration falls steadily until birth. The general characteristics of this protein are shown in Table V. The background reading for AFP research can be found in the many recent reviews by Seppälä and Ruoslahti (1976), Adinolfi (1979), Hirai (1982), Sell (1980), and Sell and Wahren (1982). We will concentrate on its current use as a test in clinical medicine. The major uses of AFP are the detection and monitoring of hepatocellular carcinoma and germinal cell tumor of yolk-sac origin as well as the detection of developmental abnormalities (Seppälä and Ruoslahti, 1976; Adinolfi, 1979).

In Western medicine radioimmunoassays or enzyme linked immunosorbent assays are the routine methods of AFP measurement that report changes near to the normal range. In third world countries, where hepatoma may be of a high frequency, simple immunoelectrophoretic tech-

TABLE V

Physical and Chemical Characteristics of Human AFP[a]

Characteristic	Physicochemical characteristics
Molecular weight	69,000 (gel filtration SDS; gel electrophoresis)
Molecular weight of polypeptide chain	64,600 (ultracentrifugation); 69,000 (SDS gel electrophoresis)
Sedimentation constant	$S_{20,w}$ 4.5
Partial specific volume	V_{20} 0.726
Diffusion constant	$D_{20,w}$ 6.18
Isoelectric point	4.75
$E^{1\%}_{1\,cm}$ (278 nm)	5.30
C-terminal amino acid	Valine
Carbohydrate	4.5%

[a] From Ruoslahti *et al.* (1979).

niques may be used, but their lower cut-off level is usually about 400 ng/ml, which will be adequate for diagnostic purposes in >80–94% of the patients.

In primary hepatocellular carcinoma (PHC), it is generally agreed that 80–90% of HPC will show an elevation of AFP. Levels >3000 ng/ml are usually considered diagnostic of PHC, those between 400 and 3000 ng/ml can still be of uncertain significance, and elevations <400 ng/ml are often observed in a variety of different liver diseases (Fig. 1) (Chen and Sung, 1977; Lehmann and Wegener, 1979; Hirai, 1982).

In North America and Europe the incidence of hepatoma is low, about 1 per 100,000, and the diagnosis may be considered far more often than it is confirmed. Bell (1982), reporting his experience from Norway of AFP level in 21 cases of hepatoma, 106 patients with hepatic metastases, and 110 alcoholics with liver disease; found 10 out of 14 cases of PHC had strongly elevated AFP, but 0 out of 17 with cholangiocarcinoma. α-Fetoprotein showed a small rise in 26% of these patients without hepatocellular cancer. This is typical of the grey zone that surrounds the interpretation of AFP levels in Western medicine. It is estimated that between 12 and 32% of Caucasian patients with liver cirrhosis will have a rised AFP, which is usually 400 ng/ml (Chayvialle and Ganguli, 1973; Ruoslahti *et al.*, 1974; Bloomer *et al.*, 1975). The range of AFP levels in a series of 57 PHC seen in London (Johnson *et al.*, 1981) is typical of the range. Eleven (27%) had AFP levels that were slightly raised (between 10 and 1000 ng/ml); in

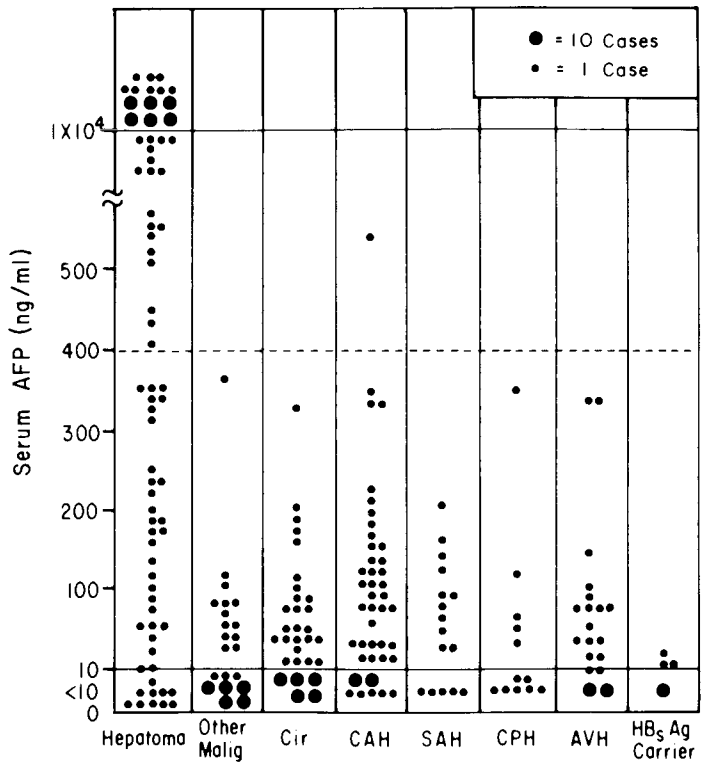

Fig. 1. Serum AFP level in liver disease. From Chen and Sung (1977).

24 cases (58%) the levels were 1,000–10,000 ng/ml and 6 cases (15%) were >100,000 ng/ml.

These figures can be compared to collective experience in Japan, where 89% of 515 patients with PHC had an AFP >20 ng/ml; and 20.7% of 395 cases of cirrhosis also showed a raised AFP (Hirai, 1982). Once again, Japanese physicians consider that an AFP of >400 ng/ml and rising is highly suspicious of PHC. The rates of change of AFP reflect the differentiation of the tumors. Matsumoto *et al.* (1982) observed that in 96 Japanese patients, poorly differentiated tumors could produce a rise of 10,000 ng/ml in 1 week. In moderately differentiated tumors the AFP tended to rise at a rate of 1000 ng/ml in 3–4 months, and in well-differentiated and anaplastic tumors both tend to have levels <200 ng/ml throughout the illness. Despite the difficulties of interpretation of small rises of AFP, it is of interest that Lehmann (1976) in Germany made a survey of 318 cases of cirrhosis, and on the basis of a raised AFP was able to lead to the detection of 15 clinically undiagnosed hepatomas. Population screening for

PHC has no place in Western medicine but has been taken up with enthusiasm in parts of China where PHC is endemic; several million persons have been screened (Coordinating Group for the Research of Liver Cancer, 1974). One such survey of 1,786,906 persons in Qidong province led to the discovery of 1026 cases of PHC. It was claimed that the pick up of stage I cases could be increased to 76% by twice yearly assays, and the 2-year survival for stage I is 69% (Zhu, 1981).

Obviously, AFP is a marker of choice for hepatoma, possibly being more effective in tropical countries, although this would depend on how thoroughly the AFP negative cases were investigated. In Western hospitals' practice, the opinion is that caution is needed in the interpretation of a modest rise of AFP; the usual battery of ancillary biochemical tests, imaging techniques, and hepatic biopsy will be needed to reach a definitive diagnosis. Most physicians agree that the serial measurement of AFP is a useful parameter in observing the progress of a patient after resection of a primary tumor or during chemotherapy. Partial hepatectomy in man is not followed by a rise of AFP (Alpert and Feller, 1978), which is in marked contrast to the temporary burst of synthesis of this protein in the rat after partial hepatectomy.

a. Gonadal and Extragonadal Germ-Cell Tumor. There is overwhelming evidence that AFP and human chorionic gonadotrophin (hCG), when present in elevated titers, are highly sensitive indicators of the presence of germ-cell malignancy [see Nørgaard-Pedersen and Raghavan (1980) for a comprehensive guide to the literature]. Their routine use in testicular tumors has considerably increased the awareness of unsuspected teratomas. These markers are not ideal for the estimate of total tumor load but are very helpful in the diagnosis. The cumulative experience of 800 cases has shown no persistent false positive, but false negative results—that is, a negative result in a patient with known active tumor—can be encountered with a frequency of 10–30% (Schultz *et al.*, 1978; Anderson *et al.*, 1979; Barzell and Whitmore, 1979; Lange *et al.*, 1980; Kohn and Raghavan, 1980; Raghavan *et al.*, 1980). The prognostic significance of the marker production in terms of sensitivity to therapy is still uncertain. Loss of sensitivity because of the selective elimination of germ-cell elements is one reason why they can become negative after prolonged chemotherapy.

3. Tennessee Antigen

Tennessee antigen [Tennagen (TAG)] is a glycoprotein extracted from a colon adenocarcinoma. It has some similarities to CEA but is antigenically distinct from CEA (Potter *et al.*, 1980). A commercial hemagglutina-

tion inhibition assay is available. Their initial results indicated that more than 90% of cancer sera gave values greater than the upper limit of normal (5.5 ng/ml serum), including colorectal Dukes's stage A. Subsequent investigations have failed to confirm these first impressions. Sampson *et al.* (1982) reported that TAG has a sensitivity of 71% and a specificity of 77% for gastric cancer; it was a less attractive test, however, in colorectal cancer with a sensitivity of 76% but with a specificity of only 44%. Pentycross and Cross (1982) has cast doubt on the upper limit of normal. They found higher values than first reported and also confirmed the low specificity of the test and its unhelpfulness in discriminating cancer from other diseases. In his series, although higher values were found in patients with active cancer, persistently raised levels were found in tumor-free patients. Gray *et al.* (1982) reported that 29 out of 31 patients with resectable colorectal cancers had a raised serum level of TAG compared to 8 with a raised CEA. However, the lack of specificity and the persistent elevation after resection of all macroscopic cancer limits the clinical usefulness of TAG. Tennessee antigen has been observed to be raised in 54% of patients with bladder cancer, rising from 30 to 80% positive with increase of stage from superficial cancer to metastatic cancer.

4. Tissue Polypeptide Antigen

Tissue polypeptide antigen (TPA) is a polymeric protein made up of units with molecular weights of 45,000–20,000, without sugar or lipid conjugates. Tissue polypeptide antigen is found in most tumors, and a hemagglutination inhibition assay (and more recently, a radioimmunoassay) has been devised for its measurement in body fluids (for a review see Björklund, 1980). A general study of TPA levels in 2028 serum samples from 1060 cancer patients showed that active cancer was associated with elevated levels in 50–100% of the patients. According to the tumor type, gynecological, urological, and head- and neck cancers tended to be raised in >60%; tumor-free patients and patients with stable disease had elevated levels in 14–35% (Skryten *et al.*, 1981).

There have been a few studies of TPA levels in colorectal cancer. Andrén-Sandberg and Isaacsson (1978) studied 157 patients and found that high preoperative levels of TPA were associated with a bad prognosis. In monitoring, TPA levels rose several months prior to the detection of recurrence in 10 out of 13 patients. A comparative study of TPA and CEA in gastric and colorectal cancer has been made by Wagner *et al.* (1982). They concluded that both TPA and CEA are not tumor-specific markers and that raised TPA levels occurred in inflammatory states. Thirty out of 39 (76.9%) cases of carcinoma of the stomach showed a raised TPA, but a raised CEA occurred in only 18 out of 39 patients

(46.1%). Tumor stage and TPA levels were related. In colorectal cancer, TPA was raised in 37 out of 60 (61.6%) and CEA elevated in 38 out of 60 (63.6%).

A comparison of TPA, CEA, and a panel of standard biochemical tests has been made in breast cancer (Schlegel *et al.*, 1981). A combination of TPA and CEA levels were shown to be the most powerful discriminants in separating tumor-free patients from those with active disease. Their combination could separate the cases with a 95% efficiency for the tumor-free group and 46 out of 62 for the tumor group.

In bladder cancer, of all stages, the serum TPA was elevated in 53%, rising from 30 to 80% with increase of stage from superficial cancer to metastatic cancer (Oehr *et al.*, 1981). However these authors also report a minor rise of CEA >2.5 ng/ml but less than 5 ng/ml as a positive test; in view of the incidence of smokers among bladder cancer patients this is of little significance. Urine TPA levels have been demonstrated to be erratic because of diurnal variation, but the concentration in 24-hour urines correlates well with the presence or absence of bladder cancer (Kumar *et al.*, 1981).

B. Hormones

1. Calcitonin

Calcitonin is a polypeptide hormone produced mainly by the parafollicular C cells of the thyroid gland. Calcitonin is present in the peripheral blood in a concentration proportional to the blood calcium levels (Potts *et al.*, 1971). Medullary thyroid carcinoma (MTC) is the classic indication for the use of calcitonin for the detection and monitoring of the disease (Tashjian *et al.*, 1970; Wells *et al.*, 1978). The levels of calcitonin will be increased in the majority of patients with MTC. Very large tumors and aggressive anaplastic tumors, however, may exhibit a fall off in their calcitonin production (Trump *et al.*, 1979; Mendelsohn and Baylin, 1982). An important aspect of MTC is that this tumor can have a familiar pattern present in 10–20% of patients with the disease. Calcitonin and calcitonin provocation tests using calcium infusions (Pathemore *et al.*, 1974) or pentagastrin infusions (Hennessy *et al.*, 1974) have identified the preneoplastic stages of MTC in families known to be at risk (Wells *et al.*, 1978; Baylin *et al.*, 1979).

Several other types of cancer can produce calcitonin or calcitonin-like proteins. In metastatic breast cancer, raised blood calcitonin has been reported in metastatic disease (Coombes *et al.*, 1974; Milhaud *et al.*, 1974). Although some tumors have been shown to produce calcitonin *in*

vitro, it is still possible that raised levels in skeletal metastases are part of a response to hypercalcemia. However, it does not appear to be of great clinical importance. Lung cancer, especially small (oat)-cell carcinomas, may produce calcitonin (Silva *et al.*, 1974; Milhaud *et al.*, 1974; Coombes *et al.*, 1974; Hansen and Hummer, 1979; Mulder *et al.*, 1981). There is a variation in the molecular weights of proteins that react as immunoreactive CT in nonthyroid cancer (Sizemore and Heath, 1975). This heterogeneity of CT, coupled with the fact that the rise in the levels is small when compared to MTC, limits its practical application as a marker.

2. Adrenocorticotropic Hormone

Ectopic, or so-called inappropriate adrenocorticotropic hormone (ACTH) secretion is a well-recognized phenomenon occurring mainly in carcinoma of the lung, especially in small-cell tumors, pancreatic carcinomas, thymomas, and pheochromocytomas. A few of these patients can present with some of the classic signs of Cushing's disease, including dependent edema, hypertension, hypercalcemia, and diffuse hyperpigmentation (Imura *et al.*, 1975).

When secreted by tumor cells, ACTH may be similar to native pituitary-derived ACTH with a molecular weight of 4500. A second type of ACTH, "big or bigger ACTH," has been recognized with higher molecular weights of 20,000 and 36,000, respectively. The big ACTH is a glycosylated form of the protein and possesses only a few percentages of the biological activity of the native ACTH (Gerwirtz and Yallow, 1974; Eipper *et al.*, 1976; Odell and Wolfsen, 1978).

Surveys of the incidence of the frequency of raised levels of ACTH or its precursors in lung cancer indicate it may be as high as 70% in all histological types (Wolfsen and Odell, 1979), but estimates of the frequency of clinical evidence of excess ACTH production range only from 0.4 to 2% (Yallow, 1979; Tortensson *et al.*, 1980).

The general experience of using ACTH (or big ACTH) levels to monitor lung cancer has been disappointing (Baylin *et al.*, 1978; Mendelsohn and Baylin, 1982). The release of ACTH from tumors into the circulation is variable: Some tumors show histochemical evidence of ACTH and normal blood levels, and, conversely, some patients with an elevated plasma ACTH show no detectable ACTH in their tumors. There are accounts of the ACTH levels mirroring the evolution of the tumor and its treatment (Gerwirtz and Yallow, 1974; Silva *et al.*, 1979; Odell and Wolfsen, 1978; Gropp *et al.*, 1979); however, these patients are the exception, and ACTH measurements have not been widely adopted as a method of monitoring lung cancer.

3. *Human Chorionic Gonadotrophin*

Human chorionic gonadotrophin is a glycoprotein hormone secreted by the placental syncytrophoblast and normally is only detectable in the serum during pregnancy. The hormone is composed of two subunits, α and β. The α hCG subunits are almost identical to the α subunits of LH, FSH, and TSH, whereas the β subunit is the basis of the immunological specificity of hCG assays. The β subunit assay has a sharp cut-off of normal at approximately 1 IU/liter in plasma; With hCG, significance can be attached only to levels >100 IU/liter.

Raised levels of serum hCG have been reported in patients with adenocarcinomas of the pancreas and stomach and various gynecological cancers. The major contribution of hCG as a tumor marker has been in gestational trophoblastic disease and in the monitoring and diagnosis of nonseminomatous germ cell tumors.

a. Trophoblastic Disease and Trophoblastic Tumors. The production of hCG bears a close relationship to the number of trophoblastic cells. The single-cell production rate for a 10-day-old human placenta is 1.4×10^{-2} IU/day (Braunstein *et al.*, 1973) and by malignant trophoblast *in vitro* 5×10^{-5} IU/day (Bagshawe, 1969) (1 ng = 5 mIU). The usual precursor of gestational neoplasm is an abnormal growth of the placenta called a hydatidiform mole, but gastational tumors can arise from normal, aborted, or ectopic pregnancies.

A rising serum β hCG level, as shown by assays at weekly intervals in patients at risk, is an important element in the diagnosis of a trophoblastic neoplasm or persistently high levels of urinary hCG. About 14–18% of patients with molar pregnancies develop trophoblastic tumor; the majority undergo spontaneous regression (Goldstein, 1979). Monitoring of urine or plasma hCG levels should continue for 6 months and be discontinued after this time if the levels are normal. This approach has led to an earlier diagnosis of germ-cell tumors and the start of chemotherapy when the tumor mass is still relatively small. Bagshawe's indications for the treatment of an invasive mole include urinary hCG values of greater than 40,000 IU/24 hr at more than 4–6 weeks after evacuation, values >25,000 IU/24 hr at more than 10 weeks after evacuation of a hydatidiform mole, or a persistently raised level 5–7 months after evacuation. In trophoblastic tumor (choriocarcinomas) the serum levels of hCG are of great importance in monitoring the results of treatment. A review of 317 patients with trophoblastic tumors treated between 1957 and 1973 (Bagshawe, 1976) showed that in those with hCG 24-hr urine excretion rates between 1,000 and 10,000 IU/liter the fatality was 3% and rose progressively with increasing hCG and was >60% when the hCG was >1,000,000 IU/liter.

b. Germinal-Cell Testicular Neoplasms. The combination of hCG and AFP will show a raised level of one or both markers in up to 80% of nonseminomatous testicular tumors, whereas in pure seminomatous tumors up to 25% may show a rise of hCG (Javadpour *et al.*, 1978), the high AFP being strongly indicative of a germinal-cell tumor. Levels of AFP and hCG are normally used to help in the clinical management of the disease; they are particularly valuable in giving early warning of recurrence and in the efficacy of various treatment modalities prescribed to attempt to eradicate the tumor.

Human chorionic gonadotrophin is excreted intact in the urine. Recent improvements in urine concentration techniques and assays suggest that urine hCG levels can be an even more sensitive method of monitoring germ-cell tumors (Javadpour and Chen, 1981).

c. Nontrophoblastic Tumor. A large survey of hCG levels in various cancers has been made by Braunstein (1979). The incidence of immunoreactive sera in patients with cancer is shown in Table VI.

The clinical significance of the relatively small rises in hCG in cancers other than germ-cell tumors is unclear. Rutanen and Seppälä (1978) examined the levels of hCG β subunit in gynecological cancers. Their data illustrates the correlations that can occur in other forms of cancer. In 276 patients, 49 (18%) had raised hCG above that seen in nonmalignant gynecological conditions. There was no relationship of hCG positives with age or tumor stage, and 29% remained positive after radical surgery.

Lung cancer is often associated with a small rise in the level of serum hCG. For example, Blackman *et al.* (1980) reported values above the 95th percentile of normal in 41% of men and 16% of women with lung cancer. Most values in this and other series (Goldstein *et al.*, 1974; Gailani *et al.*, 1976; McIntire, 1982) are below 10 ng/ml. Other investigators consider the incidence of positive levels to be more in the region of 5–15%. Rising levels in sequential measurements are associated with tumor growth, but hCG levels can give discordant results. Human chorionic gonadotrophin has been included in batteries of markers used to discriminate tumors from nonmalignant conditions, but this approach is too expensive for routine use, and it is a weak discriminant.

C. Milk Proteins

Four milk proteins, casein, lactoferrin, lactalbumin, and the sweat α_2-globulin (GCDPF), have been examined for their role in the diagnosis and monitoring of breast cancer. Caseins are phosphoproteins, a major component of normal milk in a concentration of 0.4 g/ml. Hendrick and Fran-

TABLE VI

Incidence of Immunoreactive hCG in Sera of Patients with Neoplasms[a]

	Number of patients	Number positive by hCG	%
Ovarian	103	42	40.8
Breast	386	115	29.8
Melanoma	210	40	19.1
Gastrointestinal	654	109	16.7
Esophagus	12	0	0
Stomach	75	17	22.7
Pancreatic	45	15	33.3
Small intestine	23	3	13.0
Biliary tract	9	1	11.1
Liver	217	36	16.6
Large intestine/rectum	129	21	16.3
Sarcoma	51	7	13.7
Lung	311	41	13.5
Renal	69	7	10.1
Hematopoietic	661	34	5.1
Leukemia	262	12	4.6
Lymphoma	283	11	3.9
Multiple myeloma	116	11	9.5
Total	2445	395	16.2

[a] According to Braunstein (1979).

chimont (1974) developed a radioimmunoassay for casein in the plasma. Initial studies showed that high concentrations of plasma casein were frequent in metastatic breast cancer; later an assay for κ-casein was developed by Hendrick *et al.* (1977) that had a narrower specificity; they reported that when using a cut-off level of 25 μg/liter, 6 out of 39 patients with primary breast cancer and 11 out of 25 with metastases were positive. Only 1 in 55 patients with benign disease showed an elevated level. More extensive studies (Cowen *et al.* 1978), however, showed that the overlap between plasma κ-casein levels in various types of breast disease was too great for its use as a marker.

The plasma levels of lactoferrin, a milk iron-binding protein, has been found to show little difference in benign and malignant disease and is produced by breast monocytes, which precludes its use as a marker (Rümke *et al.*, 1971). Lactalbumin, a low molecular weight protein (15,000) acts as a controlling mechanism of lactose synthetase in milk

(Fitzgerald *et al.*, 1970). The levels of lactalbumin in the blood reflect breast activity, but it is not found in the tissue-culture fluid of breast-cancer cells grown *in vitro* or their cytosols and thus has no value as a potential marker of breast cancer (Zangerle *et al.*, 1982). Gross cystic disease fluid protein (GCPF 70) is found in an increased level in the plasma in 27 out of 49 cases (55%) of breast cystic disease and 64 out of 105 (60%) of women with breast cancer with a very low incidence of raised levels of nonbreast benign disease.

This line of research is an important exploration for potential markers. Unfortunately, none are sufficiently specific to warrant their regular use in monitoring breast cancer.

D. Pseudouridine and Other Modified Nucleosides

The methylated nucleosides are now readily separable and quantifiable by high-performance liquid chromatography (HPLC), using reverse phase columns, in urine, serum, and amniotic fluid (Davis *et al.*, 1977; Gehrke *et al.*, 1978). The release of methylated nucleosides is a measure of nucleic acid degradation; pseudouridine and the methylated nucleosides is a breakdown product of transfer RNA. Tumor tissue, with its high rate of cell division, would be expected to release large amounts of these metabolic end products. Indeed, urinary methylated nucleosides have been suggested as a good indicator of the effectiveness of chemotherapy (Borek, 1980).

Elevated levels of urinary nucleosides have been found in patients with newly diagnosed and recurrent nasopharyngeal carcinoma. Nasopharyngeal carcinoma is difficult to diagnose at the early stages, so a marker of this type could be useful (Trewyn *et al.*, 1982). In chronic myeloid leukemia (CML) seven modified nucleosides were quantified in urine. In the stable phase of the disease, levels were 1–2 times normal, but in the blastic phase they reached up to 7 times normal. The most significant difference in levels between the two phases were seen in ψ-uridine, 1-methylinosine, and N^2,N^2-dimethylguanosine. These markers are, therefore, suitable for the detection of early blast transformation (Heldman *et al.*, 1982). On the other hand, measurement of urinary nucleosides has been found to be of limited value in following the course of breast cancer (Tormey *et al.* 1980b).

The measurement of methylated nucleosides has been slow to gain a large-scale investigation. This could be a result of the specialist skills required for the assay. Some encouraging results have been seen, however, and it is a candidate marker system for use as a monitor of chemotherapy.

E. Polyamines

These low molecular weight organic polycations are found in all nucleated cells in relatively large amounts (frequently in millimolar concentrations). They have a strong affinity for the negatively charged phosphates of nucleic acid backbones and are instrumental in the regulation of nuclear metabolism. Rapidly growing tumor tissue would be expected to produce large quantities of polyamines (Russell and Durie, 1978) because the increased amount of ribosomal RNA during growth and proliferation is accompanied by increased polyamine synthesis.

Techniques for the assay of urinary and serum polyamines now include the use of automatic amino acid analyzers, gas–liquid chromatography, and thin-layer chromatography. A method has also been developed to detect tissue polyamines and to demonstrate significant differences between benign prostatic hyperplasia and renal adenocarcinoma and their corresponding normal tissues (Dunzendorfer and Russell, 1980). The elevation of polyamines in the serum and urine of cancer patients with active disease is now well known (Nishioka and Romsdahl, 1974, 1977; Waalkes *et al.*, 1975; Desser, 1980; Milano *et al.*, 1980a; Sadananda *et al.*, 1980; Bakowski *et al.*, 1981; Pastorini *et al.*, 1981; Horn *et al.*, 1982). In a study of urinary polyamines in breast carcinoma, elevations of one or more of the polyamines putrescine, spermidine, spermine, and cadaverine were seen in 50% of patients with metastases, 38.5% of preoperative patients, and 35.7% of 5–24 weeks postoperative patients with nodal involvement (Tormey *et al.*, 1980a).

An indicator of polyamine release more sensitive than the methods mentioned above may be the assessment of erythrocyte polyamines. Erythrocytes serve as carriers for spermine and spermidine in the blood. Using an HPLC method, erythrocyte polyamines showed promising results as a monitor of response to therapy in disseminated prostatic carcinoma (Killian *et al.*, 1981).

The polyamines, probably in combination with other markers, can assist the clinician in diagnosis, assessment of response to therapy, staging of tumors, and monitoring of remission and relapse (Russell, 1982). The only disadvantage at present in their widespread use is the lack of a rapid and inexpensive assay suitable for use in a routine laboratory.

Russell and Durie (1978) have set out five major areas for the further evaluation of the clinical usefulness of polyamines; they are summarized as follows:

1. Use of plasma spermidine to evaluate the effectiveness of drugs in killing tumor cells.
2. Use of urine and plasma putrescine to evaluate rates of cell division.

3. Use of extracellular polyamines to monitor remission and relapse.
4. Use of polyamines to evaluate tumor status and burden.
5. Development of radioimmunoassay as a more suitable method for polyamine assay in clinical studies.

F. Pregnancy Proteins and Placental Proteins

This group of proteins consists of pregnancy-specific proteins, seen only in pregnancy sera and absent from normal sera; pregnancy-associated proteins, which occur in trace amounts in normal sera as well as in pregnancy; and placental proteins, which are tissue proteins produced by the placenta. Three pregnancy proteins (SPs) and more than 20 soluble placental tissue proteins (PPs) have been identified by Hans Bohn. Some of these have been put forward as possible markers in oncology (Bohn, 1980), although poor results have been seen in colorectal carcinoma using a series of placental proteins (Szymendera *et al.*, 1981). The synonyms and physicochemical properties of the pregnancy proteins, as defined by Bohn (1982), are summarized in Table VII. Another major placental protein, human chorionic gonadotrophin, has been discussed in Section B,3 of this chapter.

1. SP1

This is a pregnancy-specific protein produced by the human trophoblast and found in the sera of pregnant women. Serum levels have been found to be useful in monitoring trophoblastic diseases (Than *et al.*, 1979). It can also be produced by nontrophoblastic malignant tissues, such as breast carcinoma, adenocarcinoma of the gastrointestinal tract, and bronchial carcinoma (Horne *et al.*, 1979). It has been found, however, to be of little use as a marker in nontrophoblastic tumors (Tsujino *et al.*, 1981). Recently, the heterogeneity of SP1 has been investigated (Sørensen, 1982). Four components can be identified on the basis of their electrophoretic mobilities, the major components having β mobility. The presence of the other components may result in variation of levels, depending on the assay system and antiserum used. Clearly, problems with the assay should be resolved before conclusions can be drawn about the clinical usefulness of the levels of SP1.

2. SP2

The concentration of SP2 is important in that it reflects the levels of the unbound sex hormones, testosterone and estrogen, in the circulation. It is the free hormones that are physiologically active. A high SP2 results in a lower free testosterone concentration in plasma while raising unbound

estrogen. Estrogens induce a rise in SP2, which acts to amplify estradiol action because free testosterone is reduced and free estrogen raised (Anderson, 1974).

Cancers of the target tissues of these hormones (e.g., breast and prostate) are well known to be hormone dependent. In prostate cancer patients on estrogen therapy, high levels of SP2 are seen (Houghton *et al.*, 1977), which may be of prognostic significance. Total testosterone levels and SP2 can be used to calculate free testosterone levels (Bowen *et al.*, 1980), and the assay of SP2 by radial immunodiffusion is straightforward (Earnshaw *et al.*, 1982). The level of free testosterone calculated in this way may prove to be a better indicator of the hormonal status of the prostate cancer patient than total testosterone. It is also a more sensitive way of detecting whether the patient has stopped the course of estrogen therapy independent of any advice from his doctor.

3. SP3

An assessment has recently been made of SP3 as a tumor marker (Müller *et al.*, 1982). There was no apparent relationship between its levels and either tumor burden or response to treatment. Its wide range in normal females made single determinations meaningless, and this is the largest factor against its usefulness as a diagnostic indicator (see Figs. 2 and 3). This has also been the conclusion of Bundschuh (1981) after a 20-year study. He stated that α_2-PAG is useful only as a diagnostic test for cancer when the individual's genetically determined initial values are known. In ovarian cancer, it may be of use in association with CEA as a prognostic indicator (Sawada *et al.*, 1982), and similar conclusions have been drawn for lung cancer (Watanabe, 1980).

4. Placental Proteins

The appearance of some of the placental proteins in tissues in which they do not usually occur can be indicative of malignancy. An increase in levels of these proteins in serum, in which they are usually present only in trace amounts, can also be useful in monitoring disease. Radioimmunoassay is usually required for their detection. A study of PP7 and PP8 (Lüben *et al.*, 1979) showed higher levels in disease than in normal sera, but not specific to malignancy. Seppälä *et al.* (1979) reported the elevation of hCG and SP1 levels in trophoblastic disease (as in pregnancy), but the absence of PP5, which is normally present in pregnancy. Placental tissue protein 5 could therefore differentiate normal pregnancy and invasive trophoblastic disease. Placental tissue proteins 11 and 12 have been found in tumor tissue and may be useful as tumor markers (Bohn and Lüben, 1981). Many of the other PPs are still under investigation.

TABLE VII

Summary of the Properties of Pregnancy-Specific, Pregnancy-Associated, and Placental Proteins

Protein	Synonyms	Electrophoretic mobility	MW	Comments
SP1 (Pregnancy specific β_1-glycoprotein)	Pregnancy-associated plasma protein C (PAPP-C); trophoblast-specific β_1 globulin (TSG)	β_1	90,000	Pregnancy-specific; may be of use as a monitor of malignancy, particularly trophoblastic disease
SP2 (Steroid-binding β-globulin, SBβG)	Sex hormone binding globulin (SHBG)	β_1	65,000	Pregnancy-associated; may be of use in assessment of hormonal status in hormone-dependent tumors
SP3 (Pregnancy-associated α_2-glycoprotein, α_2-PAG)	Pregnancy zone protein (PZ); pregnancy-associated macroglobulin (PAM)	α_2	360,000	Pregnancy-associated; unlikely to be useful as a tumor marker
PP1	—	α_1	160,000	Solitary tissue protein
PP2 (Ferritin)	—	α_2	500,000	Solitary tissue protein[c] (See Section III,d)
PP3	—	α_2	~100,000	Solitary tissue protein[c]
PP4	—	Albumin	~ 30,000	Solitary tissue protein[c]
PP5	—	β_1	36,000[a]	Placenta-specific protein; possible diagnostic indicator of invasive trophoblastic disease

PP6	—	α_1	1,000,000[a]	Solitary tissue protein[c]
PP7	—	$\alpha_2\beta_1$	40,000	Ubiquitous tissue protein (i.e., present in all tissues), serum levels elevated in disease
PP8	—	α_2	45,000[a] 55,000[b]	Ubiquitous tissue protein (i.e., present in all tissues); serum levels elevated in disease
PP9	—	β_1	35,000[a] 40,000[b]	Ubiquitous tissue protein (i.e., present in all tissues)
PP10	—	α_1	48,000[a] 65,000[b]	Placenta-specific protein
PP11	—	α_1	44,300[a] 62,000[b]	Placenta-specific protein
PP12	Chorionic α_1-microglobulin (CAG-1)	α_1	25,200[a] 51,000[b]	Placenta-specific protein
PP14	Chorionic α_2-microglobulin (CAG-2)	$\alpha_2\alpha_1$	43,000[a] 42,000[b]	Solitary tissue protein[c]
PP15		Albumin	30,700[a]	—
PP16		Albumin	46,000[b]	Solitary tissue protein[c]

[a] Determined by ultracentrifugation.
[b] Determined by SDS-PAGE.
[c] Occurs only in certain other tissues.

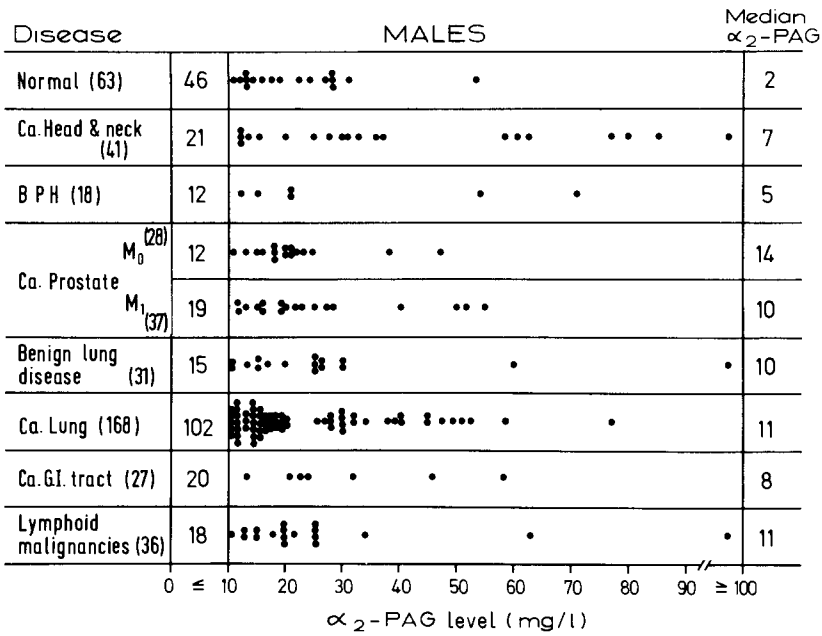

Fig. 2. α_2-PAG levels in various diseases in males. Numbers in parentheses are the number of patients; all patients with prostate cancer were treated by orchiectomy. Müller *et al.* (1982).

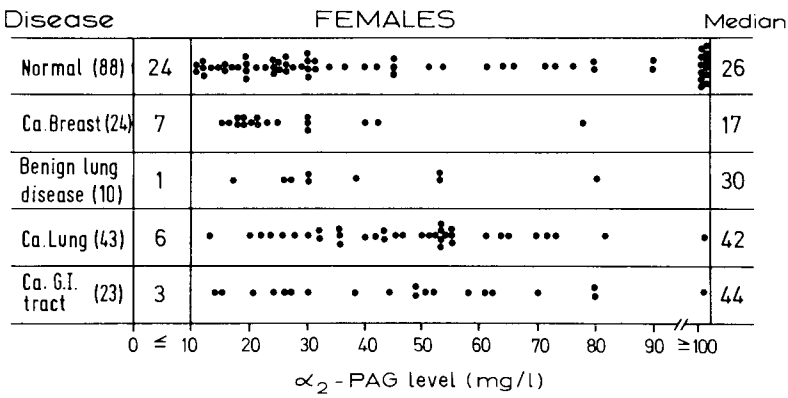

Fig. 3. α_2-PAG levels in various diseases in females. Numbers in parentheses are the number of patients. Müller *et al.* (1982).

With the development of more-sensitive assays for these proteins, it seems hopeful that more of them will be of use as markers of malignancy in the future.

III. TUMOR-ASSOCIATED CHANGES

A. Enzymes

1. Glycosyltransferases and Sialic Acid

The glycosyltransferases are members of a family of enzymes catalyzing the sequential addition of monosaccharides during glycoprotein synthesis. They are named according to the monosaccharide involved; Galactose, for example, is added by galactosyltransferases.

A major feature in the mechanism of cell proliferation in neoplasia is the loss of contact inhibition in neoplastic cells because of an alteration in the properties of the cell surface. Cell-surface glycoproteins are important in cell–cell interactions: They are mainly responsible for the negative cell-surface charge of most cells and generally have sialic acid as a terminal residue. Lectins—for example, Concanavalin A (Con A), which binds to glycoproteins—have been used in the study of the glycosylation of cell-surface proteins. In chronic lymphocytic leukemia (CLL), the lymphocytes are characterized by a specific pattern of altered cell-surface glycoproteins as detected by such studies (Speckart *et al.*, 1978). The authors speculate that the alterations may be related to decreased glycosyltransferase activities. However, studies of galactosyltransferase activity (Chatterjee and Kim, 1977) and fucosyltransferase activity (Chatterjee and Kim, 1978) in rat mammary carcinoma showed higher levels in metastasizing tumor tissue than nonmetastasizing and larger numbers of incomplete glycopeptides on the cell surface. It was suggested that the alteration of cell-surface properties associated with metastatic growth may be the result of a high rate of shedding of cell-surface glycoproteins into the circulation, requiring a greater turnover of glycoproteins. This would result in an increased requirement for glycosyltransferases by the cells, which would themselves be released into the circulation in greater quantities. [The release of galactosyltransferase into the cell-culture media of transformed cell lines has been demonstrated (Klohs *et al.*, 1981)]. Indeed, cell surface shedding is now known to be an important feature of malignant transformation (Black, 1980). It has therefore been suggested that serum levels of glycosyltransferase may be important as monitors of malignancy, and several methods have been used for their detection. These largely involve the measurement of the rate of transfer of the ra-

diolabeled monosaccharide from a nucleotide donor to a suitable glycoprotein acceptor. The methods are intricate and unsuitable for use in other than a research laboratory. As we shall see, the choice of glycoprotein acceptor in these assays is very important: Fetuin is most commonly used. Serum itself contains incompletely glycosylated proteins, possibly more so during an acute phase response, where there is hypersynthesis of glycosylated proteins such as acid glycoprotein and antichymotrypsin (Raynes, 1982), and these may accept the labeled sugars, reducing the binding to the acceptor in the assay and resulting in a falsely low level.

The biological and clinical implications of the use of glycosyltransferases as indicators of cancer have been reviewed (Weiser and Wilson, 1981).

The three major glycosyltransferases and sialic acid will be considered separately.

a. Sialyltransferase. Because sialic acid is the most ubiquitous terminal monosaccharide, sialyltransferases are important in the posttranslational modification of proteins.

In a study of 256 patients, Ronquist *et al.* (1980) did not find any general difference in serum sialyltransferase activity between benign and malignant diseases. Malignant diseases could have increased activity (e.g., colorectal cancer), decreased activity (e.g., gastric and pancreatitic cancer) or no difference (e.g., breast cancer) compared to the benign diseases. Another study of 50 breast cancer patients (Lee *et al.*, 1980), however, showed 50% of patients with disseminated breast cancer and normal CEA had elevated sialyltransferase levels. It may be of value in advanced breast tumors that do not produce CEA. In a more recent study (Berge *et al.*, 1982) only those patients with metastatic tumors had significantly elevated sialyltransferase levels. The levels are compared to some benign diseases in Table VIII. It can be seen that cystic fibrosis, rheumatoid arthritis, and acute hepatitis also have raised levels.

In combination with 5′-nucleotidase, the enzyme levels were found to be useful for monitoring disease activity and the success or failure of treatment in breast cancer (Dao *et al.*, 1980). In women with primary tumors who had elevated enzyme levels, these fell to within the normal range after mastectomy, unless there was nodal involvement. Again, in women undergoing palliative therapy for metastatic disease, those *who had elevated enzyme levels* showed a fall of levels if they were responding to treatment.

An improved assay method, using Con A sepharose as the receptor for radiolabeled acceptor molecules, showed a higher diagnostic sensitivity in

TABLE VIII

Level of Sialyltransferase in Normal and Pathologic Sera[a]

Group	Age (yrs)	Number	Sialyltransferase levels (U/ml)		
			Median	Arithmetic mean (±SE)	Range
Adult control	>20	39	98	97 ± 15	73–127
Children control	<2	3	70	69 ± 8	60–76
	>2 <6	4	81	80 ± 16	59–98
	6–15	15	99	100 ± 19	60–135
Tumor patients					
Subgroup I[b]	>20	38	91	93 ± 19	64–154
Subgroup II[c]	>20	39	94	103 ± 32	60–187
Subgroup III[d]	>20	30	108.5	111 ± 28	57–155
Children with malignant disease	6–15	9	89	92 ± 36	42–164
Cystic fibrosis	6.15	17	121	119 ± 29	74–164
Rheumatoid arthritis	>20	22	111.5	112 ± 22	82–173
Acute hepatitis	>20	10	111.5	108 ± 17	67–124

[a] From Berge *et al.* (1982).
[b] Low activity of disease.
[c] Localized tumor.
[d] Metastatic tumor.

colorectal cancer than other methods, that involve, for example, acid precipitation of the acceptors (Cervén *et al.*, 1981).

Although levels of sialyltransferase have not generally seemed too promising as valuable markers in cancer, its isoenzymes may prove more interesting. Work by Kessel *et al.* (1981) has paved the way for the study of isoenzymes by isoelectric focusing. Sialyltransferases focusing at pH 5.2 and 5.6 were always present in plasma. In an inflammatory response, an isoenzyme of pI = 7.5 was seen, and in untreated primary or recurrent breast cancer, one of pI = 4.7 was demonstrated. The preparation of plasma samples for this type of work, however, is critical: Fresh plasma (rather than serum) or plasma stored for short times at −70°C must be used. Altered focusing patterns may otherwise be seen.

b. Galactosyl Transferase. Serum galactosyl transferase activity was shown to be higher in the serum of cancer patients than age-matched controls in a recent study (see Table IX; Capel *et al.*, 1982). Highest levels

TABLE IX

Serum Galactosyl Transferase Activity in Controls and in Patients with Various Types of Cancer[a]

Group	Number of subjects	Galactosyl transferase activity[b]	Significance ($p<$)[c]
Controls	55	50.5 ± 2.8	—
Prostatic	48	62.3 ± 6.7	n.s.[d]
Breast	126	70.8 ± 5.3	.001
Ovarian	21	68.9 ± 8.5	.05
Respiratory tract	48	70.9 ± 7.1	.01
Gastrointestinal tract	56	66.0 ± 6.3	.02

[a] From Capel *et al.* (1982).

[b] Expressed as nmol/hr/ml serum; values represent mean ± SE.

[c] Significance of difference between means of control subjects and patients with differing cancer type.

[d] n.s., Not significant ($p > .05$).

were seen in breast and respiratory cancer, but no elevation was seen in prostatic cancer patients. The authors noted that other studies have found little or no elevation of enzyme activity in cancer sera, but that this could be explained by the difference in levels of tumor-produced glycoprotein acceptors, including cancer-associated galactosyl transferase acceptor (CAGA; Podolsky and Isselbacher, 1980), which are secreted into serum by disseminated tumors. These compete with the acceptors used in the assay for galactosyl transferase. The choice of acceptor used for the nucleotide-linked monosaccharide transferred by the enzyme, usually UDP-galactose, is therefore crucial. Fetuin was used in Capel's study. A study using *N*-acetylglucosamine as acceptor (Paone *et al.*, 1980) showed significant elevations of enzyme activity in 14.3% (3 out of 21) stage-I, 66.7% (8 out of 12) stage-II, 78.6% (11 out of 14) stage-III, and 96.5% (28 out of 29) stage-IV breast cancer patients. It therefore correlated with the stage of the disease in breast cancer and may be useful in the detection of recurrence. The same group went on to show that galactosyl transferase was more sensitive than CEA as a marker for breast cancer (Paone *et al.*, 1981).

In 1975 a cancer-associated isoenzyme of galactosyl transferase was detected by discontinuous polyacrylamide electrophoresis (PAGE) in sera of cancer patients (Podolsky and Weiser, 1975). This was named galactosyl transferase isoenzyme (GT-II). Podolsky extended the initial results in 1978, confirming its presence in a wide range of cancer sera (Podolsky *et al.*, 1978) and found that it was more sensitive than a range of

other tumor markers in discriminating benign and malignant pancreatic disease (Podolsky *et al.*, 1981).

c. Fucosyl Transferase. Elevated activities of two different fucosyl transferases (depending on the acceptor to which the fucose is transferred) have been seen in cancer sera (Bauer *et al.*, 1977). The choice of acceptor distinguished infectious hepatitis from cancer. In a study involving patients with colon and breast cancers, Bauer *et al.* (1978) concluded that fucosyl transferase activity can facilitate the diagnosis of neoplasia and the success of treatment.

Recently, plasma fucosyl transferase has been used as an indicator of imminent blastic crisis in chronic granulocytic leukemia (Shah-Reddy *et al.*, 1982). Enzyme activity in plasma of patients in blast-cell crisis was 1630 ± 570 units, 509 ± 110 units in the stable phase, and 354 ± 57 in normal controls.

d. Sialic Acid. It is appropriate here to mention some recent work concentrating on sialic acid (*N*-acetylneuraminic acid or NANA).

i. TOTAL SIALIC ACID

Elevated serum sialic acid levels have previously been seen in malignant disease (Silver *et al.*, 1978; Lipton *et al.*, 1979). A recent study suggests that it is a better marker in bronchial carcinoma than other neoplasms, although raised levels of sialic acid are still seen (Prignitz *et al.*, 1981). Silver *et al.* (1981a) have developed a rapid HPLC method for its quantitation that is more sensitive than the more conventional thiobarbituric acid method (TBA). They also found it to be a better discriminant between tumor burden groups in breast cancer than the TBA method or CEA determination. It was also found by these workers to be useful as a monitor in ovarian carcinoma (Silver *et al.*, 1981b). In general, sialic acid levels were found to be useful in monitoring the course of neoplastic disease but not helpful in diagnosis (Cunietti *et al.*, 1981).

ii. PLATELET SIALIC ACID

Martin *et al.* (1982) noted that platelet sialic acid may be important as a marker of malignancy because pathological thrombosis and hemostasis are well recognized features of malignant disease, and sialic acid on platelet membranes is involved in cell–cell contact between platelets. This group found a mean platelet sialic acid of 24% below normal and a serum sialic acid 68% above normal in malignant disease. The authors see this as an area for further investigation.

iii. LIPID-BOUND SIALIC ACID

A straightforward method has recently been devised (Katopodis and Stock, 1980) for the determination of serum or plasma lipid-bound sialic acid (LBSA). This could prove to be a better indicator of malignancy than total sialic acid (TSA). For example, Horgan (1982) studied levels in 40 patients with various malignancies, 45 normal patients, and 15 patients other than cancer patients. Results are shown in Table X. The difference between cancer and noncancer is more obvious using LBSA.

Another study of LBSA in cancer patients showed raised values in 12 out of 20 patients with breast cancer, 12 out of 18 with colon cancer, 13 out of 18 with lung cancer, 19 out of 21 with leukemia, 14 out of 18 with lymphoma, 17 out of 18 with Hodgkin's disease, and 10 out of 12 with melanoma (Dnistrian and Schwartz, 1981). A more recent study showed that LBSA was of limited value in the detection of breast cancer, but serial measurements could aid the assessment of progression (Dnistrian *et al.*, 1982).

Further work may prove LBSA to be a valuable marker of neoplasia, not least because the assay is inexpensive and requires no sophisticated equipment and is therefore available to laboratories anywhere in the world. At this stage, however, the interpretation and usefulness of the test has yet to be clarified.

2. *Prostatic Acid Phosphatase*

The enzymatic measurement of serum acid phosphatase is a well established routine test in clinical chemistry, introduced some 40 years ago. Many laboratories employ substrates such as 4-nitrophenyl phosphate, thymolphthalein monophosphate, or 1-naphthyl phosphate that have an increased specificity for prostatic acid phosphatase; they also use L-(+)-tartrate in association with less specific substrates. The characteristics of the various substrates have been reviewed (Bowers, 1982).

TABLE X

Total Sialic Acid and Lipid-Bound Sialic Acid Levels in Cancer Patients: Benign Diseases and Normal Controls[a]

Group	TSA (nmol/liter)	LBSA (μmol/liter)
Malignant	4.00 ± 1.5	88.7 ± 52.7
Noncancer diseases	2.88 ± 0.6	22.3 ± 9.2
Normal controls	1.94 ± 0.2	28.6 ± 5.9

[a] From Horgan (1982).

The development of a radioimmunoassay for prostatic acid phosphatase (PAP) by Cooper and Foti (1974), using PAP isolated from prostatic fluid as the antigen, introduced a new chapter in the study of this enzyme in prostatic cancer. Foti's early results (Foti *et al.*, 1977) suggested that a raised PAP was present in 43% of 44 patients with stage I and II carcinoma of the prostate and 94% of the 65 patients with stage III and IV tumors; coincidental enzymatic assays were positive only in 9 and 46% of these two groups of patients, respectively. Double antibody radioimmunoassays were also developed by Choe *et al.* (1978), Belleville *et al.* (1978) and Vihko *et al.* (1978).

An alternate approach has been the counter immunoelectrophoresis (CIEP) for prostatic acid phosphatase and its antisera to migrate in opposite directions in an electric field so that they meet and form a precipitation line. The PAP in the precipitate is revealed by an appropriate enzyme-staining technique. The method is rapid and simple. This study has been evaluated in a multicenter trial (Wajsman *et al.*, 1979). However, neither CIEP nor any other radioimmunoassay or enzyme-linked immunosorbent assay have been able to repeat Foti's results of such high positivity for the detection of cancer localized to the prostate.

The effect of immunological assays has been considerable because several commercial kits have become available and have been used in many laboratories.

The role of PAP in diagnosis of prostatic cancer is unclear, especially in early disease. Cooper and Finkle (1980) screened 6320 men over 45 years old attending general outpatient clinics: 444 had a raised PAP, and 62 of these 444 had carcinoma of the prostate; 67% of the men with raised values had benign prostatic hypertrophy (BPH). Several authors report that BPH can be accompanied by moderate rise of PAP, and this can be higher if there is acute retention or microinfarction of the prostate (Bruce and Mahan, 1982; Cooper *et al.*, 1981, 1982). The present position of serum PAP assays in detecting cancer localized within the prostate (stages A and B) have been summarized by Bruce and Mahan (1982) and is shown in Table XI. The table also contrasts their data with the higher incidence of positivity found in disease outside the capsule of the prostate (stage C) and metastatic cancer (stage D). Our experience of the Behringwerke Enzygnost PAP commercial assay kit in untreated patients showed raised levels (>1.8 ng/ml) in 30% of disease localized in the prostate, 48% in nonmetastic disease extending outside the prostate, and 91% in metastatic disease (Cooper *et al.*, 1982). However, the variations of positivity according to the different immunological assays are still wide enough to cause hesitation by urologists to turn to this type of assay as a routine.

TABLE XI

Elevations of Serum PAP (%)[a]

Stage	Murphy *et al.* (1979)	Griffiths (1980)	Foti *et al.* (1977)	Lee *et al.* (1980)	Bruce and Mahan (1982)
A	38	12	33	73	13
B	35	32	79	56	21
C	49	47	71	82	37
D	69	86	92	86	74

[a] From Bruce and Mahan (1982).

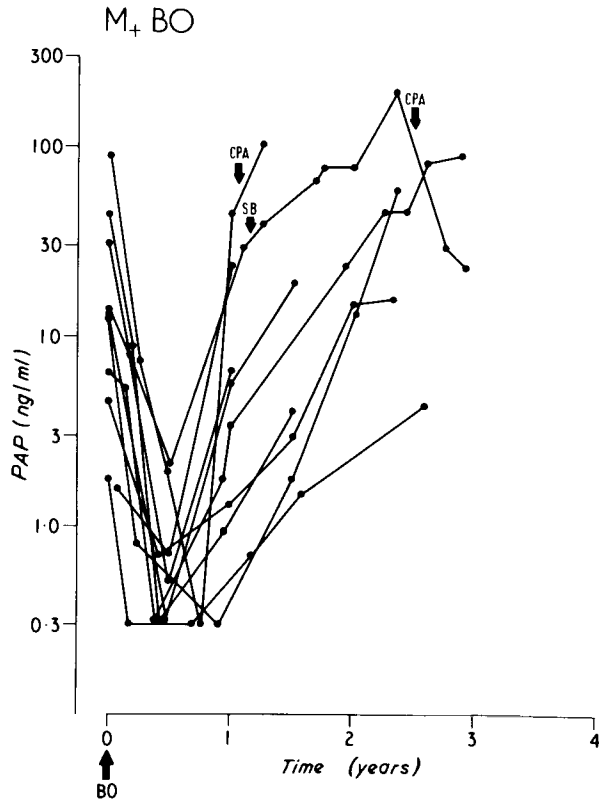

Fig. 4. The change in sPAP levels following orchidectomy (M_+ = metastatic disease, D). Eleven of 34 patients showed a similar response. SB = stilboestrol; CPA = cyproterone acetate; BO = bilateral orchidectomy. Cooper *et al.* (1983).

Even though PAP assays cannot provide a reliable test for the diagnosis of prostatic cancer, it is of interest to see how they perform as a method for patient monitoring. It is generally agreed that PAP is more sensitive than routine enzymological tests, and slow exponential rises of PAP can be appreciated several months ahead of routine enzymes (Killian *et al.*, 1982; Cooper *et al.*, 1983). Figures 4 and 5 show typical rates of change of this assay after orchidectomy in metastatic disease. Whether the increased sensitivity and early warning of a rising PAP in an asymptomatic patient can lead to more effective measurement of the disease has not been demonstrated. As often as not, it occurs months or years ahead of any further symptoms. One possible advantage of an immunological PAP test is that it is very useful for stratification in multicenter trials, so that the vagaries of local variations in methods for PAP determination can be avoided by using a uniform technique.

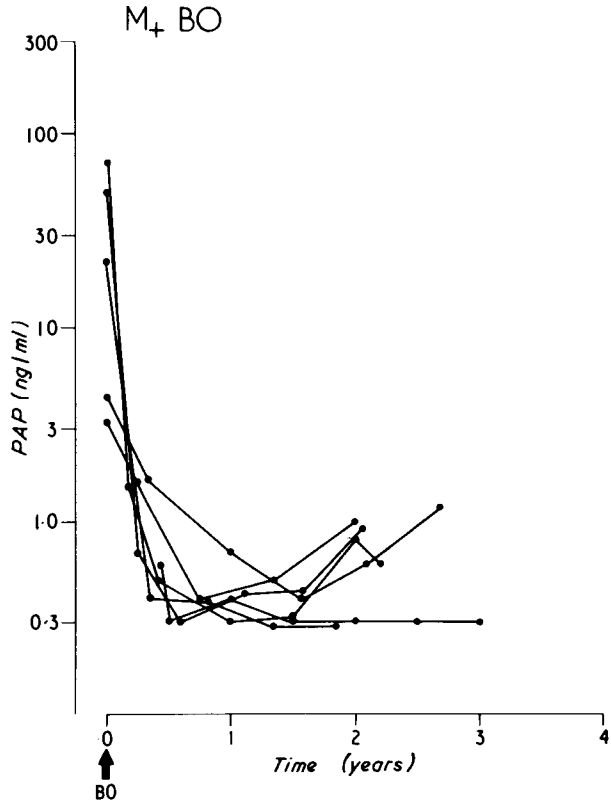

Fig. 5. The change in sPAP levels after treatment by estrogens (M_+ = metastatic disease, D). This pattern was shown by 12 of 29 patients. BO = bilateral orchidectomy. Cooper *et al.* (1983).

3. Phosphohexose Isomerase

Early work by Bodansky (1954) set the scene for the use of this enzyme of the glycolytic pathway (whose modern name is glucose phosphate isomerase) as an indicator of tumor mass and activity. Cancer cells, actively dividing, require more energy than normal cells, and so glycolysis occurs at a faster rate.

Phosphohexose isomerase (PHI) is of little use in cancer detection because only extensive tumors will release enough to raise the level above the normal range. Its sequential measurement in metastatic cancer, however, is valuable as an index of the effectiveness of therapy. This has been demonstrated in breast cancer by Bodansky (1954), Griffith and Beck (1963), and Maity and Burma (1973), who also showed that PHI was more useful than lactate dehydrogenase (LDH) as a monitor of treatment. Lactate dehydrogenase catalyzes the last step of the glycolytic pathway, so it would be expected to reflect tumor activity in the same way as does PHI. But it is a much more unstable enzyme than PHI, which is therefore more suitable for laboratories where samples must be stored before assay. In patients with gastrointestinal and breast cancer, raised and fluctuating PHI levels were indicative of advanced cancer under poor control (Bowen *et al.*, 1981). Munjal *et al.* (1976) found that PHI, γ-glutamyl transpeptidase and LDH were a useful adjunct to CEA in the detection of liver metastases, the enzymes being particularly important in patients not producing CEA.

It has been known for a long time that PHI is useful in monitoring advanced cancer. The relative stability and high activity of the enzyme simplify assay procedures. It certainly warrants consideration as an alternative to LDH, the assay of which is widespread in routine hospital biochemistry laboratories.

B. Acute Phase-Reactant Proteins

Acute phase-reactant proteins (APRPs) mainly are glycoproteins produced in the liver that increase their levels in the blood as part of the systemic response to a variety of forms of tissue injuries, including wounding, burns, tissue infarction, acute and chronic inflammation, and cancer. The general properties of this reaction have been reviewed extensively (see Owen, 1967; Koj, 1974; Fisher and Gill, 1975; Cooper and Stone, 1979; Pepys, 1981; Kushner, 1982). Despite the lack of specificity of this response, experience has shown that the levels of certain acute phase reactants, notably C-reactive protein (C-RP), α_1-acid glycoprotein (AGP), and α_1-antichymotrypsin (ACT) can reflect events in cancer-bearing patients that are of value in the assessment of prognosis and the

monitoring of the evolution of the tumor and its complications. The general properties of these three proteins and serum amyloid A protein (SAA), a more recently discovered acute phase reactant (Benditt *et al.*, 1982; Benson, 1982), are shown in Table XII.

The other members of the APRP family of proteins, α_1-antitrypsin (AT), haptoglobin (Hp), ceruloplasmin, and fibrinogen generally tend to follow the pattern of change of C-RP, ACT, and SAA. But they react at a slower rate, and AT and Hp have the disadvantage of genetic variability that affects the interpretation of their blood levels. Similarly, it can be shown there is a general tendency for the acute phase response and the erythrocyte sedimentation rate (ESR) to rise together. But the ESR is prone to be influenced by many extraneous factors such as anemia and immunoglobulin concentration, which reduce its usefulness in cancer; it also lacks the speed of response of C-RP and SAA to an inflammatory stimulus. C-reactive protein and SAA in addition return to normal very soon after the stimulation is withdrawn.

The levels of acute phase reactants, notably, C-RP, ACT, and SAA, both of which closely correlate with C-RP, can contribute to the assessment of prognosis in several types of cancer. Raised levels of APRPs prior to treatment have been shown to be significantly correlated with a shorter survival time in metastatic prostatic cancer (Seal *et al.*, 1978), in invasive bladder cancer (O'Quigley *et al.*, 1980), and advanced lung cancer (Bradwell *et al.*, 1979) and can be an important factor in a multivariate approach to tumor assessment and monitoring (Coombes *et al.*, 1980; Rashid *et al.*, 1982; de Mello *et al.*, 1983). Certain tumors such as kidney carcinoma show a relationship between the tumor stage and the APRP

TABLE XII

Acute Phase Reactants Valuable for Monitoring and Assessing Cancer Patients

Protein	MW	Normal serum levels	Maximum response in cancer	Glycosylation
C-reactive protein	105,500	<5 mg/l	100–300 mg/l	Nil
α_1-acid glycoprotein (orosomucoid)	44,000	0.6–1.4 g/l	2.0–4.0 g/l	++[b]
α_1-antichymotrypsin	69,000	0.3–0.6 g/l	1.0–2.0 g/l	++
Serum amyloid A protein[a]	12,000–14,000	10–60 mg/l	3–5 g/l	Nil

[a] Bound to high density lipoprotein, effective molecular weight approximately 100,000–200,000 Note the very large increases of C-RP and SAA compared to the proportional increases of the other proteins.

[b] ++ = glycosylated protein.

reaction that can be helpful in the preoperative assessment of the likelihood of spread (Richards *et al.*, 1982). Metastatic tumors in the liver and hepatomas are usually accompanied by a rise in the levels of AGP, which tends to be <1.0 g/liter in most forms of chronic benign liver disease and in combination with alkaline phosphatase makes a most useful test for the evaluation of hepatomegaly in the elderly (Rashid *et al.*, 1981).

Acute phase-reactant proteins have been used in monitoring several types of cancer such as prostate (Trautner *et al.*, 1980), colon (Cooper and Turner, 1980), cervix (Te Velde *et al.*, 1979), and ovary (Meerwaldt *et al.*, 1983). In advanced cancer, rising levels of APRPs tend to reflect the increase of tumor burden; a slowly rising level of APRPs tends to be the prelude to the terminal phase of a malignant disease, as shown in Table XIII. The measurement of APRPs can provide a rapid and cheap addition to the routine assessment of a cancer-bearing patient. Patients with normal levels of APRPs in cancer usually will be at a relative advantage when compared to patients with raised levels for a similar tumor burden. In our experience measurement of C-RP in parallel with new substances under investigation has often led to a better appreciation of the potential contribution of the new substance. C-reactive protein levels in lymphoma and leukemia are often raised in active disease, especially in Hodgkin's disease, with general symptoms (Pepys, 1981); a rise of C-RP >100 mg/liter in acute leukemia is also strongly suggestive of septicemia.

C. β_2-Microglobulin

Free β_2-microglobulin (β_2-m) is present in all body fluids. It originates from nucleated cells, where it forms the light chain of the histocompatibility surface antigens (Berggard *et al.*, 1980). β_2-Microglobulin is a low molecular weight protein (11,300), and in a fashion similar to other low molecular weight proteins in the blood, its clearance from the body is by glomerular filtration. The β_2-m in the glomerular filtrate is normally reabsorbed by the proximal tubule cells and catabolized. This extraction is

TABLE XIII

Serum C-Reactive Protein in Advanced Colorectal Cancer Treated with Chemotherapy[a]

Time	Number	<10 mg/l (normal)	11–50 mg/l	>50 mg/l
6–12 months before death	26	18(69%)	3(16%)	3(16%)
3–5 months before death	50	19(38%)	20(40%)	11(22%)
Within 3 months of death	55	6(10.7%)	14(25.4%)	35(63.6%)

[a] From Cooper and Turner (1980).

about 99% efficient, so that of the 150 mg of β_2-m produced per 24 hr only about 0.3 mg will appear in the urine. The serum levels of β_2-m are 0.6–2.4 mg/liter in young adults, tending to rise to an upper limit of about 3 mg/liter in the elderly. In the absence of a source of hyperproduction of β_2-m, there is a close relationship between the serum β_2-m level and the glomerular filtration rate (GFR) as measured formally (Wibell *et al.*, 1973) or reflected by the serum creatinine level. In diseases in which there may be a coincidental reduction of GFR and hyperproduction of β_2-m, the rise of β_2-m will be disproportionately high for a given level of GFR. The extent of this departure from the accepted β_2-m level can be measured using a formula devised by Cassuto *et al.* (1978).

Initial reports suggested that levels of serum β_2-m are elevated in a wide variety of neoplastic diseases, and it may be useful as a cancer marker (Shuster *et al.*, 1976; Kin *et al.*, 1977; Teasdale *et al.*, 1977). However, detailed assessment of β_2-m levels, with careful attention to tumor stage, the change of levels with tumor progression, and assessment of β_2-m levels as a prognostic factor, failed to confirm its usefulness in common solid tumors. Detailed studies on β_2-m in gastrointestinal cancer have been reported by Rashid *et al.* (1981) and Staab *et al.* (1981) and in lung cancer by Hällgren *et al.* (1980). In breast cancer Papaioannou *et al.* (1979) felt that β_2-m levels corrected for creatinine levels were a useful index of metabolic cancer in about one half of the patients with the disease. Adami *et al.* (1979) found no difference in β_2-m levels between women with breast cancer and age-matched controls, all of whom concluded that β_2-m levels cannot be used as a tumor marker in these common types of solid tumors. Furthermore, chronic liver disease, especially alcoholic cirrhosis, chronic active hepatitis, and primary biliary cirrhosis, tend to be accompanied by a raised serum β_2-m level (Rashid *et al.*, 1981; Berochia *et al.*, 1981).

Despite this lack of specificity there is growing evidence that the level of serum β_2-m is a powerful index of prognosis in myelomatosis (Norfolk *et al.*, 1980; Bataille *et al.*, 1982; Child *et al.*, 1983). Furthermore, this measurement can still add information when the clinical, hematological, and renal factors as incorporated into the staging system of Durie and Salmon (1975) are taken into account (Child *et al.*, 1983). Cassuto *et al.* (1978) have proposed a polynominal equation to allow the ratio between the observed β_2-m level and the theoretical level calculated according to the level of serum creatinine to be estimated. Using this equation an estimate of the overproduction of β_2-m can be made. In our experience, this ratio tends to be 0.5–5.0 in myelomatosis. This equation is as follows:

$$\log \beta_2\text{-m}(\mu\text{g/ml}) = 3.934 - 5.96Y + 2.94Y^2 - 0.476Y^3 + 0.0252Y^4$$

where Y = creatinine in μg/ml. Using this equation, Bataille *et al.* (1982) found the following measured-(M)-to-calculated (CTD) ratios (mean ± SD): normal individuals n = 37, 1.03 ± 0.31; benign monoclonal gammapathies n = 8, 0.94 ± 0.19; untreated myelomatosis n = 54, 2.05 ± 1.22; and untreated low mass myelomatosis n = 9, 1.10 ± 0.26.

Acute lymphoblastic leukemia in children and adults and blast-cell transformation in chronic granulocytic leukemia tend to have normal or only slightly raised β_2-m levels. Very high levels are found in chronic and acute myelomonocytic leukemia, and in chronic lymphocytic leukemia β_2-m levels are related to the stage of the disease. The rate of change in the blood levels reflects the increase or decrease of tumor burden (Späti *et al.*, 1980; Simonsson *et al.*, 1980). In lymphomas, raised levels are found in advanced Hodgkin's disease and in non-Hodgkin's lymphoma, especially in patients with unfavorable histology, where the level may have prognostic significance (Amlot and Adinolfi, 1979; Cooper and Child, 1981), although this still awaits confirmation. Studies in infectious mononucleosis and mixtures of B and T lymphocytes *in vitro* now strongly indicate that T-cell activation is a major source of a raised serum β_2-m *in vivo* (Lamelin *et al.*, 1982).

In summary, the changes of serum β_2-m level in most solid tumors are variable and may be a reflection of alterations of the activity of lymphoid [especially T cells] and reticuloendothelial systems rather than derived from the cancer per se. So far these changes are unrelated to any particular pattern of clinical evolution of the tumor. Some investigators incorporate β_2-m in a battery of tests for cancer and in established cancer patients; but this logic is unclear.

At present it is in lymphoid malignancies and, especially, myelomatosis that β_2-m has emerged as a powerful marker. It will probably be used in the assessment and stratification of these diseases.

D. Ferritin

The determination of serum ferritin levels is now widespread because of the development of many methods and the wide availability of assay kits. Ferritin is now well known to reflect body iron stores (Jacobs, 1977; Craswell *et al.*, 1978), increased-marrow iron stores being associated with high serum ferritin. High values are often found in chronic inflammatory states and acute infections (Jacobs and Worwood, 1975; Birgegård *et al.*, 1978). In addition, elevated serum ferritin levels are a good indicator of liver disease; the circulating ferritin is dependent on both the degree of hepatocellular injury and liver iron stores and is largely derived from damaged hepatocytes (Prieto *et al.*, 1975).

Several studies now indicate the use of ferritin as a monitor of malignancy. Elevated serum ferritin has been reported in acute leukemia (Parry *et al.*, 1975) and Hodgkin's disease (Jacobs *et al.*, 1976), and Tisman *et al.* (1977) demonstrated its use as a monitor of chemotherapy in tumors of the colon, lung, pancreas, and in patients with melanoma, myeloma, and testicular carcinoma. In a recent study of 306 patients with various malignancies (71 Hodgkin's disease, 146 non-Hodgkin's lymphomas, 10 myelomas, and 79 patients with miscellaneous solid tumors) 60% had serum ferritin levels above 200 μg/liter (the upper limit of normal) and 20% over 1000 μg/liter. Ferritin increased with increasing stage in Hodgkin's disease. The ferritin levels were also a good indicator of iron stores in these patients (Jacobsen *et al.*, 1982).

Serum ferritin has thus shown potential as a monitor of malignancy, but it is probable that most assays, which are at present largely directed against normal human liver or spleen ferritin, are underestimating levels in patients with ferritin-producing tumors. Different "isoferritins," observed by isoelectric focusing, are produced by different tissues. Tumor-specific isoferritins also have been demonstrated that are generally more acidic than the "normal" ferritins produced by the liver or spleen (Halliday *et al.*, 1976; Bullock *et al.*, 1980). Although these tumor-type ferritins have been found in some normal tissues (mainly in the heart, kidney, and placenta) the current development of antisera to, for example, isoferritins isolated from Hela cells (Albertini *et al.*, 1981) may mean the future use of ferritin as a more specific marker of neoplasia.

REFERENCES

Abelev, G. I., Perova, S., Khramkoba, P. N., Postnikova, Z. A., and Irlin, I. S. (1963). *Transplantation* **1**, 174–180.

Adami, H. O., Hallgren, R., and Lundquist, G. (1979). *Clin. Chim. Acta* **93**, 43–50.

Adinolfi, M. (1979). *In* "Advances in Human Genetics" (H. Harris and K. Hirschhorn, eds.), Vol. 9, pp. 165–212. Plenum, New York.

Albertini, A., Lacobello, C., Ghielni, S., and Arosio, P. (1981). *J. Clin. Chem. Clin. Biochem.* **19**, 598.

Alexander, J. C., Chretien, P. B., Dellon, A. L., and Snyder, J. (1978). *Cancer (Philadelphia)* **42**, 1492–1497.

Alpert, E., and Feller, E. R. (1978). *Gastroenterology* **74**, 856–858.

Al-Sarraf, M., Baker, L., Talley, R. W., Kithier, K., and Vaitkevicius, V. K. (1979). *Cancer (Philadelphia)* **44**, 1222–1225.

Amlot, P. L., and Adinolfi, M. (1979). *Eur. J. Cancer* **15**, 791–796.

Anderson, D. C. (1974). *Clin. Endocrinol.* **3**, 69–96.

Anderson, T., Waldmann, T. A., Javadpour, N., and Glatstein, E. (1979). *Ann. Intern. Med.* **90**, 373–385.

Andrén-Sandberg, A., and Isaacson, S. (1978). *In* "Clinical Application of Carcinoembry-

onic Antigen Assay" (B. P. Krebs, C. M. Lalanne, and M. Schneider, eds.), pp. 139–143. Excerpta Med., Amsterdam.
Arnaud, J. P., Koehl, C., and Adloff, M. (1980). *Dis. Colon Rectum* **23,** 141–144.
Asseo, P. P., and Tracopoulos, G. D. (1982). *Am. J. Clin. Pathol.* **77,** 66–71.
Astler, V. B., and Coller, F. A. (1954). *Ann. Surg.* **139,** 846–852.
Attiyeh, F. F., and Stearns, M. W. (1981). *Cancer (Philadelphia)* **47,** 2119–2125.
Bagshawe, K. D. (1969). "Choriocarcinoma: The Clinical Biology of the Trophoblast and Its Tumors." Arnold, London.
Bagshawe, K. D. (1976). *Cancer (Philadelphia)* **38,** 1373–1385.
Bakowski, M. T., Toseland, P. A., Wicks, J. F., and Trounce, J. R. (1981). *Clin. Chim. Acta* **110,** 273–286.
Barzell, W. E., and Whitmore, W. F., Jr. (1979). *Semin. Oncol.* **6,** 48–52.
Bataille, R., Magub, M., Grenier, J., Donnadios, D., and Sany, J. (1982). *Eur. J. Cancer Clin. Oncol.* **18,** 59–66.
Bauer, C., Köttgen, E., and Reutter, W. (1977). *Biochem. Biophys. Res. Commun.* **76,** 488–493.
Bauer, C. H., Reutter, W. G., Erhart, K. P., Köttgen, E., and Gorek, W. (1978). *Science (Washington, D. C.)* **201,** 1232–1233.
Baylin, S. B., Weisburga, W. R., Eggleston, J. C., Mendelsohn, G., Beaven, M. A., Abeloff, M. D., and Ettniger, D. S. (1978). *N. Engl. J. Med.* **229,** 105–110.
Baylin, S. B., Mendelsohn, G., and Levine, M. A. (1979). *Johns Hopkins Med. J.* **145,** 201–206.
Beart, R. W., Metzger, P. P., O'Connel, M. J., and Schutt, A. J. (1981). *Dis. Colon Rectum* **24,** 585–588.
Bell, H. (1982). *Scand. J. Gastroenterol.* **17,** 898–903.
Belleville, W. D., Cox, H. D., Mahan, D. E., Olmert, J. P., Mittenmeyer, B. T., and Bruce, A. W. (1978). *Cancer (Philadelphia)* **41,** 2286–2291.
Benditt, E. P., Hoffman, J. S., Eriksen, N., Parmelee, D. C., and Walsh, K. A. (1982). *Ann. N.Y. Acad. Sci.* **389,** 183–189.
Benson, M. D. (1982). *Ann. N.Y. Acad. Sci.* **389,** 116–120.
Berge, P. G., Wilhelm, A., Schriewer, H., and Wüst, G. (1982). *Klin. Wochenschr.* **60,** 445–449.
Berggard, B., Björck, L., Cigen, R., and Lödberg, L. (1980). *Scand. J. Clin. Lab. Invest.* **40,** (Suppl. 154), 13–25.
Berochia, S., Vincent, C., Revillard, J. P., and Trepo, C. (1981). *Clin. Chim. Acta* **109,** 245–255.
Birgegård, G., Hällgren, R., Killander, A., Strömberg, A., Verge, P., and Wide, L. (1978). *Scand. J. Haematol.* **21,** 333–340.
Björklund, B. (1980). *Tumor Diagnostik* **1,** 9–19.
Black, P. H. (1980). *Adv. Cancer Res.* **32,** 75–199.
Blackman, M. R., Weintraub, B. D., Rosen, S. W., Korides, I. A., Steinwascher, K., and Gail, M. H. (1980). *J. Natl. Cancer Inst. (US)* **65,** 81–93.
Blake, K. E., Dalbow, M. H., Concannon, J. P., Hodgson, S. E., Brodmerkel, G. J., Panahendeh, A. H., Zimmerman, K., and Headings, J. J. (1982). *Dis. Colon Rectum* **25,** 24–32.
Bloomer, J. R., Waldmann, T. A., McIntire, R., and Klatskin, G. (1975). *Gastroenterology* **68,** 342–350.
Bodansky, O. (1954). *Cancer (Philadelphia)* **7,** 1200–1226.
Bodansky, O. (1975). "Biochemistry of Human Cancer." Academic Press, New York.
Bohn, H. (1980). *Klin. Wochenschr.* **58,** 489–492.

Bohn, H. (1982). *Ric. Clin. Lab.* **12,** 221–230.
Bohn, H., and Lüben, G. (1981). *Oncodev. Biol. Med.* **2,** 141–153.
Borek, E. (1980). *In* "Cancer Markers" (S. Sell, ed.), pp. 445–462. Humana Press, Clifton, New Jersey.
Bowen, M., Earnshaw, R. J., Levell, M. J., and Hancock, K. (1980). *J. Steroid Biochem.* **17,** iv.
Bowen, M., de Mello, J., and Cooper, E. H. (1981). *Tumor Diagnostik* **2,** 22–26.
Bowers, G. N., Jr. (1982). *Ann. N.Y. Acad. Sci.* **390,** 89–99.
Bradwell, A. R., Burnett, D., Newman, C. E., and Ford, C. H. J. (1979). *Protides Biol. Fluids Proc. Colloq.* **27,** 327–330.
Braunstein, G. D. (1979). *In* "Immunodiagnosis of Cancer" (R. B. Herberman and K. R. McIntire, eds.), Part I, pp. 383–408. Dekker, New York.
Braunstein, G. D., Vaitukaitis, J. L., Carbone, P. P., and Ross, G. T. (1973). *Ann. Intern. Med.* **78,** 39–45.
Bruce, A. W., and Mahan, D. E. (1982). *Ann. N.Y. Acad. Sci.* **390,** 110–121.
Bullock, S., Bomford, A., and Williams, R. (1980). *Biochem. J.* **185,** 639–645.
Bundschuh, G. (1981). *Dtsch. Gesundheitswes.* **36,** 1193–1199.
Capel, I. D., Dorrell, H. M., Williams, D. C., Hanhann, I. W. F., and Levitt, H. N. (1982). *Oncology* **39,** 193–196.
Cassuto, P. P., Krebs, B. P., Viot, G., Dujardin, P., and Masseyeff, R. (1978). *Lancet* **ii,** 108–109.
Cervén, E., Ronquist, G., Rimsten, A., and Agren, G. (1981). *Upsala J. Med. Sci.* **86,** 237–247.
Chatterjee, S. K., and Kim, U. (1977). *J. Natl. Cancer Inst. (US)* **58,** 273–280.
Chatterjee, S. K., and Kim, U. (1978). *J. Natl. Cancer Inst. (US)* **61,** 151–162.
Chayvialle, J. A. P., and Ganguli, P. C. (1973). *Lancet* **1,** 1355–1357.
Chen, D. S., and Sung, J. L. (1977). *Cancer (Philadelphia)* **40,** 779–783.
Child, J. A., Crawford, J. M., Norfolk, D. R., O'Quigley, J., Scarffe, J. H., and Struthers, L. P. L. (1983). *Br. J. Cancer.* **47,** 111–114.
Choe, B. K., Pontes, E. J., Morrison, K., and Rose, N. R. (1978). *Arch. Androl.* **1,** 227–233.
Chu, M. T. (ed.) (1982). "Biochemical Markers for Cancer." Dekker, New York.
Chu, M. T., and Nemoto, T. (1973). *J. Natl. Cancer. Inst. (US)* **51,** 1119–1122.
Colnaghi, M. I., Buraggi, G. L., and Ghione, M. (eds.) (1982). "Markers for Diagnosis and Monitoring of Human Cancer." Academic Press, New York.
Concannon, J. P., Dalbow, M. H., Hodgson, S. E., Headings, J. J., Markopoulos, E., Mitchell, J., Cushing, W. J., and Liebler, G. A. (1978). *Cancer (Philadelphia)* **42,** 1477–1483.
Coombes, R. C., Greenberg, P. B., Hillyard, C., and MacIntyre, I. (1974). *Lancet* **1,** 1080–1083.
Coombes, R. C., Powles, T. J., Gazet, J. C., Nash, A. G., Ford, H. T., McKinna, A., and Neville, A. M. (1980). *Lancet* **1,** 296–297.
Cooper, E. H., and Child, J. A. (1981). *Tumor Diagnostik* **2,** 167–171.
Cooper, E. H., and Giles, G. R. (1983). *In* "Gastrointestinal Malignancy" (J. J. De Cosse and P. Sherlock, eds.), in press. Nijhoff, The Hague.
Cooper, E. H., and Stone, J. (1979). *Adv. Cancer Res.* **30,** 1–44.
Cooper, E. H., and Turner, R. (1980). *In* "Colorectal Cancer, Prevention Epidemiology and Screening" (S. Winawer, D. Schottenfeld, and P. Sherlock, eds.), pp. 211–218. Raven, New York.
Cooper, E. H., Glashan, R., Robinson, M. R. G., Morgan, D. B., and Trautner, K. (1981). *Clin. Chim. Acta* **113,** 27–34.

Cooper, E. H., Pidcock, N. B., Glashan, R., Smith, P. H., Robinson, M. R. G., Richards, B., and Newling, D. (1982). *Clin. Chem.* **28,** 1729–1730.

Cooper, E. H., Pidcock, N. B., Daponte, D., Glashan, R. W., Rowe, E., and Robinson, M. R. G. (1983). *Eur. J. Urol.* **9,** 17–23.

Cooper, J. F., and Finkle, W. D. (1980). *Prostate* **1,** 441–450.

Cooper, J. F., and Foti, A. G. (1974). *Invest. Urol.* **12,** 98–102.

Coordinating Group for the Research of Liver Cancer (1974). *Chin. Med. J.* **2,** 241–245.

Cowen, D. M., Searle, F., Ward, A. M., Benson, E. A., Smiddy, F. G., Eaves, G., and Cooper, E. H. (1978). *Eur. J. Cancer* **14,** 885–893.

Craswell, P., Hunt, F., Russo, A., Goethart, J., and Halliday, J. (1978). *Aust. N.Z. J. Med.* **8,** 38–42.

Cunietti, E., Palma, R., Gandini, M. C., Viola, P., Gandini, R., and Fasoli, A. (1981). *Tumori* **67,** 198.

Dao, T. L., Ip, C., and Patel, J. (1980). *J. Natl. Cancer Inst. (US)* **65,** 529–534.

Davis, G. E., Suits, K. D., Kuo, K. C., Gehrke, C. W., Waalkes, T. P., and Borek, E. (1977). *Clin. Chem.* **23,** 1427–1435.

de Jong-Bakker, M., Hart, A. A. M., Persijn, J. P., and Cleton, J. F. (1981). *Eur. J. Cancer Clin. Oncol.* **17,** 1307–1313.

de Mello, J., Struthers, L., Turner, R., Cooper, E. H., and Giles, G. R. (1983). *Br. J. Cancer* **48,** 341–348.

Dent, P. B., and McCulloch, P. B. (1979). *In* "Compendium of Assays for Immunodiagnosis of Human Cancer" (R. B. Herberman, ed.), pp. 273–277. Elsevier-North Holland, New York.

Desser, H. (1980). *In* "Polyamines in Biomedical Research" (J. M. Gangas, ed.), pp. 415–433. Wiley, New York.

Dnistrian, A. M., and Schwartz, M. K. (1981). *Clin. Chem.* **27,** 1737–1739.

Dnistrian, A. M., Schwartz, M. K., Katopadis, N., Fracchia, A. A., and Stock, C. C. (1982). *Cancer (Philadelphia)* **50,** 1815–1819.

Dukes, C. E. (1932). *J. Pathol. Bacteriol.* **35,** 323–332.

Dunzendorfer, U., and Russell, D. H. (1980). *Arzneim. Forsch.* **30,** 1801–1804.

Durie, B. G. M., and Salmon, S. E. (1975). *Cancer (Philadelphia)* **36,** 842–854.

Earnshaw, R. J., Bowen, M., and Levell, M. J. (1982). *Clin. Chim. Acta* **123,** 175–180.

Eipper, B. A., Mains, R. E., and Guenzi, D. (1976). *J. Biol. Chem.* **251,** 4121–4126.

Ellis, D. J., Spiers, C., Kingston, R. D., Brookes, V. S., Leonard, J., and Dykes, P. W. (1978). *Cancer (Philadelphia)* **42,** 623–625.

Evans, J. T., Mittleman, A., Chu, T. M., and Holyoke, E. D. (1978). *Cancer (Philadelphia)* **42,** 1419–1421.

Finlay, I. G., and McArdle, C. S. (1982). *Br. J. Surg.* **69,** 583–584.

Fisher, B., and Gill, L. W. (1975). *In* "Serum Protein Abnormalities" (S. E. Ritzmann and J. L. Daniels, eds.), pp. 331–350. Little, Brown, Boston, Massachusetts.

Fitzgerald, D. K., Colvin, B., Mawal, R., and Ebner, K. E. (1970). *Anal. Biochem.* **36,** 43–61.

Ford, C. H., Newman, C. E., and Anderson, I. G. (1979). *In* "Carcino-Embryonic Proteins" (F. G. Lehmann, ed.), Vol. 2, pp. 169–172. Elsevier-North Holland, Amsterdam.

Foti, A. G., Cooper, J. F., Herschman, H., and Malvaez, R. R. (1977). *N. Engl. J. Med.* **297,** 1357–1361.

Fraser, R. A., Ravry, M. J., Sequra, J. W., and Go, V. L. W. (1975). *J. Urol.* **114,** 226–229.

Fuks, A., Banjo, C., Shuster, J., Freedman, S. O., and Gold, P. (1975). *Biochim. Biophys. Acta* **417,** 123–152.

Gailani, S., Chu, T. M., Nussbaum, A., Ostrander, M., and Christoff, N. (1976). *Cancer (Philadelphia)* **38,** 1684–1686.
Gehrke, C. W., Kuo, K. C., Davis, G. E., and Suits, R. D. (1978). *J. Chromatogr.* **150,** 455–476.
Gerwirtz, G., and Yallow, R. S. (1974). *J. Clin. Invest.* **53,** 1022–1032.
Gold, P., and Freedman, S. O. (1965). *J. Exp. Med.* **121,** 439–462.
Goldstein, D. P. (1979). "Gestational Neoplasms in Endocrinology." Grune & Stratton, New York.
Goldstein, D. P., Kosasa, T. S., and Skarin, A. T. (1974). *Surg. Gynecol. Obstet.* **138,** 747–751.
Goslin, R., Steele, G., MacIntyre, J., Mayer, R., Sugarbaker, P. H., Cleyhorn, K., Wilson, R., and Zamcheck, N. (1980). *Ann. Surg.* **192,** 747–751.
Goslin, R. H., Skarin, A. T., MacIntyre, J., and Zamcheck, N. (1981). *JAMA J. Am. Med. Assoc.* **246,** 2173–2176.
Gray, B. N., Walker, C., Baynard, R., and Bennett, R. C. (1982). *Dis. Colon Rectum* **25,** 539–541.
Griffith, M. M., and Beck, J. C. (1963). *Cancer (Philadelphia)* **16,** 1032–1041.
Griffiths, J. C. (1980). *Clin. Chem.* **26,** 433–000.
Gropp, C., Havemann, K., Scheuer, A., and Grun, R. (1979). *Protides Biol. Fluids Proc. Colloq.* **27,** 331–334.
Guinan, P., Dubin, A., Bush, I., Alsheik, H., and Ablin, R. J. (1975). *Oncology* **32,** 158–168.
Hall, R., Laurence, D., Neville, A. M., and Wallace, D. M. (1973). *Br. J. Urol.* **45,** 88–92.
Hällgren, R., Nou, E., and Lundqrist, G. (1980). *Cancer (Philadelphia)* **45,** 780–785.
Halliday, J. W., McKeening, L. V., and Powell, L. W. (1976). *Cancer Res.* **36,** 4486–4490.
Hansen, M., and Hummer, L. (1979). *In* "Lung Cancer" (F. Muggia and M. Rosencweig, eds.) pp. 199–207, Raven Press, New York.
Heldman, D. A., Grever, M. R., and Trewyn, R. W. (1982). *Proc. Am. Soc. Clin. Oncol.* **1,** C–24.
Hendrick, J. C., and Franchimont, P. (1974). *Eur. J. Cancer* **10,** 725–730.
Hendrick, J. C., Thirion, A., Zangerle, P. F., and Franchimont, P. (1977). *In* "Tumour Markers" (K. Griffiths, A. M. Neville, and C. G. Plerrpoint, eds.), pp. 121–127. Alpha-Omega, Cardiff, Wales.
Hennessy, J. F., Wells, S. A., Jr., Outjes, D. A., and Cooper, C. W. (1974). *J. Clin. Endocrinol. Metab.* **39,** 487–495.
Herberman, R. B. (ed.) (1979). "Compendium of Assays for the Immunodiagnosis of Cancer." Elsevier-North Holland, New York.
Herberman, R. B. (1982). *In* "Clinical and Biochemical Analysis" (T. Ming Chu, ed.), Vol. 11, pp. 1–23. Dekker, New York.
Herberman, R. B., and McIntire, K. R. (eds.) (1979). "Immunodiagnosis of Cancer." Dekker, New York.
Hirai, H. (1982). *In* "Biochemical Markers for Cancer" (M. T. Chu, ed.), pp. 26–59. Dekker, New York.
Hobbs, J. R. (1982). *In* "Human Cancer Markers" (S. Sell and B. Wahren, eds.) pp. 165–178. Humana Press, Clifton, New Jersey.
Horgan, I. E. (1982). *Clin. Chim. Acta* **118,** 327–331.
Horn, Y., Beal, S. L., Walach, N., Lubich, W. P., Spigel, L., and Marton, L. J. (1982). *Cancer Res.* **42,** 3248–3251.
Horne, C. H. W., Pugh-Humphreys, R. G. P., and Brenner, R. D. (1979). *In* "Carcino-Embryonic Proteins" (F. G. Lehmnann, ed.), Vol. 1, pp. 301–311. Elsevier-North Holland, Amsterdam.

Houghton, A. L., Turner, R., and Cooper, E. H. (1977). *Br. J. Urol.* **49,** 227–232.
Hunter, R., Gelder, F., Chu, M. T., and Moosa, A. R. (1982). *In* "Markers for Diagnosis and Monitoring of Human Cancer" (M. I. Colnaghi, G. L. Burragi, and M. Chione, eds.), pp. 135–140. Academic Press, New York.
Imura, H., Matsukura, S., Yamamoto, H., Hirata, Y., and Nakai, Y. (1975). *Cancer (Philadelphia)* **35,** 1430–1437.
Jacobs, A. (1977). *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **36,** 2024–2027.
Jacobs, A., and Worwood, M. (1975). *N. Engl. J. Med.* **292,** 951–956.
Jacobs, A., Slater, A., Whitaker, J. A., Carellos, G., and Wiernik, P. H. (1976). *Br. J. Cancer* **34,** 162–166.
Jacobsen, E., Engeset, A., Sandstad, B., and Aas, M. (1982). *Scand. J. Haematol.* **28,** 264–271.
Javadpour, N., McIntire, R., and Waldman, T. A. (1978). *Cancer (Philadelphia)* **42,** 2768–2772.
Javadpour, N., and Chen, H. C. (1981). *J. Urol.* **126,** 176–178.
Johnson, P. J., Melia, W. M., Palmer, M. K., Portman, B., and Williams, R. (1981). *Br. J. Cancer* **44,** 502–505.
Kalser, M. H., Barkin, J. S., Redlhammer, D., and Heal, A. (1978). *Cancer (Philadelphia)* **42,** 1468–1471.
Katapodis, N., and Stock, C. C. (1980). *Res. Commun. Chem. Pathol. Pharmacol.* **30,** 171–180.
Kessel, D. H., Chou, T. H., and Coombes, R. C. (1981). *Eur. J. Cancer Clin. Oncol.* **17,** 1035–1040.
Killian, C. S., Vargas, V. P., Partes, J. E., Beckley, S., Wajsman, Z., Slack, N. H., Murphy, G. P., and Chu, T. M. (1981). *Prostate* **2,** 187–206.
Killian, C. S., Vargas, F. P., Slack, N. H., Murphy, G. P., and Chu, T. M. (1982). *Ann. N.Y. Acad. Sci.* **390,** 122–132.
Kin, K., Sakurabayashi, I., and Kawai, T. (1977). *Gann* **68,** 427–434.
Kjorstad, K. E., and Orjasaester, H. (1982). *Cancer (Philadelphia)* **50,** 283–287.
Klohs, W. D., Mastrangels, R., and Weiser, M. M. (1981). *Cancer Res.* **41,** 2611–2615.
Koch, M., and McPherson, T. A. (1979). *J. Surg. Oncol.* **12,** 319–325.
Kohn, J., and Raghavan, D. (1980). *In* "The Management of Testicular Tumours" (M. J. Peckham, ed.), pp. 50–69. Arnold, London.
Koj, A. (1974). *In* "Structure and Function of Plasma Proteins" (A. C. Allison, ed.), pp. 73–132. Plenum, London.
Kumar, S., Costello, C. B., Glashan, R. W., and Bjorklund, B. (1981). *Br. J. Urol.* **53,** 578–581.
Kushner, I. (1982). *Ann. N.Y. Acad. Sci.* **389,** 39–45.
Lamelin, J. P., Vincent, C., Legrand, C. F., and Revillard, J. P. (1982). *Clin. Immunol. Immunopathol.* **24,** 55–62.
Lamerz, R. A., Leonhardt, A., Ehrhart, H., and Lieven, H. V. (1980). *Oncodev. Biol. Med.* **1,** 123–135.
Lange, P. H., McIntire, K. R., and Waldmann, T. A. (1980). *Natl. Cancer Inst. Monogr.* **49,** 215–217.
Lavin, P. T., Day, J., Holyoke, E. D., Mittelman, A., and Chu, T. M. (1981). *Cancer (Philadelphia)* **47,** 823–826.
Lawton, J. O., Giles, G. R., and Cooper, E. H. (1980). *Proc. R. Soc. Med.* **73,** 23–28.
Lee, Y. T. N., Tokes, Z. A., and Csipke, C. P. (1980). *J. Surg. Oncol.* **14,** 159–165.
Lehmann, F. G. (1976). *In* "Onco-Developmental Gene Expression" (W. H. Fishman and S. Sell, eds.), pp. 407–415. Academic Press, New York.

Lehmann, F. G., and Wegener, T. (1979). *J. Toxicol. Environ. Health* **5,** 281–299.
Lipton, A., Harvey, H. A., Delong, S., Allegia, I., White, D., Allegia, M., and Davidson, E. A. (1979). *Cancer (Philadelphia)* **43,** 1766–1771.
Lüben, G., Schütze, D., and Bohn, H. (1979). *Protides Biol. Fluids Proc. Colloq.* **27,** 253–255.
Mach, J. P., Jaeger, P. H., Bertholet, M. M., Ruegsegger, C. H., Loosli, R. M., and Pettard, J. (1974). *Lancet* **2,** 535–540.
Maity, C. R., and Burma, D. P. (1973). *Indian J. Cancer* **10,** 356–360.
Mariani, G., Carmellini, M., Bonaguidi, F., Benelli, M. A., and Toni, M. G. (1980). *Eur. J. Cancer* **16,** 1099–1103.
Martin, J. F., Kilbey, R. S., and Paterson, A. D. (1982). *Haemostasis* **11,** 128–131.
Martinez-Vea, A., Artigas, J. M. G., Garcia, M. E., and Pascuchi, R. F. (1981). *Acta Cytol.* **25,** 726–727.
Matsumoto, Y., Suzuki, T., Asada, I., Ozawa, K., Tobe, T., and Honjo, I. (1982). *Cancer (Philadelphia)* **49,** 354–360.
McIntire, K. R. (1982). *In* "Human Cancer Markers" (S. Sell and B. Wahren, eds.), pp. 359–380. Humana Press, Clifton, New Jersey.
Medical Research Council Tumour Products Clinical Subcommittee (1980). *Br. J. Cancer* **41,** 976–979.
Meerwaldt, J. H., Haije, W. G., Cooper, E. H., Pidcock, N. B., and v. d. Burg, M. E. L. (1983). *Gynecol. Oncol.,* **16,** 209–218.
Mendelsohn, G., and Baylin, S. B. (1982). *In* "Human Cancer Markers" (S. Sell and B. Wahren, eds.), pp. 321–358. Humana Press, Clifton, New Jersey.
Milano, G., Cassuto, J. P., Schreider, M., Viguier, E., Lesbats, G., Caubon, P., and Lalanne, C. M. (1980a). *Nouv. Rev. Fr. Hematol.* **22,** 249–255.
Milano, G., Krebs, B. P., Duplay, H., and Lalanne, C. M. (1980b). *Clin. Chem.* **26,** 1632.
Milhaud, G., Calmette, C., Taboulet, J., Juliene, A., and Moukhtar, M. S. (1974). *Lancet* **1,** 462–463.
Minton, J. P., James, K. K., Hurtubise, P. E., Rinker, L., Joyce, S., and Martin, E. W. (1978). *Surg. Gynecol. Obstet.* **147,** 208–214.
Moertel, C. G., Schutt, A. J., and Go, V. L. W. (1978). *JAMA J. Am. Med. Assoc.* **239,** 1065–1066.
Mulder, H., Hackeng, W. H. L., Silberbusch, J., den Ottolander, G. J. H., and van deer Meer, L. (1981). *Br. J. Cancer* **43,** 786–792.
Müller, T., Bowen, M., de Mello, J., Pidcock, N. B., and Cooper, E. H. (1982). *Eur. J. Cancer Clin. Oncol.* **18,** 327–332.
Munjal, D., Chawla, P. L., Lokich, J. J., and Zamcheck, N. (1976). *Cancer (Philadelphia)* **37,** 1800–1807.
Murphy, G. P., Chu, T. M., and Karr, J. P. (1979). *Clin. Biochem.* **12,** 226–000.
Myers, R. E., Sutherland, D. J., Meakin, J. W., Kellen, J. A., Malkin, D. G., and Malkin, A. (1978). *Cancer (Philadelphia)* **42,** 1520–1526.
Myers, R. E., Sutjerland, D. J. A., Meakin, J. W., Malkin, D. G., Kellen, J. A., and Malkin, A. (1979). *Protides Biol. Fluids Proc. Colloq.* **27,** 285–288.
National Institute of Health Consensus Development Conference (1981). *Ann. Intern. Med.* **94,** 407–409.
Neville, A. M., and Cooper, E. H. (1976). *Ann. Clin. Biochem.* **13,** 283–305.
Nishioka, K., and Romsdahl, M. M. (1974). *Clin. Chim. Acta* **57,** 155–161.
Nishioka, K., and Romsdahl, M. M. (1977). *Cancer Lett.* **3,** 197–202.
Norfolk, D., Child, J. A., Cooper, E. H., Kerruish, S., and Milford Ward, A. (1980). *Br. J. Cancer* **42,** 510–515.

Nørgaard-Pedersen, B., and Raghavan, D. (1980). *Oncodev. Biol. Med.* **1,** 327–358.
Odell, W. D., and Wolfsen, A. R. (1978). *Annu. Rev. Med.* **29,** 379–406.
Oehr, P., Adolphs, H. D., Wustrow, A., Klar, R., Schlosser, T., and Winkler, C. (1981). *Tumor Diagnostik* **2,** 27–33.
O'Quigley, J., Haworth, S., Cooper, E. H., Haije, W., Van der Werf-Messing, B., Richards, B., and Robinson, M. R. G. (1980). *Eur. J. Cancer* **17,** 251–255.
Owen, J. A. (1967). *Adv. Clin. Chem.* **9,** 1–41.
Paone, J. F., Waalkes, T. P., Baker, R. R., and Shaper, J. H. (1980). *J. Surg. Oncol.* **15,** 59–66.
Paone, J. F., Baker, R. R., Waalkes, T. P., and Shaper, J. H. (1981). *J. Surg. Res.* **31,** 269–273.
Papaioannou, D., Geggie, P., and Klassen, J. (1979). *Clin. Chim. Acta* **99,** 37–41.
Parry, D. H., Worwood, M., and Jacobs, A. (1975). *Br. Med. J.* **1,** 245–247.
Pastorini, P., Milano, G., and Toubol, J. (1981). *Urol. Res.* **9,** 13–16.
Pathemore, J. G., Bronzert, D., Roberts, G., and Deftos, L. J. (1974). *J. Clin. Endocrinol. Metab.* **39,** 108–111.
Pentycross, E. Y., and Cross, C. R. (1982). *Br. J. Cancer* **45,** 223–229.
Pepys, M. B. (1981). *Lancet* **1,** 653–656.
Persijn, J. P., and Hart, A. A. M. (1981). *In* "CEA und andere Tumor Marker" (G. Uhlenbruck and G. Wintzer, eds.), pp. 98–105. Tumor Diagnostik Verlag, Leonberg.
Peto, R., Pike, M. C., Armitage, P., Breslow, N. E., Cox, D. R., Howard, S. V., Mantel, N., McPherson, K., Peto, J., and Smith, P. G. (1977). *Br. J. Cancer* **35,** 1–39.
Plow, E. F., and Edgington, T. S. (1979). *In* "Immunodiagnosis of Cancer" (R. B. Herberman and K. R. McIntire, eds.), Part 1, pp. 181–239. Dekker, New York.
Podolsky, D. K., and Isselbacher, K. J. (1980). *Cancer (Philadelphia)* **45,** 1212–1217.
Podolsky, D. K., and Weiser, M. M. (1975). *Biochem. Biophys. Res. Commun.* **65,** 545–551.
Podolsky, D. K., Weiser, M. M., Isselbacher, K. J., and Cohen, A. M. (1978). *N. Engl. J. Med.* **299,** 703–705.
Podolsky, D. K., McPhee, M. S., Alpert, E., Warshaw, A. L., and Isselbacher, K. J. (1981). *N. Engl. J. Med.* **304,** 1313–1318.
Potter, T. P. J. R., Jordan, T., Jordan, J. G., and Lassater, H. (1980). *In* "Prevention and Detection of Cancer" (H. E. Nieburgs, ed.), Part 2, pp. 467–490. Dekker, New York.
Potts, J. T. Jr., Keutman, H. T., Niall, H. D., and Tregear, G. W. (1971). *Vitam. Horm.* **29,** 41–93.
Prieto, J., Barry, M., and Sherlock, S. (1975). *Gastroenterology* **68,** 525–533.
Prignitz, R., Rainer, K., Biller, H., and Prignitz, I. (1981). *Strahlentherapie* **157,** 37–40.
Pritchard, D. G., and Todd, C. W. (1979). *In* "Immunodiagnosis of Cancer" (R. B. Herberman and K. R. McIntire, eds.), Part 1, pp. 165–180. Dekker, New York.
Raghavan, D., Gibbs, J., Nogueira Costa, R., Kohn, J., Orr, A. H., Barrett, A., and Peckham, M. J. (1980). *Br. J. Cancer* **41** (Suppl. IV), 191–194.
Rashid, S. A., Axon, A. T. R., Bullen, A. W., and Cooper, E. H. (1981). *Clin. Chim. Acta* **114,** 83–91.
Rashid, S. A., O'Quigley, J., Axon, A. T. R., and Cooper, E. H. (1982). *Br. J. Cancer* **45,** 390–394.
Ravry, M., McIntire, K. R., Moertel, C. G., Waldmann, T. A., Schutt, A. J., and Go, V. L. M. (1974). *J. Natl. Cancer Inst. (US)* **52,** 1019–1021.
Raynes, J. G. (1982). *Biomedicine* **36,** 77–86.
Richards, B., Robinson, M. R. G., Pidcock, N. B., and Cooper, E. H. (1982). *Eur. J. Urol.* **8,** 32–36.

Ronquist, G., Rimsten, A., Westman, M., and Cerven, E. (1980). *Acta Chir. Scand.* **146,** 247–252.

Rümke, P. H., Bisser, D., Kwa, H. G., and Hart, A. A. M. (1971). *Folia Med. Neerl.* **14,** 156–168.

Ruoslahti, E., Piliko, H., and Seppala, M. (1974). *Transplant Rev.* **20,** 38–60.

Ruoslahti, E., Enguall, E., and Kessler, M. J. (1979). *In* "Immunodiagnosis of Cancer" (R. B. Herbermann and K. R. McIntire, eds.), Part 1, pp. 101–117. Dekker, New York.

Russell, D. H. (1982). *In* "Biochemical Markers for Cancer" (T. M. Chu, ed.), pp. 241–266. Dekker, New York.

Russell, D. H., and Durie, B. G. M. (1978). "Polyamines as Biochemical Markers of Normal and Malignant Growth." Raven, New York.

Rutanen, E. M. (1978). *Int. J. Cancer* **22,** 413–421.

Rutanen, E. M., and Seppälä, M. (1978). *Cancer (Philadelphia)* **41,** 692–696.

Sadananda, B., Natarajan, R. S., Vittal, C., and Kalyankar, G. D. (1980). *Indian J. Cancer* **17,** 220–225.

Sampson, J., Wangl, J., and Hamis, O. D. (1982). *Aust. N.Z. J. Surg.* **52,** 39–40.

Satake, K., Kitamura, T., Ishikawa, T., Mukai, R., Yoshimoto, T., Chung, Y. S., and Umeyama, K. (1981). *J. Surg. Oncol.* **17,** 225–232.

Sawada, M., Shimizu, Y., Okudaira, Y., Matsui, Y., Nishiura, H., and Hayakawa, K. (1982). *Gynecol. Oncol.* **13,** 229–236.

Schlegel, G., Lüthgens, M., Eklund, G., and Bjorklund, B. (1981). *Tumor Diagnostik* **2,** 6–11.

Schultz, H., Sell, A., Nørgaard-Pedersen, B., and Arends, J. (1978). *Cancer (Philadelphia)* **42,** 2182–2186.

Seal, U. S., Doe, R. P., Byar, D. P., Corle, D. K., and Veterans Administration Cooperative Urology Research Group (1978). *Cancer (Philadelphia)* **42,** 1720–1729.

Searle, F., Louesay, A. C., Roberts, B. A., Rodgers, G. T., and Bagshawe, K. D. (1974). *J. Immunol. Methods* **4,** 113–119.

Sell, S. (1980). "Cancer Markers—Diagnostic and Developmental Significance." Humana Press, Clifton, New Jersey.

Sell, S., and Wahren, B. (eds.) (1982). "Human Cancer Markers." Humana Press, Clifton, New Jersey.

Seppälä, M. (1982). *In* "Human Cancer Markers" (S. Sell and B. Wahren, eds.), pp. 233–257. Humana Press, New Jersey.

Seppälä, M., and Ruoslahti, E. (1976). *Contrib. Gynecol. Obstet.* **2,** 143.

Seppälä, M., Wahlström, T., and Bohn, H. (1979). *Int. J. Cancer* **24,** 6–10.

Shah-Reddy, I., Kessel, D. H., Chou, T., Mirchandani, I., and Khilanani, U. (1982). *Am. J. Haematol.* **1,** 29–37.

Shuster, J., Gold, P., and Poulik, M. D. (1976). *Clin. Chim. Acta* **67,** 307–313.

Silva, O. L., Becker, K. L., Primack, A., Doppman, J. L., and Snider, R. H. (1974). *N. Engl. J. Med.* **290,** 1122–1124.

Silva, O. L., Broder, L. E., Doppman, J. L., Snider, R. H., Moore, C. F., Cohen, M. H., and Becker, K. L. (1979). *Cancer (Philadelphia)* **44,** 680–684.

Silver, H. K. B., Rangel, D. M., and Morton, D. L. (1978). *Cancer (Philadelphia)* **41,** 1497–1499.

Silver, H. K. B., Karim, K. A., Gray, M. J., and Salinas, F. A. (1981a). *J. Chromatogr.* **224,** 381–388.

Silver, H. K., Karim, K. A., Salinas, F. A., and Swenerton, K. D. (1981b). *Surg. Gynecol. Obstet.* **153,** 209–213.

Simonsson, B., Wibell, L., and Nilsson, K. (1980). *Scand. J. Haematol.* **24,** 174–180.
Sizemore, G. W., and Heath, H. (1975). *J. Clin. Invest.* **55,** 1111–1118.
Skryten, A., Unsqaard, B., Bjorklund, B., and Eklund, G. (1981). *Tumor Diagnostik* **2,** 117–121.
Sørensen, S. (1982). *Clin. Chim. Acta* **121,** 199–208.
Späti, B., Child, J. A., Kerrush, S. M., and Cooper, E. H. (1980). *Acta Haematol.* **64,** 79–86.
Speckart, S. F., Boldt, D. H., and MacDermott, R. P. (1978). *Blood* **52,** 681–695.
Staab, H. J., Anderer, F. A., Stumpf, E., and Fisher, R. (1978). *Am. J. Surg.* **136,** 322–327.
Staab, H. J., Anderer, F. A., Brümmendorf, T., Stumpf, E., and Fisher, R. (1981). *Br. J. Cancer* **44,** 652–662.
Staab, H. J., Anderer, F. A., Brümmendorf, T., Hornung, A., and Fisher, R. (1982). *Br. J. Cancer* **45,** 718–727.
Steel, G., Ellenberg, S., Ramming, K., O'Connell, M., Moertel, C., Lessner, H., Bruckner, H., Horton, J., Schein, P., Zamcheck, N., Novak, J., and Holyoke, E. D. (1982). *Ann. Surg.* **196,** 162–169.
Stevens, D. P., Mackay, I. R., and Cullen, K. J. (1975). *Br. J. Cancer* **32,** 147–151.
Steward, A. M., Nixon, D., Zamcheck, N., and Aisenberg, A. (1974). *Cancer (Philadelphia)* **33,** 1246–1252.
Stokes, T. C., Stevens, J. F., Long, P., Lockey, E., and Miller, A. L. (1980). *Br. J. Dis. Chest* **74,** 390–394.
Sugarbaker, P. H., Bloomer, W. D., Corbett, E. D., and Chaffey, J. T. (1976). *Am. J. Roentgenol.* **127,** 641–644.
Szymendera, J. J., Kaminska, J. A., Nowacki, M. P., Szawlowski, A. W., and Gadek, A. (1981). *Eur. J. Cancer Clin. Oncol.* **17,** 1047–1052.
Tashjian, A. H., Jr., Howland, B. G., Melvin, K. E. W., and Hill, C. S. (1970). *N. Engl. J. Med.* **283,** 890–895.
Teasdale, C., Mander, A. M., Fifield, R., Keyser, J. W., Newcombe, R. C., and Hughes, L. E. (1977). *Clin. Chim. Acta* **78,** 135–143.
Te Velde, E. R., Faber, J. A. J., Roebersen, W., Berghuys, M., Zegers, B. J. M., and Ballieux, R. E. (1979). *Protides Biol. Fluids Proc. Colloq.* **27,** 335–338.
Than, G. N., Bohn, H., Csaba, I. F., Karg, N. J., and Mann, V. (1979). *In* "Carcino-Embryonic Proteins" (F. G. Lehmann, ed.), Vol. 2, pp. 481–486. Elsevier-North-Holland, Amsterdam.
Tisman, G., Wu, S. G., and Hittle, T. (1977). *Clin. Res.* **25,** 580A.
Tomoda, H., Furusawa, M., Ohmachi, S., Seo, Y., Matsukuchi, T., Miyazaki, M., and Kanashima, R. (1981). *Jpn. J. Clin. Oncol.* **11,** 69.
Tormey, D. C., Waalkes, T. P., Ahmann, D., Gehrke, C. W., Zumwatt, R. W., Synder, J., and Hansen, H. (1975). *Cancer (Philadelphia)* **35,** 1095–1100.
Tormey, D. C., Waalkes, T. P., and Gehrke, C. W. (1980a). *J. Surg. Oncol.* **14,** 267–273.
Tormey, D. C., Waalkes, T. P., Kuo, K. C., and Gehrke, C. W. (1980b). *Cancer (Philadelphia)* **46,** 741–747.
Tortensson, S., Thoren, M., and Hall, K. (1980). *Acta Med. Scand.* **207,** 353–357.
Trautner, K., Cooper, E. H., Haworth, S., and Milford Ward, A. (1980). *Scand. J. Urol. Nephrol.* **14,** 143–149.
Trewyn, R. W., Glaser, R., Kelly, D. R., Jackson, D. G., Graham, W. P., and Speicher, C. E. (1982). *Cancer (Philadelphia)* **49,** 2513–2517.
Trump, D. L., Mendelsohn, G., and Baylin, S. B. (1979). *N. Engl. J. Med.* **301,** 253–255.
Tsujino, D., Sasaki, Y., Fukushina, M., Someya, K., and Saito, K. (1981). *Gann no Rinsho* **27,** 1355–1358.

Union Internationale Contre le Cancer (UICC) (1978). "Classification of Malignant Tumours," 3rd ed. Geneva, Switzerland.
Vihko, P., Sanjati, E., Janne, O., Peltonen, L., and Vohko, R. (1978). *Clin. Chem.* **24,** 1915–1919.
Vincent, R. G., Chu, T. M., and Fergen, T. B. (1975). *Cancer (Philadelphia)* **36,** 2069–2076.
Vincent, R. G., Chu, T. M., and Lane, W. W. (1979). *Cancer (Philadelphia)* **44,** 685–691.
Vladutiu, A. O., Adler, R. H., and Brason, F. W. (1979). *Am. J. Clin. Pathol.* **71,** 210–214.
Waalkes, T. P., and Tormey, D. C. (1978). *Semin. Oncol.* **5,** 434–444.
Waalkes, T. P., Gehrke, C. W., Tormey, D. C., Zumwalt, R. W., Hueser, J. N., Kuo, K. C., Lakings, D. B., Ahmann, D. L., and Moertel, C. G. (1975). *Cancer Chemother. Rep.* **59,** 1103–1116.
Waalkes, T. P., Abeloff, M. D., Woo, K. B., Ettinger, D. S., Ruddon, R. W., and Aldenderfer, P. (1980). *Cancer Res.* **40,** 4420–4427.
Wagner, W., Husemann, B., Becker, H., Groite, H., Koerfgen, H. P., and Hammerschmidt, M. (1982). *Aust. N.Z. J. Surg.* **52,** 41–43.
Wahren, B., Lidbrink, E., Wallgren, A., Eneroth, P., and Zajicek, J. (1978). *Cancer (Philadelphia)* **42,** 1870–1878.
Wahren, B. J., Harmenberg, J., Edsmyr, F., Jackobsson, P., and Ingimarsson, S. (1979). *Scand. J. Gastroentol.* **14,** 361–365.
Wajsman, Z., Chu, T. M., Saroff, J., Slack, N., and Murphy, G. P. (1979). *Urology* **13,** 8.
Wanebo, H. J., Rao, B., Pinsky, C. M., Hoffman, R. G., Stearns, M., Schwartz, M. K., and Oettgen, H. F. (1978). *N. Engl. J. Med.* **299,** 448–451.
Wang, D. Y., Bullrook, R. D., Hayward, J. L., Hendrick, J. C., and Franchimont, P. (1975). *Eur. J. Cancer* **11,** 615–618.
Watanabe, T. (1980). *Iwate Igaku Zasshi* **32,** 519–530.
Weiser, M. M., and Wilson, J. R. (1981). *CRC Crit. Rev. Clin. Lab. Sci.* **14,** 189–239.
Wells, J. A., Jr., Baylin, S. B., Linehan, W. M., Farell, R. E., Cox, E. B., and Cooper, W. (1978). *Ann. Surg.* **188,** 139–141.
Wibell, L., Evrin, P. E., and Berggard, I. (1973). *Nephron* **10,** 320–331.
Wolfsen, A. R., and Odell, W. D. (1979). *Am. J. Med.* **66,** 765–772.
Wood, C. B., Ratcliffe, J. G., Bert, R. W., Malcolm, A. J., and Blumgart, L. H. (1980). *Br. J. Surg.* **67,** 46–48.
Wood, R. A. B., and Moossa, A. R. (1977). *Br. J. Surg.* **64,** 718–720.
Yallow, R. S. (1979). *In* "Lung Cancer" (F. M. Muggia and M. Rosencweig, eds.), pp. 209–216. Raven, New York.
Yu, H., Glashan, R., Robinson, M. R. G., Richards, B., Fryszman, A., Daponte, D., and Cooper, E. H. (1982). *J. Urol.* **128,** 272–280.
Zamcheck, N. (1975). *Cancer (Philadelphia)* **36,** 2460–2468.
Zamcheck, N. (1976). *Clin. Gastroenterol.* **5,** 625–638.
Zamcheck, N., and Martin, E. V. (1981). *Cancer (Philadelphia)* **47,** 1620–1627.
Zangerle, P. F., Collette, J., Hendrick, J. C., Miller, W. B., and Franchimont, P. (1982). *In* "Markers for Diagnosis and Monitoring of Human Cancer" (M. I. Colnaghi, G. L. Buraggi, and M. Ghione, eds.), pp. 35–49. Academic Press, New York.
Zellen, M. (1975). *In* "Cancer Therapy Prognostic Factors and Criteria of Response" (M. J. Staquet, ed.), pp. 1–6. Raven, New York.
Zhu, Y. (1981). *Zhonghua Zhongliu Zazhi* **3,** 35–38.
Zimmerman, R., Wahren, B., and Edsmyr, F. (1980). *Cancer (Philadelphia)* **46,** 1802–1809.

2

The Use of Chemical and Physiochemical Approaches to Detecting and Identifying Etiological Agents in Clinical Specimens

EDWARD V. SAVARD

CLINICAL BIOCHEMISTRY
Contemporary Theories and Techniques, Vol. 3

ISBN 0-12-657103-1

I. INTRODUCTION

In the last few decades there have been extraordinary advances in instrumentation and automation in the clinical laboratory. In contrast with other fields of laboratory medicine, microbiology has lagged behind in achieving the levels of sophistication seen in chemistry and hematology, and, more recently, in the areas of immunochemistry and immunology. This technological lag exists despite the importance of identifying the causative agent in an infectious disease as quickly as possible in order to initiate appropriate therapy.

The inability to rapidly diagnose many life-threatening infections has been one of the major factors of the persistently high mortality still seen in infectious diseases. Many physicians treat patients with therapeutic agents prior to collection of appropriate specimens for laboratory examination. When such materials finally are submitted for analysis, the responsible agent may be nonviable, greatly suppressed, or even obscured by the overgrowth of other organisms and normal flora. Consequently, misinformation may be generated by the analysis that does not reflect the clinical condition.

Often the clinician submits material to confirm his or her diagnosis and thus contributes little to the diagnostic armamentarium that one sees in clinical chemistry and hematology. The principal reason for using microbiology as a confirmatory service is a result of the time lag between submission of the specimen and when results are available. This is entirely a result of using techniques that have undergone little change since the last century.

In recent time, there have been several developments using more modern techniques that provide rapid, sensitive, and specific information for the diagnosis of infectious diseases. These have been derived from advances in analytical chemistry and immunology. The developments involve the detection of microbial metabolites or antigens in body fluids. The techniques employed include chromatography, agglutination, immunodiffusion, electrophoresis, immunofluorescence, and enzyme-linked immunosorbent assay (ELISA), to mention a few. A second approach is

the detection of the etiological agent itself, using methods such as radiorespirometry, impedance, bioluminescence, and calorimetry.

In addition to the detection of etiological agent, there is the need to identify the agent requiring multiple tests that are difficult to mechanize or automate. Many specimens contain mixtures of microorganisms adding to the problem. They must be separated in order to differentiate normal microbial flora from those that may be pathogenic. Often the disease-producing agents in the specimen may not be present in sufficient numbers to ensure their identification through instrumentation and must be enhanced through culture. For this reason, much attention in microbiological instrumentation has been directed to detection of organisms in blood, urine, and spinal fluid that should normally be sterile. Other instruments perform automated antibiotic susceptibility tests that provide results more rapidly than conventional methods. They still require initial isolation using conventional techniques. Various biochemical and physiochemical techniques are being developed in an attempt to achieve more rapid identification in 4–6 hr versus the typical 48 hr. If early detection is combined with rapid identification and antibiotic susceptibility, a substantial improvement in the efficacy of the clinical microbiology laboratory would be achieved (*1*).

Numerous immunological tests can be employed in the detection of infectious diseases and the diagnosis of noninfectious pathological conditions. More progress will be shown as monoclonal antibodies are developed where sensitivity and specificity in the past could not be achieved. Efforts must be made to adapt these immunological tests to instrumentation or automation. Sight must not be lost of the fact that these goals must be achieved in a cost-effective manner that will be commercially feasible. These factors will continue to be the major obstacles to the technology in reaching the clinical microbiological laboratory.

In the following discussion we will review the current technology and those techniques currently in research or development stages.

II. CONVENTIONAL MICROBIOLOGICAL TECHNIQUES

A. Isolation

The fundamental principles of diagnostic microbiology essentially are collecting the specimens and transporting them to the laboratory still viable, and selecting the appropriate primary isolation media, inoculating it, and incubating under appropriate conditions.

B. Identification

A second phase occurs 18–24 hr later when the microbiologist inspects the media for growth to decide if any further work is necessary. If significant growth is observed, a decision is made either to subculture in order to obtain a pure culture or to select and inoculate additional tests to better characterize the agent. These tests require an additional 18–24 hr, at which time the tests are interpreted and either a final report is issued or additional tests are ordered or the culture is sent to a reference laboratory (*2*).

Prior to the last 10 years, the selection of the test battery to identify the isolate was at the discretion of each laboratory. The results of these tests either were interpreted from tables of expected results or by the use of flow charts from which dichotomous keys originated. These approaches were fraught with considerable error. Two frequent errors were a failure to address the biological variability of the organisms and treating each test in the sequence with equal weight and reliability.

C. Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing was devised as a means of making a rational selection of the appropriate chemotherapeutic agent to assure a favorable prognosis in the treatment of the patient. In antimicrobial susceptibility testing, a specimen must be obtained before a probable drug of choice is administered. The probable drug is selected by the physician, using tentative diagnosis based on clinical grounds. This selection is based on the assumption that certain etiological agents have expected drug susceptibilities and generally include groups of antibiotics with a broad spectrum of activity. Should the isolated agent prove not to be the presumptive one or to have a different spectrum of sensitivity, then the antibiotic is changed to a more appropriate one.

There are several techniques for performing susceptibility tests. The Kirby–Bauer method, the most widely used, consists of inoculating plates of Mueller–Hinton sensitivity agar with a standardized inoculum. Disks containing various concentrations of antibiotics are placed onto the agar surface and the plate is incubated for 18 hr. Following incubation, the zones of inhibition are measured and compared to a standard established for each antibiotic. Each antibiotic must be compared to itself because the zones are a function of the molecular characteristics of the drug. This test measures the ability of the agent to inhibit growth of the microorganism and generally correlates well with the therapeutic response to the drug in the patient (*3*).

In the agar dilution method, various concentrations of antibiotic are prepared directly in the sensitivity agar. Plates of the different antibiotic concentrations are prepared and inoculated using a standardized inoculum and a replicator. This permits large numbers of isolates to be tested simultaneously. The plates are then examined for growth following incubation. The therapeutic drug concentration is in that plate containing a concentration of the antibiotic on which the isolate fails to grow (*4*).

A similar technique is employed in the minimal inhibitory concentration method (MIC). In this procedure, tubes containing diluted amounts of antibiotic are inoculated with a standardized inoculum. Following incubation, the tubes are examined for turbidity. The tube in the series that remains clear is the end point, and this value provides an estimate of the smallest *in vivo* dose necessary to inhibit the growth of the organism. This method gives a semiquantitative value. The tube dilution method has the additional advantage of permitting the determination of the bactericidal effect of the antibiotic being tested. This is simply accomplished by subculturing the first tube without growth onto antibiotic-free medium. No growth indicates that the agent is bactericidal; growth would indicate that the agent is bacteriostatic (*4, 5*).

D. Serological Methods

Closely related to the MIC is the serum drug level. Here, patient blood is collected, and the serum is assayed to determine if the MIC levels are being achieved with the selected therapeutic agent. The procedure is to serially dilute the serum in a broth medium and inoculate it with a standardized inoculum of a strain of bacteria of a known susceptibility. The tubes are incubated and inspected for growth. By comparing the highest dilution of serum that inhibits growth with that of the known standard test organism, it is possible to compute the patient's blood level of the antibiotic (*6*).

Serological methods are frequently employed as an adjunct to the diagnosis of infectious disease. It has been observed that during the course of an infectious disease, antibodies to the specific agents frequently appear relatively early in the disease. These antibodies may be of short duration or last for months or years. The demonstration of an antibody in a patient's serum does not necessarily indicate an association with the current illness but that the individual at some time was infected with the agent. To use serological methods to diagnose a current infection, it is necessary to run two serum samples in parallel. One is collected during the acute phase of the illness and a second sample is drawn 10–14 days later. It is indicative of current infection if the test shows a twofold or higher increase in

antibody titer. This diagnostic tool is somewhat limited to relatively few diseases. In acute life-threatening disease, it is not possible to wait 10 days to secure paired sera, but these procedures are useful in some bacterial diseases and in certain mycotic diseases. In some diseases, serology is very useful because it is often difficult to isolate the etiological agent or the agent cannot be cultured in the routine diagnostic laboratory (*7*).

Although these methods continue to be widely used, we should see their replacement in the near future with more rapid and sophisticated technologies.

III. SYSTEMATIC MICROBIOLOGICAL IDENTIFICATION

Systematic methods of microbial identification have been developed in the past 10 years and are based on selecting a battery of tests by the manufacturer, consisting of conventional microbiological tests in a miniaturized version. These tests remain manual but generally employ some form of multiple inoculation. The majority of these systems are designed for identification of the Enterobacteriaceae and, to a lesser degree, nonfermentative gram-negative rods, yeasts, and certain gram-positive bacteria. Although these systems may appear to be relatively simple, they are the result of a considerable amount of research. They provide the user with a powerful tool to achieve a high level of reliability and accuracy to identify members of the family Enterobacteriaceae without the need of extensive training (*8–13*).

Test selection is based on computer analysis of published data like that of Ewing (*14–18*), which was accumulated in large reference centers. From this data a matrix consisting of selected genera and species of the Enterobacteriaceae and their reactions to the largest number of possible tests is compiled. These matrices are then analyzed using programs aimed at selecting the fewest number of tests that yield the largest number of pair separations. These tests are then evaluated to determine their feasibility for inclusion into the system. The first analysis is polythedic, with all tests of equal weight. If a test, such as motility, ranked high in the analysis but is not readily adaptable and could not be included, test ranking is then introduced. When test ranking is employed, the undesirable test is ranked as only a last choice during the sequenced analysis. By such computer analyses, a test battery can be selected that is compatible with any physical limits of the device.

These devices provide the user with a data interpretation system. Positive or negative test results are converted to a numerical score based on octal numbers. Those numbers are then listed in a computer-generated

key listing the names of the organisms giving such a reaction profile. These codes are generated using a polythedic approach; that is, all tests in the sequence are given equal weight thus minimizing the old problem with dichotomous "keys." These programs for generating the keys can also take into consideration the biological variability of an organism for each biochemical test parameter being tested. The lack of this ability with the older dichotomous keys was a serious source of error in identification with that taxonomic approach.

Those computer-generated keys can also provide valuable information regarding the statistical probability of such a biotype, the measure of the reliability of the answer, the likelihood of the appearance of an organism in the cluster, and the number of improbable tests. The octal identification values also can provide the user with a sophisticated means of biotyping individual isolates. Employment of this data by the hospital epidemiology department as an adjunct to hospital infection control can be helpful in spotting patterns of etiological agents that may be a source of nosocomial infection. These computer-assisted identification systems also offer the user a periodic means of updating their proficiency in identification. As new information on taxonomic groups of the family Enterobacteriaceae becomes available, these systems can respond quickly by updating the data base through published information from large reference centers.

An advantage of these systems is that they can be used by any size of institution without the need for expensive instrumentation or computer access. Institutions with large numbers of cultures to be tested desire more automated equipment to reduce labor costs. In recent times, these have become available either as a specifically designed automated system such as Vitek's Auto Microbic System or mechanized reading devices for microtiter technology such as Micro-Media, Micro-Scan, and Dynatech. These provide a broad appeal to both the low- and high-volume user and employ the same consumable goods.

After primary isolation, these systems provide results 4–18 hr after inoculation. Some do not provide antimicrobial susceptibility data, but others provide both MIC values and identification. Fully automated systems such as Abbott's MS-2 were designed primarily for antimicrobial susceptibility testing but are now developing identification capabilities to increase their scope and usefulness.

All these systems depend on conventional measurements of bacterial growth, fermentation, or other metabolic activities—all requiring considerable time. Methods for more rapid analysis, either starting with clinical specimens directly or using techniques for detection of constitutive enzymes or microbial antigens, are still in the research stages. These newer approaches are based on methodologies resembling those of clinical

chemistry and enzymology, using sophisticated centrifugal analyzers and spectrophotometry. Although these newer techniques will be based on biochemical or immunochemical procedures, their ultimate ability to provide an answer will rely heavily on pattern-recognition approaches currently in use with simpler systematic devices. This systematic approach has provided an important breakthrough in rapid data interpretation where multiple tests are used. During the interval between research and the commercialization of the automated analyzers, the simpler systems will continue to provide important application for some time. They will be useful where economics can not tolerate highly instrumented approaches, as in developing third world countries.

IV. DIRECT METHODS OF DETECTION

A. Radiometry

The radiometric technique is designed to detect the presence of microorganisms by their metabolism of a ^{14}C-labeled substrate forming radioactive carbon dioxide ($^{14}CO_2$). Blood, cerebrospinal fluid (CSF), or other body fluids are inoculated into a sealed ampule containing sterile ^{14}C-labeled growth medium and incubated. The gas phase in the head space of the ampule is sampled at regular intervals for the presence of radioactive $^{14}CO_2$. When the amount of $^{14}CO_2$ reaches a predetermined threshold, the instrument signals a positive ampule. The instrument is commercially available as the BACTEC system by Johnston Laboratories (Cockeysville, MD) and is used primarily for monitoring blood cultures. BACTEC has been used for rapid identification of pathogenic *Neisseria*, biological assays, and detection of tuberculosis. It has several industrial applications in monitoring product sterility and food microbiology.

The BACTEC system has undergone numerous evaluations (*19*). The principal advantage of the system is repetitive monitoring of individual specimens, which may shorten the time required for detection of a positive culture. Results published by Randall (*20*) indicate that the time required for detection of positive blood cultures containing aerobic, facultative anaerobic, and anaerobic bacteria was similar to that of conventional methodologies (*21, 22*). The main disadvantages of this technology are the relatively short period when the ampules are periodically monitored, the use of a small volume of blood, and the need for radioisotopes with the problems of their disposal. This method still requires blind subculture and flags only positive cultures. Once a positive culture is detected, conventional techniques of recovery, culture, identification, and antibiotic susceptibilities remain to be performed.

The system requires instrumentation that can range from moderately expensive to a major investment in capital equipment. This equipment is mostly dedicated to determining a negative from a positive blood culture, with only limited usefulness in conducting other diagnostic microbiological tasks. Other less costly methods are available that offer the same sensitivity without the need for radioisotopic monitoring, providing results in approximately the same amount of time and with colony isolation.

B. Electrical Impedance

It has been demonstrated that bacteria may be detected by measuring the impedance of a liquid medium in which bacteria are liberating metabolites. The impedance changes are a function of altered resistance and capacitance in accordance to the equation

$$z = [R^2 + (2\pi/cf)^2]^{1/2}$$

where z is impedance, R is resistance, c is capacitance, and f is the frequency of the alternating current. The measurement of impedance correlates with bacterial growth curves in colony-forming units per milliliter as a function of time. It has been shown that these measurements can be employed for detecting the presence of bacteria in certain clinical specimens (*23*).

In the clinical area are a variety of specimens normally sterile or low in colony-forming units, such as CSF, blood, peritoneal fluid, and urine. Urine comprises a substantial number of the daily specimens submitted for analysis. The majority of these yield negative findings but require a substantial effort to screen. Impedance seemed to offer a relatively easy procedure to quickly identify the positives and provide a semiquantitative answer. It would provide same-day results, preventing unnecessary therapy, while standard methods require overnight incubation.

The system consists of adding a premeasured volume of urine to a recommended bacteriological medium and then monitoring the impedance, using automatic measuring and recording devices. Two such devices are the Bactometer by Bactomatic Inc. and the Malthus System by Malthus Instruments, Ltd. Impedance is measured for a period of 4–5 hr, and if a change in conductance of 10 μS (the reciprocal of resistivity) is detected, the sample is considered positive. This technique has been evaluated by a number of investigators (*24–26*). Although the methodology is both simple and relatively inexpensive, there are several drawbacks. The two most serious are the inability to detect slow-growing organisms in sufficient time to make the test useful in screening. Second, there is a failure to detect significant cases of bacteriuria. This method detects only the presence of bacteria and can not identify the agent. It has also been

evaluated by Hadley and Kazinka (*27*) in screening blood cultures; they showed that it failed to detect bacteremia detected by conventional means.

It is doubtful whether this technology will ever make any substantial impact in the diagnostic microbiological laboratory. It may have considerably more usefulness in the industrial environment in monitoring large-scale fermentation operations or in quality-control procedures by measuring the microbial burden of various products. It does not offer the versatility of other existing instruments or techniques currently under development.

C. Microcalorimetry

Calorimetry is based on the principle that all reactions—physical, chemical, or biological—involve the production or consumption of heat. By monitoring the heat flow, one is able to show that a reaction is taking place (analytical calorimetry) or to measure the amount of heat exchange (quantitative calorimetry). Calorimetry provides a method for detecting life processes without the need for biochemical tests or photometric measurements. This methodology provides graphs, called thermograms, showing heat flow versus time. It was believed that these thermograms in a fixed medium could provide species-specific "fingerprints" that could be used for the identification of microbes. To provide the complex fingerprint, a thermogram would be necessary. Johansson (*28*) devised a computer analysis for transforming these thermograms to a fixed maximum height and time. These standard thermograms could then be easily compared with thermograms of known microbial strains. Regrettably, these so-called species-specific profiles have been shown not to be species specific. Numerical analysis of a larger number of profiles has demonstrated that the spectra of profile variations among various organisms overlap; thus this method does not provide the separatory power originally proposed by Russell *et al.* (*29*). It has also been shown by Boling *et al.* (*30*) that small changes in any of the growth conditions—pH, oxygen tension, osmotic pressure, or even minute differences in medium components—can give quite different results.

Microcalorimetry can be used to diagnose bacteriuria (*31*) and can be used for bacterial enumeration without the need for performing conventional plate counts (*32*). Using microcalorimetry, Harju-Jeanty (*33*) has shown that it is possible to detect nearly instantaneous changes in the typical thermogram when adding bacterostatic or bacterocidal agents to the medium. These responses are observable in only a few minutes as compared to the hours or days required for interpreting antibiotic activity by conventional means.

Some 5 years ago, there were scientists who believed that the era of calorimetry in the clinical laboratory had not yet begun; it has become apparent that their opinions were overoptimistic. In a recent review of current methods in microbiology, Bergan (*34*) devoted but five short sentences to this entire technology. Microcalorimetry remains unused and has gained little acceptance in the clinical laboratory, although it may have a more suitable place in monitoring large-scale industrial processes. The technique has several drawbacks. It does not work in mixed cultures, and only one thermogram per time interval can be evaluated. Although multichannel calorimeters have been constructed, no such instruments are on the market. Another drawback is its cost. This technology is not predicted to make any important contribution to rapid microbial detection or identification in the future.

V. INDIRECT METHODS OF DETECTION

A. Immunological

Serological diagnosis is not new to microbiology, having been used for decades. In recent times, these basic techniques have become modified or adapted to new technology or completely replaced by entirely new methods. Many of the older serological tools—such as agglutination, complement fixation, hemagglutination, hemagglutination inhibition, direct or indirect immunofluorescence, and virus neutralization—were effective but burdened with numerous labor-intensive procedural steps requiring hours to complete and lacking sensitivity and specificity. Recent advances in instrumentation have played an important part in adapting some of the older techniques by automating manual procedures. Many of the older agglutination and compliment fixation tests have been adapted for use in various microtiter apparatuses now available. In recent years, there has been a sudden proliferation of serological instrumentation and automation in the serological laboratory. All these changes have speeded up the test procedures, providing earlier answers and bringing the serological laboratory into the mainstream of the biomedical services. With the advent of hybridoma monoclonal antibody technology, we will see even greater test speed and accuracy as high-quality serodiagnostic reagents become available. Many of these improvements are already available commercially (see Table I). These tests are not restricted to diagnosis, but may be used to answer the immunological status of the patient, the efficacy of vaccination, and the stage of the disease state (*7*).

TABLE I

Immunological Tests Developed and Commercially Available Sources[a]

Test methodology	Etiological agent	Classification	Detects		Commercial name	Manufacturer
			Antibodies	Antigen		
ELISA	*Salmonella*	Bacterial	x		—	—
	Yersinia	Bacterial	x		—	—
	Brucella	Bacterial	x		—	—
	Rickettsia	Rickettsial	x		—	—
	Treponema	Bacterial	x		—	—
	Vibrio cholerae	Bacterial		x	—	—
	Escherichia coli	Bacterial		x	—	—
	Legionella pneumophila	Bacterial	x		—	—
	Mycobacterium tuberculosis	Bacterial	x		—	—
	Mycobacterium leprae	Bacterial	x		—	—
	Streptococcus mutans	Bacterial	x		—	—
	Staphylococcus enterotoxin	Bacterial		x	—	—
Immunofluorescence	Cytomeglovirus	Viral	x	x	IFA Test Kit	Immuno Diagnostic Products, Inc.
	Herpes	Viral	x	x	IFA Test Kit	Immuno Diagnostic Products, Inc.
	Rubella	Viral	x	x	IFA Test Kit	Immuno Diagnostic Products, Inc.
	Toxoplasmosis	Protozoan	x	x	IFA Test Kit	Immuno Diagnostic Products, Inc.
Microagglutination	*Francisella tularensis*	Bacterial	x		—	—
	Brucella abortus	Bacterial	x		—	—
Microflocculation	*Treponema pallidum*	Bacterial	x		RPR	Hynson, Wescot & Dunning
Particle agglutination	Rubella	Viral	x		Rubelisa (IgG, IgM)	Abbott
Hemagglutination	Rubella	Viral	x		—	—
Latex agglutination	*Legionella pneumophila*	Bacterial		x	—	—
	Streptococci (A–D)	Bacterial		x	Strep Tex	Wellcome Diagnostics
	Clostridium difficile	Bacterial		x	—	—

	Streptococcus pneumoniae	Bacterial		x	—	—
	Haemophilus influenzae	Bacterial		x	—	—
	Cryptococcus neoformans	Fungal		x	Crypto Detection System	Immuno-Micrologics, Inc.
	Neisseria meningitidis	Bacterial		x	—	—
Coagglutination	*Haemophilus influenzae*	Bacterial		x	Directogen	Hynson, Wescot & Dunning
	Streptococcus pneumoniae	Bacterial		x	—	—
	Neisseria meningitidis	Bacterial		x	Directogen	Hynson, Wescot & Dunning
	Streptococcus (A-D)	Bacterial		x	Directogen	Hynson, Wescot & Dunning
	Staphylococcus aureus	Bacterial		x	Sero-STAS Staph	Scott
Immunodiffusion	*Aspergillus*	Mycotic	x		—	—
	Candida albicans	Mycotic	x		—	—
	Blastomyces dermatitidis	Mycotic	x		—	—
	Histoplasma capsulatum	Mycotic	x		—	—
	Coccidioides immitis	Mycotic	x		—	—
CIF	*Aspergillus*	Mycotic	x		—	—
	Candida albicans	Mycotic	x		—	—
	Blastomyces dermatitidis	Mycotic	x		—	—
	Histoplasma capsulatum	Mycotic	x		—	—
	Coccidioides immitis	Mycotic	x		—	—
	Streptococcus Groups A–G	Bacterial		x	—	—
	Legionella pneumophila	Bacterial		x	—	—
	Streptococcus pneumoniae	Bacterial		x	—	—
	Haemophilus influenzae	Bacterial		x	—	—
	Neisseria meningitidis	Bacterial		x	—	—
	Pseudomonas aeruginosa	Bacterial		x	—	—
	Klebsiella pneumoniae	Bacterial		x	—	—
	Escherichia coli K1	Bacterial		x	—	—
	Staphylococcus aureus	Bacterial		x	—	—
	Mycoplasma	Bacterial		x	—	—
	Listeria monocytogenes	Bacterial		x	—	—
	Clostridium difficile	Bacterial		x	—	—

[a] Many serodiagnostic tests have been developed and reported but few are commercially available.

B. Bioluminescence

Chappell and Levin in 1968 (*35*) described a procedure where a bioluminescence assay for adenosine 5′-triphosphate (ATP) using firefly luciferase could be employed for detection and enumeration of bacteria. The greatest effort has been made on detecting bacteriuria with this methodology. It is based on the principle that bacteria contain ATP, and their presence in urine can be detected by measuring the amount of ATP using the luciferase assay. The first problem was to differentiate nonhost ATP from host cells in the specimen. The procedure to rid the specimen of nonbacterial ATP was time consuming, but a simplified procedure has been developed (*36, 37*). Further improvements were made in the recovery of somatic-cell ATP (*38*). A method to minimize the quenching properties of urine on the luciferin–luciferase assay has been developed (*39*). The time required for processing the urine has been reduced to approximately 10 min using an automated procedure (*40*).

Despite these advancements, this methodology has gained little acceptance in the microbiology laboratory. This may be caused by the disparity of results obtained with bioluminescence and those obtained using conventional plate-counting procedures. Although bioluminescence can detect reliably 5×10^4–5×10^5 colony-forming units (CFU) per milliliter using pure cultures, this cannot be directly extrapolated to urines where organisms may cluster giving lower CFUs. Bioluminescence has difficulties in giving clear-cut results when the number of bacteria is close to the threshold of sensitivity of this methodology. When compared with other automated methods, such as the Abbott MS-2 and Autobac, the frequency of false negatives is approximately the same, 5.5–6% (*39*). Comparison of standard microbiological methods shows an even greater disparity of 76% false negatives (*39*). The most probable reason for a lack of acceptance is attributed to other factors. The procedure requires a piece of dedicated, expensive instrumentation, which, like radiorespirometry, only excludes the negatives. Once a positive is detected, conventional culture techniques are needed to process the specimen. Bioluminescence does not identify the infectious agent or give information to which appropriate chemotherapeutic agent should be used in therapy. It is difficult to justify expensive instrumentation dedicated to performing only a single screening procedure.

C. *Limulus* Lysate

Bang (*41*) and Shirodkor (*42*) reported on the similarity of intravascular coagulation in the horseshoe crab (*Limulus*) with that seen in mammals during gram-negative sepsis. Exposure of *Limulus* blood to endotoxin

resulted in amebocyte aggregation and degranulation. From these reports, Levin in 1970 (*43*) developed an assay to detect endotoxin in the blood of patients with gram-negative bacterial sepsis. Gram-negative sepsis is associated with fever, hypertension, and intravascular coagulation (*44–46*) attributed to the presence of endotoxin. The ability to demonstrate the presence of endotoxin in clinical materials such as blood has been difficult because of technical reasons and inadequate sensitivity (*47, 48*). It is imperative to note that bacteremia and endotoxemia are neither synonymous nor do they necessarily occur simultaneously. The most frequent proof of endotoxemia is derived from routine blood culture when a gram-negative organism is isolated. Such procedures generally require 18 hr or longer before results are available. Because the frequency of death among patients with gram-negative sepsis is 70%, early diagnosis of the condition has a profound effect on mortality (*49*).

Levin's (*49*) original test consisted of collecting plasma extracted with chloroform and reacted with *Limulus* amebocyte lysate. Following incubation for 4 hr at 37°C, the mixture gels in the presence of endotoxin. He reported that endotoxin or endotoxin-like material in human plasma can be detected in concentrations from 5–0.5 ng/ml. Since the original report, numerous others have appeared in the literature, some with test refinements (*49–51*).

This test has several important advantages: good sensitivity and specificity. Rojas-Cerona (*52*) has shown that neither normal plasma nor its constituents produce false positive reactions, and Reinhold (*50*) has shown the same for calcium, hemoglobin, and thrombin. It provides results faster than blood culture, does not depend upon the presence of live organisms in the blood, and is not affected by the administration of antibiotics. The technique does not require expensive instrumentation and could be valuable in third world countries. It may also be used to assist in the early diagnosis of gram-negative meningitis, where CSF is used in place of blood.

Although this test is valuable in the early detection of gram-negative sepsis, it can not replace routine blood cultures. It also fails to identify the causative agent and gives no information on the appropriate antimicrobial therapy. This procedure will be quickly replaced by other immunological methods that will provide better diagnosis with greater test simplicity, sensitivity, and specificity.

D. Enzyme-Linked Immunosorbent Assay

Enzyme-linked immunosorbent assay technology is based on the principle that an enzyme can be coupled to an antigen or antibody, retaining

its activity; the complex can act as an antigen or antibody (*53*). It can be employed for detecting antigen or antibodies. When detecting an antigen, the homologous antibody is attached to a solid support. The solid support is then incubated with its homologous antigen, forming an antigen–antibody complex. Following incubation, any unreacted antigen is removed and the antibody coupled to the enzyme is added. This second antibody binds to the first antigen–antibody complex. The solid support is washed free of any unreacted second antibody–enzyme complex. An enzyme chromogenic substrate is added to this antibody–antigen–antibody enzyme "sandwich" and the reaction can be visually seen as a colorimetric reaction. This type of assay has the sensitivity and specificity of a radioimmunological assay (RIA) without the need for the use of radioisotopes or expensive counting instrumentation.

There are several variations of this basic technique. In the indirect method the antigen is bound to the support and reacted with its homologous antibody. The support is washed and a second antispecies enzyme-conjugated antibody is added. If antibody to the antigen is present in the sample, it will form a complex with the antigen. The second antibody then will react with antigenic determinants of the first antibody to form an antigen–antibody–antibody–enzyme complex. Addition of the enzyme substrate will result in the development of a color if any antibody is present in the sample.

Although this technology appears to be straightforward, it has difficulties that make it necessary to ascertain the variable parameters of each assay. These include binding antigen or antibody to the solid phase, buffers, temperature and incubation time, optimal concentration of antigen and antibody, washing steps, pretreatment of specimens, choice of enzyme, choice of substrate, and method of conjugating the enzyme. These represent some of the many parameters which must be carefully defined for each assay.

Several ELISA assays have been developed for detecting various bacterial antibodies and antigens (*54–66*). This technology has become very popular with new assays appearing frequently in the literature, but the number of commercially available assays is limited to a relatively few bacterial diseases. The methodology has gained some acceptance with available commercial reagents for the diagnosis of viral infections where there are assays for cytomegalovirus, measles, rubella, adenovirus, mumps, picornavirus, and Epstein–Barr virus. The reason may be that the current techniques for the isolation and identification of the bacterial agents are considerably easier than for the isolation of viral agents.

This technology represents a major advancement in immunomicrobiology for detecting disease or disease-producing agents. Its use will

undoubtedly continue to expand because it neither requires expensive equipment nor generates radioactive waste with its disposal problem. The basic problem seems to be in the variability of procedures in each different assay. These types of problems may be resolved as a more unified technology is applied to this immunodiagnostic approach.

E. Chemiluminescent Immunoassay

This procedure resembles an ELISA assay except that detection is not dependent on a chromogenic substrate but on chemiluminescence. In the chemiluminescent assay, an antibody is linked to peroxidase (*67*). When the conjugate binds with its homologous antigen, an antigen–antibody–enzyme complex is formed. If a chemiluminescent material such as luminol or pyrogallol is added in the presence of hydrogen peroxide, light is emitted. The emitted light can be measured using a biometer. This assay can be modified like the ELISA or RIA to be a sandwich assay using a second antibody. The chemiluminescent assay resembles RIA in sensitivity without the need for radioisotopes. It is either a qualitative or a quantitative assay with the sensitivity superior to ELISA. Any ELISA test can be adapted to a chemiluminescent immunoassay.

This methodology is just beginning to be used and few microbiological tests have been developed. With the advent of monoclonal antibodies, this methodology may play a more important role in diagnostic and public health laboratories as a substitute for the RIA. It may be especially useful for screening a large number of clinical samples for a single or small group of etiological agents such as those of the sexually transmitted diseases, hepatitis, or group-A Streptococcus. Its primary function will be to detect and identify but give no information regarding the selection of appropriate antimicrobial agents. In situations where multiple etiological agents may be responsible for the clinical symptoms, multiple tests would be required. This would be time consuming and expensive, and not enough clinical material might be available to conduct all of the tests necessary.

F. Radioimmunoassay

An isotope is coupled to an antigen that competes with the unlabeled antigen in the clinical specimen for a binding site to a specific antibody (*68*). A known amount of labeled antigen is added to the test, and the level of bound antigen is measured by assaying the amount of radioactive antigen bound to the antibody. The antigen–antibody complex is removed from solution by precipitation, using one of several techniques. The higher the level of unlabeled antigen in the sample, the lower the radioac-

tivity of the bound complex. With the exception of certain antibiotic assays, commercial kits do not exist for most of the bacterial assays developed. The RIA technique is very sensitive, consistently higher than latex, agglutination, coagglutination, or counterimmunoelectrophoresis (CIF). But RIA tests are time consuming and require very good high-titer monospecific antisera, which are scarce and expensive. Radioimmunological assay has not been extensively used in the microbiology laboratory, because multiple agents can cause a disease and multiple RIAs would have to be run on each specimen. This procedure also requires expensive instrumentation in the form of an isotope counter.

Because of the labor intensive aspects of an RIA, the equipment costs, the lack of commercially available reagents, and the production of waste—which is radioactive and also infectious—this methodology has little future in the bacteriology laboratory. Equally sensitive and easier assay procedures are becoming available.

G. Chromatography

A variety of gas chromatographic techniques have been found to be useful in the microbiological laboratory. These include head-space gas chromatography (HSGC), frequency-pulsed, electron-capture gas–liquid chromatography (FPEG–GLC), and conventional gas–liquid chromatography (GLC). A big advantage of HSGC is that it lends itself to automation. It involves placing the material to be analyzed into a glass ampule, sealing the ampule, heating it to increase the concentration of volatiles in the head space, and analyzing the vapors. This chromatographic technique permits the detection of volatile alcohols and fatty acids. In the identification of anaerobes, HSGC has several advantages over conventional GLC because extraction and methylation are unnecessary. Additionally, HSGC can detect peaks of short-chain alcohols masked by the large solvent peaks seen with GLC, providing more informative chromatograms.

Use has been made of HSGC for analysis of positive blood cultures. A sample of the turbid medium is acidified, and organic volatiles are concentrated by salting out with magnesium sulfate prior to analysis (*69*). Analysis of blood cultures containing anaerobes gives peaks corresponding to short-chain fatty acids, and facultative anaerobes and aerobes produce volatiles with shorter retention times (*70*). Although this technique did not yield any false positives, it did yield a substantial number of false negative results (*71*). Similar approaches have been employed using pus as the material for analysis in diagnosing anaerobic infections (*70*). Attempts at identification of urinary pathogens using HSGC patterns, employing a

computerized pattern-recognition program to identify the agent, has been tried with limited success (*72*). It is important to note that this is not a direct analysis of the clinical material but is performed on specimens following conventional overnight culture. Similar results can be obtained using more conventional rapid methodologies without the need for such costly instrumentation.

Although HSGC offers advantages over conventional GLC, it is primarily a complimentary method of analysis for assisting the microbiologist in detecting organisms in clinical materials by separating species from each other having similar biochemical and phenotypic characteristics. It may differentiate an anaerobic from a facultative anaerobic or aerobic infection, but it still fails to identify definitively the causative agent.

When the technique of GLC is combined with a frequency-pulsed electron-capture detector, one has a very sensitive tool for the detection of bacterial metabolites. The technique can use spent culture medium (*73*), or body fluids (*74*). The use of this technology permits the detection and often the identification of the etiological agent without the need for primary isolation. Such analyses are, however, restricted to those body fluids normally sterile and in which the number of potential pathogens encountered are restricted (*74*). If chromatograms were computerized and a program for data reduction and sorting employed, the important differential peaks of such chromatograms would make it possible to employ pattern recognition to separate and identify the causative agents. With the current revolution in microcomputers, this type of analysis would not be restricted to institutions with large computer facilities. This technology is still limited in its usefulness to a narrow spectrum of diseases and is concerned with analysis of only a few specimen types. In many human diseases, the causative agent is only one of a mixture of other agents with similar metabolisms. Conventional culture is necessary again to isolate the causative agent before any analytical procedure can be conducted.

Conventional GLC is used in the microbiology laboratory as an adjunct to conventional identification of anaerobic bacteria following conventional biochemical, morphological, and serological techniques (*75*). Here, GLC provides a rapid method for differentiating closely related microorganisms based on the GLC chromatograms of the fatty acid composition of their cell walls and membranes. In more recent times, this technique has been extended to nonanaerobic bacteria (*76–80*).

The problems inherent with all these methodologies are that they do not provide data concerning antibiotic susceptibilities. The knowledge of the infectious agent involved in the disease is of substantial importance in initiating therapy and assisting in a preliminary or presumptive diagnosis. The problem with multiple analytical and conventional techniques is that

manual procedures or other expensive instrumentation must be used. Such instrumentation is generally available in larger hospitals or research-oriented institutions because the instrumentation is not cost effective. Sixty-nine percent of health-care facilities have less than 200 beds. It must, however, be remembered that the larger institutions do serve 78% of the patient population (*81*). Instrumented analysis may also increase in usefulness because of the growing trend to use large, centralized commercial diagnostic laboratories, thus making this technology more cost effective. What remains to be developed is a more unified approach to bacterial detection, enumeration, identification, and antimicrobial susceptibility using more versatile instrumentation. A possible reason for the lack of this unified approach is that current efforts are directed at employing existing analytical equipment designed for chemical analysis rather than designing instruments committed to microbiological analysis.

H. Counterimmunoelectrophoresis

This is a modification of immunodiffusion of an antigen and its homologous antibody in an agar gel. When the antigen and it homologous antibody reach optimal concentration, a precipitin line is formed. By driving the antigen and antibody toward each other, the reaction can be observed in approximately 90 min. The negatively charged antigen, when placed in an electrophoretic chamber, diffuses to the anode; the antibody, also negatively charged, diffuses to the cathode, driven by the streaming of buffer ions to the cathode. This streaming is termed *endosmosis*. Various physiochemical parameters affect this test, including gel strength, the isoelectric nature of the antigen and antibody, the buffer, the current, voltage, and endosmotic flow. A detailed description of the procedure is given by Greenwood (*82*). There have been a number of modifications of the procedure, but these have not contibuted any significant improvement of the procedure's sensitivity or selectivity.

This technique is employed for diagnosing various diseases by detecting microbial antigens or their antibodies present in various body fluids (*83*). Many of these analyses can be performed only in large institutions because there are no commercially available sources of antigen or antisera in most cases. High-titered specific antisera are generally not readily available, but this problem may be alleviated as monoclonal antibodies become more available from commercial sources.

This technique has several advantages over conventional culture. It offers rapid detection, does not require the need to recover viable organisms, and detects disease even after antimicrobial therapy has been instituted. In diseases such as meningitis, early detection is paramount to

institute appropriate therapy. In such conditions, even a difference of a few hours can be critical as to the outcome of the disease. These procedures are more frequently performed in the chemistry or immunology laboratory than in the microbiology lab.

This technique also has limitations of assay sensitivity. The range of antigen detection by counterimmunoelectrophoresis is between 0.01 and 0.05 μg/ml (*83*). A negative result cannot be equated with absence of disease, but rather it can only suggest that the antigen is below the threshold of sensitivity. One can minimize this by concentrating the specimen sample prior to analysis by ultrafiltration or cold ethanol precipitation. These methods are applicable only where there is a large sample of material. Another problem may be that the antigen is present but lacks a negative charge, as is seen with *Streptococcus pneumoniae* type 7 and 14 capsular polysaccharide. Positive results must be interpreted with caution, because immunological cross-reactivity is known to occur between some of the antigens routinely detected. Lines may also occur as a result of precipitated protein around the antigen or antibody wells.

Since electrophoresis is frequently performed for other analytical purposes, this procedure will continue to be employed. But there are the easier, simpler, less time consuming, and more sensitive techniques of latex agglutination and coagglutination (*84–86*). As monoclonal antibodies become available to a wider range of etiological agents, they will broaden the spectrum of infectious diseases to be detected using these simpler and more direct analyses.

I. Agglutination

These tests were used extensively in the clinical laboratory as an aid in diagnosing enteric fevers, brucellosis, and rickettsial diseases. These tests were termed febrile agglutination tests or "Widal" tests. Their use has diminished considerably over the years as more sophisticated techniques have become available to recover, isolate, and identify these etiological agents. The tests were subject to great variability even when laboratories used standardized methodologies and reagents. This variability increased when modifications such as the slide test were used instead of the tube test (*7*).

J. Coagglutination and Latex Agglutination

These techniques are replacing the older bacterial agglutination methods, because they are more direct, easier to read, and give more reliable results faster.

Coagglutination is based on the principle that the Cowan strain of *Staphylococcus aureus*, its outer surface rich in protein A, binds IgG. This protein binds the Fc portion of the IgG of the subclasses 2 and 4, leaving the Fab portion of the molecule to function as a specific antibody for its homologous antigen. In the presence of the homologous antigen, cross-linking occurs between antibody-coated staphylococcal cells, resulting in agglutination of the suspension (*7*). This reagent is easily prepared from an overnight broth culture of *Staphylococcus*. The cells are harvested, washed free of growth medium, and fixed with formalin. The cell suspension is adjusted to 10% in phosphate-buffered saline (PBS) and heated at 80°C for 1 hr. The preparation is sensitized by mixing 0.1 ml of undiluted antiserum with 1 ml of the 10% suspension. The suspension is then adjusted to 10 ml with PBS and stored at 4°C until use. The test consists of placing a drop of the clinical specimen or a bacterial suspension on a slide and adding an equal volume of the sensitized staphylococcal suspension. After mixing for 2 min, agglutination occurs if the test is positive. *Staphylococcus* mixed with undiluted serum from an unimmunized animal of the same species from which the antisera originated is an appropriate control.

This coagglutination reagent has been used for the detection of bacterial antigens, bacteria in body fluids, and the identification of bacteria.

The technique is easy, requires no instrumentation, is sensitive, and results are available in minutes. More extensive use is limited by the small number of bacterial diseases for which the reagents are available. This shortage is primarily attributed to the lack of high-titered specific antisera. These will become more readily available as monoclonal antibodies to specific antigenic epitopes of other important disease-producing agents are produced. A problem with these reagents is the tendency of nonspecific agglutination that may occur when testing body fluids. This can be minimized by the addition of protein A to the clinical sample prior to adding the sensitized *Staphylococcus*.

Latex agglutination consists of a specific antibody globulin coupled to polystyrene latex particles in the 0.8-μm size range. The reagent is prepared by mixing equal volumes of antibody with latex of an optical density of 0.3 at 650 nm. One drop of the latex reagent is added to 2 drops of a bacterial suspension or the body fluid to be tested. The drops are mixed for 2 min and inspected for agglutination. A control consisting of a latex suspension coupled to normal serum should be run each time the test is performed. A known positive control should be run for comparison. Latex reduces the frequency of autoagglutination problems as are seen with staph coagglutination. Similar cautions observed with coagglutination apply to latex when the specimen is a body fluid. As with staph coagglutination, the lack of reagents is due to the unavailability of good antisera (*7*).

Both *Staphylococcus* coagglutination and latex agglutination offer significant advantages over counterimmunoelectrophoresis. As more specific antibodies become available, we will see an increase in the use of these techniques for detection of disease and identification of etiological agents.

K. Fluorescent Antibody

The use of the fluorescent antibody (FA) technique has been in existence for more than two decades and continues to be used widely (*87*). Numerous applications have been developed since these early reports, but because of the large number of publications involved, it is not practical to include these references in this chapter. The test is based on labeling an antibody molecule with a fluorescent compound, such as fluorescein isothiocyanate. In the direct FA test, the labeled antibody is reacted with its homologous antigen, and the resulting antigen–antibody fluorescein-labeled complex is viewed under ultraviolet light at 360 nm; the complex will fluoresce in the visible light spectrum. The test can be modified also to be used as an indirect FA, where the second antibody, which is species specific to the first antibody, is fluorescently tagged. The technique can be adapted as an inhibition test where the presence of unlabeled antibody inhibits the staining of the antigen by the second fluorescent antibody. These procedures have been widely used for the detection and identification of numerous etiological agents.

Although these methods are still extensively used, there are several drawbacks. One problem is nonspecific staining, especially if the samples contain extraneous materials, such as occur in tissue. Another drawback is the need for considerable technical skill in performing the time-consuming test. The procedure requires a fluorescent microscope and is subject to variations in the interpretations by the observer. The frequent problem of cross-reactivity requires that the user be informed of these problems with each test. Although many antisera are commercially available and suitable for use in the FA procedure, they are often low titer, lack specificity, and vary widely from lot to lot and from supplier to supplier. The advent of monoclonal antibodies will help a great deal to minimize these problems.

L. Fluorometric Assays

This is a modification of the old indirect FA technique, substituting a fluorometer for the fluorescent microscope to make the readings. This eliminates the subjectivity of the user. Because the instrument reads relative fluorescence, it is possible to make a standard curve. Specimen titers

can then be interpolated from a linear scale rather than the formerly used twofold dilutions. A commercial system called FIAX (International Diagnostic Technology) is available, consisting of a fluorometer, a microprocessor, and a plastic Sti Q sampler containing a fiber pad with the test antigen bound to the fiber. The serum to be tested for antibody is incubated with the Sti Q antigen-coated pad, washed, and reacted with a fluorescently tagged second antibody. The pad is washed and placed into the fluorometer, where the degree of fluorescence is read. The system reads as many as 72 samples in 6 min; most assays require 30–90 min, and the system allows for batch processing.

At present, FIAX is primarily used in detecting antibodies to viral agents and includes rubella, cytomegalovirus, herpes simplex, and *Toxoplasma,* and is given the acronym TORCH. The system detects antibodies, but could be adapted to detect antigen by absorbing specific antibodies to the Sti Q sampler to allow assaying for viral antigens in the clinical specimens. Such a system is not available at this time.

The principal advantages of the system are simplicity to perform, accuracy, rapidity, the ability to process samples in batch, and the removal of subjectivity—which is inherent in reading immunofluorescence with a microscope. At present, its use is primarily restricted to the diagnosis of a few viral diseases. As more specific antibodies are developed, we might see the use of this method of antigen detection directly on clinical specimens. The limiting factor might be the relatively low levels of viruses or bacteria in clinical specimens that might be beyond the range of sensitivity of the immunofluorometric technique.

VI. ENZYMATIC PROFILES

The principles of microbial identification are achieved by determining the metabolic activities of an unknown organism and comparing it with the metabolic pattern of a known bacterium. The traditional methodologies rely on the observation of the color change of a pH indicator, or growth, showing how the unknown organism metabolizes a particular substrate. Such observations require prolonged incubations of 18 hr to 2 days. A number of methods have been discussed for the detection of bacterial growth such as impedance, microcalorimetry, radiometry, turbidity, or a new approach, the detection of characteristic compounds by chromatographic techniques. A more direct method, measuring bacterial enzymatic activity, will be discussed. These techniques, discussed earlier, all depended on growth of the inoculum for results. There are inherent disadvantages when growth-dependent methods are used, including

the need to maintain aseptic conditions, the need to provide optimal growth conditions, and the fact that there is a substantial time lag before any changes are detectable. When the enzymatic activities of bacterial suspensions are analyzed, there is an advantage that all constituents of the bacterial cells and all changes in the growth medium are products of enzymatic activity. These enzymatic activities can be increased by the optimization of the assay conditions. Incubation time for the measurement of enzymatic activity is relatively short. It is assumed that enzymatic assays could be theoretically substituted for the conventional tests employed for bacterial identification. This approach also lends itself to automation and makes use of equipment similar to that used in the clinical chemistry laboratory.

Automation has followed three basic approaches. In the continuous-flow system of Technicon, air-segment samples and reagents flow through a very long tube where addition of reagents can be made at various points. The stream is eventually passed through a flow cell where the various parameters are measured. This system is flexible in the number of reagents used, the ability to separate various fractions of the reaction mixture, and the types of measurements possible. It is subject to carry-over that restricts the sample throughput. Although enzymatic profiles generated can be used to identify bacteria, its carryover and incubation time limit its usefulness. It cannot be employed for antibiotic susceptibility determinations.

In discrete sample analysis (DSA) used by Abbott in their system, the reactions occur in individual containers where samples and reagents are added by automatic dispensers. These containers act as cuvettes for absorbance readings. In other systems the reading of absorbance is achieved using a flow cell similar to that used in continuous-flow analysis. The DSA systems lend themselves to both identification and antibiotic susceptibility determinations. The principal limitations are their inability to handle small sample volumes, and they are essentially semiautomated. Increasing throughput often requires more pieces of expensive equipment.

In centrifugal analyzers such as the COBAS Roche, centrifugal force is used to dispense the samples, mix the samples and reagents, and permit frequent and multiple measurements, ranging from 8 to 12 measurements per sample per second. The instruments can measure optical density and absorbance at multiple wavelengths. These analyzers are best suited for kinetic assays of enzymatic activity, but they can also determine multiple enzymatic activities of a single sample, do bacterial growth curves, enumerate bacterial concentrations, and perform antibiotic susceptibility determinations. The centrifugal analyzers have a high throughput, can perform multiple determinations (such as identification, susceptibility, and

enumeration) do not require batch operations, and can handle individual samples as they occur.

When these systems are applied to microbial analysis, they must handle a small sample size. The microbiological sample and the amount of enzyme activity present are many times smaller than those seen in clinical chemistry. The systems must be tailored to handle small reaction volumes and aim for maximum sensitivity. With the advent of microprocessors, these systems can process data quickly and compare results with those in the data bank so that pattern recognition can be employed to yield direct answers. Organism identification is possible at the same time its antibiotic susceptibility profile is obtained. These systems can be used for direct microbial detection and identification. Such instruments, especially the centrifugal analyzers, seem to offer the greatest potential for true automation in the microbiological laboratory by providing the greatest diversity of possible analyses. They can also provide for adaptability to the other new technologies such as direct immunological detection using agglutination, latex agglutination, coagglutination or by adapting to bio or chemiluminescent technologies, or fluorometry. For these technologies, the basic instrument would remain the same but the detector systems would have to be modified to perform these measurements.

VII. SEMIAUTOMATED AND AUTOMATED METHODS

Three of the automated or semiautomated systems currently available have gained some degree of success. These include Autobac (General Diagnostics), MS-2 (Abbott Laboratories), and the Auto Microbic System (AMS; Vitek Systems, Inc.). The original aim of these systems was to mechanize the method for testing clinical bacterial isolates for susceptibility to antimicrobial agents. This task represents a considerable part of the daily work load in the diagnostic microbiology laboratory. For this reason, automation offers the best market entry for dedicated instrumentation that could reduce the laboratory work load and would reduce the turnaround time for obtaining antibiotic profiles, thus justifying capital outlay. Presently, these instruments are directed at susceptibility testing in the family Enterobacteriaceae and other gram-negative rods.

The Autobac is a semiautomated system utilizing light-scattering measurements to determine inhibition of bacterial growth response to test agents. Antibiotic disks of various antimicrobial agents are dispensed into a multiple compartmented cuvette. Antibiotic selection is at the discretion of the user. The prepared cuvette is inoculated manually and transferred manually from the incubator to the reading station. Readings are made

3–5 hr following incubation at 37°C and after comparing each antibiotic-containing cuvette to the one containing only growth medium. Susceptibility is determined when the ratio between the control cuvette and the cuvette containing antibiotic reaches a predetermined ratio. The system displayed a 90% correlation when compared to disk diffusion and 95% with microbroth dilution (*88*).

The AMS makes use of a plastic template that contains microwells containing selected dried antibiotics. The template is inoculated with a suspension of bacteria, using a specially designed vacuum chamber. Following inoculation, the unit is placed into an incubator–reader module. From this point on, the system is automatic. It performs readings and reports the results of antibiotic susceptibilities in 4 hr. Comparison of AMS results with those obtained using disk diffusion or microdilution have shown agreement of 83–90% (*88*).

The MS-2 system consists of a series of plastic cuvettes into which paper disks containing antibiotics selected by the user are placed. The cuvettes are then inoculated with a standardized suspension of bacteria, and the unit is placed into an incubator–analyzer; the growth of bacteria is monitored by photometric detectors. The analysis mode is automatic, and the results of antimicrobial susceptibilities are reported in 3–5 hr. Evaluation of the system with conventional disk diffusion and microdilution methods shows an agreement between 91 and 98% (*88*).

There are discrepancies between the automated and reference methods with all of these systems. These range from very major (reference method, resistant; automated method, susceptible), to major (reference method, susceptible; automated method, resistant), to minor (reference method, intermediate; automated method, resistant or susceptible). These values range from 1.2 to 3.1% for very major, 0.6 to 2.1% for major, and 4 to 12.4% for minor discrepancies between the different systems. The discrepancies between different antimicrobial agents occurred between 3 and 35%. Overall, the highest values are seen with ampicillin, carbenicillin, cephalothin, tetracyclines, and nitrofurantoin. Discrepancies are seen less often with amikacin, gentamicin, tobramycin, and trimethoprim-sulfomethoxazole. Discrepancies predominate among certain genera of the Enterobacteriaceae (*88*). Despite these problems, these automated or semiautomated susceptibility testing systems provide an acceptable level of accuracy, making the systems attractive for use in the clinical laboratory. When susceptibility results are available in 3–5 hr combined with a rapid identification system, they offer an attractive system.

Although these systems are currently available on the commercial market, they are still in an early stage of development. These instruments are

costly for the microbiological laboratory where they are primarily devoted to a single operation. Much research is being performed by the manufacturers to develop units to do other important microbiological tasks. The systems are not designed to detect bacteria initially but start at the point following primary isolation. The closest application to bacterial detection without primary isolation is seen in the urine screens for detection of bacteriuria, as an Autobac (*89, 90*). Autobac is currently developing a rapid identification system for gram-negative rods by measuring their growth response to different antibacterial agents (*91*). This has been achieved by redesigning the test cuvette to contain 19 chambers, combined with a modification of the photometer, which will generate inhibition profiles in place of conventional biochemical tests. These profiles when submitted to multivariate statistical procedures generate test profiles that can be analyzed, giving an identification (*92*). A similar extension of the MS-2 is being evaluated for a rapid 5-hr identification system for the family Enterobacteriaceae, using rapid biochemical tests. Results reported by Cooper (*93*) show a 95% agreement between MS-2 identification and those obtained with conventional tests. The Autobac and MS-2 include systems for the rapid identification of medically important yeasts (*93, 94*). Both systems are based on a modification of the conventional assimilation patterns to obtain results in 12–24 hr versus the usual 4–5 days with a reported accuracy in the MS-2 system of around 95% (*93*).

Harrington and Gaydos (*95*) have reported on efforts to adapt the Autobac for use in performing antibiotic susceptibilities for anaerobes. By the addition of agar to Schaedler broth, the authors were able to obtain a 99% correlation in the Autobac cuvette broth-disk method to that of conventional broth-disk susceptibility testing. The cuvettes had to be stored in an anaerobic box overnight to degas the polystyrene and provide optimal conditions for growth. There were some disparities between Autobac readings and visual readings with *Bacteroides fragilis* and *Clostridium perfringens* with some of the cell wall active drugs. Antibiotic susceptibility testing of fastidious organisms using Autobac has been reported. Ryan (*96*) has reported favorable results testing antibiotic susceptibilities of strains of *Haemophilus influenzae* in 5–6 hr using MIC broth supplemented with 10% Levinthal extract.

Johnston and Griffiths (*97*) have reported on the use of the MS-2 system to perform MIC determinations on organisms isolated from patients having life-threatening infections where the organisms showed multiple drug resistance. They used the instrument not only to determine conventional MICs, but also to investigate for synergy or antagonism between antibiotic combinations. These results were found to be beneficial in selecting the drug or drug combinations for these patients who would otherwise

have to be treated empirically. Although there were discrepancies between MS-2 results and those of conventional testing, it was pointed out that conventional test methodologies are a poor reference for comparison. Conventional methods are difficult to standardize, and it is difficult to obtain reproducible results from them; but reproducibility in the MS-2 has been reported to be consistently good.

All these systems represent a substantial step forward in attempting to instrument and automate the clinical microbiological laboratory. They have addressed a more holistic approach by designing an instrument with the capabilities of performing more than a single function. Most of the previously described instruments were mere extensions of an existing instrument to another application. It is for this reason that these automated devices offer a substantial improvement over all the other technologies that had a very limited scope. Despite this dedication of design to perform microbiological functions, these instruments have had difficulty in replacing more conventional technologies. There are several basic reasons for the lack of success in replacing older technology. One is the lack of available reagent disks for performing susceptibilities on newly available antibiotic agents. Another is the initial cost, together with the high cost of consumables. Such factors are especially important in the smaller institution which does not have the specimen load to justify such an expenditure. Although other uses, such as bacterial identification and urine screening, are being developed, they have been slow in coming, making it difficult to justify such a large expense for an instrument to perform a single operation. These instruments are relatively recent in the microbiological laboratory, yet the majority are all based on older technologies. The most serious drawback is that the instruments do not offer sufficient versatility to adapt to methodologies not dependent on light transmission. The inherent limitations in such instruments will be their inevitable demise. A more flexible instrument such as the centrifugal analyzer—which can be adapted to perform a variety of operations such as spectral absorbance, luminescence and fluorescence—will continue to be the instrument of the future. It will be better able to address a larger range of functions, including microbial detection, identification, and susceptibility. Direct analysis of clinical specimens without the need for primary culture and isolation is an area of great promise.

Because of the large diversity of etiological agents and infectious diseases, many routine methodologies will continue to be employed in the microbiological laboratory for a considerable time. Many diseases are seen infrequently, and to develop sophisticated techniques will not prove economical with respect to the research required and the usefulness of the test.

REFERENCES

1. Balows, A., *in* "Rapid Methods and Automation in Microbiology" (R. C. Tilton, ed.), pp. 76–79. Amer. Soc. for Microbiology, Washington, D.C., 1982.
2. Isenberg, H. D., Washington, J. A., II, Balows, A., and Sonnerwirth, A. C., *in* "Manual of Clinical Microbiology" (E. H. Lennette, A. Balows, W. J. Hausler, Jr., and J. P. Truant, eds.), 3rd ed, pp. 52–82. Amer. Soc. for Microbiology, Washington, D.C., 1980.
3. Barry, A., and Thornsberry, C., *in* "Manual of Clinical Microbiology" (E. H. Lennette, A. Ballows, W. J. Hausler, Jr., and J. P. Truant, eds.), 3rd ed., pp. 463–474. Amer. Soc. for Microbiology, Washington, D.C, 1980.
4. Sutter, V. L., and Washington, J. A., *in* "Manual of Clinical Microbiology" (E. H. Lennette, A. Ballows, W. J. Hausler, Jr., and J. P. Truant, eds.), 3rd ed., pp. 475–477. Amer. Soc. for Microbiology, Washington, D.C, 1980.
5. Anhalt, J. P., Sabath, L. D., and Barry, A. L., *in* "Manual of Clinical Microbiology" (E. H. Lennette, A. Balows, W. J. Hausler, Jr., and J. P. Truant, eds.), 3rd ed., pp. 478–484. Amer. Soc. for Microbiology, Washington, D.C, 1980.
6. Sabath, L. D., and Anhalt, J. P., *in* "Manual of Clinical Microbiology" (E. H. Lennette, A. Balows, W. J. Hausler, Jr., and J. P. Truant, eds.), 3rd ed., pp. 485–490. Amer. Soc. for Microbiology, Washington, D.C, 1980.
7. Farmer, S. G., and Tilton, R. C., *in* "Manual of Clinical Microbiology" (E. H. Lennette, A. Balows, W. J. Hausler, Jr., and J. P. Truant, eds.), 3rd ed., pp. 521–532. Amer. Soc. for Microbiology, Washington, D.C, 1980.
8. MacDonald, J. C., *Am. Lab.* **10,** 78–85 (1978).
9. Bascomb, S., LaPage, S. P., Curtis, M. A., and Willcox, W. R., *J. Gen. Microbiol.* **77,** 291–315 (1973).
10. LaPage, S. P., Bascomb, S., Willcox, W. R., and Curtis, M. A., *J. Gen. Microbiol.* **77,** 291–315 (1973).
11. Willcox, W. R., LaPage, S. P., Bascomb, S., and Curtis, M. A., *J. Gen. Microbiol.* **77,** 317–330 (1973).
12. Morse, L. E., Peters, J. A., and Hamel, P. B., *BioScience* **21,** 174–181 (1971).
13. Lockhart, W. R., and Liston, J. (eds.), "Methods for Numerical Taxonomy." Amer. Soc. for Microbiology, Washington, D.C., 1970.
14. Ewing, W. H., "Isolation and Identification of Salmonella and Shigella." Center for Disease Control, Atlanta, Georgia, 1972.
15. Ewing, W. H., *Public Health Lab.* **30,** 146–160 (1972).
16. Ewing, W. H., "Differentiation of Enterobacteriaceae by Biochemical Reactions," revised ed. Center for Disease Control, Atlanta, Georgia, 1973.
17. Ewing, W. H., and Davis, B. R., "Biochemical Characterization of *Citrobacter freundii* and *Citrobacter diversus*." Center for Disease Control, Atlanta, Georgia, 1971.
18. Ewing, W. H., and Martin, W. J., *in* "Manual of Clinical Microbiology" (E. H. Lennett, E. H. Spaulding, and J. P. Truant, eds.), 2nd ed., pp. 189–221. Amer. Soc. for Microbiology, Washington, D.C., 1974.
19. Anhalt, J. P., *in* "The Detection of Septicemia" (J. A. Washington, ed.), pp. 109–144. CRC Press, West Palm Beach, Florida, 1978.
20. Randall, E. L., *in* "Microbiology 1975" (D. Schlessinger, ed.), pp. 39–44. Amer. Soc. for Microbiology, Washington, D.C., 1975.
21. Paisley, J. W., Rosenblatt, J. E., Hall, M., and Washington, J. A., II, *J. Clin. Microbiol.* **8,** 764–766 (1978).
22. Washington, J. A., II, *in* "The Detection of Septicemia" (J. A. Washington II, ed.), pp. 41–87. CRC Press, West Palm Beach, Florida, 1978.

23. Leigh, D. A., and Williams, J. D., *J. Clin. Pathol.* **17,** 498–503 (1964).
24. Derek, F., Brown, J., and Warner, M., *in* "Rapid Methods and Automation in Microbiology" (R. C. Tilton, ed.), pp. 171–175. Amer. Soc. for Microbiology, Washington, D.C., 1981.
25. Cady, P., Dufour, S. W., Lawless, P., Nunke, B., and Kraegar, S. J., *J. Clin. Microbiol.* **7,** 273–278 (1978).
26. Leigh, D. A., and Williams, J. D., *J. Clin. Pathol.* **17,** 498–503 (1964).
27. Hadley, W. K., and Kazinka, W., *Abstr. Annu. Meet. Am. Soc. Microbiol. No.* C69, p. 37 (1976).
28. Johansson, A., Nord, C. E., and Wadstrom, T., *Sci. Tools* **22,** 19 (1975).
29. Russell, W. J., Zettler, J. F., Blanchard, G. C., and Boling, E. A., *in* "New Approaches to the Identification of Microorganisms" (C. G. Heden and T. Illeni, eds.), pp. 101–121. Wiley, New York, 1975.
30. Boling, E. A., Blanchard, G. C., and Russell, W. J., *Nature (London)* **241,** 472 (1973).
31. Beezer, A. E., Bettelheim, K. A., Newell, R. D., and Stevens, J., *Sci. Tools* **21,** 13 (1974).
32. Beezer, A. E., Bettelheim, K. A., Al-Salihi, S., and Shaw, E. J., *Sci. Tools* **25,** 6 (1978).
33. Harju-Jeanty, P., LKB Application Note 268, LKB Instruments, Gaithersburg, Maryland, 1977.
34. Bergan, T., *in* "Rapid Methods and Automation in Microbiology" (R. C. Tilton, ed.), pp. 36–40. Amer. Soc. for Microbiology, Washington, D.C., 1981.
35. Chappell, E. W., and Levin, G. V., *Biochem. Med.* **2,** 41–51 (1968).
36. Thore, A., Ansehn, S., Lundin, A., and Bergman, S., *J. Clin. Microbiol.* **1,** 1–8 (1975).
37. Ansehn, S., Lundin, A., Nillsson, L., and Thore, A., *in* "Proceedings International Symposium on Analytical Applications of Bioluminescence and Chemiluminescence" (E. Schram and P. Stanley, eds.), pp. 438–445. State Printing and Publ., Westlake Village, California, 1979.
38. Lundin, A., and Thore, A., *Appl. Microbiol.* **30,** 713–721 (1975).
39. Johnston, H. H., Curtis, G. D. W., and Nichols, W. W., *in* "Rapid Methods and Automation in Microbiology" (R. C. Tilton, ed.), pp. 162–165. Amer. Soc. for Microbiology, Washington, D.C., 1981.
40. Johnston, H. H., Mitchell, C. J., and Curtis, G. D. W., *Lancet* **ii,** 400–402 (1976).
41. Bang, F. B., *Bull. Johns Hopkins Hosp.* **98,** 325–351 (1956).
42. Shirodkar, M. V., Warwick, A., and Bang, F. B., *Biol. Bull. (Woods Hole, Mass.)* **118,** 324–327 (1960).
43. Levin, J., Poore, T. E., Zauber, N. P., and Oser, R. S., *N. Engl. J. Med.* **283,** 1313–1316 (1970).
44. Hjort, P. F., and Rapaport, S. I., *Annu. Rev. Med.* **16,** 135–168 (1965).
45. Zweifach, B. W., and Janoff, A., *Annu. Rev. Med.* **16,** 201–220 (1965).
46. Harlan, W. R., *N. Engl. J. Med.* **281,** 1127–1128 (1969).
47. Braude, A. I., *in* "Bacterial Endotoxins" (M. Landy and W. Braun, eds.), pp. 98–109. Rutgers Univ. Press, New Brunswick, New Jersey, 1964.
48. McGill, M. W., Porter, P. J., and Kass, E. H., *J. Infect. Dis.* **121,** 103–112 (1970).
49. Levin, J., Poore, T. E., Young, N. S., Margolis, S., Zauber, N. P., Townes, A. S., and Bell, W. R., *Ann. Intern. Med.* **76,** 1–7 (1972).
50. Reinhold, R. B., and Fine, J., *Proc. Soc. Exp. Biol. Med.* **137,** 334–340 (1971).
51. Yin, E. T., Galanos, C., Kinsky, S., Bradshaw, R. A., Wessler, S., Luderitz, O., and Sarmiento, M. E., *Biochim. Biophys. Acta* **261,** 284–289 (1972).
52. Rojas-Corona, R. R., Skarnes, R., Tamakuma, S., and Fine, J., *Proc. Soc. Exp. Biol. Med.* **132,** 599–601 (1969).

53. Engvall, E., and Perlmann, P., *Immunochemistry* **8,** 871–874 (1971).
54. Bidwell, D. E., Buck, A. A., Diesfeld, H. J., Enders, B., Haworth, J., Huldt, G., Kent, N. H., Kirsten, C., Mattern, P., Ruitenberg, E. J., and Voller, A., *Bull. World Health Org.* **54,** 129–139 (1976).
55. Buxton, T. B., Crockett, J. K., and Rissing, J. P., *Lab. Med.* **10,** 630–634 (1979).
56. Saunders, G. C., Clinard, E. H., Bartlett, M. L., and Sanders, W. M., *J. Infect. Dis.* (Suppl.), **136,** 258–266 (1977).
57. Ruitenberg, E. J., and VanKnapen, F., *J. Infect. Dis.* (Suppl.), **136,** 267–273 (1977).
58. Bidwell, D. E., Bartlett, A., and Voller, A., *J. Infect. Dis.* (Suppl.), **136,** 274–278 (1977).
59. Bullock, S. L., and Walls, K. W., *J. Infect. Dis.* (Suppl.), **136,** 279–285 (1977).
60. Gilman, S. C., and Docherty, J. J., *J. Infect. Dis.* (Suppl.), **136,** 286–293 (1977).
61. Leinikki, P. O., and Passila, S., *J. Infect. Dis.* (Suppl.), **136,** 294–299 (1977).
62. Gravell, M., Dorsett, P. H., Gutenson, O., and Ley, A. C., *J. Infect. Dis.* (Suppl.), **136,** 300–303 (1977).
63. Miranda, Q. R., Bailey, G. D., Fraser, A. J., and Tenoso, H. J., *J. Infect. Dis.* (Suppl.), **136,** 304–310 (1977).
64. Wolters, G., Kuijpers, L. P. C., Kacaki, J., and Schuurs, A. H. W. M., *J. Infect. Dis.* (Suppl.), **136,** 311–317 (1977).
65. Halbert, S. P., and Anken, M., *J. Infect. Dis.* (Suppl.), **136,** 318–323 (1977).
66. Castellano, G. A., Hazzard, G. T., Madden, D. L., and Sever, J. L., *J. Infect. Dis.* (Suppl.), **136,** 337–340 (1977).
67. Aurameas, S., and Ternynck, T., *Immunochemistry* **8,** 1175–1179 (1971).
68. Addison, G. M., and Hales, C. N., *in* "Clinical Aspects of Immunology" (G. H. Gell, R. R. A. Coombs, and P. J. Lachmann, eds.), 3rd ed., pp. 259–281. Blackwell, Oxford, 1975.
69. Larsson, L., Mardh, P. A., and Odham, G., *J. Clin. Microbiol.* **7,** 23–27 (1978).
70. Mardh, P. A., Larsson, L., and Odham, G., *Scand. J. Infect. Dis.* (Suppl. 26), 14–18 (1981).
71. Larsson, L., *in* "Rapid Methods and Automation in Microbiology" (R. C. Tilton, ed.), pp. 151–154. Amer. Soc. for Microbiology, Washington, D.C., 1981.
72. Larsson, L., Mardh, P. A., and Odham, G., *Curr. Microbiol.* **4,** 143–146 (1980).
73. Alley, C. C., Brooks, J. B., and Kellogg, D. S., Jr., *J. Clin. Microbiol.* **9,** 97–102 (1979).
74. Brooks, J. B., Craven, R. B., Schlosberg, D., Alley, C. C., and Pitts, F. M., *J. Clin. Microbiol.* **8,** 203–208 (1978).
75. Moss, C. W., *J. Chromatogr.* **203,** 337–347 (1981).
76. Herbert, G. A., Moss, C. W., McDougal, L. K., Bozeman, F. M., McKinney, R. M., and Brenner, D., *Ann. Intern. Med.* **92,** 45–52 (1980).
77. Moss, C. W., and Dees, S. B., *J. Clin. Microbiol.* **10,** 390–391 (1979).
78. Moss, C. W., *in* "Glucose Nonfermentative Gram-negative Bacteria in Clinical Microbiology" (G. L. Gilardi, ed.), pp. 171–201. CRC Press, Inc., Boca Raton, Florida, 1978.
79. Moss, C. W., and Dees, S. B., *J. Clin. Microbiol.* **8,** 772–774 (1978).
80. Dees, S. B., and Moss, C. W., *J. Clin. Microbiol.* **8,** 61–66 (1978).
81. MacLowry, J. D., *in* "Rapid Methods and Automation in Microbiology" (R. C. Tilton, ed.), pp. 6–9. Amer. Soc. for Microbiology, Washington, D.C., 1981.
82. Greenwood, B. M., Whittle, H. C., and Dominic-Raj Kovic, O., *Lancet* **2,** 519 (1971).
83. Tilton, R. C., *CRC Crit. Rev. Clin. Lab. Sci.* **9,** 347–365 (1978).
84. Leinonen, M., and Kayhty, H., *J. Clin. Pathol.* **31,** 1172–1176 (1978).

85. Ingram, D. L., Suggs, D. M., and Pearson, A. W., *J. Clin. Microbiol.* **16,** 656–658 (1982).
86. Welch, D. F., and Hensel, D., *J. Clin. Microbiol.* **16,** 905–908 (1982).
87. Cherry, W. B., *in* "Manual of Clinical Microbiology" (E. H. Linnette, A. Balows, W. J. Hausler, Jr., and J. P. Traunt, eds.), 3rd ed., pp. 501–508. Amer. Soc. for Microbiology, Washington, D.C., 1980.
88. Kelly, T. M., Latimer, J. M., and Balfour, L. C., *in* "Rapid Methods and Automation in Microbiology" (R. C. Tilton, ed.), pp. 302–306. Amer. Soc. for Microbiology, Washington, D.C., 1982.
89. Randall, E. L., *in* "Rapid Methods and Automation in Microbiology" (R. C. Tilton, ed.), pp. 295–297. Amer. Soc. for Microbiology, Washington, D.C., 1982.
90. McCarthy, L. R., *in* "Rapid Methods and Automation in Microbiology" (R. C. Tilton, ed.), pp. 316–320. Amer. Soc. for Microbiology, Washington, D.C., 1982.
91. Matsen, J. M., Sielaff, B. H., and Buck, G. E., *in* "Rapid Methods and Automation in Microbiology" (R. C. Tilton, ed.), pp. 286–289. Amer. Soc. for Microbiology, Washington, D.C., 1982.
92. Sielaff, B. H., Matsen, J. M., and McKie, J. E., *in* "Rapid Methods and Automation in Microbiology" (R. C. Tilton, ed.), pp. 280–285. Amer. Soc. for Microbiology, Washington, D.C., 1982.
93. Cooper, B. H., *in* "Rapid Methods and Automation in Microbiology" (R. C. Tilton, ed.), pp. 311–315. Amer. Soc. for Microbiology, Washington, D.C., 1982.
94. Snyder, J. W., *in* "Rapid Methods and Automation in Microbiology" (R. C. Tilton, ed.), pp. 290–294. Amer. Soc. for Microbiology, Washington, D.C., 1982.
95. Harrington, B. J., and Gaydos, J. M., *in* "Rapid Methods and Automation in Microbiology" (R. C. Tilton, ed.), pp. 298–301. Amer. Soc. for Microbiology, Washington, D.C., 1982.
96. Ryan, W. R., *in* "Rapid Methods and Automation in Microbiology" (R. C. Tilton, ed.), pp. 307–310. Amer. Soc. for Microbiology, Washington, D.C., 1982.
97. Johnston, H. H., and Griffiths, D. T., *in* "Rapid Methods and Automation in Microbiology" (R. C. Tilton, ed.), pp. 321–324. Amer. Soc. for Microbiology, Washington, D.C., 1982.

3

Monoclonal Antibodies in Clinical Investigations

PATRICK C. KUNG, TSE-WEN CHANG, AND VINCENT R. ZURAWSKI, JR.

I. INTRODUCTION

Throughout the history of medicine, technological breakthrough has often brought forth major advances in the diagnosis, monitoring, or therapeutic intervention of diseases. To date, many diseases, such as cancer, autoimmune disorders, and microbial infections remain difficult to prognose or diagnose. In many instances ideal treatment for the disease is still

CLINICAL BIOCHEMISTRY
Contemporary Theories and Techniques, Vol. 3

ISBN 0-12-657103-1

unavailable. The recent emergence of hybridoma technology for monoclonal antibody (MOab) production promises some solutions to these medical problems.

The antibody molecule is a remarkable product of evolution. The mechanism of its action was first postulated in the late nineteenth century. Ehrlich (1900) was one of the first to suspect that the mysterious substances (antibodies) remaining in the serum after disease or immunization were produced by cells carrying antibodies on their surface. Through the pioneering work of Landsteiner (1945) and others (Edelman, 1973; Potter, 1972), it gradually became clear that the antibody–antigen reaction possessed exquisite specificity and very high affinity. These two properties and the enormous antibody repertoire form the cornerstones of modern immunoassays and serotherapy.

Antisera have been routinely produced *in vivo* in laboratory and farm animals and sometimes in human subjects. Using this *in vivo* (or conventional) method, high-quality reagents can usually be obtained, provided that a pure antigen is employed as immunogen. But the antisera are typically polyspecific and consist of immunoglobulins of several classes and subclasses, recognizing several distinct determinants on the antigens and having a range of affinities to these determinants. The *in vivo* method of antibody production, however, has some inherent problems, such as the difficulty of obtaining specific antibodies with such a crude antigen preparation as immunogen. The batch variation in titer as well as in the composition of antibody molecules is another problem. As a consequence of these problems, the procurement of a "good batch" of antisera has often been considered somewhat of an art.

The recent emergence of hybridoma technology (Köhler and Milstein, 1975) has transformed antisera production into comprehensible and reasonably reproducible science. Using this technology an individual B-cell clone can now be immortalized through cell fusions. The resultant hybrid produces predefined antibody indefinitely.

Perhaps one of the most revolutionary aspects of the technology lies with the fact that a pure and monospecific antibody against a given antigen can be obtained even when with a very crude antigen preparation is used as immunogen. This has provided medical researchers with an extremely powerful tool for defining new hormones, lymphokines, membrane antigens of human and microbial cells, and tumor-associated antigens. These MOabs are now being tested in serotherapy for immunosuppression, controlling tumor growth, localizing tumors, and in developing novel diagnostic tests for many human diseases (Sever *et al.*, 1981).

This chapter reviews basic concepts and the process of MOab produc-

tion and examines the use of MOabs in well-documented clinical studies. Other monographs dealing with perspectives and technical advances relating to MOabs are provided in the appendix.

II. DEFINITION AND PRODUCTION OF MONOCLONAL ANTIBODY

According to Burnet's clonal-selection theory (1959), the theory most immunologists favor, a B cell is programmed by its DNA to make antibody molecules with one specificity. An antigen reacts with a surface receptor (membrane antibody) on a B cell. This reaction triggers the B cell to multiply and to secrete a high concentration of the antibody in the serum. Although each individual organism produces many different antibodies, any particular B cell (or clone of B cells) makes one kind of light chain and, essentially, one kind of heavy chain. Therefore, each cell or clone is restricted in its recognition ability to one (or a few) antigen(s) out of the millions that the individual can respond to.

The antibodies elicited by an antigen are highly diverse. Antigens often are large molecules and possess many distinct antigenic determinants. Furthermore, a single determinant usually evokes the formation of a family of antibody molecules of overlapping specificity and different affinity. Studies on myeloma proteins in the last two decades have substantiated the complexity of antibody response to an antigen (Schilling *et al.*, 1980).

It was accidentally discovered in the 1950s that myeloma proteins were produced by plasma cells (B cells at the terminal stage of differentiation) that became cancerous. The cells in each myeloma tumor (or plasmacytoma) apparently derive from a single clone (hence monoclonal). Each tumor usually produces a homogeneous antibody, called a myeloma protein, sometimes in huge amounts in serum or in other body fluids (Potter, 1972). The monoclonal nature of myeloma proteins was proved by its uniform amino acid sequence of the light and heavy chains and by its physiochemical properties (Wu and Kabat, 1970; Poljak, 1975).

Taking advantage of the inducible plasmacytomas in mice by repetitive injection of mineral oils, Porter and co-workers (1972) developed a series of murine myeloma cell lines that produced MOabs with random reactivity. To obtain a MOab with predefined specificity, the laboratories of Scharff (Yelton and Scharff, 1981) and Milstein (Köhler and Milstein, 1975) ingeniously developed the methods of cell fusion. When two different parental B cells are incubated with certain surface-active reagents such as inactivated Sendi virus (or, more popularly, polyethylene

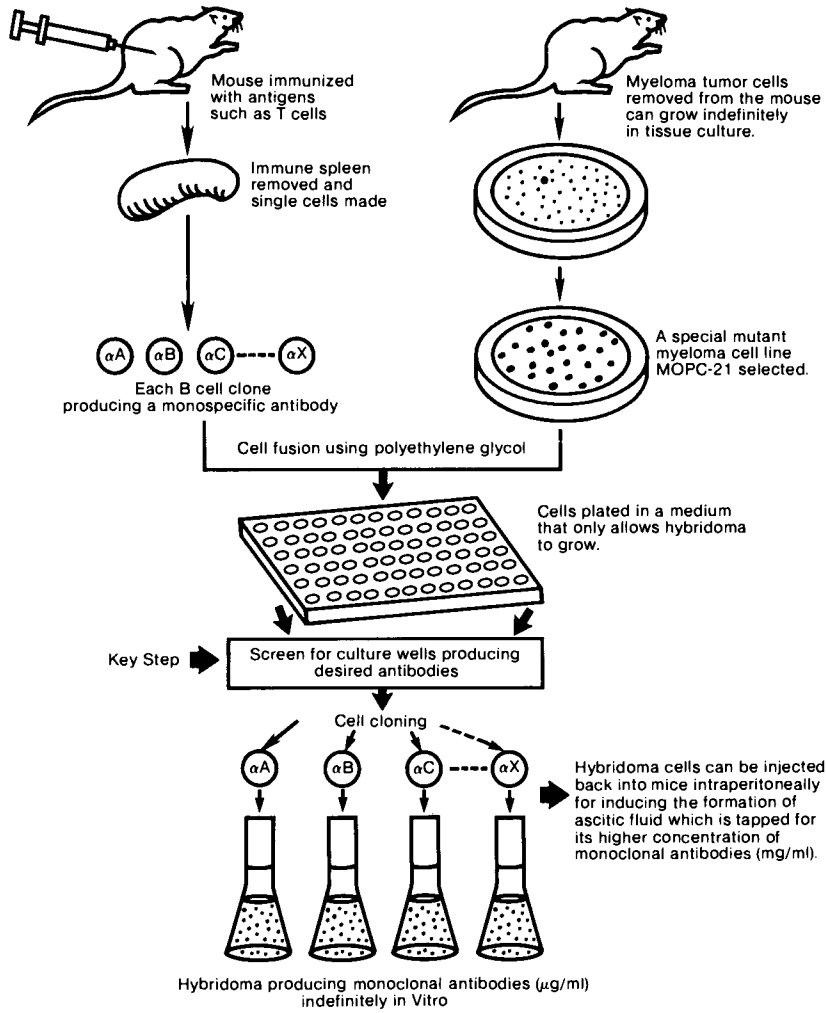

Fig. 1. Production of monoclonal antibodies in mouse. αA, αB, αC, and αD means that a B cell secretes a monospecific antibody against the antigenic determinant A, B, C, D, respectively.

glycol), cells fuse to form heterokaryons: single cells with a nucleus from each parent. Some heterokaryons become hybrids, that is, the nuclei fuse. If one of the parents is immortal and the other is a normal mortal cell, hybrids can be generated and maintained indefinitely as immortal cells that have both parental phenotypes.

Monoclonal antibody-producing immortal hybrids are prepared by fus-

ing a cultured mouse myeloma cell line with spleen B cells from an immunized mouse that serves as a source of specific antibody-producing normal parental cells. Because only a small portion of spleen cells can make antibodies to a particular antigen, only a small portion of the hybrid cells can produce antibodies to the antigen of interest. These few can be detected by testing culture media from individual clones (usually thousands) of hybrid cells. When a clone that produces an antibody of interest is detected, the antibody can be mass produced by passage of that clone as a tumor (hybridoma) in histocompatible or immunosuppressed mice. Serum or ascites fluid from mice carrying a transplanted hybridoma can have extraordinarily high levels of the MOab [e.g., 5–15 mg/ml; Kung *et al.*, (1983) and Fig. 1].

With further genetic modification and selection, some myeloma or hybridoma cells, that produce no antibody, can still function well as fusion partners in generating hybrids. Hybrids established from such partners produce only monoclonal antibodies of the spleen B-cell origin (Schulman *et al.*, 1978). Otherwise, myeloma antibody chains may freely recombine with spleen B-cell antibody chains to form hybrid molecules in the hybridized cells. Incidentally, fusions between spleen B cell with T cell or fibroblast lines yield no hybrids producing antibody (Köhler *et al.*, 1977). Fibroblasts and T cells suppress the B cells from producing antibodies. The monoclonal nature of the cloned hybridoma antibody can be verified by biochemical techniques or by sequencing of amino acids in the individual antibody chains (Schilling *et al.*, 1980).

III. MONOCLONAL ANTIBODIES FOR STUDYING CELL DIFFERENTIATION AND MONITORING DISEASES

A. Human T-Cell Development Studies

We know that T cells are involved in the regulation of immune functions and consequently are implicated as potential mediating factors in many disease processes. Using MOabs, the complexity of the T-cell system is being unraveled.

1. Total T Cells Defined by Monoclonal Antibodies

Human thymocytes and peripheral T cells can be enumerated and identified by their ability to form rosettes with sheep red blood cells (E rosette test). With the use of a panel of monoclonal antibodies, a more precise

delineation of the human T-cell compartments can now be achieved (Kung *et al.*, 1983).

Three distinct pan-T antibodies (OKT1, OKT3, and OKT11) have been derived from fusions of splenocytes obtained from mice immunized with peripheral human T lymphocytes. The antibody OKT11 defines the same lymphocyte population as the E-rosette test, whereas OKT1 and T3 react with about 90% of the $OKT11^+$ peripheral lymphocytes (Kung *et al.*, 1979; Zarling *et al.*, 1981; Ip *et al.*, 1982). The $OKT3^+$ ($T1^+$) population contains the cells involved in all known T-cell functions, as defined by our current *in vitro* tests for these functions. This would seem to be an adequate definition of a T cell (Kung and Goldstein, 1980). The $OKT11^+$ $T1^-$, $T3^-$ population has been shown to contain natural killer (NK) activity and supressor activity against autologous mixed lymphocyte reaction (AMLR) (Rohowsky *et al.*, 1983). Whether these activities are closely related to thymus (T)-derived functions will remain the subject of further investigations (Zarling *et al.*, 1981).

Biochemically, OKT1 immunoprecipitates a membrane antigen of 67,000 daltons, OKT3 an antigen of 20,000 daltons (Borst *et al.*, 1982), and OKT11 an antigen of 52,000 daltons (Cotner *et al.*, 1981). All three antigens are composed of a single polypeptide chain. The antibody OKT11, but not OKT1 nor OKT3, blocks E-rosette formation of lymphocytes (Verbi *et al.*, 1982; Van Wauwe *et al.*, 1982). The antibody OKT3 exhibits many interesting properties. It is a very potent T-cell mitogen, induces the secretion of γ-interferon, and blocks cytotoxic T-cell activity (Chang *et al.*, 1981, 1982). Because of these properties, it was postulated that the OKT3 antigen might be associated with the T-cell antigen receptor. Meuer *et al.* (1983a,b) recently supported this claim. They showed that OKT3 antigen was linked to idiotypic structures defined by MOabs against antigen-specific cytotoxic T-cell clones. Although some of the more recent data from several laboratories contradicted this postulate, they demonstrated that OKT3 antibody did not inhibit the recognition of target cells by killer T cells but blocked the ensured lethal hit phase of the cell killing by the T cells (Tsoukas *et al.*, 1982; Landegren *et al.*, 1982).

The antigens recognized by OKT1, OKT3, and OKT11 antibodies are stable surface markers of T lineage cells, although OKT1 also has been reported to be present on B-chronic lymphocytic leukemia cells (Schrof *et al.*, 1982).

2. T-Cell Subsets Defined by Monoclonal Antibodies

Two distinct groups of antibodies have been developed to define nonoverlapping human T-cell subsets (Kung and Goldstein, 1980; Zarling and Kung, 1980); they were named the OKT4 series, and OKT5, OKT8 monoclonal antibodies.

a. Defining Helper–Inducer Subset. The OKT 4 antibody reacts with approximately 65% of peripheral T cells (Kung and Goldstein, 1980; Ip *et al.*, 1982). This subset contains helper cells capable of inducing B-cell differentiation and of augmenting the generation of cytotoxic T cells and cells involved in delayed type hypersensitivity (Kung and Goldstein, 1980). Recently, it was reported that lectin activated $OKT4^+$ cells behaved as radiosensitive suppressor cells (Thomas *et al.*, 1982). Several laboratories also claimed that precursors and effectors of killer T cells against HLA-D antigens were associated with the $OKT4^+$ subset (Biddison *et al.*, 1982). These findings suggest the existence of finer subsets within the $OKT4^+$ subset.

In addition to the first OKT4 antibody, we generated four other distinct OKT4-like antibodies, namely OKT4A, T4B, T4C, and T4D (Kung *et al.*, 1981a). The OKT4 series all apparently identify the same T-cell subset and have immunoprecipitated membrane molecules of a similar molecular weight (50,000). Recently, we observed that T cells from a small fraction of the black ethnic group displayed unusual OKT4 phenotypes; their T cells are $OKT4^-$ but react with T4A, T4B, T4C, or T4D. T cells from other ethnic groups studied to date are reactive with all the OKT4 antibodies. Based on this and the biochemical data mentioned we propose that the OKT4 antigen possesses polymorphic determinants (Bach *et al.*, 1981). Biddison *et al.* (1982) proposed that OKT4 antigen was a part of T-cell recognition structure because some of the OKT4 antibodies were capable of blocking the killing of DR-bearing target cells by the $OKT4^+$-killing T cells.

The $OKT4^+$ subset secretes a number of soluble factors upon proper stimulation. The $OKT4^+$ cells stimulated by the OKT3 antibody and lectins were demonstrated to be the source of immune interferon (Chang *et al.*, 1982).

b. Defining Cytotoxic–Suppressor Subset. The antibodies OKT5 and OKT8 react with approximately 35% peripheral T lymphocytes (Kung and Goldstein, 1980). Both antibodies immunoprecipitate antigens composed of two polypeptides of 30,000 and 32,000 daltons under denaturing conditions (Cotner *et al.*, 1981); they recognize distinct antigenic determinants of the surface marker because one antibody does not block the binding of the other antibody to T cells and the two antigenic determinants exhibit differential sensitivity to reduction and alkylation (Snow *et al.*, 1983). The authors suggested, however, that the monoclonal reagents detect a dimeric structure which migrates as a 76,000- or 67,000-dalton glycoprotein; under reducing conditions the monomeric structure has a molecular weight of 34,000.

The $OKT8^+$ cells contain precursors and effectors of cytotoxic T

cells against allogeneic HLA-A and -B antigens, as well as against altered-self antigens. The latter conclusion was reached in three separate systems, including influenza and Epstein–Barr virus (EBV)-infected cells and trinitrophenol-modified cells (Biddison *et al.*, 1981). Several groups observed that the OKT8 antibody could inhibit, to a lesser degree than OKT3 antibody, cell lysis by cytotoxic T cells (Chang and Gingras, 1981). It was postulated that the OKT8 antigen is associated with the recognition structure of the killer cells.

The $OKT8^+$ population was further shown to contain suppressor cells as assayed in the suppression of mixed lymphocyte reaction (MLR; Kung and Goldstein, 1980) and of B-cell differentiation (Thomas *et al.*, 1982). The antibody does not neutralize the suppression when added to the assay culture. It was claimed that the $OKT8^+$ cells did not interact directly with B cells but exerted their suppressive effect on the radiosensitive $OKT4^+$ cells. The $OKT8^+$ suppressor cells are also radiosensitive (Thomas *et al.*, 1982).

Unlike the $OKT4^+$ cells, the $OKT8^+$ cells did not respond to soluble antigens tested *in vitro* but proliferated well upon the presentation of allogeneic cells and lectins (Kung and Goldstein, 1980).

c. T-cell Subsets Occupy a Distinct Microenvironment. The $OKT4^+$ and $OKT8^+$ cells were found to be localized in distinct microenvironments in several tissues studied (Janossy *et al.*, 1980, 1981). In tonsils and nodes the $OKT4^+$ cells occupy areas around the B-cell corona and in the germinal center and are closely associated with DR^+ dendritic cells scattered in the tissues. In the intestine, the $OKT8^+$ cells were preferentially situated in the epithelium, and the $OKT4^+$ cells were found in the lamina propria. The ratio of $OKT4^+$ to $OKT8^+$ cells in the lymph nodes was found to be higher than that found in the peripheral blood.

A few percentages of peripheral T cells in blood and nodes are both $OKT4^+$ and $T8^+$ (Janossy *et al.*, 1981). The function of these cells is unknown.

d. Activated T Cells Defined by Monoclonal Antibodies. Several monoclonal antibodies produced in our laboratories and laboratories of others appeared to react with alloantigen- and lectin-stimulated T cells (Cotner *et al.*, 1981; Yokoi *et al.*, 1982). Although further studies indicated that their reactivities were not limited to T-lineage cells, they have been useful in describing the physiology of T-cell activation.

It was reported by two separate laboratories that OKT9 antibody recognized transferrin receptors, which are expressed during the S, G_2, and M phases of the cell cycle (Greaves *et al.*, 1981). The antibody OKIal, which reacts with B cells, monocytes, and activated T cells, identifies the non-

polymorphic determinant of the HLA-DR antigen. We recently reported that OKT10 immunoprecipitated an HLA-like class I antigen and that it was normally expressed on all thymocytes, bone marrow B cells, and some peripheral NK cells (Reinherz *et al.*, 1980a; Cotner *et al.*, 1981). Several laboratories produced MOabs against the IL-2 receptor, which is restricted to activated T cells and absent from resting T cells nor other proliferating cell types (Yokoi *et al.*, 1982; Cotner *et al.*, 1983).

A likely question: "How many more unique anti–T-cell monoclonals will be forthcoming?" An understanding of T-cell idiotypes is necessary for this question to be answered (Suciu-Foca *et al.*, 1982, 1983; Meuer *et al.*, 1983a,b). The T cells, like B cells, express a single clonal response; that is, a T cell will respond only to a given antigenic determinant. The antigen recognition domain on the T-cell receptor molecule is called an idiotype, idiotypes are as numerous as antigens. Therefore, the potential exists for the production of numerous MOabs against these individual T-cell idiotypes. This should provide us with important reagents for monitoring immune responses to a specific antigen.

3. Cell Differentiation Studies With Monoclonal Antibodies

Using the panel of MOabs we have described, Reinherz *et al.* (1980a) have outlined the developmental stages of human T cells (Greaves *et al.*, 1981). Bone marrow prothymocytes express OKT10, but lack OKT3, T4, T5, T6, T8, and T11 antigens. As they enter the thymus and multiply, their progenitor cells (in the cortex) acquire, concomittantly, OKT3, T4, T5, T6, T8, and T11 antigens on their cell surface. These cortical thymocytes move to the medulla, and there they differentiate either into $OKT3^+$, $T4^+$, $T6^-$, $T8^-$, $T11^+$ or $OKT3^+$, $T4^-$, $T6^-$, $T8^+$, $T11^+$ cells resembling the phenotypes of peripheral T-cell subsets (Table I). It is

TABLE I

Change of Cell Surface Markers during Human T-Cell Differentiation

Developmental stage	OKT4	OKT6	OKT8	OKT11/OKT3	OKT10
Prothymocytes	−	−	−	−	+
Cortical thymocytes	+	+	+	+	+
Medullary thymocytes	+ or −	−	−	+	+
		−	+	+	+
Peripheral T cells[a]	+ or −	−	−	+	+
		−	+	+	+

[a] Activated T cells express OKT9, OKT10, Tac, and HLA-DR antigens.

noted that OKT6 antigen (thymus leukemia antigen) is restricted to cortical thymocytes. But, this antigen was recently found on epidermal Langerhans cells (Fithian *et al.*, 1981; Chu *et al.*, 1982) and cells of neural crest origin (Kemshead *et al.*, 1982).

B. Pathology and Diagnostic Research Using Anti–T-Cell Antibodies

1. T-Lymphocytic Neoplasms

The availability of the panel of MOabs against human T cells and flow cytometry have enabled clinical researchers:

1. To define and enumerate T cells and their precursors more precisely.
2. To derive the OKT4/OKT8 cell ratio and the absolute number of circulating T cells and T-cell subsets in subjects studied.
3. To correlate the T-cell alteration with onset, progression, and resolution of diseases in patients.
4. To investigate immunosuppressive therapy using these antibodies.

The aforementioned studies form the basis for classifying leukemias and lymphomas of T-cell origin into recognizable maturational states equivalent to those of normal T-lineage cells. It was found that tumor cells of cutaneous T-cell lymphomas (CTCL) were $OKT3^+$, $T4^+$, $T6^-$, $T8^-$, a phenotype corresponding to the peripheral helper–inducer T cells (Kung *et al.*, 1981b). The observation concurs with the results previously obtained by *in vitro* functional studies. In the advanced stage of this disease $OKT4^+$ cells account for virtually all circulating T cells in CTCL patients. These antibodies however, are not useful for detecting CTCL tumor cells in early stages of the disease. Other MOabs such as BE1 and BE2, developed by Berger *et al.* (1982), seem more promising for such uses. The antigens BE1 and BE2 are not expressed on normal resting lymphocytes, but are present on a small percentage of abnormal circulating lymphocytes in early CTCL.

In situ studies on T cells infiltrating the epidermis in CTCL patients have shown that, in some cases of this disease, activated T lymphocytes predominate in the epidermal lesions. These activated T cells in one case lost the mature helper T-cell phenotype and were reactive with antigens identifying activated or immature T cell [expressing OKT9, OKT1, OKT10, and complement receptor; Kung *et al.*, (1981b)]. This observation was extended in other studies using MOabs to detect Thy-1 antigen, Ia-like antigens, and the receptor for transferrin (Haynes *et al.*, 1981). These antigens were present on epidermal lymphocytes and absent from

circulating peripheral blood T lymphocytes in patients with CTCL. These observations suggest subclone formation of the malignant lymphocytes in the epidermis of CTCL patients, perhaps caused by influences found in the epidermal microenvironment (Kung *et al.*, 1981b).

Several laboratories reported that lymphoblastic lymphomas, T-cell acute lymphoblastic leukemia, and tumors with features of T-immunoblastic sarcoma or the multilobated lymphoma of Pinkus expressed prothymic and intrathymic phenotypes (Knowles and Halper, 1982).

2. Herpes Infections and Acquired Immunodeficiency

In mononucleosis induced by acute EBV, absolute numbers of $OKT3^+$ T cells are increased. The $OKT4^+$ T cells make up only a small percentage of total T cells in the patient, but their absolute number is in the normal range. The number of $OKT8^+$ T cells is increased several times, and these cells form the majority of the T-cell population. OKT4/OKT8 cell ratios in these patients averaged 0.49; the controls averaged 1.69, clearly showing the imbalance of immunoregulation. During the acute phase there is also an increase of activated T cells expressing HLA–DR antigens. These observations correlate well with the presence of cytotoxic T cells for EBV-infected lymphocytes and acquired immunodeficiency in these patients. Convalescence, however, is associated with a return to normal T-cell subsets and immune functions (De Waele *et al.*, 1981).

Similar immunoregulatory imbalance was observed in patients with acute cytomegalovirus (CMV) infections (Rubin *et al.*, 1981). There was an absolute and relative decrease in the $OKT4^+$ cells and a simultaneous increase in the $OKT8^+$ cells. The prolonged period of clinical convalescence noted in the CMV patient is reflected in the lymphocyte subset measurement with only partial return of normal T cell toward normal levels as long as 300 days after the acute episode of illness.

Very recently, Kornfeld *et al.* (1982) studied a large number of male homosexuals. They found that both symptomatic and asymptomatic homosexuals had reduced OKT4/OKT8 ratios and that there was a significant trend toward the lower ratios in the symptomatic subjects who developed Kaposi's sarcoma and opportunistic infections. The immunoregulatory imbalance in these subjects were apparently not associated with the use of inhaled nitrites.

3. Autoimmune Diseases

Previous studies have suggested that many immune disorders in patients with autoimmune diseases occur as a consequence of defects in suppressor T-cell function. The recent availability of a range of MOabs

against human T-cell surface markers also has prompted the investigations of T-cell imbalance in these patients in many laboratories.

Systemic lupus erythematous (SLE) and rheumatoid arthritis (RA) have long been considered two prototypic autoimmune diseases. In active SLE patients the percentage of $OKT3^+$ cells has been found to be reduced in contrast to normal and inactive SLE patients (Morimoto *et al.*, 1980). In addition, a selective decrease in $OKT8^+$ T cells in most patients was observed. Serial analysis of several patients showed a significant correlation between the level of $OKT8^+$ cells and clinical disease activity. A decrease in the number of $OKT8^+$ T cells in the peripheral blood of patients with multiple sclerosis (Reinherz *et al.*, 1980b), RA (Fox *et al.*, 1982), and primary biliary cirrhosis (Bhan *et al.*, 1982a) also has been observed.

It has been observed that synovial fluids from patients with RA contained significantly more activated T cells and increased $OKT8^+$ T cells. These findings demonstrated that the lymphocytes at the site of inflammation differ significantly from lymphocytes present in the peripheral blood of the same patients. The $OKT8^+$ T cells may migrate to synovial tissues, where they become activated (Fox *et al.*, 1982).

These studies suggest that aberrations in the $OKT8^+$ subset may be critical for the pathogenesis of autoimmune diseases.

4. Monitoring Organ Transplantation

The role of T-cell subset monitoring in renal allograft recipients has been extensively studied. Investigators at the Massachusetts General Hospital observed a highly significant correlation between allograft rejection and the OKT4/OKT8 ratios in peripheral blood. The recipients with a normal T4/T8 ratio had a higher incidence of rejection than those with a lower T4/T8 ratio in the first few months after HLA-nonidentical renal transplantation, when they were treated with azathioprine and prednisone, with or without antithymocyte globulin (Cosimi *et al.*, 1981). Recently they suggested that the T4/T8 ratio might also correlate with the reversibility of graft injury and the presence of a distinctive glomerulopathy (Cosimi, 1983).

Platt *et al.* (1982) examined interstitial mononuclear cell populations in renal graft rejection in tissue sections and reported that most infiltrating cells are T cells, the greater proportions of which are $OKT8^+$ cells. They postulated that these $OKT8^+$ cells may play an important role in mediating renal graft rejections.

In human skin allografts, $OKT4^+$ cells were 1.5–3 times more numerous than $OKT8^+$ cells in the graft dermis and graft bed. In contrast, $OKT8^+$ cells were relatively abundant in the epidermis and hair follicles.

The distinct subset distribution pattern may be relevant to the elucidation of the rejection mechanism (Bhan *et al.*, 1982b).

These studies represent the exploratory phase of organ transplantation. Different immunosuppressive protocols used at each transplant center and other variables, including infection and disease, may result in different types of T-cell subset alterations. Additional studies should enhance the utility of this new approach to the immunologic analysis of patients with perturbed immune states.

Since 1978, methodology for T-cell enumeration has evolved from the single E-rosette test to a panel of monoclonal antibody tests. The E-rosette test identifies a cell of thymus lineage but does not distinguish thymocytes from peripheral T cells. With MOabs we can now define T-lineage cells at distinct stages of their maturation, infer their functional properties, and readily obtain reproducible and consistent results. This monoclonal testing represents a major advance in clinical immunology.

The ability to enumerate major T-cell functional subsets has provided insight into the immunoregulatory alterations associated with the onset, progression, and convalescence of many disorders—including infection, aging, autoimmunity, and cancer. Monoclonal reagents also may prove to be highly efficacious for immunosuppression therapy in organ transplantation and in the control of autoimmune problems.

C. Monoclonal Antibodies for Diagnosis of Infectious Diseases

With the dramatic rise of antibiotic-resistant bacteria and prevalence of viral infections, the need for more specific and sensitive diagnostic tests and targeted therapy has become of paramount importance to medical research. Monoclonal antibodies of diagnostic potential have been prepared in research laboratories against a battery of viruses (Robert Guroff *et al.*, 1981; Schmaljohn *et al.*, 1982), bacteria (Hansen *et al.*, 1982; Hasty *et al.*, 1982) and parasites (Cruise *et al.*, 1981; Yoshida *et al.*, 1981; Wood *et al.*, 1982). But most of these studies are still preliminary. For the following review we focus on the MOabs intensively investigated in clinical laboratories.

1. *Diagnosis of* Neisseria gonorrhoeae

Tam and co-workers (1982) have developed many MOAb against the principle outer membrane protein (prI; 30,000–37,000 daltons) of *N. gonorrhoeae*. Among these antibodies selected for very broad reactivity against strains of *N. gonorrhoeae*, many were found to cross-react with other *Neisseria* species, decreasing their diagnostic value. Whereas those

antibodies recognize only a subset of the *N. gonorrhoeae*, reference strains are pooled into a mixture and found to be more specific for the diagnosis. To identify the entire spectrum of *N. gonorrhoeae* without compromising the selective reactivity of each antibody, three monoclonal antibodies reacting with mutually exclusive serotypes (1 MOab against prI4, 2 MOabs against prIB) were pooled into a cocktail as diagnostic reagents. When the antibody cocktail was tested on the 719 isolates with immunofluorescence (IF) assay, 99.6% of the isolates were correctly identified.

2. *Diagnosis of* Chlamydia trachomatis

Direct diagnosis of chlamydial infection is presently not possible. Since chlamydiae are obligate intracellular parasites, cultures diagnosis require the infection of mammalian cell lines *in vitro*. To improve sensitivity, cultures are generally performed in duplicate. One culture is stained with iodine 3 days after infection. The other culture is used for secondary passage onto yet another culture, which is then stained 3 days postinfection. Iodine reacts with the glycogen-rich inclusion body of the *Chlamydia*-infected cells. Of all specimens detected by culture, approximately 65–80% are detected in the first passage, and 20–35% are detected by the second passage.

Stephens *et al.* (1982) recently reported an MOab (I-H8) that reacted with a 39,000-dalton outer-membrane protein of all members of *C. trachomatis* without yielding cross reaction to the closely related *C. psittaci*.

In preliminary studies, the IF test with the I-H8 antibody was compared to the iodine test for the detection of *C. trachomatis* infections of the culture. The IF method detected 8–11 times more inclusions than the iodine method and allowed an accurate account of the inclusion at 2 hr postinfection. Staining of a parallel set of infected cells with iodine, however, failed to yield visual evidence of infection until 48 hr, with 72 hr required for routine quantitation. Similar enhanced sensitivity (approximately fourfold) has been demonstrated by comparing IF MOab stains to Giemsa stains of *Chlamydia*-infected cells.

Immunofluorescence tests with MOabs have also been performed directly on patients' urethra or cervix specimens streaked onto a microscope slide for the testing. This direct IF test seems to be very good in sensitivity and in predicting positive and negative smears.

3. *Diagnosis of Herpes Simplex Viruses*

For the purposes of typing herpes simplex viruses (HSV) in culture, Goldstein *et al.* (1983) have developed a panel of four MOabs that clearly distinguish HSVI from HSV2. Monoclonal antibody 3 G11 reacts with an

HSV1 specific 80,000–120,000 dalton glycoprotein complex, MOAb 6-A6 with an HSV2 specific protein of 140,000 daltons, MOab 6-E12 with our HSV2 specific protein of 55,000 daltons, and MOab 6H11 with an HSV2 protein of 38,000 daltons. A comparative analysis by independent methods, namely, immunoperoxidase labeling with HSV type specific rabbit antisera, IF with MOab, and restriction endonuclease analysis of viral DNA, was carried out. Results of MOab typing demonstrated 100% concordance with restriction endonuclease analysis of viral DNA in 117 out of 122 isolates; the test was unambiguously typed a either HSV1 or HSV2. In 5 different isolates from 3 patients, the MOab typed a mixed infection of HSV1 and HSV2. The rabbit antisera, however, were capable of typing only 66% of the 122 isolates; of the remaining isolates, 34% yielded indeterminate antigen patterns for which a definitive diagnosis could not be made. In addition to their utility in culture system, the MOab may also provide sufficient specificity to enable typing of HSV directly or of primary clinical specimens. For this purpose, cells were obtained by scraping herpes lesions with swabs and then smearing the cells onto slides for the IF tests. Forty-eight of the 54 specimens (88%) in which HSV was isolated in tissue culture had HSV antigens detected in the IF tests. In addition, the MOab detected HSV antigens in six clinically suspect specimens from which the culture method did not reveal infectious virus. The MOab did not detect HSV antigens in any of the 43 specimens obtained from the 43 control patients with unsuspected HSV infection (Nowinski *et al.*, 1983).

4. Diagnosis of Hepatitis B Virus Infection

Hepatitis B surface antigen (HBsAg) is found in the serum of (HBV) hepatitis B virus-infected patients during the acute phase of the disease and also in the serum of patients with chronic active and chronic persistent hepatitis of HBV origin. The presence of HBsAg in patient serum is considered to be a reliable marker of HBV infection.

Wands and Zurawski (1981) have produced and characterized high-affinity MOab, IgM, and IgG to specific determinants on HBsAG. Some of these antibodies (5D3, A5C3, and A5C11), broadly reactive with HBV serotypes, have been utilized to develop highly sensitive immunoradiometric assays (IRMA) for detection of HBsAg in human serum at subnanogram levels (Wands *et al.*, 1981). Goodall *et al.* (1981) have also devised a radioimmunoassay (RIA) that can measure the presence of HBsAg in the serum of HBV-infected individuals. The monoclonal IRMA using 5D3, A5C3, and A5C11 can detect viral determinants in HBsAg–anti-HBsAg immune complexes formed in anti-HBsAg excess. The presence of HBsAg in such complex is frequently undetectable using RIA

which relies on conventionally produced polyclonal anti HBsAg (Shafritz *et al.*, 1982). It is therefore possible that presumably undetected exacerbation of HBV-related hepatitis could be detected by the IRMA. Moreover, the presence of such HBsAg-positive individuals in the blood donor population would likely be revealed.

A monoclonal test for HBsAg detection in blood banking has been approved by the FDA.

The previous discussion clearly demonstrates that use of MOabs provides exceeded diagnostic specificity and reproducibility when compared with conventionally prepared antisera. One can foresee the continued development of MOab products and a concomitant improvement in the quality of microbiological diagnosis.

D. Monoclonal Antibodies Reactive with Tumor-Associated Antigens

Numerous monoclonal antibodies against human cancer cells have been reported in the literature (Yeh *et al.*, 1979; Bast *et al.*, 1981; Woods *et al.*, 1982). Most of the antigens have, however, not yet been well characterized by their normal cell distribution, specificity among various tumor types, correlation of their presence with tumor progression, and regression. To illustrate the utility of monoclonal antibody in cancer research, we discuss one extensively studied system.

1. Monoclonal Antibody against Colorectal Tumor Cells

Koprowski *et al.* (1979) established a hybridoma synthesizing an antibody designated 1116 NA-19-9 (19-9) by immunization of BALB/c mice with a human colorectal carcinoma cell line. The antibody was found to react with a sialylated lacto-*N*-fucopentaose II, an oligosaccharide biochemically related to Lewis a-blood group substance (Magnani *et al.*, 1982). This carbohydrate antigenic determinant (CA 19-9) was found on a glycolipid isolated from meconium and tumor cells. Recent studies suggest that CA 19-9 may also be found on a mucin-like glycoprotein in the sera of cancer patients (J. Magnani and T. Klug, personal communication). The distribution of CA 19-9 in normal and malignant tissues has been described by Atkinson *et al.* (1982) using immunohistopathological methods. In normal tissues, staining was found in some cells of the columnar epithelium in the pancreas, stomach, liver, gall bladder, and in bronchial glands of the lung. Positive staining of tumor cells was observed in most patients with adenocarcinomas of the pancreas (86%), stomach (89%), and with tumors of the colon (59%) and gall bladder (40%). Addi-

tionally, Charpin *et al.* (1982) have also identified CA 19-9 in 57 of 121 (47%) ovarian tumors by immunohistological staining. Among these, 28 of 37 (76%) mucinous tumors were found to have CA 19-9 present; CA 19-9 was also present on normal ovarian epithelial cells.

Although the CA 19-9 is associated with normal cells, its presence in the sera of healthy subjects appears to be nearly always at a very low level. Less than 0.5% of blood bank donors, for example, have been found to have higher than normal CA 19-9 level in their sera. Elevated serum concentrations of CA 19-9, however, have been demonstrated in many patients with gastrointestinal tumors. Del Villano *et al.* (1983) have developed a forward sandwich RIA for the quantitative measurement of serum CA 19-9. The RIA has detected elevated levels of CA 19-9 in 79% of patients with adenocarcinomas of the pancreas and 50% of patients with adenocarcinoma of the stomach (Table II). Patients with colorectal adenocarcinoma may also have elevated levels of CA 19-9 in their serum; relatively few patients with early-stage disease, however, have been found with CA 19-9 levels above 37 units/ml (Table II). The CA 19-9 seem also much less likely to be found in the sera of lung cancer or breast cancer patients. The CA 19-9 has also been used in conjunction with carcinoembryonic antigen (CEA). The CA 19-9 and CEA were used to monitor the reoccurrence of colorectal tumors in 10 out of the 20 cancer patients. Increases in both markers occurred at the same time with an average lead time before identification of recurrence of 4.9 months. In five patients

TABLE II

Cancer Patients with Elevated Serum Levels of CA 19-9[a]

Diagnosis	No. of patients positive/total examined	Serum levels elevated (%)
Pancreatic adenocarcinoma		
Resectable	4/5	80.0
Nonresectable	63/80	79.0
Gastric adenocarcinoma	9/18	50.0
Colorectal adenocarcinoma		
Dukes's A	2/27	7.5
Dukes's B	5/30	17.0
Dukes's C	9/19	47.0
Advanced untreated	40/69	58.0
Lung cancer	4/32	12.0
Breast cancer	6/57	10.0

[a] Patients whose sera had ≥37 units/ml were considered to have elevated CA 19-9 levels.

CA 19-9 serum level increased prior to CEA. Similarly, in another five patients, CEA levels increased prior to CA 19-9. Five patients were negative for both markers prior to recurrence. These data suggest that CA 19-9 and CEA can be used together for monitoring resected colorectal cancer patients with some advantage. But, further studies will be required to verify these findings (Sears *et al.*, 1982).

Levels of CA 19-9 below 37 U/ml have also been found in most benign diseases (Del Villano and Zurawski, 1983; Del Villan *et al.*, 1983). In fact, <2% of sera are found to have elevated CA 19-9 levels. Among patients with such diseases as primary biliary cirrhosis, chronic active hepatitis, alcoholic liver diseases, and cholecystitis with obstructive jaundice, however, 16–33% of sera from these patients contain elevated levels of CA 19-9. Therefore, with appropriate precautions relating to certain patient groups, it seems that determination of CA 19-9 levels in patient sera may provide useful information to physicians in diagnosing adenocarcinomas of the pancreas, stomach, or hepatobiliary system.

Most human tumor antigens (except known viral antigens) identified to date by MOabs may turn out to be expressed on fetal cells or on prescursor cells of a given cell lineage. During tumorigenesis the level of tumor-associated antigens may rise significantly in a specific body compartment, such as blood (Del Villano and Zurawski, 1983; Del Villano *et al.*, 1983) or serous fluid (Woods *et al.*, 1982). Quantitative measurement of the tumor antigen levels in body fluids may provide physicians with important information on tumor burden, type of tumor, and tumor metastases. It is foreseeable that MOab-based tests will play an important role in the management of cancer patients.

IV. MONOCLONAL ANTIBODIES FOR SEROTHERAPY

A. Organ Transplantation

Heterologous antilymphocyte globulins (ALG) or antithymocyte globulins (ATG) have been shown to be effective in reversing established renal rejection. When used alone it can significantly prolong skin-graft survival in allografted burn patients; and it can reverse bone marrow aplasia. When used in combination with other agents it delays the onset of acute rejection and reduces significantly the amount of steroids required in the early postoperative period (Cosimi, 1983). These antisera, being polyspecific, often react with other hemopoietic cell types in addition to T-lineage cells. The polyspecific reactivities are major problems in dissecting the immunosuppressive mechanism of ALG. Additionally, the titers and the

composition of these antisera vary from batch to batch. The availability of the monoclonal antibodies promise improvements in production method, and, more importantly, offers a selective and specific abolition of T cells and T-cell subsets to achieve immunosuppression.

Cosimi and co-workers (1981) first reported that the infusion of OKT4 MOab alone into kidney-grafted cynomolgus monkeys significantly prolonged the viability of the grafts. They subsequently treated several human renal-allograft recipients with OKT3 and observed reversal of the first rejection episode that occurred, despite the standard protocol of prednisone and immuran given (Cosimi *et al.*, 1982). A randomized trial to test the efficacy of OKT3 antibody in renal transplant patients has been initiated, and preliminary data look very encouraging. Many of the OKT3-treated patients developed antimouse antibody, a factor that will apparently limit the duration of such therapy.

Two groups also reported their very preliminary findings on the use of OKT3 in preventing acute graft-versus-host disease (GVH) in bone marrow transplantation. Prentice *et al.* (1982) indicated that acute GVH in transplant recipients was reduced from 79 to 18% when OKT3 treatment was added to prophylactic methotrexate. In their studies, the marrows from the donors were incubated with OKT3 before infusion. Under this condition the OKT3-coated T cells were presumably opsonized and removed *in vivo*.

Using mismatched donor marrow treated with OKT3 plus rabbit complement *in vitro,* Hayward *et al.* (1982) reported a failure of the antibody to prevent GVH in a patient with severe combined immunodeficiency.

The mechanism of cyclosporin A has been investigated with MOabs. The drug induces immunosuppression by selectively affecting the $OKT4^+$ T cells with relative sparing of the $OKT8^+$ population (Cosimi, 1983).

B. Treating T- and B-Cell Lymphomas

Miller and co-workers (1981) reported treatment of three CTCL patients with an anti-pan T-cell antibody. They noted a dramatic fall in white blood cells (WBC) with return to pretreatment levels over 24 hr, but in one case the WBC count stabilized after 2 weeks of therapy. There was clearing of skin lesions and a decrease in lymphadenopathy. Remission, however, was not achieved. This lack of efficacy may be related to antigenic modulation of the leukemic cells, occurrence of host anti-mouse antibody, and insufficient dosage.

In another study (Miller *et al.*, 1982), MOabs were raised to idiotypic determinants on B-cell lymphoma cells. Following intravenous infusions with the antiidiotypic antibody, the patient with the tumor went into a

remission. His blood content, liver, spleen, and enlarged lymph nodes normalized. The patient has remained in remission for more than 1 year.

V. *IN VITRO* TREATMENT OF BONE MARROW FOR AUTOLOGOUS TRANSPLANT

Patients with blood cancers are sometimes given supralethal doses of chemoradiotherapy to eliminate tumor cells. After the chemoradiotherapy, suitable marrows are infused into the patients to regenerate their blood and immune systems. The major obstacle to aggressive exploration of autologous bone marrow transplantation in the lymphohematopoietic malignancies is the problem of removing tumor cells in the marrow of patients in remission. Ritz *et al.* (1982) reported some encouraging data on the use of anti-CALLA (common acute lymphocytic leukemia antigen) MOab in leukemic patients. Of the non-B, non-T or common ALL (acute lymphocytic leukemia) cells, 85% were CALLA$^+$. Autologous bone marrows were collected from the ALL patients in second remission, treated *in vitro* with anti-CALLA MOab and rabbit complement, and cryopreserved in liquid nitrogen. Patients then received oblative chemotherapy and total body irradiation prior to infusion of the treated marrow. All four patients treated in this fashion successfully engrafted their bone marrow, and two have remained in remission for more than 1 year after the transplantation.

Kaizer *et al.* (1982) presented a case report involving *in vitro* treatment of marrow with a pan–T-cell MOab (anti-Leu-1). The patient under study had a testicular and hematologic relapse. After reinduction therapy, marrow was collected from the patient, treated *in vitro* with anti-Leu-1$^+$ rabbit complement, and reinfused after lethal doses of chemoradiotherapy. The patient exhibited prompt hematologic recovery and his posttransplant course has been benign over more than 20 months of follow up. Most of the marrow lymphocytes are morphologically mature and do not bear the surface antigens characteristic of his initial tumor. The authors suggest that the antibody treatment may have been effective in eliminating all clonogenic tumor from the remission marrow.

In summary several problems are associated with serotherapy using MOabs:

1. Patients often developed antibodies against mouse monoclonals, sometimes directed toward the idiotype of the mouse antibody. This problem might be overcome by tolerizing the patient with large doses of murine antibody. The availability of human MOab (Olsson and Kaplan, 1980;

Eisenbarth *et al.*, 1982) may offer some advantage over murine reagent, although the possibility of antiidiotypic responses to the human monoclonals would still exist.

2. During serotherapy many monoclonals induce antigenic modulation on tumor cells, and their effects are thus diminished (Ritz *et al.*, 1982). This problem may be circumvented by spacing treatments or by selecting a different isotype of the same antibody with minimal modulation effect (Foon *et al.*, 1982). Antigens on primary and metastatic tumors are heterogeneous. Multiple antibodies that recognize different antigens on the same tumor cells would be required to solve this problem. In the next few years, mouse monoclonal antibody–based serotherapies for immune suppression and for controlling cancerous growth may finally gain acceptance.

VI. MONOCLONAL ANTIBODIES FOR *IN VIVO* RADIOIMAGING OF TUMORS

The *in vivo* diagnosis with radioimaging of tumors has been an important segment of medical investigation (Mach *et al.*, 1981). The major problems associated with radioimaging have been (1) the choice of radionuclides and their conjugation to antibodies, and (2) the availability of suitable antibodies against the tumor cells in question. The advent of hybridoma technology has offered a solution to the second problem. For routine analysis, radiologists prefer the use of $F(ab')_2$ and Fab of an antibody because the fragments are cleared sooner from the circulation of patients. Because MOabs are pure antibody fragments and can readily be prepared, the specific uptake of the labeled antibody by tumor cells is higher.

J. F. Chatal, (personal communication) reported a preliminary scintigraphic study in patients injected with ^{131}I-radiolabeled whole monoclonal antibody (17-1A) to colorectal carcinoma. The aim of their study was to test the differential uptake of the labeled antibody in colonic and noncolonic cancers. They observed that the photoscanning was considered positive in 19 out of 23 colorectal carcinoma sites, of which 4 were primary, 3 were local recurrences, 9 were liver metastases, and 3 were lung metastases. In general, tumor uptake showed the best contrast 4–5 days after injection.

In another study Epenetos *et al.* (1982) used two ^{123}I-radiolabeled MOabs, HMFG1 and HMFG2, to detect primary and metastatic ovarian, breast, and gastrointestinal neoplasms by external body scintigraphy in 20 patients with advanced disease. They claimed that tumor became visible

3–18 hr after injection of labeled antibody. The presence of antibody in the tumors was confirmed by immunoperoxidase staining of surgically removed tissues. The mean tumor uptake of radiolabel was 0.6% of the injected amount.

The field of monoclonal antibody in radioimaging is still at an early stage of development (Mach *et al.*, 1981). A number of antibody-related problems, such as false negative results, antigen modulation, and heterogenicity of tumor-associated antigens, need to be addressed.

VII. DISCUSSION

Monoclonal antibody reagents have brought forth a revolution in immunology, cell biology, molecular genetics, medicine, and essentially all levels of biomedical science since the initial report of Köhler and Milstein (1975). The literature is now full of alphanumerics describing thousands of MOabs. In this regard the hybridoma technology has already transformed biological and medical practices into a digitized world, a powerful revolution comparable to the transition from analog to digital computers.

The hybridoma technology has the potential to provide us with an inexhaustible, pure source of MOabs with specific reactivity, high avidity, and selected isotype. Additionally, if an antigen is unavailable in pure form, is weakly immunogenic, or is costly to purify, the use of monoclonal antibody for its detection, characterization, and extraction provides very significant advantages (Sever *et al.*, 1981). The production of monoclonal antibodies against hormones, immunoglobulin idiotype (Bona *et al.*, 1982), cell surface differentiation, and tumor-associated antigens has provided excellent examples of the utility of hybridoma technology.

Within the context of the stated advantages, however, it should be emphasized that MOabs also possess unique problems. As clonal products it is not uncommon to observe differences between individual MOabs for properties such as solubility, isoelectric point, stability and retention of activity after chemical labeling with isotope, fluorochromes, and other ligands. These problems imposed by the individuality of MOabs can be rather formidable. Furthermore, the hybridoma cells can mutate at relatively high rates (Morrison and Scharff, 1981). For routine production purposes it is necessary to select more stable clones, freeze adequate vials of cells for future regeneration, and monitor the antibody production very closely—a labor-intensive endeavor.

A number of laboratories (Neuberger and Rajewsky, 1981; Dangl and Herzenberg, 1982; Thammana and Scharff, 1983) have, however, taken

advantage of the problem of hybridoma mutation. Like normal B cells, myeloma and hybridoma cells produce heavy-chain switch variants at a rate of 10^{-5} to 10^{-7}. Dangl and Herzenberg (1982) generated families of such heavy-chain switch variants, each expressing the same heavy-chain variable-region gene and light chain in conjunction with a different one of the eight possible murine isotypes. For example, IgG_{2b}, IgG_{2a}, and IgE variants could be derived sequentially from an IgG_1 producer. These variant families should add more flexibility to monoclonal applications and lead to a more refined understanding of the molecular genetic events involved in the class switch as well as the functional properties rendered onto the immunoglobulin molecule by each isotype in combination with an identical variable region.

The first major wave of monoclonal antibodies were produced against cell surface–associated antigens such as human T cells, bacterial cells, parasites, and tumor cells. New diagnostic tests and serotherapy using these monoclonal antibodies are forthcoming. Monoclonal reagents against human viruses, idiotypes of lymphocyte receptors, novel hormones, oncogene products, and other biological molecules should also open exciting areas in clinical investigation.

VIII. APPENDIX: GENERAL REVIEWS ON PERSPECTIVES, METHODOLOGY, AND APPLICATIONS

Fellows, R. E., and Eisenbarth, G. S. (eds.) (1981). "Monoclonal Antibodies in Endocrine Research." Raven, New York.

Hammerling, G. J., Hammerling, U., and Kearny, J. F. (eds.) (1983). *Res. Monogr. Immunol.* **3.**

Hurrell, J. G. R. (ed.) (1982). "Monoclonal Hybridoma Antibodies: Techniques and Applications." CRC Press, Boca Raton, Florida.

Katz, D. (ed.) (1981). "Monoclonal Antibodies and T Cell Products." CRC Press, Boca Raton, Florida.

Kennet, R. H., McKearn, J. J., and Bechtol, K. B. (eds.) (1980). "Monoclonal Antibodies. Hybridomas: A New Dimension in Biological Analysis." Plenum, New York.

Kohler, G. (1980). "Hybridoma Techniques: A Step to Step Manual." Cold Spring Harbor Lab., Cold Spring Harbor, New York.

McKay, R., Raff, M. C., and Reichardt, L. S. (eds.) (1981). "Monoclonal Antibodies to Neural Antigens." Cold Spring Harbor Lab., Cold Spring Harbor, New York.

McMichael, A., and Fabre, W. (eds.) (1981). "Monoclonal Antibodies in Clinical Medicine." Academic Press, London.

Melcher, F., Potter, M., and Warner, N. L. (eds.) (1978). *Curr. Top. Microbiol. Immunol.* **81.**

Setlow, J. K., and Hollander, A. (eds.) (1980). "Production of Monoclonal Antibodies." Plenum, New York.

REFERENCES

Atkinson, B. F., Ernst, C. S., Herlyn, M., Steplewski, Z., Sears, H. R., and Koprowski, H. (1982). *Cancer Res.* **42,** 4820–4823.

Bach, M. A., Phan-Dink-Dinh-Tuy, F., Bach, J. F., Wallach, D., Biddison, W. G., Sharrow, S. O., Goldstein, G., and Kung, P. C. (1981). *J. Immunol.* **127,** 980–982.

Bast, R. C., Jr., Feeney, M., Lazarus, H., Nasler, L. M., Coloin, R. B., and Knapp, R. C. (1981). *J. Clin. Invest.* **68,** 1331–1337.

Berger, C. L., Morrison, S., and Chu, A. (1982). *J. Clin. Invest.* **70,** 1205–1209.

Bhan, A. K., Dienstag, J. L., and Wands, J. R. (1982a). *Clin. Exp. Immunol.* **47,** 351–358.

Bhan, A. K., Mihm, M. C., Jr., and Dvorak, H. F. (1982b). *J. Immunol.* **129,** 1578–1583.

Biddison, W. E., Sharron, S. O., and Shearer, G. W. (1981). *J. Immunol.* **127,** 487–491.

Biddison, W. E., Rao, P. E., Talle, M. A., Goldstein, G., and Shaw, S. (1982). *J. Exp. Med.* **156,** 1065–1076.

Bona, C. A., Finley, S., Waters, S., and Kunkel, H. G. (1982). *J. Exp. Med.* **156,** 986–999.

Borst, J., Prendiville, M. A., and Terhorst, C. (1982). *J. Immunol.* **128,** 1560–1565.

Burnet, F. M. (1959). "The Clonal Selection Theory of Acquired Immunity." Cambridge Univ. Press, London.

Chang, T. W., and Gingrass, S. P. (1981). *Int. J. Immunopharmacol.* **3,** 183–186.

Chang, T. W., Kung, P. C., Gingras, S. P., and Goldstein, G. (1981). *Proc. Natl. Acad. Sci. USA* **78,** 1805–1808.

Chang, T. W., Testa, D., Kung, P. C., Perry, L., Dreskin, H. J., and Goldstein, G. (1982). *J. Immunol.* **128,** 585–589.

Charpin, C., Bhan, A. K., Zurawski, V. R., Jr., and Scully, R. E. (1982). *Int. J. Gynecol. Pathol.* **1,** 231–238.

Chu, A., Berger, C. L., Kung, P. C., and Edelson, R. (1982). *J. Am. Acad. Dermatol.* **6,** 350–354.

Cosimi, A. B. (1983). *Transplant Proc.,* 15:583–589.

Cosimi, A. B., Colvin, R., Burton, R., Goldstein, G., Kung, P. C., Hansen, W. P., Delmonico, F. L., and Russell, P. S. (1981). *N. Engl. J. Med.* **305,** 308–314.

Cosimi, A. B., Burton, R. C., Kung, P. C., Colvin, R., Goldstein, G., Lifter, J., Rhodes, W. E., and Russell, P. S. (1982). *Transplant. Proc.* **13,** 499–503.

Cotner, T., Hemler, M., and Strominger, J. L. (1981). *Int. J. Immunopharmacol.* **3,** 255–268.

Cotner, T., Williams, J. M., Christenson, L., Shapiro, H. M., Strom, T. B., and Strominger, J. L. (1983). *J. Exp. Med.* **157,** 461–472.

Cruise, K. M., Mitchell, G. F., Tapalaer, E. P., Garcia, E. G., and Huant, S. R. (1981). *Aust. J. Exp. Biol. Med. Sci.* **59,** 503–514.

Dangl, J. L., and Herzenberg, L. A. (1982). *J. Immunol. Methods* **52,** 1–14.

Del Villano, B. C., and Zurawski, V. R., Jr. (1983). *In* "Proceedings of the 1982 National Conference on Immune Diagnostics" (T. Hyam and R. Aloisi, eds.), in press. Liss, New York.

Del Villano, B. C., Brennan, S., Brock, P., Bucher, C., Victor, L., McClure, M., Rake, B., Space, S., Westrick, B., Schoemaker, H., and Zurawski, V. R., Jr. (1983). *Clin. Chem.* **29**:549–553.

De Waele, M., Thielmans, C., and Van Camp, B. K. G. (1981). *N. Engl. J. Med.* **304,** 460–462.

Edelman, G. M. (1973). *Science (Washington, D.C.)* **180,** 830–841.

Ehrlich, P. (1900). *Proc. R. Soc. London Ser. B* **66,** 424–434.

Eisenbarth, G. S., Linnenbach, A., Jackson, R., Scearce, R., and Croce, C. M. (1982). *Nature (London)* **300,** 264–267.

Epenetos, A. A., Britton, K. G., Mather, S., Shepherd, J., Gramowsker, M., Taylor-

Papciditimitriou, J., Nimmon, C. C., Durbin, H., Hawkins, L. R., Malper, J. S., and Bodmer, W. F. (1982). *Lancet* **ii,** 999–1004.

Fithian, E., Kung, P. C., Goldstein, G., Rubenfeld, M., Fenoglio, C., and Edelson, R. (1981). *Proc. Natl. Acad. Sci. U.S.A.* **78,** 2541–2544.

Foon, K. A., Bernhard, M. I., and Oldham, R. K. (1982). *J. Biol. Response Modif.* **1,** 277–304.

Fox, R. I., Fong, S., and Sabharwal, N. (1982). *J. Immunol.* **128,** 351–354.

Goldstein, L. C., Corey, L., McDougall, J. K., Tolentino, E., and Nowinski, R. C. (1983). *J. Infect. Dis.* **147,** 829–837.

Goodall, A. H., Miescher, G., Meek, F. M., Janossy, G., and Thomas, H. C. (1981). *Med. Lab. Sci.* **38,** 349–354.

Greaves, M., Delia, D., and Sutherland, R. (1981). *Int. J. Immunopharmacol.* **3,** 283–300.

Hansen, E. J., Robertson, S. M., Grulig, P. A., Frisch, G. F., and Hanes, G. J. (1982). *Lancet* **1,** 366–368.

Hasty, E. L., Beachey, E. H., Simpson, W. A., and Dale, J. B. (1982). *J. Exp. Med.* **155,** 1010–1018.

Haynes, B. F., Metzgar, R. S., Minna, J. D., and Bumn, P. A. (1981). *N. Engl. J. Med.* **304,** 1319–1323.

Hayward, A. R., Murphy, S., and Githens, J. (1982). *J. Pediatr.* **100,** 665–668.

Hunter, K. W., Fischer, G. W., and Memming, V. G. (1982). *Lancet* **II,** 798–799.

Ip, S. H., Rittershaus, C. W., Struzziero, C. C., Hoffman, R. A., and Hansen, P. W. (1982). *Blood* **60,** 795–799.

Janossy, G., Tidman, N., Selby, W. S., Kung, P. C., and Goldstein, G. (1980). *Nature (London)* **288,** 81–84.

Janossy, G., Panayi, G., and Duke, O. (1981). *Lancet* **ii,** 839–842.

Kaizer, H., Levy, R., Brovall, C., Civin, C. I., Fuller, D. J., Hsu, S. H., Leventhal, B. G., Miller, R. A., Milveham, E. S., Santor, G. W., and Wharam, M. D. (1982). *J. Biol. Response Modif.* **1,** 233–243.

Kemshead, J. T., Fritschy, J., and Asser, U. (1982). *Hybridoma* **1,** 109–123.

Knowles, D. M., II, and Halper, J. P. (1982). *Am. J. Pathol.* **106,** 187–203.

Köhler, G., and Milstein, C. (1975). *Nature (London)* **256,** 495–498.

Köhler, G., Pearson, T., and Milstein, C. (1977). *Somatic Cell Genet.* **3,** 303–309.

Koprowski, H., Steplewski, Z., Mitchell, K., Herlyn, M., Herlyn, D., and Fuhrer, P. (1979). *Somatic Cell Genet.* **5,** 957–972.

Kornfeld, H., Vande Stouwe, R. A., Lange, M., Reddy, M. M., and Grieco, M. (1982). *N. Engl. J. Med.* **307,** 729–731.

Kung, P. C., and Goldstein, G. (1980). *Vox Sang.* **39,** 121–127.

Kung, P. C., Goldstein, G., Reinherz, E. L., and Schlossman, S. F. (1979). *Science (Washington, D.C.)* **206,** 347–349.

Kung, P. C., Talle, M. A., DeMaria, M., Ziminski, N., Look, R., Lifter, J., and Goldstein, G. (1981a). *Int. J. Immunopharmacol.* **3,** 175–182.

Kung, P. C., Berger, C. L., Goldstein, G., and Edelson, R. (1981b). *Blood* **57,** 261–266.

Kung, P. C., Berger, C. L., Estabrook, A., and Edelson, R. L. (1983). *Int. J. Dermatol.* **22,** 67–74.

Landegren, U., Ramstedt, U., Axberg, I., Ullberg, M., Jondal, M., and Wigzell, H. (1982). *J. Exp. Med.* **155,** 1579–1584.

Landsteiner, K. (1945). "The Specificity of Serological Reactions." Harvard Univ. Press, Cambridge, Massachusetts.

Mach, J. P., Buchegger, F., Ritschard, J., Berche, C., Lumbrow, J. D., Schreyer, M., Girardet, C., Accolla, R. S., and Carrel, S. (1981). *Immunol. Today,* **1,** 239–249.

Magnani, J. L., Nilson, B., Brockhaus, M., Smith, D. F., Ginsburg, V., Blaszcyk, M.,

Mitchell, D. F., Steplewski, Z., and Koprowski, H. (1982). *J. Biol. Chem.* **257,** 14365–14369.

Meuer, S. C., Fritzgerald, K. A., Hussey, R. E., Hodgor, J. C., Schlossman, S. F., and Reinherz, E. L. (1983). *J. Exp. Med.* **157,** 705–719.

Meuer, S. C., Aeuto, O., Hussey, R. E., Hodgon, J. C., Fitzgerald, K. A., Schlossman, S. F., and Reinherz, E. L. (1983b). *Nature (London)* **303,** 808–810.

Miller, R. A., Maloney, D. G., and McKillop, J. (1981). *Blood* **58,** 78–82.

Miller, R. A., Maloney, D. G., Warnicki, E. R., and Levy, R. (1982). *N. Engl. J. Med.* **306,** 517–522.

Morimoto, C., Reinherz, E. L., Schlossman, S. F., Schur, P. H., Mills, J. A., and Steinberg, A. D. (1980). *J. Clin. Invest.* **66,** 1171–1174.

Morrison, S. L., and Scharff, M. D. (1981). *CRC Crit. Rev. Immunol.* **3,** 1–22.

Neuberger, M. S., and Rajewsky, K. (1981). *Proc. Natl. Acad. Sci. USA* **78,** 1138–1142.

Nowinski, R. C., Tam, M. R., Goldstein, G., Strong, L., Kuo, C. C., Carey, L., Stamar, W. E., Hardsfila, H. H., Knapp, J. S., and Holmes, K. K. *Science (Washington, D.C.)* **219**:637–643.

Olsson, L., and Kaplan, H. S. (1980). *Proc. Natl. Acad. Sci. USA* **77,** 5429–5473.

Platt, J. L., LeBien, T. W., and Michael, A. F. (1982). *J. Exp. Med.* **155,** 17–30.

Poljak, R. J. (1975). *Adv. Immunol.* **21,** 1–21.

Porter, R. R. (1973). *Science (Washington, D.C.)* **180,** 713–724.

Potter, M. (1972). *Physiol. Rev.* **52,** 631–678.

Prentice, H. G., Janossy, G., and Skeggs, D. (1982). *Lancet* **1,** 700–703.

Reinherz, E. L., Kung, P. C., Goldstein, G., and Schlossman, S. F. (1980a). *Proc. Natl. Acad. Sci. USA* **77,** 1588–1592.

Reinherz, E. L., Weiner, H. L., Hauser, S. L., and Schlossman, S. F. (1980b). *N. Engl. J. Med.* **303,** 125–129.

Ritz, J., Salken, S. E., Bast, R. C., Reinherz, E. L., and Schlossman, S. F. (1982). *Lancet* **2,** 60–63.

Robert-Guroff, M., Ruscetti, F. W., Posner, L. E., Poiesz, B. J., and Gallo, R. C. (1981). *J. Exp. Med.* **154,** 1957–64.

Rohowsky, C., Suciu-Foca, N., Kung, P. C., Tang, T. F., Reed, E., and King, D. W. (1983). *Transplant. Proc.,* **15,** 765–770.

Rubin, R. H., Carney, W. P., and Schooley, R. T. (1981). *Int. J. Immunopharmacol.* **3,** 307–312.

Schilling, J., Clevinger, B., Davie, J. M., and Hood, L. (1980). *Nature (London)* **285,** 35–38.

Schmaljohn, A. L., Johnson, E. D., Dalrymple, J. M., and Cole, G. A. (1982). *Nature (London)* **297,** 70–72.

Schrof, R. W., Foon, A. K., and Billing, R. J. (1982). *Blood* **59,** 207–215.

Schulman, M., Wilde, C. D., and Kohler, G. (1978). *Nature (London)* **276,** 269–271.

Sears, H., Herlyn, J., Del Villano, B., Steplewski, Z., and Koprowski, H. (1982). *J. Clin. Immunol.* **2,** 141–148.

Sever, E. D., David, G. S., Martinis, J., Desmond, W. J., Bartholomew, R. M., and Wang, R. (1981). *Clin. Chem.* **27,** 1297–1306.

Shafritz, D. A., Lieberman, H. M., Isselbacher, K. J., and Wands, J. R. (1982). *Proc. Natl. Acad. Sci. USA* **79,** 5675–5679.

Snow, P., Spits, H., de Vries, J., and Terhorst, C. (1983). *Hybridoma,* **2,** 187–200.

Stephens, R. S., Tam, M. R., Kuo, C. C., and Nowinski, R. C. (1982). *J. Immunol.* **128,** 1083–1089.

Suciu-Foca, N., Rohowsky, C., Kung, P. C., and King, D. W. (1982). *J. Exp. Med.* **150,** 283–288.

Suciu-Foca, N., Reed, E., Rohowsky, C., Kung, P. C., and King, D. W. (1983). *Proc. Natl. Acad. Sci. USA* **80,** 830–834.

Tam, M. R., Buchanan, T. M., Sandstrom, E. G., Holmes, K. K., Knapp, J. S., Siadak, A. W., and Nowinski, R. C. (1982). *Infect. Immun.* **36,** 1042–1053.

Thammana, P., and Scharff, M. D. (1983). *Eur. J. Immunol.* **13,** 614–619.

Thomas, Y., Rognozinski, L., Irigoyen, O. H., Shen, H. H., Talle, M. A., Goldstein, G., and Chess, L. (1982). *J. Immunol.* **128,** 1386–1390.

Tsoukas, C. D., Carson, D., Fong, S., and Vaughan, J. H. (1982). *J. Immunol.* **129,** 1421–1425.

Van Wauwe, J., Goossens, J., Decock, W., Kung, P. C., and Golstein, G. (1981). *Immunology* **44,** 865–871.

Verbi, W., Greves, M. F., Schneider, C., Koubek, K., Janossy, G., Stein, H., Kung, P., and Goldstein, G. (1982). *Eur. J. Immunol.* **12,** 81–86.

Wands, J. R., and Zurawski, V. R., Jr. (1981). *Gastroenterology* **80,** 225–231.

Wands, J. R., Carlson, R. I., Schoemaker, H. J. P., Isselbacher, K. J., and Zurawski, V. R., Jr. (1981). *Proc. Natl. Acad. Sci. USA* **78,** 1214–1218.

Wood, J. N., Hudson, L., Jessell, T. M., and Yamamoto, M. (1982). *Nature (London)* **296,** 34–38.

Woods, J. C., Spriggs, A. I., Hartris, H., and McGee, J. (1982). *Lancet* **2,** 512–514.

Wu, T. T., and Kabat, E. (1970). *J. Exp. Med.* **132,** 211–217.

Yeh, M. Y., Hellstrom, J. P., Brown, G. A., Warner, J. A., Hansen, J., and Hellstrom, K. E. (1979). *Proc. Natl. Acad. Sci. USA* **76,** 2927–2931.

Yelton, D. E., and Scharff, M. D. (1981). *Annu. Rev. Biochem.* **50,** 657–715.

Yokoi, T., Miyawaki, T., and Yachie, A. (1982). *J. Immunol.* **129,** 1441–1445.

Yoshida, N., Potocnjak, P., Nussenzweig, V. A., and Nussenzweig, R. S. (1981). *J. Exp. Med.* **154,** 1225–1236.

Zarling, J. M., and Kung, P. C. (1980). *Nature (London)* **394–396.**

Zarling, J. M., Clouse, K. A., Biddison, W. E., and Kung, P. C. (1981). *J. Immunol.* **127,** 2575–2580.

4

Serologic Methods in Disease Diagnosis

ALLEN L. PUSCH

I. INTRODUCTION

Knowledge in the field of immunology has been expanding tremendously in the past 25–30 years, especially in regard to immunoglobulin (Ig) structure, the cellular components of the immune response (the B and T lymphocytes), and antigen–antibody interaction. The details of this expansion are beyond the scope of this chapter; details can be found in one of the many texts on general immunology, clinical immunology, or immunopathology (Rose *et al.*, 1979, Sell, 1980). This chapter will discuss the general principles of diagnostic serology, with selected examples of the use of serologic data in clinical medicine. Detailed instructions for most serologic procedures and a discussion of clinical interpretation can be found in recent publications by the American Society for Microbiol-

CLINICAL BIOCHEMISTRY
Contemporary Theories and Techniques, Vol. 3

ISBN 0-12-657103-1

ogy, the U.S. Public Health Association, and others (Lennette and Schmidt, 1969; Nakamura, 1974; Rose and Friedman, 1980; Balows and Hausler, 1981).

Classical serology developed to demonstrate the presence of antibodies in a patient's serum that reacted with various microorganisms to indicate infection. Antibodies are the immunoglobulins produced by the B lymphocytes (also known as B cells). These B cells are stimulated to multiply and to produce antibodies by the presence of foreign material and cellular activity on the part of macrophages and members of the T lymphocyte group (T cells). The substances initiating this process, which in turn combine with the antibody to form immune complexes, are called *antigens*.

There is usually a time delay between the entry of the antigen into an animal and the ability to demonstrate antibody in the serum. This may vary from several days to a few weeks and is generally put at 1 week. A serologic diagnosis of acute infection is made by demonstrating a significant increase in antibody levels. This is done by collecting two serum samples from a patient, separated by at least a 2-week interval. One sample is collected during the first week of the illness (called the acute-phase serum), and a second is collected 2–3 weeks later (called the convalescent serum). The earlier sample is saved and tested in the same "run" as the second serum. To be considered presumptive evidence of an acute infection, the convalescent sample should have a fourfold or greater titer of antibody to the antigen in question than the acute sample. In some diseases a longer time interval may be required to demonstrate a significant titer rise. The time interval may thus be varied or a third, later, sample collected and tested with the acute serum.

The serologic diagnosis of infection is often retrospective and, as such, may not be helpful where specific and prompt antibiotic therapy is desired. Ideally, isolation, identification, and perhaps antibiotic sensitivity testing are preferred. Situations arise, however, where obtaining diagnostic material would be overly invasive to the patient or where the methods to isolate the organism are not immediately available or where the cultures are negative. Here, serology may be used to generate diagnostic data.

In chronic infections the infecting organism has been present in the patient's tissues for a prolonged period, and the serum antibody titer has reached a plateau by the time the patient seeks medical attention. Here, one serum sample is sufficient, and a "high" titer is considered diagnostic when associated with the appropriate symptoms. A low or intermediate titer may be present in a person who has been exposed to this antigen or organism in the distant past and who no longer has active infection. Thus, knowledge of patients' medical history and of their exposure to microor-

ganisms in employment or hobbies must be considered in the interpretation of antibody titers. The numerical value of high and low titers is determined by experience with each infectious disease.

Serologic reaction with a given organism may not be an indicator of exposure to that same organism in some situations. In nature there are multiple examples of different species of microorganisms sharing the same or similar chemical structures. This in turn results in the individual producing an antibody that reacts with multiple species following stimulation by a single species. This is called *cross-reactivity*. In these situations it is best to test all the known cross-reacting antigens representing species that may cause the clinical symptoms demonstrated by the patient and to consider the one giving the highest titer as the most probable cause of the infection.

II. SEROLOGIC METHODS

The serologic methods used in the routine laboratory try to demonstrate the presence of immunoglobulins in serum reactive with a given antigen. The amount of antibody is quantitated in a relatively crude way, usually by a serial doubling dilution of the patient's serum. The starting point of the serial dilution is established empirically, with most starting between 1 : 8 and 1 : 20. The results are usually expressed as a proportion (i.e., 1 : 128, 1 : 256) and referred to as the titer. Occasionally, the results are expressed as the reciprocal of the greatest serum dilution showing a positive reaction (i.e., 128, 256).

The antigen frequently is a suspension of an intact microorganism such as a bacterium or, in a few cases, a specific fraction or constituent extracted from the organism. A whole organism has many different antigens on its surface and in its cytosol, each of which can stimulate antibody formation. Thus a patient's serum is a heterogeneous mixture of antibody specificities and, using a whole organism in testing measures, a summation of antibody activity. Traditionally the reactions have been carried out in serum serially diluted within a test tube with saline or buffer and incubated with a constant amount of antigen. Many new methods have been developed to improve sensitivity, to ease performance of the test, and to reduce the volume of sample required.

In most testing procedures the reaction between antigen and antibody occurs rapidly but may not be apparent until a secondary reaction occurs, giving it visibility. It is the great variety of secondary reactions that gives rise to the long list of procedures now in use. Often, the name of the method is applied to the antibody itself, i.e., "Salmonella agglutinins" or

"indirect fluorescent antibody to Herpes virus," and so forth. But all procedures measure the same thing: namely, the presence of immunoglobulin in the serum reactive with the antigen.

In the so-called precipitin methods the antigen and antibody complexes unite into large aggregates that form a visible precipitate at the appropriate pH, ionic strength, and temperature in a liquid medium. Precipitation within gels such as agar is a more sensitive method than in liquid. Here the two reactants passively diffuse into the gel from wells in the agar and form a precipitin line where the two meet at an appropriate concentration. This method, known as Ouchterlony double diffusion, may take several days before a precipitin line is visible. The time interval can be shortened to a period of hours by driving the reactants toward each other in an electrical field, a procedure called counter immunoelectrophoresis (CIE). In CIE the antigen and antibody must have opposite charges at the pH used, and in some cases chemical alteration of one of the reactants is required to achieve this.

In solutions where the antibody or antigen are in significant excess, the immune complexes may not unite in aggregates large enough to form a precipitate and thus remain in solution. When such occurs in antibody excess it is called a "prozone" phenomena and can lead to false negative results in serum with a very high antibody titer. To guard against this in screening for serum antibody, one should use two different serum dilutions. The prozone phenomena can also cause false negative results in some of the gel precipitin methods.

In procedures where the antigen is part of a cell, exemplified by a bacterium or a red blood cell (RBC), a smooth suspension of the cells is incubated with the diluted serum. The reaction is identified by agglutination of the cells with resultant change in the visual appearance of the suspension or altered light-scattering properties. These agglutination procedures may be performed in test tubes, on a solid surface such as a glass slide, or in a moving stream as in autoanalyzer tubing.

Nonparticulate antigens can be adsorbed to particles such as RBCs or latex beads. When RBCs are treated with tannic acid (so-called tanned RBC), a wide variety of substances readily adhere to their surface. These are used in the indirect hemagglutination (IHA) methods in contrast to the direct hemagglutination (HA) methods in which the antigen is naturally part of the RBC. Chicken, rabbit, sheep, and human RBC may be used in the IHA procedures. Care must be taken, however, to rule out false positive reactions caused by the presence of antibodies in the serum reacting with the red cell itself rather than the adsorbed antigen. A control tube with each patient's serum and RBC without antigen is required.

Several antigens will adsorb to latex beads. Beads of a uniform small

size will form a smooth suspension that persists for a moderately long period. In the presence of antibody to the adsorbed antigen, the beads agglutinate with a visible change in the character in the suspension. These are called "latex fixation" procedures and are adapted to both tube and slide methods.

The complement system consists of several serum proteins that participate in a sequential reaction that can be initiated by antigen–antibody complexes. One product of this reaction, when it occurs on cell membranes, is an enzymatic alteration of the membranes with the resultant lysis or destruction of the cells. Early in the development of clinical serology this system was utilized as an indicator of antigen–antibody reaction and known as the "complement fixation" procedure. It is carried out in two distinct steps. First, the antigen, serum, and complement are mixed and incubated to allow any immune reaction to occur. In a second step, "sensitized" sheep RBCs are added to this mixture and it is incubated again. These sensitized RBCs have previously been reacted with antisheep RBC antibody with resultant immune complexes on their surface. If complement remains after the first incubation, the sheep RBCs are lysed (i.e., hemolyzed) giving a visible reaction. Thus, lysis indicates that no complexes are formed in the first step to consume the complement. The absence of lysis in the second step is considered a positive indicator of an immune reaction in the first step consuming complement. These are referred to as complement-fixing (CF) antibodies. Complement fixation is a lengthy procedure requiring experience, careful attention to detail, and several different controls. Today it is performed only in more specialized laboratories.

Experience has shown that the presence of CF antibodies has different significance in different infections. For example, in rubella virus infections CF antibodies appear early in the disease and then disappear in 18–24 months. They are generally considered indicative of acute or recent rubella infection. In certain deep fungal infections the CF titers may rise to very high levels in individuals who are experiencing disseminated disease and whose immune system is not controlling the infection. But patients whose defenses have localized and contained the infection have low or absent CF titers. Observations such as these are empirical and have been documented for individual infectious diseases.

Fluorescent antibody (FA) methods have become very popular for demonstrating antibodies to various cell constituents, to organisms that are intracellular parasites such as viruses, or to bacteria smeared on a slide. There is an ever-increasing number of such antigens commercially available. Conveniently, the same reagent can be used to demonstrate the binding of patient's antibody to all of the antigens; namely a fluorescein–

isothiocyanate-labeled antihuman immunoglobulin. This general reagent reacts with all the major classes of immunoglobulin (IgG, IgA, and IgM). Class specific reagents, such as labeled antihuman IgG or antihuman IgM, can be used to demonstrate a specific class of immunoglobulin attached to the antigen. This is helpful to determine if a given infection is acute or has occurred in the past.

The class of immunoglobulin reactive with a specific antigen goes through an evolutionary cycle during an acute infection. Early in the response, IgM antibody appears first, peaks in a matter of weeks, and falls to undetectable levels in months. Immunoglobulin G antibody appears slightly later, peaks in several months, falls somewhat, but persists in the serum for years to life. Thus the presence of mostly IgM antibody is indicative of acute or recent infection and a titer of IgG antibody alone suggests infection in the distant past. This type of information is usually sought where significant therapeutic decisions hinge on differentiating an acute from a remote infection. An example is the problem of rubella or toxoplasmosis early in pregnancy and the consideration of therapeutic abortion if there is a significant probability of transplacental infection of the fetus. Unfortunately, reagents for specific classes of immunoglobulins have been difficult to produce commercially and such testing has been limited. We hope that improved reagents can be produced with the monoclonal antibody techniques.

The indirect fluorescent (IF) antibody method is being more widely used in routine serodiagnosis each year. The antigens, such as cells or organisms, are fixed to a microscope slide, and the serum to be tested is then overlaid and incubated. This allows any specific antibodies in the serum to combine with the antigen. After the slide is washed free of excess serum, the fluorescein-tagged antihuman immunoglobulin reagent is overlaid and allowed to react with human immunoglobulins attached to the antigen. The slide is washed again and then examined under a fluorescent microscope for fluorescence of the appropriate antigenic structure. It is essential for the observer to be familiar with the structure or cell used as antigen in order to determine that the appropriate portions fluoresce.

III. SYPHILIS SEROLOGY

Syphilis is an infectious disease caused by the spirochete *Treponema pallidium*. The organism penetrates the skin or mucous membranes, usually following sexual contact with an infectious individual, and invades the local lymphatics. The organisms multiply, enter the bloodstream, and are disseminated throughout the body. They have a special propensity to

multiply and to cause tissue damage in the wall of the aorta and in the blood vessels of the central nervous system, but they can be found in almost any tissue on occasion. Dissemination of the organisms is usually accomplished before the primary lesion (called the *chancre*) appears at the site of entry in the skin. The chancre usually heals spontaneously regardless of whether or not treatment is instituted. Then weeks or months later the untreated patient may break out in a rash, develop generalized swollen lymph nodes, and, perhaps, lesions on the mucous membranes—a stage known as secondary syphilis. The patient again appears to recover spontaneously and passes into the latent or asymptomatic stage, which may last for years or decades until the late stage, in which the ongoing damage to nervous system or aorta becomes clinically manifest. In most series it appears that a third of untreated individuals develop symptomatic late syphilis, indicating that a majority of those infected are able to contain and defend against the disease process (Rein, 1983).

It is in the primary and secondary stages that the organisms are available for identification by direct examination. *Treponema pallidium* cannot be cultured *in vitro*, but is identified by darkfield microscopy of material obtained from active lesions. Because relatively few patients seek medical aid in these early stages and because darkfield examinations are often unsuccessful, physicians rely heavily on serum serology to make the diagnosis (Wallace and Norins, 1970).

Two general categories of tests have evolved for use in syphilis: the nontreponemal antigen tests and the treponemal antigen procedures. The nontreponemal antigen procedures in use today, the VDRL and the rapid plasma reagin (RPR) circle card test, have evolved from Wasserman's original method described in 1906. The VDRL (the letters refer to the Venereal Disease Research Laboratory of the U.S. Public Health Service which has standardized the method) uses as antigen a balanced suspension of "cardiolipin," lecithin, cholesterol, and alcohol. The cardiolipin is a lipid-containing material extracted from beef heart with which the serum antibody reacts. The cholesterol is present to provide adsorption centers for the agglutination, which is visualized microscopically on glass slides. The results are read as nonreactive, weakly reactive, or reactive. All reactive sera are then serially diluted (i.e., 1 : 1, 1 : 2, 1 : 4) and the highest dilution showing agglutination reported as the titer. The serum antibody reacting with this antigen was named "reagin," which, interestingly, is the same term applied early in this century to the antibody thought responsible for allergic reactions. But these are two very different antibodies (Sparling, 1971; Newman, 1974; Felman and Nikitas, 1980).

The RPR circle card test uses essentially the same antigen with the addition of charcoal particles that provide a macroscopic agglutination

reaction on a white plastic-coated card. The RPR is comparable to the VDRL in sensitivity and specificity. The titers, however, are not always comparable, and if one is following a patient with serial titers, it is important to use the same method. This antigen with the charcoal has been adapted to the autoanalyzer with the result of the reaction being applied to a filter paper strip so one can visualize the agglutination of the charcoal particles. This is known as the automated reagin test (ART) and is used in centers screening large numbers of sera (Newman, 1974).

The search for a procedure that incorporates antigen from the organism *T. pallidium* has been hindered by the inability to grow the organism *in vitro*. But organisms cultured in rabbit testes were employed in the 1940s for the *T. pallidium* immobilization test (TPI). In this procedure the immobilization of live spirochetes suspended in a dilution of patients' serum on a glass slide is considered evidence of antibodies reactive with the organism. For decades this test was considered the standard against which other newer procedures were compared. Today, because of the technical problems of keeping cultures of live treponemes in living rabbit testes, it is seldom used and is generally not available (Wallace and Norins, 1970).

Adaptation of the IF antibody method to syphilis serology has led to its replacing the TPI (Tuffanelli *et al.*, 1967). Here the antigen consists of the Nichols strain of *T. pallidium* that originally was transferred from a human case to the rabbit testes and subsequently maintained. In the FTA–ABS (fluorescent treponemal antibody–absorbed) procedure the serum is first mixed with an extract from a culture of Reiter treponemes (a nonpathogenic strain that can be cultured *in vitro*) to remove any nonspecific antibodies reactive with general species antigens. Next, the adsorbed serum is laid over the Nichol's treponemes, fixed to a glass slide, and incubated. The slide is then washed, the fluorescent antihuman globulin applied and incubated. The slide is then examined for evidence of human antibody coating the teponemes. The test is reported as reactive or nonreactive and not titered. There are rare patients whose serum produces an atypical or beaded fluorescence in this procedure; these persons often have diseases of the collegen–vascular group, such as systemic lupus erythematosus (SLE) and do not have evidence of syphilis (Kraus *et al.*, 1970; McKenna *et al.*, 1973). The FTA–ABS reagents are commercially available, and this test has become the standard for confirming a reactive VDRL or RPR screening test.

Recently, tanned RBCs coated with *T. pallidium* antigen have been incorporated into a microhemagglutination procedure performed in microliter plates (the MHA–TP test). This treponemal antigen test can be semiautomated and is now replacing the FTA–ABS as a confirmatory test in several laboratories (Lesinski *et al.*, 1974; Ravel, 1976; Wormser, 1982).

Current practice in syphilis serology is to use a nontreponemal antigen test to screen and then confirm all reactive samples with a treponemal test before making a diagnosis of syphilis (Felman and Nikitas, 1980). The percentage of patients having reactive results with the several methods in the various stages of syphilis are noted in Table I. Generally, the FTA–ABS becomes reactive first and the MHA–TP last in patients with primary syphilis. All tests are reactive in secondary disease. In latent and late syphilis, reactivity with the VDRL and RPR fall off in untreated patients, and the treponemal tests maintain their reactivity. Thus, in patients with clinical symptoms of late syphilis, a treponemal test should be requested regardless of the VDRL results (Wallace and Norins, 1970; Sparling, 1971).

High titers in the VDRL (1 : 16 or greater) are suggestive of active infection; low titers (1 : 2 or 1 : 4) may be seen in treated disease or in false positive reactions. The accepted method to judge the effectiveness of treatment is to follow the VDRL titer serially. With successful therapy the titer drops to low values or becomes nonreactive, a process that may take as long as 18 months to occur. The treponemal antigen tests, however, remain positive for life if they are positive before treatment is started (Wallace and Norins, 1970; Schroeter *et al.*, 1972).

The individual with a reactive VDRL but a negative treponemal test is designated as having a false positive serology. In the earlier literature these were called "biologic false positive" (BFP) reactions (Moore and Mohr, 1952; Tuffanelli *et al.*, 1967). The titers are usually low, in the 1 : 1 to 1 : 8 range, and are divided into acute and chronic BFPs depending upon the persistence of the BFP for longer than 6 months. Table II lists some of the diseases associated with a BFP. Leprosy and SLE are notorious for having an associated chronic BFP (Kraus *et al.*, 1970). The acute BFP has been reported with a variety of acute infections, but its occur-

TABLE I

Reactivity of Various Tests in Different Stages of Syphilis (%)

Stage of Syphilis	VDRL–RPR[a]	FTA–ABS[b]	TPI[c]	MHA–TP[d]
Primary	50–70	60–80	50–60	50–60
Secondary	95–98	99	95	99
Latent	60–70	98	90–95	98
Late	70–80	98	90–95	98

[a] Venereal Disease Research Lab–rapid plasma reagin.
[b] Fluorescent treponomal antibody-absorbed.
[c] Treponema palladium immobilization.
[d] Microhemagglutination–Treponema pallidium.

TABLE II

Diseases Associated with Biologic False Positive (BFP)

Acute or Transient BFP	Chronic BFP
Acute infection	Systemic lupus erythematous
Immunizations	Yaws infection
Infectious mononucleosis	Pinta infection
Pregnancy	Leprosy
"Atypical" pneumonia	Hashimoto thyroiditis
	Drug addiction
	Rheumatoid arthritis

rence is quite rare, especially with the newer antigen preparations. Occasionally one finds a pregnant individual with a rough or weakly reactive VDRL and a nonreactive treponemal test. This BFP type reaction is attributed by some to pregnancy itself since the VDRL usually becomes nonreactive following delivery. Others claim that pregnancy is not a cause of the BFP (Sparling, 1971). Any reaction, however, in a test for syphilis during pregnancy is of concern because of the potential for transplacental infection of the fetus, which can be prevented with early treatment of the mother. There is also a high rate of false positives among drug addicts (Felman and Nikitas, 1980).

Central nervous system (CNS) syphilis requires larger doses of antibiotics than infection without CNS involvement. Thus cerebrospinal fluid (CSF) serologic testing is common in patients with syphilis. But VDRL and FTA–ABS procedures performed on CSF have a low sensitivity, being less than 50% reactive in some series of patients with diagnosed CNS syphilis (Escobar *et al.*, 1970; Jaffe *et al.*, 1978). On this basis one can seriously question the almost routine request for VDRL on CSF samples when serum serology has not been done. A modified procedure, the FTA–CSF, which does not use the absorption step, has been suggested as an alternative. It has a higher sensitivity, nearing 100% in some series (Hooshmand *et al.*, 1972), but produces false positive results (i.e., low specificity) and because of this is not recommended for use. Thus, if late CNS syphilis enters into a physician's consideration, the serum FTA–ABS or MHA–TP offer the best sensitivity and specificity and the CSF total protein and cell count the best laboratory indicators of a reactive process within the CNS. A reactive CSF–VDRL is additional evidence, but this test will be negative in CSF from some cases with active disease.

Serologic assessment of the newborn is also not as precise as desired, because maternal IgG can cross the placenta and render the newborn's serum reactive in the VDRL and FTA–ABS tests comparable to the mother's serum. If the VDRL titer in the infant's serum is higher than that in the mother, it suggests intrauterine infection. An IgM-specific FTA–ABS procedure has been recommended for this situation. Maternal IgM does not normally cross the placenta, thus the presence of IgM antibody reactive with *T. pallidium* in the newborn's serum indicates his own immune system has been stimulated by infection (Mamunes *et al.*, 1970). There are reports however, of newborns with IgM antibody reactive with maternal IgG (rheumatoid factor-like activity) giving a false positive reaction when the infant's serum is tested by this method. In this situation the treponemes are first coated with maternal IgG that has crossed the placenta, next the infants IgM attaches to the maternal IgG, and finally the fluorescent anti-IgM attaches to the infant's IgM with the result of fluorescence of the organisms. An alternative is to perform serial VDRL titers on the baby over a 3–6 month period. A rising titer suggests infection of the infant. If the titer falls, as expected with the normal catabolism of maternal IgG, the infant's reactive serology results from the maternal antibody. Of course, if there are any physical signs suggesting infection in the infant, he or she will be treated without awaiting serologic changes (Kaufman *et al.*, 1974; Felman and Nikitas, 1980).

Very few studies have been done on the predictive value of a positive serologic test for syphilis. Physicians have accepted that a patient with both a positive VDRL and a positive FTA–ABS has been infected. Recent studies on laboratory error suggest that an individual from the general population (where the prevalence of syphilis is 0.1%), with both tests positive and the test being done in the best of laboratories, has an 80% probability of being infected. If the tests are done in an average laboratory, the probability of syphilis is <20%. Such data are sobering and emphasize the problem of testing an unselected group with a very low prevalence of disease, a subject well understood in clinical chemistry. It should be noted that syphilis serology is probably the best standardized and controlled methodology in the serology laboratory (Goodhart *et al.*, 1981).

IV. SEROLOGY OF STREPTOCOCCAL INFECTIONS

Group-A streptococcal infections of the pharynx and skin are common and can be easily diagnosed by bacteriological culture. In many individuals the infection is mild, and the person recovers without antibiotic

therapy and without having visited a physician. Thus the infection is not documented by culture during the acute phase. In rare individuals these strep infections initiate a process, thought to be immunological, that damages the kidneys (acute glomerulonephritis) or the heart muscle and heart valves (rheumatic fever; Nissenson *et al.*, 1979; Unny and Middlebrooks, 1983). These nonsuppurative sequellae of the group-A strep infection usually become manifest weeks after the infection when the organisms may no longer be demonstrable by culture and the physician must turn to serological methods to document a recent strep infection (Bisno and Stollerman, 1975).

Streptococci produce many antigenic substances that are capable of stimulating an immune response in infected persons. Several of these have enzymatic activity blocked by specific antibody binding to the molecule. This property has been adapted as an indicator of antibody in a patient's serum. Antistreptolysin O (ASO), antihyluronidase (AH), antideoxyribonuclease B (anti-DNAse B), antistreptokinase, and antiphosphopyridine nucleotidase (anti-DPNase) are among those studied (Bisno and Stollerman, 1975). But only three—ASO, anti-DNAse B, and AH—have been developed for routine laboratory use.

Streptolysin O, an oxygen-labile hemolysin that lyses RBCs of several species, is produced by most strains of group-A strep and a few strains of groups C and G. The antigen is obtained from the broth of an 18-hr culture of the bacteria. It is mixed with dilutions of the patient's serum and incubated. Next, a suspension of RBCs is added as an indicator of remaining enzyme activity. The titer is the greatest dilution of serum completely inhibiting hemolysis. On the basis of experience, the jumps with doubling dilutions are too great for diagnostic purposes, and a dilution scheme with 1.25-fold or 0.1 log increases in dilution is used. The reciprocal of the dilution is reported and named "Todd units" (Wannamaker and Ayoub, 1960).

Materials for the anti–DNAse-B titer determination have become commercially available in the last several years, and this has significantly improved strep serology—especially following skin infections (Kaplan *et al.*, 1970). Group-A strep produces at least four distinct antigenic deoxyribonucleases which are labeled A, B, C, and D. Antibodies reactive with the B "iso-enzyme" are found more frequently than antibody to the others and thus this antigen is used for testing (Wannamaker, 1959). Patient's serum is serially diluted at 0.1 log increments and incubated with the antigen preparation. Next a solution of DNA is added, incubated, and finally the dye methyl green added to demonstrate the presence of any remaining intact DNA. The color fades promptly in the absence of polymerized DNA (Bisno and Stollerman, 1975).

Antibodies to streptococcal hyaluronidase are demonstrated in a similar manner. Here doubling dilutions of serum are incubated with a standard enzyme preparation followed by addition of the enzyme substrate to indicate remaining uninhibited enzyme activity.

Titers of antibody begin rising in the second week following strep infection. The ASO titer usually rises first with AH and anti–DNAse-B titers lagging behind by a week or so. The ASO peaks in 3–5 weeks and then falls to preinfection levels in 6–12 months. The AH titers fall rapidly to base-line levels, sometimes as early as 5 weeks after the infection. Anti–DNAse-B titers peak later, often in 4–6 weeks, and may remain elevated several months longer than ASO (Bisno and Stollerman, 1975).

Streptococcal antibody titers are usually obtained several weeks after the acute infection when the patient develops symptoms of kidney or heart disease and when the antibody levels may have reached a plateau. This may negate the use of paired serum samples and requires that one interpret a single titer. Here, one compares the patient's titer to a reference value defined as the titer found in the eighty-fifth percentile of the general population with no history of recent strep infection. The reference values are numerically similar with ASO and anti-DNAse B and tend to vary somewhat in different geographic areas. The school-age population experiences more frequent strep infections than adults or preschool children and thus have higher reference values, in the range of 170–200 units. Values between 85 and 125 are considered normal for the other two age groups. The values for AH are uniform in all ages with titers of 1 : 128 and below considered negative, 1 : 256 borderline, and 1 : 512 and above indicative of recent infection (Bisno and Stollerman, 1975).

The presence of an elevated titer of at least one of the three antibodies indicates a recent strep infection but not necessarily glomerulonephritis or rheumatic fever. It does appear, however, that individuals who develop rheumatic fever have significantly higher ASO titers than patients with uncomplicated strep infections (Roy *et al.*, 1956). The sensitivity with which these measurements accurately indicate a recent strep infection varies with the body site infected and with the antibody specificity. Following pharyngitis 80% of patients demonstrate a rise in ASO or anti-DNAse B; following streptococcal skin infections, only 25% demonstrate an elevated ASO and about 60% an elevated anti-DNAse B. The AH titer elevations are found in 60% of patients following pharyngitis and in lower incidence following skin infection. From experience, if a single test is done following pharyngitis, sensitivity as high as 80% can be observed; by utilizing two different tests the sensitivity rises to 90% (i.e., at least one of the two titers being elevated). Performing a third test only increases sensitivity a few percentage points and is not considered cost effective. The

general rule is to use two tests, the ASO and anti-DNAse B. In addition to the greater sensitivity of the two tests, this combination offers the better diagnostic yield of anti-DNAse B with recent skin infections (Kaplan *et al.*, 1970; Wannamaker, 1970).

An "antigen" consisting of a suspension of RBCs to which multiple different streptococcal antigens have been attached is available commercially as a test for elevated titers of strep antibodies. This is known as "streptozyme" and is set up to demonstrate a positive reaction with antibody titers in the range of 150–200 units for any one of the several antigens. There are reports both of false positives and false negatives with this procedure, which cause some to question its use. It is felt that high normal antibody levels to several different strep antigens may summate to give a false positive result. Study of sera giving false negative results usually demonstrates slight elevations of a single antibody titer, with the others being low. But when this "antigen" is used to test patients with a definite diagnosis of streptococcal sequellae, it nears 100% sensitivity. This probably relates to these latter patients usually having a marked elevation in antibody levels and suggests that the streptozyme test is adequate to identify persons who are candidates for developing strep sequellae (Bisno and Ofek, 1974; El Kholy *et al.*, 1974).

V. OTHER BACTERIAL SEROLOGY

Antibody determinations for other bacterial infections usually employ whole bacteria as antigens. Formalin-killed suspensions of *Salmonella typhi* have been used to demonstrate antibody titers in patients with typhoid fever, a method known as the Widal test. In recent decades this procedure has been expanded to include other members of the *Salmonella* species that today are more commonly isolated in deep *Salmonella* infections or the so-called enteric fevers (Wilson and Miles, 1975; Rose and Friedman, 1980). Serology is not useful in acute gastroenteritis caused by *Salmonella* because this is usually a self-limited disorder that resolves before antibody titers appear and in addition is easily diagnosed by stool culture. Antibodies in serum of patients with brucellosis and tularemia are also measured using suspensions of killed whole organisms as antigen. The fortuitous observation that patients with infection by some of the rickettsial species have antibodies that agglutinate certain strains of bacteria of the *Proteus* species led to the development of the Weil–Felix reaction (Wilson and Miles, 1975). Three strains of killed *Proteus* (OX-19, OX-2, and OX-K) are used as antigen, and the pattern of reaction is used to support a diagnosis of one of the rickettsial diseases (see Table III).

TABLE III

The Weil–Felix Reaction (*Proteus* Antigens) in Rickettsial Diseases

Rickettsial Disease	Semiquantitative Response to *Proteus* Antigens		
	OX-19	OX-2	OX-K
Rocky mountain spotted fever	++++	+	0
Other tick-borne spotted fevers	+	++++	0
Epidermic typhus	++++	+	0
Murine typhus	++++	+	0
Scrub typhus	0	0	++++
Rickettsial pox	0	0	0
Q fever	0	0	0
Trench fever	0	0	0

This method, however, is nonspecific and lacks sensitivity. Specific rickettsial antigens have become available and are now used in indirect hemagglutination (IHA), IF antibody and latex agglutination procedures. These are usually available in larger, centralized laboratories (Osterman and Eisemann, 1980). Rocky mountain spotted fever may be diagnosed by demonstration of the organisms in frozen sections of skin lesions with IF antibody techniques. Such is preferable because early treatment may be life saving, and one cannot withhold treatment until a serologic diagnosis is made. Early therapy may also prevent the development of a diagnostic titer rise (Walker *et al.*, 1978).

In evaluating such bacterial agglutinin titers, one must remember that cross-reacting antibodies may arise. For example, infection caused by other members of the *Enterobacteriaceae* family of bacteria (such as *E. coli*) can stimulate antibodies that agglutinate *Salmonellae*. Cross-reactions also occur between *Brucella* and *Pasteurella tularensis*. The number of known cross-reactions is ever increasing as additional experience is gained; this adds to the complexity of interpreting titers (Rose and Friedman, 1980).

During the first half of this century these several bacterial antigens were grouped as a panel known as the "febrile agglutinins" and used to screen the serum of patients with fever of unknown origin. This panel consisted of serologic titers for *S. typhi* O and H antigens, *S. paratyphi* A, *S. paratyphi* B, *Brucella, Pasteurella tularensis,* and the three *Proteus* species. It no longer makes a great deal of sense to test these antigens as a group or panel, because careful history and physical examination can usually lead to more efficient ordering of single agglutinin titers. But such

traditions die slowly, and one still receives requests for the febrile agglutinins.

Occasionally a patient is encountered who has two or more blood cultures positive for *Staphylococcus aureus* but only inconclusive symptoms of a deep staphylococcal infection. Because staph are occasionally found as contaminants in blood cultures, another indication of infection is sought. One procedure involves identifying elevated titers of teichoic acid antibodies in the patient's serum. Antibodies against this cell-wall constituent of the bacterium are usually found in elevated titer in persons with staph aureus endocarditis and occasionally in other deep staph infections. Their presence in the serum, in conjunction with a positive blood culture, suggests that the staph isolate should not be written off as a contaminant (Nagel *et al.*, 1975). The presence of such antibodies is identified by CIE and then titered by Ouchterlony double diffusion using sonicates of the organism as antigen. Titers above 1 : 4 have been associated with endocarditis. Elevated titers have been found in a few infections caused by other species, which suggest this test is not as specific for Staph aureus infections as one might like it to be (Tuazon and Sheagren, 1976).

VI. SEROLOGY IN ATYPICAL PNEUMONIA

The usual case of pneumonia is caused by one of several different bacteria that can usually be isolated on sputum culture. A few cases of lung infection follow an atypical and prolonged course with no pathogen demonstrated on routine cultures. Viruses such as influenza and adenovirus, *Mycoplasma pneumonia, Chlamydia psittacii, Coxiella burneti* (a rickettesia-like organism associated with various farm animals and cause of "Q fever"), and the *Legionella* species of bacteria represent some of the causes of "atypical pneumonia" that can be identified by special cultural methods or in most instances by serology.

For many years an elevated cold-agglutinin titer has been used to indicate a high probability of pneumonia with *M. pneumoniae*. Here, human type-O RBCs are incubated with serial dilutions of the patient's serum at 4°C and then examined for evidence of agglutination of the RBCs. Titers above 1 : 32 are considered significant. The mycoplasma apparently contain antigens that cross-react with the human blood group-I antigens. This antigen is present on virtually all human adult RBCs, but usually absent from the cells of newborns. Umbilical-cord cells are considered to have "i" antigen. Thus the specificity of an antibody that reacts with virtually all adult group-O RBCs in the cold is confirmed by showing that it fails to agglutinate umbilical-cord RBCs. In most sera the titer of cold agglutinins

falls as the temperature of incubation is raised above 4°C and is absent at 37°C. Rarely, the range of thermal activity of these antibodies in some patients may extend up to 37°C with coating of the patient's RBCs *in vivo* and the development of a hemolytic anemia. Occasionally, one finds an elevated cold-agglutinin titer in diseases caused by other organisms, such as adenoviruses, indicating a lack of specificity for this determination. The wider availability of *Mycoplasma*-specific serology will no doubt reduce the use of the cold agglutinins titer (Griffin and Crawford, 1965; George *et al.*, 1966; Menonna *et al.*, 1977).

Titers of antibodies to *Chlamydia* antigens are measured by complement fixing (CF)-techniques and are thought to react with group-specific and not species-specific antigens. Patients with lymphogranuloma venereum (a systemic venereal disease) or psittacosis (a pulmonary infection usually contracted from birds) would thus both have high titers in this test, and one must correlate results with the patient's clinical history. These chlamydial infections and the species causing them are distinct from the chlamydial urethritis and cervicitis so prevalent today in the sexually active population. Patients with the latter infections do not develop antibodies to the group-specific antigens (Schachter, 1978). Antibodies raised in response to infection by *Coxiella burneti* do not cross-react with the *Proteus* antigens, and one must resort to a CF, IF antibody, or microagglutination procedures using a specific *Coxiella* antigen to diagnose this infection (Warren and Hornick, 1979).

One would expect that all causes of pulmonic infection would have been identified by now. But our experience with legionnaires disease suggests that we may expect the list to grow. Regarding legionella pneumonia, isolating the organism or demonstrating them in smears of clinical material represents the optimal method of diagnosis. A serologic diagnosis is possible, but a diagnostic rise in titer is not demonstrable until at least 21 days after the onset of infection, which is too long to wait before starting appropriate therapy. A fourfold titer rise with paired serum samples or a single titer of 1 : 256 or greater is considered presumptive evidence of legionellosis. It is not known what effect early treatment for *Legionella* has upon the development of serologic titers, thus early empiric therapy may prevent development of antibody titers and inhibit making a diagnosis retrospectively. *Legionella* antibody titers are measured by IF antibody or IHA methods (Edson *et al.*, 1979; Fallon and Abraham, 1979). An alternate diagnostic procedure is to demonstrate the excretion of *Legionella* antigens in the urine by RIA, enzyme-linked immunoabsorbent assay (ELISA), or CIE methods. Such can be used early in the course of infection and once reagents become available may be the most efficient method of diagnosis (Tilton, 1979; Kahler *et al.*, 1981).

VII. VIRAL SEROLOGY

Serologic methods are commonly used in the diagnosis of individual viral infections, in epidemiologic studies, and in assessing an individual's immune status to a given virus. The method and the type of antigen used vary with the clinical situation, the information desired, and the specific virus involved. There are hundreds of different viruses, and one cannot reasonably screen by serology for all possible agents. The laboratory must know what viruses are suspected or what type of clinical syndrome exists in the patient. A laboratory will have different panels of viral antigens used to test sera from patients with respiratory infection, CNS infection, and the like. Thus, a request for "viral serology" not otherwise specified is inappropriate. In addition, the serologic methods used to screen for immunity may not be appropriate to determine if an acute infection is present. Communication between the clinician and the laboratory is essential if the necessary data are to be generated.

The CF methods have long been used in viral serology. The antigen preparations for CF are usually from infected tissue-culture cells, material from animal inoculation, or from infected embryonated eggs. A few viruses have surface antigens that attach to certain types of RBCs and can cause agglutination of the RBCs, i.e., a hemagglutination. Antibodies directed against these antigens can coat the virus and prevent the hemagglutination. These are known as hemagglutination inhibition (HAI) antibodies. Another technique is to demonstrate the inhibited ability of a suspension of virus to infect a cell culture after incubation with an antibody-containing serum. Such are called viral neutralization titers. They are seldom used, however, because the culturing of viruses and the need for appropriate controls is rather cumbersome. More recently, viral-infected cells fixed to glass slides have become commercially available for IF-antibody procedures for some of the more commonly tested viruses. Here, the patient's serum is laid over the cells, incubated, the cells washed, and the fluorescent-tagged antihuman immunoglobulin applied to demonstrate the human gamma globulin attached to the viral inclusions. The observer must be familiar with the cell structure and where in the cell the viral antigen is found. Care must be taken to rule out false positive readings resulting from the presence of an antinuclear antibody in the patient's serum (often the viral antigen appears in the nucleus (Lenette and Schmidt, 1969; Rose *et al.*, 1979; Rose and Friedman, 1980).

The rubella virus causes special problems when an infected woman is pregnant. This has provided the impetus to develop a vaccine to induce immunity and the recommendation that all women of child-bearing age, and especially those who are pregnant, document their immunity by sero-

logic titer. The rubella virus causes hemagglutination, and thus the HAI method is the standard for testing immunity. When evaluating a pregnant individual with no past documentation of immunity, who has a rash or a recent exposure to someone thought to have rubella, the timing of specimen collection relative to the date of exposure is critical. The HAI titer may start to rise at about the time the rash of acute rubella infection appears (14–21 days after exposure), and thus a single low-to-intermediate titer at this time may represent immunity resulting from infection in the distant past or may represent the rising titer of an acute infection. A second titer, 2–3 weeks later, may resolve this question. If the patient is approaching the end of the first trimester of pregnancy, such a wait may be undesirable regarding a decision for a therapeutic abortion. The CF titer, which rises early in acute rubella and then usually falls to undetectable levels in 12–18 months, or an IgM-specific rubella antibody titer is recommended here. A demonstrable HAI titer with a negative CF or absent IgM antibodies is interpreted as indicating rubella infection in the distant past and probable immunity; the presence of a demonstrable CF titer or IgM titer suggests an acute infection (Marymont and Herrmann, 1982).

An IF antibody method in which rubella virus antigen is fixed to one side of a dipstick has been developed. The dipstick is incubated in a dilute sample of the patient's serum, washed, incubated with the fluorescein-labeled antihuman immunoglobulin, and then read in a fluorometer. The reverse side of the dipstick, on which no viral antigen is attached, serves as a control for the individual's serum. This method correlates well with the standard methods and semiautomates rubella serology (Weissfeld *et al.*, 1982).

In 1932 the fortuitous observation that patients with the viral infection infectious mononucleosis had an elevated titer of antibodies that agglutinated sheep RBCs, horse RBCs, ox RBCs, and RBCs of a few other species was reported. Such antibody is known as heterophile antibody, to indicate that its formation is stimulated by one antigen and the resulting antibody reacts with other quite different antigens. Other disease states were found that also were associated with serum sheep cell agglutinins. One of these was serum sickness in which the serum antibody reacted with guinea pig kidney tissue in addition to sheep RBCs. This latter antibody was named the Forsman antibody and was recognized as being distinct from the heterophile antibody, which does not react with the guinea pig kidney (Paul and Bunnell, 1982). Such observations led to the differential agglutination test to differentiate these two antibodies. Here, the sheep-cell or heterophile titer is performed first, and if it is above 1 : 56 an aliquot of serum is extracted with guinea pig kidney antigen and the

sheep cell titer repeated. If the sheep-cell titer in the extracted serum is 1:28 or below it is indicative of Forsman antibody in the serum. No significant fall in titer suggests the presence of heterophile antibody. To confirm this a third aliquot of serum is extracted with beef RBC stroma and another sheep-cell titer performed. The titer in this test should be 1:28 or below to be consistent with the heterophile antibody of mononucleosis (Davidsohn, 1937).

The differential series of extractions and reactions have been incorporated into a much simpler and more rapid test called the monospot test, in which the horse RBC is used as antigen instead of sheep RBC. The reaction, including the extraction steps, is carried out on the surface of a glass slide and read as positive or negative with no titer being performed (Lee *et al.*, 1968a,b). Because there is no association between the severity of the disease and the level of the heterophile titer, a positive–negative result is sufficient. In the majority of mononucleosis cases there is excellent correlation between these two procedures. There are a few cases, however, in which the results are discordant, and generally the monospot test is positive when the heterophile titer is negative in a case that is otherwise mononucleosis by clinical and hematologic parameters. It is believed that the horse cell is more sensitive than the sheep cell in demonstrating the heterophile antibody in mononucleosis (Crumpacker, 1982).

The Epstein–Barr virus (EBV) was isolated in the 1960s from tumor tissue obtained from African children who suffered from a distinctive type of lymphoma (Burkitt's lymphoma). Samples of this virus were sent to laboratories in the United States and fortuitously shown to be associated with infectious mononucleosis. The Epstein–Barr virus is now accepted as the causative agent of mononucleosis, and EBV viral antigen preparations are used to measure specific serum antibody titers. The viral capsid antigen (VCA) titer, which begins to rise in the first week of illness and peaks near the end of the second week, is the most commonly used procedure. It is measured by an IF antibody method using viral-infected cells on glass slides as antigen. The diagnosis of acute infection is best made by demonstrating an elevated IgM titer to VCA. A large portion of the population have IgG antibodies to VCA because of infection in the past. Antibody to Epstein–Barr nuclear antigen (EBNA) may be demonstrated by the third or fourth week of illness in a few patients, but in most do not appear until a few months after onset of disease and then probably persist for life. Thus one may be able to diagnose an acute or current infection by noting an elevated titer to VCA and the absence of anti-EBNA antibody. The more frequently used alternative is to demonstrate IgM anti-EBV antibodies. (Andiman, 1979; Evans and Niederman, 1982).

Today, the monospot test and the heterophile titer are the most com-

monly used procedures. But, about 5–10% of all cases, and sometimes a higher proportion in preschool children, are negative with these methods. Here the physician may desire EBV specific serology. Once EBV antigen preparations become widely available they will become the method of choice because of the greater specificity. The clinical mononucleosis syndrome may be associated with cytomegalovirus infection or with toxoplasmosis. In both the heterophile titer and serology specific for acute EBV infection are negative (Crumpacker, 1982).

The discovery of the hepatitis B virus surface antigen (HBsAg) and its rapid application in screening for blood donors who potentially may transmit hepatitis is a good example of putting new discoveries to practical use. There are now recognized three sets of antigen and antibody specificities that can be used in the diagnosis of hepatitis B infection:

HBsAg	anti-HBs
Core antigen (HBcAg)	anti-HBc
"e" antigen (HBeAg)	anti-HBe

These are usually all measured by radioimmunoassay (RIA) or enzyme immunoassay (EIA) methods.

In the usual infection, HBsAg is found in the serum during the incubation period and during the symptomatic phase of hepatitis. It usually disappears in the mid or late symptomatic phase. The core and "e" antigens are present during the same period but for a shorter time; thus their measurement is redundant in the usual infection. After the disappearance of serum HBsAg there may be a period of several weeks or even months before anti-HBs is demonstrable. This is known as the serologic window. Fortunately, anti-HBc routinely appears before HBsAg disappears and persists in high titer for a year or more before falling to low levels, thus filling in the "window." This provides a trio of diagnostic tests—HBsAg, anti-HBc, and anti-HBs—to cover all stages of the infection. Anti-HBs with little or no anti-HBc indicates infection in the distant past and immunity to infection. There is a small set of patients with acute hepatitis infection (3–5%) in whom the HBsAg persists indefinitely and no anti-HBs appears. These are at very high risk to develop chronic hepatitis and, possibly, cirrhosis. In this group the anti-HBc levels are very high (Bianchi and Gudat, 1979; Deinhardt, 1980; Overby, 1980; Ganem, 1982).

An antigen associated with hepatitis A virus (HAV) infection has recently become available for demonstrating the presence of serum antibody in this variety of hepatitis. Demonstration of IgM-specific antibodies, however, is needed to diagnose acute HAV infection because a significant proportion of the population has IgG titers from old, subclinical infection. This is routinely done by RIA or EIA. The HAV antigen

may be demonstrated in the feces (but not the serum) in the early acute infection in 50% of cases (Deinhardt, 1980; Overby, 1980).

The TORCH complex is an acronym for a group of infectious agents that can cause severe damage to the fetus and newborn. These are mostly (but not all) viral infections where the mother is acutely infected during pregnancy with the agent reaching the placenta through the maternal blood stream and infecting the fetus. Some of these agents such as herpes virus and cytomegalovirus may also infect the cervix and be picked up by the baby during passage through the birth canal. The agents involved are T-Toxoplasma, O-Other (syphilis, varicella virus), R-Rubella, C-Cytomegalovirus, H-Herpes virus. Infection with many of these agents is relatively common, and paired sera or IgM specific titers thus are necessary to document acute infection with serology. Titers can be done on both the mother and infant with due consideration being given to the presence of maternal IgG in the newborn's serum when interpreting the latter's titers. There has been some criticism of using this grouping as a panel instead of testing with selected antigens as the clinical signs and symptoms suggest. Originally, the acronym was suggested in regard to the organisms one should consider in evaluating cases of intrauterine infection and not as a testing panel. There are no good standards generally available for controlling these procedures and interlaboratory surveys have indicated wide variability of results among laboratories. This is especially worrisome if major therapeutic decisions, such as interruption of pregnancy, are to be made on the basis of serology alone. In this situation clinicians may be asking for more than can be provided with the current state of the art (Nahmias, 1974).

VIII. ANTIBODY TO TISSUES

There has long been an interest in antibody-mediated disease in man. Asthma and the other allergic diseases are obvious examples and are associated with activity of IgE. These are not considered here. There are several other diseases characterized by tissue infiltrations of lymphocytes and plasma cells, serum antibody reactive with tissue components, and tissue deposits of immunoglobulin and complement. These are suspected as having an autoimmune mechanism in their pathophysiology, and demonstration of such serum antibody in a patient is important in making the diagnosis. In most of these conditions the stimulus for antibody formation and its precise role, if any, in the pathophysiology of the disease is uncertain (Rose *et al.*, 1979).

Several classifications of the autoimmune diseases are available. Many employ an organ system classification. But I prefer one based on the antigen–antibody specificity as suggested by Steffen (1970); group I—diseases associated with organ specific antibodies; group II—non–organ-specific antibodies in which the antigen is found in many different tissues; and group III—antibodies specific for one of the formed elements of the blood.

A. Antithyroid Antibodies

Diseases of the endocrine glands are most frequently encountered in group I with antibody reactive with thyroid, adrenal, gonad, and, occasionally, pancreatic islet-cell antigens demonstrable in patients with disease of these respective organs. The thyroid gland and the disorder chronic thyroiditis have been extensively studied. Histologically, the gland is often densely infiltrated with lymphocytes and plasma cells, and in virtually every patient one or more of the several antithyroid antibodies is demonstrable in the serum.

Antithyroglobulin antibody is most frequently demonstrable by (1) IF-antibody using tissue sections of human or monkey thyroid gland as antigen, (2) IHA of tanned RBCs coated with thyroglobulin antigen, (3) radioimmunoassay, or (4) ELISA methods. Approximately 80% of thyroiditis patients have serum antibody to thyroglobulin as measured by IHA, the most commonly used method. Antibody to thyroid colloid and antibody to the cytoplasmic organelles of thyroid epithelial cells, the microsomes, may also be demonstrated in patients' serum. These are demonstrated by IF-antibody methods using microscopic sections of human thyroid gland as antigen. In thyroiditis both of these latter antibodies may occur in addition to thyroglobulin antibody, or only one of the three appear alone. It is generally believed that the absence of any thyroid antibody activity in a patient's serum makes the diagnosis of chronic thyroiditis unlikely (Balfour *et al.*, 1961).

Patients with Graves' disease, primary myxedema, adenomatous goiter, thyroid carcinoma, pernicious anemia, and some allergic disorders may have serum antibody to thyroglobulin and thyroid microsomal antigen in addition to patients with thyroiditis. Also, healthy persons in the older age groups may have low titers of such antibodies. Thus, the specificity of the determination is rather low. But patients with thyroiditis generally have significantly higher titers than individuals without thyroid disease. This presence of thyroid antibodies in normal persons suggests that humoral immunity does not necessarily cause tissue damage but may

only be an indicator of such. There is increasing interest in examining the role of cellular immunity, i.e., the T lymphocytes, as the vehicle of tissue damage in this process (Strakosch *et al.*, 1982).

Immunoglobulin G reactive with the site on the thyroid cell membrane to which thyroid-stimulating hormone (TSH) attaches has been found in most patients with Graves' disease (hyperthyroidism). This immunoglobulin has a greater affinity for this site than TSH and appears in most but not all instances to stimulate the gland to produce and release excess hormone. The original demonstration of this IgG involved *in vivo* stimulation of the mouse thyroid by human IgG, which was named long acting thyroid stimulator (LATS). Today the designation LATS is applied only to IgG demonstrated by this specific method. It is found in 60–70% of cases of Graves' disease. Sera from LATS-negative cases have revealed IgG reactive with the TSH receptor by other methods. Thus it appears there are several specificities for the IgG in this disease, and as a group they are called thyroid-stimulating antibody (TSAb) or thyroid-stimulating immunoglobulin (TSIg). Other methods of demonstrating TSIg include using human thyroid cells in tissue culture and observing increased intracellular colloid droplets or by measuring altered c-AMP levels as an indicator of increased metabolic activity. Although these determinations are not routinely performed, they serve to expand our knowledge of autoimmunity in thyroid disease (Volpe, 1977; Brown *et al.*, 1978; Strakosch *et al.*, 1982).

Antibody directed against the insulin receptors on human tissue cells have been described as blocking the binding of insulin to the membrane receptors and thus inhibiting its action of moving glucose from the plasma into cells. This results in hyperglycemia and a diadetic glucose tolerance curve. Some patients with receptor antibody also have serum immunoglobulin that stimulates glucose oxidation, suggesting that the antibody not only blocks insulin binding but in some cases acts like insulin (Kahn *et al.*, 1977a,b; Kasuga *et al.*, 1978; Flier *et al.*, 1979). In other patients, antibody that binds human insulin has been described as associated with hypoglycemia. In this situation the antibody is thought to bind insulin in the postprandial state and later to release it from its low-affinity binding sites on the immunoglobulin at a time when this results in excess free insulin and hypoglycemia. These antibodies should be distinguished from the antibody to bovine and porcine insulin demonstrable in many diabetics receiving exogenous insulin. The latter antibodies may inactivate the injected insulin resulting in a hyperglycemia (Anderson *et al.*, 1978).

Table IV lists several of the tissue antibodies associated with disease of specific organs. Many of these are measured in the more specialized

TABLE IV

Commonly Encountered Tissue-Specific Antibodies

Organ	Antibody specificity	Disease association	Patients reactive (%)
Thyroid	Thyroglobulin	Thyroiditis	75–95
	Microsomal (thyroid)	Thyroiditis	70–90
	Thyroid colloid	Thyroiditis	40–70
	TSH receptor	Grave's disease	50
Adrenal	Adrenal cell cytoplasm	Addison's disease	40–70
Parathyroid	Parathyroid cell cytoplasm	Idiopathic hypoparathyroidism	40
		Addison's disease	25
Pancreatic Islet	Islet cell cytoplasm	Insulin-dependent diabetes	60–85
Stomach	Parietal cell cytoplasm	Pernicious anemia	90
		Atrophic gastritis	50
	Intrinsic factor	Pernicious anemia	40–60
Skin	Basement membrane (skin)	Bullous pemphigoid	70
	Intracellular bridge substance of epithelial cells	Pemphigus group	90
Kidney	Glomerular basement membrane	Goodpasture's disease	90
Skeletal Muscle	Acetylcholine receptor	Myasthemia gravis	60–85
	Striated muscle fiber	Myasthemia gravis	30–50

laboratories by IF-antibody techniques using tissue sections on glass slides to demonstrate the specificity.

B. The Rheumatic Diseases

In Steffen's group II the antibody reacts with antigens of a general nature found in many tissues. The rheumatic diseases (also known as collagen vascular diseases and immune complex diseases) with SLE as the prototype, are the most extensively studied of this group. In SLE the clinicopathological picture is one of multiple organ damage with kidney, brain, heart, serous surfaces (pleura, etc.), skin, and joints being involved. It appears that tissue damage starts around small blood vessels and progresses to parenchymal cell damage and scarring (thus the early designation as a collagen vascular disease). Antinuclear antibodies (ANA) are regularly found in the serum of these patients. The kidney damage has, in turn, been shown to be related to glomerular deposits of ANA, nuclear antigens, and, often, complement—thus the postulate that immune complexes with complement are formed, or are caught in, the glo-

merulus and in turn excite an inflammatory reaction causing tissue destruction and renal failure. This is the major cause of death in SLE (Klippel and Decker, 1983).

The elucidation of the ANA story received a major boost in 1947 with the chance observation of the LE-cell phenomena in SLE. It was demonstrated that blood neutrophils would phagocytose altered nuclear material, usually an altered lymphocyte nucleus, when whole blood of these patients was incubated *in vitro*. Later studies showed that a serum antibody with antinuclear activity was responsible for this reaction. Presently, ANA is identified by IF-antibody techniques in which whole cells fixed to glass slides are used as antigen. Many different cells may be used: tissue culture cells, smears of human peripheral blood, or tissue sections of human or animal organs (liver, lymph nodes, or kidney are sometimes used). Patients' serum is laid over the cells, incubated, and the cells washed to remove the excess serum proteins not attached to the cells. The fluorescent tagged antihuman gamma globulin is then laid over the cells, incubated, and the cells washed again. The cells are examined microscopically for the presence and pattern of nuclear fluorescence. The patterns commonly observed are (1) the diffuse or homogeneous pattern; (2) rim or peripheral pattern; (3) speckled pattern; and (4) nucleolar pattern. The various patterns result from differing antigen specificities to constituents of the cell nucleus (Nakamura, 1974; Friou and Quismorio, 1975).

The presence of ANA is not specific for SLE. It is also found in most other members of the rheumatic disease group (rheumatoid arthritis, scleroderma, dermatomyositis, etc.) in low or intermediate titers and may be found in 5–10% of normal individuals in the over-60 age group. It is quite sensitive, however, and the absence of ANA almost rules out a diagnosis of SLE. The SLE patients as a group demonstrate a variety of patterns of ANA, and some individuals simultaneously have more than one pattern. Table V shows the more common disease-pattern associations (Husain *et al.*, 1974; Notman *et al.*, 1975; Rose and Friedman, 1980).

High titer of antibody with specificity for native DNA (also called double-stranded DNA) is quite specific for SLE. This antibody is commonly demonstrated by IF-antibody methods using the microorganism, *Crithidia luciliae*, a hemoflagellate, as antigen. The kinetoplast, which consists of mitochondrial DNA, is the organelle within the cell demonstrating this specificity. The observer needs to be familiar with the morphology of the organism to identify this. An RIA procedure has also been developed to identify anti-DNA antibodies. In the literature this is often referred to as serum DNA-binding capacity (Pincus *et al.*, 1969).

The speckled pattern of ANA, when demonstrable in high titer, has

TABLE V

Comparison of Antinuclear Antibody (ANA) with Nuclear Component Specificity and Variety of Rheumatic Disease

ANA pattern	Antibody specificity[a]	Primary disease association
Homogeneous	Double-stranded DNA	SLE
	Soluble deoxynucleoprotein (DNA-histone complex)	SLE; drug-induced SLE
	Histone	Drug-induced SLE
Rim	Soluble deoxynucleoprotein (DNA-histone complex)	SLE; drug-induced SLE
Speckled	Sm (soluble glycoprotein)	SLE, MCTD
	RNP (RNA-protein complex)	MCTD (high levels); other rheumatic diseases (low levels)
	Scl-1 atypical speckled	Progressive systemic sclerosis; SLE
	SSB	Sjögren's syndrome; SLE
Nucleolar	Specificity unknown	Progressive systemic sclerosis; Sjögren's syndrome
Negative	SSA	Sjögren's syndrome
	RAP	Rheumatoid arthritis; Sjögren's syndrome
	Single-stranded DNA	

[a] Many of these antibody specificities are demonstrable in low titer in serum of patients with rheumatic diseases other than their primary association.

been strongly associated with a subset of SLE called mixed connective tissue disease (MCTD). These patients also often have a very high titer of antibody with specificity for antigens that can be extracted from nuclei by saline. This is referred to as extractable nuclear antigen (ENA) and the antibody as anti-ENA. There are two antigenic specificities that can be distinguished within anti-ENA; these are anti-Sm and antideoxyribonucleoprotein (anti-RNP). Some authors maintain that patients with anti-ENA have a different and more benign clinical course than the usual SLE patient, respond better to steroids, and do not develop the degree of renal damage (Reichlin and Mattioli, 1972; Sharp *et al.*, 1976). Longer follow-up of such patients, however, has suggested such is not the case and that they are no different clinically (Nimelstein *et al.*, 1980).

The ANA and immune complexes are now well accepted as playing a role in the pathophysiology of SLE, but the initiator for the production of ANA in high titer is not known. Several viruses have been suggested as

playing an etiologic role and that, perhaps, individuals who develop SLE do so because they react to the challenge of a virus infection in an atypical manner (Phillips, 1975). Immune complex diseases "run" in some families, and thus some persons may be genetically predisposed to this different reaction (Miller and Schwartz, 1979). In addition, the regulatory balance of the T and B lymphocytes may be altered as a common pathway to such disease (Weigle, 1981).

The development of a positive serum ANA and of an SLE-like syndrome has been associated with the taking of certain medications. At least 35 different drugs have been reported in this association, with most being used in heart disease or hypertension or as anticonvulsants. The phenothiazines and hydralizine, procainamide, and isoniazid are commonly used drugs causing such problems. Approximately 50% of the patients taking procainamide develop a positive ANA titer, but only 10% develop the lupus syndrome. In most of these cases the lupus symptoms disappear when the drug is withdrawn (Bloomgren *et al.*, 1969; Hess, 1981).

The specificity of the ANA in drug-induced SLE is thought to be different than that in the spontaneous form of SLE. Antinative DNA is usually absent, and instead one often finds antibody to histones. Also there are indications that immune complexes are not involved in the symptom generation. Regarding the pathophysiology of the drug-induced disease, it has been suggested that some drugs act as haptens by combining with self-antigens and inducing antibody that reacts with the self-antigen alone (Schoen and Trentham, 1981). Alternatively, there is evidence that procainamide alters the balance of T and B lymphocytes, with the resultant production of antibody to self-antigens by B-cell clones no longer held in check by suppressor T cells (Bluestein *et al.*, 1981).

Rheumatoid arthritis is another of the rheumatic disease group in which an immune mechanism seems to play a role. There is an ongoing search for an infectious organism that initiates the reaction in the joints and the production of the unusual serological markers. Viruses, mycoplasma, and chlamydia have been suggested, but none are found consistently in human cases. Rheumatoid factor (RF), which is strongly associated with the disease, is an IgM antibody that reacts with the Fc portion of an IgG molecule, especially an IgG that has combined with its antigen. The IgG molecule unfolds somewhat in the process of combining with antigen, and this exposes the sites on the Fc portion that combine with rheumatoid factor. It is not clear why this serum factor should be associated with joint disease. But demonstrating its presence in the serum of a patient with the appropriate clinical symptoms is an important diagnostic aid (Christian, 1975).

Rheumatoid factor is frequently measured by the latex fixation method. Here, Cohn fraction II of serum proteins is fixed to latex beads of a specific size. The suspension of coated beads is incubated with serum and observed for visible agglutination. This method is quite sensitive but less specific for rheumatoid arthritis. Another method, employing sheep RBCs coated with antibody (usually rabbit antisheep RBC antibody), is more specific but less sensitive. The rheumatoid factor is another serum antibody found in low titer in 5–10% of healthy elderly persons. It is also found in other members of the rheumatic disease group and occasionally in a chronic infection such as bacterial endocarditis and large abscesses (Waller, 1973). All of this serves to emphasize the lack of specificity and need to interpret results in light of the clinical presentation. In this regard it is interesting that in the childhood form of rheumatoid arthritis, known as Still's disease, rheumatoid factor is found in as few as 10% of cases (Cathcart, 1975).

Not all rheumatoid factor is IgM. A small fraction of cases have only an IgG rheumatoid factor, which does not react in the latex or sheep-cell methods. This can be identified by special procedures and shown to account for some but not all of the cases that are "sero-negative" by the usual methods that react with IgM RF (Christian, 1975).

Other members of the rheumatic disease group—such as dermatomyositis, which is associated with antibody to antigens in skeletal muscle, and Sjogren's syndrome, in which antibody reactive with portions of salivary gland may be found—often have rheumatoid factor and ANA in addition to an antibody fairly specific to that entity. This emphasizes the overlap and diversity in this group of diseases. The average hospital laboratory limits itself to determination of ANA, RF, and antinative DNA.

C. Immunohematology

Steffen's group III consists of antibody reactive with the various blood elements usually considered under the subsection of immunology known as immunohematology. Antibodies with specificity for antigens of RBCs, leucocytes, and platelets can lead to altered numbers and function of these elements in patients in whom they occur. Within the clinical laboratory such studies are usually carried out in the blood bank and not the serology laboratory.

Consideration of the antigens on RBCs and their antibodies plays a major role in matching donor and patient for transfusion. There are at least 22 sets of blood group antigens, and 10 of these may present problems in transfusion therapy. The ABO system is the only one in which we routinely find serum antibody. Supposedly, as infants, we are all stimu-

lated either by cross-reacting or identical antigens on intestinal bacteria, such as *Escherichia coli,* or other environmental sources to produce antibody to those antigens of the ABO system absent from our own cells. Thus patient and donor must be matched for antigens of the ABO system to prevent a transfusion reaction. Virtually all other antigen groups require injection of RBCs containing the foreign antigen for antibody production to result. Transfusion and pregnancy are the two clinical situations likely to result in such. During delivery of an infant there may be a mini-infusion (usually 30 ml or less) of fetal blood into the mother, which can in turn stimulate antibody formation. Experience indicates that the Rh-D antigen is the single most potent of these other antigens, and, thus, donor and patient are routinely matched for it also. The remaining antigens induce antibody so infrequently that it is considered impractical to also match for these routinely (Huestis *et al.,* 1981).

The presence of the Rh-D antigen on an individual's RBCs designates the person as Rh positive and its absence as Rh negative. The Rh blood group system is rather complex, with more than 20 antigen specificities recognized in it. The separation of persons into two groups, positive and negative, is a gross oversimplification used for expediency in the everyday transfusion service (Race and Sanger, 1975; Huestis *et al.,* 1981).

The remaining blood-group antigens may cause a transfusion reaction in a recipient who has developed an antibody. These antibodies are called atypical antibodies to distinguish them from the anti-A or anti-B which are typically found in 95% of persons (individuals of blood group AB have no anti-A or anti-B). Serum of both patient and donor are routinely screened for atypical antibody by using as antigen a suspension of several human group-O RBCs that in the aggregate contain most known RBC antigens. Group-O cells are used to exclude a reaction resulting from anti-A and anti-B. The test is performed by incubating serum with the suspensions of RBC, centrifuging briefly to pack the cells close together, and then resuspending the cells and observing for visible agglutination. If this reaction is negative, the cells are washed to remove all nonadherent serum proteins and then incubated with serum containing antihuman γ-globulin, so-called Coombs serum, which is essentially the same antibody used in IF-antibody procedures except it does not contain the fluorescent label. Following incubation the suspension is centrifuged briefly, and the cells are resuspended and visually inspected for agglutination. If none is seen, the suspension is checked microscopically before the reaction is called negative (Huestis *et al.,* 1981).

Donors whose serum contains atypical antibodies are not used for whole-blood transfusions, but their cells could be used if washed free of plasma protein. If the patient's serum has an antibody, the specificity is

determined by using a panel of human group-O RBCs with known surface antigens. A donor is sought who is ABO, Rh-D compatible, and who lacks the antigen in question. This procedure of screening for serum atypical antibody is known as the indirect Coombs test to distinguish it from the direct Coombs test (Huestis *et al.*, 1981).

Occasionally, patients produce antibody reactive with their own red cells, which leads to shortened survival of the RBCs and anemia—known as autoimmune hemolytic anemia (AIHA). The direct Coombs test is used to identify the presence of antibody on the RBC. Here, the patient's RBCs are washed with saline and then incubated with Coombs serum and examined microscopically. Agglutination indicates the presence of γ-globulin on the surface of the patient's cells, who in turn is said to have a Coombs positive anemia. The AIHA is usually associated with tumors of the lymphoma group or with SLE (Wintrobe *et al.*, 1981).

IX. TRANSPLANTATION IMMUNOLOGY

The tremendous achievements in organ transplantation have been intimately associated with developments in immunogenetics. This involves the understanding of human transplantation or human leucocyte antigens (HLA antigens). Inheritance of these antigens in man is controlled by at least four closely spaced loci on chromosome six. These loci are designated as *A*, *B*, *C*, and *D/Dr* with multiple alleles (between 8 and 39) at each and which in turn are usually designated by numbers. The antigens controlled by the *A*, *B*, and *C* loci appear on most tissue cells in humans with the exception of the RBCs. These are usually identified by testing the individual's leucocytes with panels of antisera specific for the antigens. Antigens controlled by the *D/Dr* locus are demonstrable on lymphocytes by the procedure of mixed lymphocyte culture and recently by serologic means. The mixed lymphocyte culture involves cultivation of lymphocytes of donor and recipient together in tissue culture to determine if one or the other has antigens that stimulate a reaction in the other. Usually, a person has two antigens from each of these groups. In transplantation, one attempts to match donor and recipient for the *A*, *B*, and *D/Dr* antigens. Antigens of the *C* locus are not sufficiently defined to be used on a routine basis (Carpenter, 1980; Dausset, 1981).

The *D/Dr* locus is thought to play a significant role in controlling a person's ability to respond to various immunologic challenges. There appears to be a functional association between this locus and the other loci such that some immunologic diseases occur more often in individuals with certain of the HLA antigens. It has been suggested that certain

antigen specificities on lymphocytes alter the immune response to certain microorganisms such that a chronic infection or a symbiotic relationship results instead of the organism being killed and removed. This is speculative but could form the basis of an association between diseases such as SLE or rheumatoid arthritis and certain infectious organisms (Moore, 1979; Dausset, 1981).

X. THE CLINICAL IMMUNOLOGY LABORATORY

The clinical immunology laboratory is one that has expanded capabilities to evaluate the broad range of a patients' immune status and is usually located in a university medical center. The procedures performed in such a laboratory vary from center to center and are partly a function of the interests and expertise of the people involved. The patients with repeated or unusual infections who may have some deficiency in their host defenses are a group extensively studied here. The B-lymphocyte function is evaluated by quantitation of the immunoglobulin classes or may extend to quantitating the amount of immunoglobulin generated by a specific antigen challenge. The function of the T lymophcytes in mounting a cellular immune reaction can be evaluated *in vivo* by the delayed skin-test reaction to the intradermal injection of mumps, candida, or similar common antigens to which most of us develop reactivity. Here, the patient is expected to react with slight swelling and redness at the site in 48 hr. *In vitro* procedures are also available to study the functions of T cells. There are several subsets of T cells now recognized on the basis of cell surface markers. These are evaluated by use of antisera specific for the various markers. This contributes to increased understanding of the immunological events occurring in patients with the rheumatic diseases and immunodeficiency states, including patients undergoing immunosuppressive therapy (Reinherz and Rosen, 1981). Overall complement activity is evaluated by the so-called CH50 level in a modified complement-fixation procedure. The individual complement components may be quantitated in patients with low CH50 levels. This laboratory's functions may overlap with hematology in the study of the granular leucocytes, i.e., the polys, in regard to phagocytosis and killing of organisms. In some centers there is also overlap with clinical chemistry in the study of serum and urine monoclonal proteins and immunoglobulin quantitation.

Expansion of our understanding of the role of immune mechanisms in disease and development of methods to test these has led to serodiagnostic methods being applied to more than the diagnosis of infectious disease.

This is perhaps best appreciated by examining the table of contents of the *Manual of Clinical Immunology* (Rose and Friedman, 1980).

REFERENCES

Anderson, J. H., Blackard, W. G., Goldman, J., and Rubenstein, A. H. (1978). *Am. J. Med.* **64,** 868–873.

Andiman, W. A. (1979). *J. Pediatr.* **95,** 171–182.

Balfour, B. M., Doniach, D., Roitt, I. M., and Couchman, K. G. (1961). *Br. J. Exp. Pathol.* **42,** 307–316.

Balows, A., and Hausler, W. J. (eds.) (1981). "Diagnostic Procedures for Bacterial, Mycotic and Parasitic Infections," 6th ed. Amer. Public Health Assoc., Washington, D.C.

Bianchi, L., and Gudat, F. (1979). *In* "Progress in Liver Diseases" (H. Popper and F. Schaffner, eds.), Vol. 6, pp. 371–392. Grune & Stratton, New York.

Bisno, A. L., and Ofek, I. (1974). *Am. J. Dis. Child.* **127,** 676–681.

Bisno, A. L., and Stollerman, G. H. (1975). *In* "Laboratory Diagnostic Procedures in the Rheumatic Diseases" (A. S. Cohen, ed.), 2nd ed., pp. 207–263. Little, Brown, Boston, Massachusetts.

Bloomgren, S. E., Condemi, J. J., Bynal, M. C., and Vaughan, J. H. (1969). *New Engl. J. Med.* **281,** 64–66.

Bluestein, H. G., Redelman, D., and Zvaifler, N. J. (1981). *Arthritis Rheum.* **24,** 1019–1023.

Brown, J., Solomon, D. H., Beall, G. N., Terasaki, P. I., Chopra, I. J., Van Herle, A. J., and Wu, S. Y. (1978). *Ann. Intern. Med.* **88,** 379–391.

Carpenter, C. B. (1980). *New Engl. J. Med.* **302,** 860–861.

Cathcart, E. S. (1975). *In* "Laboratory Diagnostic Procedures in the Rheumatic Diseases" (A. S. Cohen, ed.), 2nd ed., pp. 107–129. Little, Brown, Boston, Massachusetts.

Christian, C. L. (1975). *In* "Laboratory Diagnostic Procedures in the Rheumatic Diseases" (A. S. Cohen, ed.), 2nd ed., pp. 95–105. Little, Brown, Boston, Massachusetts.

Crumpacker, C. S. (1982). *Rev. Infect. Dis.* **4,** 1069–1070.

Dausset, J. (1981). *Science (Washington, D.C.)* **213,** 1469–1474.

Davidsohn, I. (1937). *JAMA J. Am. Med. Assoc.* **108,** 289–295.

Deinhardt, F. (1980). *J. Infect. Dis.* **141,** 299–305.

Edson, D. C., Stiefel, H. E., Wentworth, B. B., and Wilson, D. L. (1979). *Ann. Intern. Med.* **90,** 691–693.

El Kholy, A., Hafez, K., and Krause, R. M. (1974). *Appl. Microbiol.* **27,** 748–752.

Escobar, M. R., Dalton, H. P., and Allison, M. J. (1970). *Am. J. Clin. Pathol.* **53,** 886–890.

Evans, A. S., and Niederman, J. C. (1982). *In* "Viral Infections of Humans, Epidemiology and Control" (A. S. Evans, ed.), 2nd ed., pp. 253–281. Plenum, New York.

Fallon, R. J., and Abraham, W. H. (1979). *Ann. Intern. Med.* **90,** 684–686.

Felman, Y. M., and Nikitas, J. A. (1980). *Arch. Dermatol.* **116,** 84–89.

Flier, J. S., Kahn, R., and Ross, J. (1979). *New Engl. J. Med.* **300,** 413–419.

Friou, G. J., and Quismorio, F. P. (1975). *In* "Laboratory Diagnostic Procedures in the Rheumatic Diseases" (A. S. Cohen, ed.), 2nd ed., pp. 159–205. Little, Brown, Boston, Massachusetts.

Ganem, D. (1982). *Rev. Infect. Dis.* **4,** 1026–1047.

George, R. B., Ziskind, M. M., Rasch, J. R., and Mogabgab, W. J. (1966). *Ann. Intern. Med.* **65,** 931–942.

Goodhart, G. L., Brown, S. T., Zaidi, A. A., Pope, V., Larsen, S., and Barrow, J. E. (1981). *Arch. Intern. Med.* **141,** 1045–1050.
Griffin, J. P., and Crawford, Y. E. (1965). *JAMA J. Am. Med. Assoc.* **193,** 1011–1016.
Hess, E. V. (1981). *Arthritis Rheum.* **24** (Suppl.), vi–ix.
Hooshmand, H., Escobar, M. R., and Kopf, S. W. (1972). *JAMA J. Am. Med. Assoc.* **219,** 726–729.
Huestis, D. W., Bove, J. R., and Busch, S. (1981). "Practical Blood Transfusion," 3rd ed. Little, Brown, Boston, Massachusetts.
Husain, M., Neff, J., Daily, E., Townsend, J., and Lucas, F. (1974). *Am. J. Clin. Pathol.* **61,** 59–65.
Jaffe, H. W., Larsen, S. A., Peters, M., Jove, D. F., Lopez, B., and Schroeter, A. L. (1978). *Arch. Intern. Med.* **138,** 252–255.
Kahler, R. B., Zimmerman, S. E., Wilson, E., Allen, S. D., Edelstein, P. H., Wheat, L. J., and White, A. (1981). *Ann. Intern. Med.* **94,** 601–605.
Kahn, C. R., Baird, K., Flier, J. S., and Jarrett, D. B. (1977a). *J. Clin. Invest.* **60,** 1094–1106.
Kahn, C. K., Meggesi, K., Bar, E. S., Eastman, R. C., and Flier, J. S. (1977b). *Ann. Intern. Med.* **86,** 205–219.
Kaplan, E. L., Anthony, B. F., Chapamn, S. S., Ayoub, C. M., and Wannamaker, L. W. (1970). *J. Clin. Invest.* **49,** 1405–1414.
Kasuga, M., Akanuma, Y., Tsushima, T., Iwamoto, Y., Kosaka, K., Kibata, M., and Kawanishi, K. (1978). *Diabetes* **27,** 938–945.
Kaufman, R. E., Olansky, D. D., and Wiesner, P. J. (1974). *J. Am. Vener. Dis. Assoc.* **1,** 79–84.
Klippel, J. H., and Decker, J. L. (1983). *In* "Internal Medicine" (J. H. Stein, ed.), pp. 1039–1047. Little, Brown, Boston, Massachusetts.
Kraus, S. J., Haserick, J. R., and Lantz, M. A. (1970). *New Engl. J. Med.* **282,** 1287–1290.
Lee, C. L., Davidsohn, I., and Slaby, R. (1968a). *Am. J. Clin. Pathol.* **49,** 3–11.
Lee, C. L., Davidsohn, I., and Panczyszynm, O. (1968b). *Am. J. Clin. Pathol.* **49,** 12–18.
Lennette, E. H., and Schmidt, N. J. (1969). "Diagnostic Procedures for Viral and Rickettsial Infections." Amer. Public Health Assoc., Washington, D.C.
Lesinski, J., Krach, J., and Kadziewicz, E. (1974). *Br. J. Vener. Dis.* **50,** 334–340.
Mamunes, P., Cave, V. G., Budwell, J. W., Andersen, J. A., and Steward, R. E. (1970). *Am. J. Dis. Child.* **120,** 17–21.
Marymont, J. H., and Herrmann, K. L. (1982). *Lab. Med.* **13,** 83–91.
McKenna, C. H., Schroeter, A. L., Kierland, R. R., Stilwell, G. G., and Pien, F. D. (1973). *Mayo Clin. Proc.* **48,** 545–548.
Menonna, J., Chmel, H., Menegus, M., Dowling, P., and Cook, S. (1977). *J. Clin. Microbiol.* **5,** 610–612.
Miller, K. B., and Schwartz, R. S. (1979). *New Engl. J. Med.* **301,** 803–809.
Moore, J. E., and Mohr, C. F. (1952). *JAMA J. Am. Med. Assoc.* **150,** 467–473.
Moore, S. B. (1979). *Mayo Clin. Proc.* **54,** 385–393.
Nagel, J. G., Tuazon, C. U., Cardilla, T. H., and Sheagren, J. M. (1975). *Ann. Intern. Med.* **82,** 13–17.
Nahmias, A. J. (1974). *Hosp. Pract.* **9,** 65–72.
Nakamura, R. M. (1974). "Immunopathology, Clinical Laboratory Concepts and Methods." Little, Brown, Boston, Massachusetts.
Newman, R. B. (1974). *CRC Crit. Rev. Clin. Lab. Sci.* **4,** 1–28.
Nimelstein, S. H., Brody, S., McShane, D., and Holman, H. R. (1980). *Medicine (Baltimore)* **59,** 239–248.

Nissenson, A. R., Baroff, L. J., Fine, R. N., and Knutson, D. W. (1979). *Ann. Intern. Med.* **91,** 76–86.
Notman, D. D., Kurata, N., and Tan, E. M. (1975). *Ann. Intern. Med.* **83,** 464–469.
Osterman, J. V., and Eisemann, C. S. (1980). *In* "Manual of Clinical Immunology" (N. R. Rose and H. Friedman, eds.), 2nd ed., pp. 707–713. Amer. Soc. for Microbiology, Washington, D.C.
Overby, L. R. (1980). *In* "Current Hepatology" (G. L. Gitnick, ed.), Vol. 1, pp. 55–94. Houghton, Mifflin, Boston, Massachusetts.
Paul, J. R., and Bunnel, W. W. (1982). *Rev. Infect. Dis.* **4,** 1062–1068.
Phillips, P. E. (1975). *Ann. Intern. Med.* **83,** 709–715.
Pincus, T., Schur, P. H., Rose, J. A., Decker, J. L., and Talal, N. (1969). *New Engl. J. Med.* **281,** 701–705.
Race, R. R., and Sanger, R. (1975). "Blood Groups in Man," 6th ed. Blackwell, London.
Ravel, R. (1976). *Lab. Med.* **7,** 22–24, 34.
Reichlin, M., and Mattioli, M. (1972). *New Engl. J. Med.* **286,** 908–911.
Rein, M. F. (1983). *In* "Internal Medicine" (J. H. Stein, ed.), pp. 1395–1398. Little, Brown, Boston, Massachusetts.
Reinherz, E. L., and Rosen, F. S. (1981). *Am. J. Med.* **71,** 511–513.
Rose, N. R., and Friedman, H. (eds.) (1980). "Manual of Clinical Immunology," 2nd ed. Amer. Soc. for Microbiology, Washington, D.C.
Rose, N. R., Milgrom, F., and van Oss, C. J. (eds.) (1979). "Principles of Immunology," 2nd ed. Macmillin, New York.
Roy, S. B., Sturgis, G. P., and Massell, B. F. (1956). *New Engl. J. Med.* **254,** 95–102.
Schachter, J. (1978). *New Engl. J. Med.* **298,** 428–435, 490–495, 540–549.
Schoen, R. T., and Trentham, D. E. (1981). *Am. J. Med.* **71,** 5–7.
Schroeter, A. L., Lucas, J. B., Price, E. B., and Falcone, V. H. (1972). *JAMA J. Am. Med. Assoc.* **221,** 471–476.
Sell, S. (1980). "Immunology, Immunopathology, and Immunity," 3rd ed. Harper & Row, New York.
Sharp, G. C., Irvin, W. S., May, C. M., Holamn, H. R., McDuffie, F. C., Hess, E. V., and Schmid, F. R. (1976). *New Engl. J. Med.* **295,** 1149–1154.
Sparling, P. F. (1971). *New Engl. J. Med.* **284,** 642–653.
Steffen, C. (1970). *In* "Progress in Clinical Pathology" (M. Stefanini, ed.), Vol. 3, pp. 226–264. Grune & Stratton, New York.
Strakosch, C. R., Wenzel, B. E., Row, V. V., and Volpe, R. (1982). *New Engl. J. Med.* **307,** 1499–1507.
Tilton, R. C. (1979). *Ann. Intern. Med.* **90,** 697–698.
Tuazon, C. U., and Sheagren, J. M. (1976). *Ann. Intern. Med.* **84,** 543–546.
Tuffanelli, D. L., Wuepper, K. D., Bradford, L. L., and Wood, R. M. (1967). *New Engl. J. Med.* **276,** 258–262.
Unny, S. K., and Middlebrooks, B. L. (1983). *Microbiol. Rev.* **47,** 97–120.
Volpe, R. (1977). *Ann. Intern. Med.* **87,** 86–99.
Walker, D. H., Cain, B. G., and Olmstead, M. (1978). *Am. J. Clin. Pathol.* **69,** 619–623.
Wallace, A. L., and Norins, L. C. (1970). *In* "Progress in Clinical Pathology" (M. Stefaniani, ed.), Vol. 2, pp. 198–215. Grune & Stratton, New York.
Waller, M. (1973). *Am. J. Med.* **54,** 731–734.
Wannamaker, L. W. (1959). *Am. J. Med.* **27,** 567–574.
Wannamaker, L. W. (1970). *New Engl. J. Med.* **282,** 23–31, 78–85.
Wannamaker, L. W., and Ayoub, E. M. (1960). *Circulation* **21,** 598–614.
Warren, J. W., and Hornick, R. B. (1979). *In* "Principles and Practice of Infectious Dis-

eases" (G. L. Mandell, R. G. Douglas, and J. E. Bennett, eds.), pp. 1516–1520. Wiley, New York.
Weigle, W. D. (1981). *Arthritis Rheum.* **24,** 1044–1052.
Weissfeld, A. S., Gehle, W. D., and Sonnenwirth, A. C. (1982). *J. Clin. Microbiol.* **16,** 82–85.
Wilson, G. S., and Miles, A. A. (1975). *In* "Topley and Wilson's Principles of Bacteriology, Virology and Immunity," 6th ed., pp. 2013–2016, 2355–2357. Williams & Wilkins, Baltimore, Maryland.
Wintrobe, M. M., Lee, G. R., Boggs, D. R., Bithell, T. C., Foerster, J., Athens, J. W., and Lukens, J. N. (1981). *In* "Clinical Hematology," 8th ed., pp. 926–948. Lea & Febiger, Philadelphia, Pennsylvania.
Wormser, G. P. (1982). *Lab. Med.* **13,** 20–27.

5

Instrumentation in Clinical Chemistry

MICHAEL L. FRANKLIN

CLINICAL BIOCHEMISTRY
Contemporary Theories and Techniques, Vol. 3

ISBN 0-12-657103-1

I. INTRODUCTION

The major objective of this chapter is to present a balance between theoretical concepts in the form of basic mathematical equations and the practical application of those theories in instruments. The basic theory or measurement concept is presented in an elementary fashion with a functional block diagram following. Some illustrations of typical components are made to demonstrate the relationship between the various aspects of theory and the instrument block diagrams. It is hoped that this method, particularly when starting with the simplest of examples, will allow the reader to enter at his particular level and to move quickly to more recent instrumentation that might be considered state of the art. Finally, the manufacturer's name and location for specific instruments is given for the reader's convenience in acquiring additional information. This should not be considered an endorsement of the product.

II. SPECTROMETRIC INSTRUMENT SYSTEMS

A. Absorption Spectrophotometry

When a beam of light is directed through a sample in a transparent container or sample cell, the phenomenon called *absorption* can occur, which diminishes the amount of light leaving the cell. This reduction in light transmission occurs in the ultraviolet and visible region of the spectrum because of light absorption by molecules in the sample. If the amount of transmitted light is plotted versus sample thickness, the curve is nonlinear. The exact nature of this curve is given by the Bouguer–Beer–Lambert Law which can be written $\log 1/T = \log I_i/I_e = abc = A$. T is defined as transmittance, which is equal to the ratio of the exiting light intensity (I_e) divided by the incident light intensity (I_i). A is absorbance, a is the absorptivity or absorbancy index, b is the cell-path thickness (by convention given in centimeters), and c is the concentration. One reason

this form of Beer's law is so useful is that A is directly proportional to concentration (l).

Figure 1 illustrates, in block diagram fashion, what is necessary to implement such an absorption photometry measurement. Some of the positions in the block diagram can be interchanged, but the functions remain the same regardless of the sophistication or simplicity of the measuring system.

The simplest examples of the types of instruments used in clinical chemistry are called colorimeters or comparators. One such unit is the Lovibond Comparator, marketed by The Tintometer Co. (Williamsburg, Virginia). A diagram is presented in Fig. 2.

Two transparent cells are used. One contains the colored material to be determined or the material reacted chemically to give a particular color. The blank reagents are placed into the second cell. A wheel containing different densities of colored glass scales is rotated with the unit held up to a light source. A prism arrangement superimposes the images, one from each cell, adjacent to one another. The eye is used to match the color and intensity by rotating the standard color disk while viewing light transmitted through the cells. The number printed on the glass filter wheel is used for readout. In this device, ambient or artificial light provides the light source. The color filter wheel provides a monochromating effect, isolating the wavelength range of light allowed to pass through the sample. The cuvettes contain the sample and blank, and the eye is used as a detector. The numbers on the standard wheel test disk provide the readout. There are standard wheels available for α-amylase, cholesterol, bili-

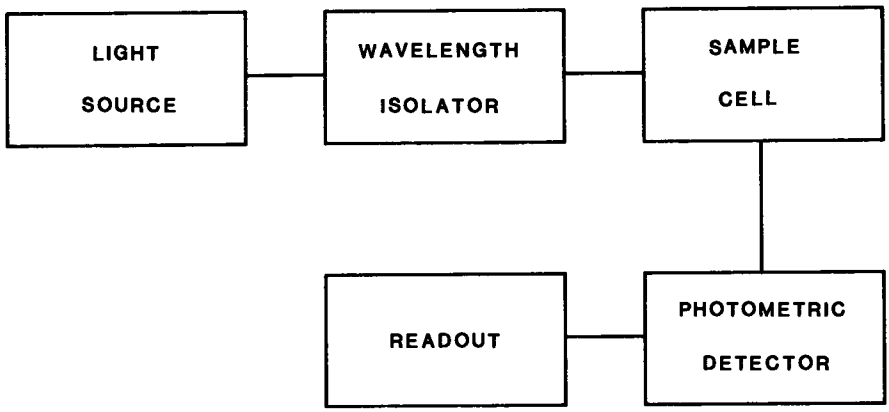

Fig. 1. Block diagram, absorption photometry.

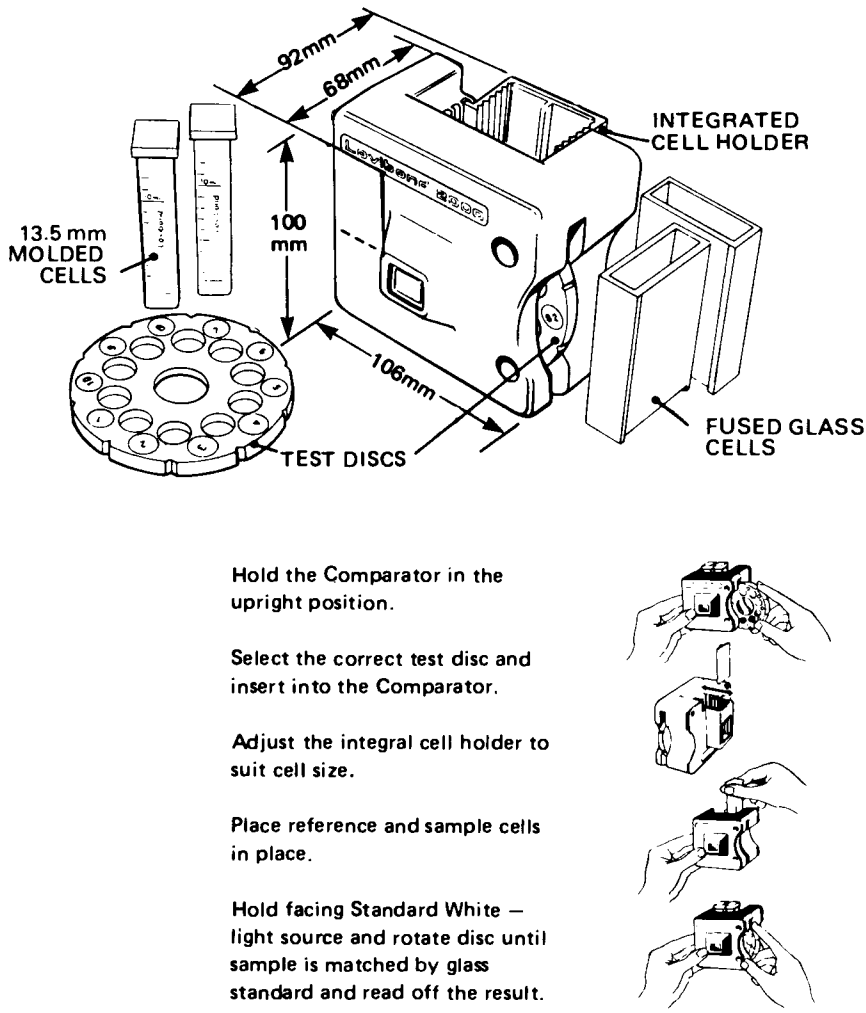

Fig. 2. Visual comparator diagram. Used with permission of Lovibond/Tintometer Company, Williamsburg, Virginia.

rubin, and protein test chemistries among others (*2*). The chemical method must result in an absorption between 450 and 650 nm for the eye to be used as a detector.

Several observations can be made at this point. First, the equation $\lambda f = C$ indicates that wavelength λ and light frequency f are related by a fundamental constant, the speed of light C. The unit chosen to measure wavelength in this region is the nanometer (nm) or 10^{-9} meters, and the

region of the electromagnetic spectrum that is examined for molecular absorption ranges from approximately 200–700 nm. The following tabulation lists colors in the visual region associated with their corresponding wavelengths in the spectrum (*3*).

Transmits (appearance, color)	Wavelength region (nm)	Absorbs (complement)
Yellow	465	Blue
Orange	490	Blue green
Red–purple	510	Green
Purple	550	Yellow green
Blue–violet	580	Yellow
Green blue	600	Orange
Blue	630	Red

From "Instrumental Methods of Chemical Analysis," G. W. Ewing, McGraw-Hill, New York (3rd ed.) with permission.

The appearance colors are listed in the first column. For example, if a solution transmits blue light, it appears or looks blue, but it has absorbed the complement of the blue color or red light. This table can be used to predict the visible region of absorbance before measurement by a spectrophotometer simply by holding the material up to white light and judging the color of the light transmitted through the sample.

A more sophisticated colorimeter, called the Unimeter, has been developed by Bio-Dynamics (Indianapolis, Indiana) to be utilized with the Unitest kits. A pictorial diagram of the colorimeter is shown in Fig. 3 along with a sketch of the instrument itself. The colorimeter employs a color filter that separates a 60-nm band centered at 525 nm for wavelength isolation. This wavelength isolator is placed directly in front of the photocell detector, and the sample is placed between the light source and the wavelength region isolator. A signal processor powers the analog meter readout, which has slide-in precalibrated scales for different chemistries. The cuvette is a 15-mm diameter Unitest test tube containing a portion of the reagents. An incubator operating at 37°C is also incorporated into the instrument. Some of the chemistries available are blood glucose, hemoglobin, cholesterol, uric acid, total bilirubin, total protein, triglycerides, serum calcium, serum salicylate, and α-amylase. Because a timer and an incubator are included in the instrument, analyses using enzymes reaction rates are also utilized. Although many of the chemistries result in an

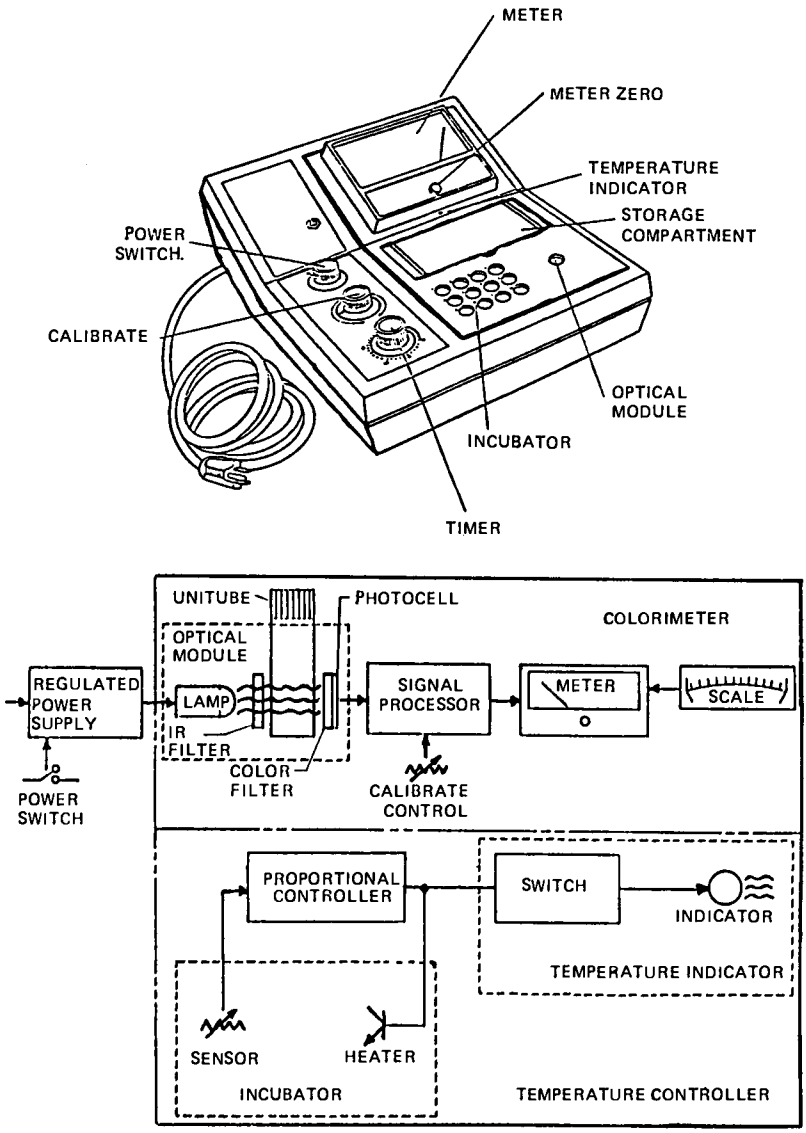

Fig. 3. Colorimeter diagram. Used with permission of Bio-Dynamics, Division of Boehringer Mannheim Diagnostics, Inc., Indianapolis, Indiana.

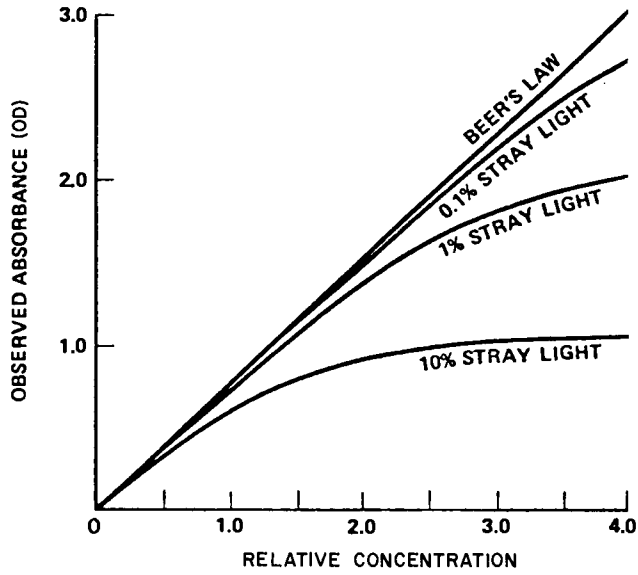

Fig. 4. Effect of stray light on absorbance. Used with permission of Varian Instruments Group, Palo Alto, California.

absorption not centered in the spectral region isolated by the color filter, there is sufficient absorption in that region to allow utilization of the test (*4*).

It should be noted that, for Beer's law to be obeyed, there is a requirement for monochromatic (single wavelength or color of light) to be employed. That wavelength region of light should be of narrower bandwidth than the absorption in the material being measured. If this does not occur, then nonlinearity in Beer's law is observed resulting from the presence of this unabsorbable stray light at the detector. A broad definition of stray light is light of a wavelength different from that which can be absorbed by the sample that gets to the detector. This includes light striking the detector which has not passed through the entire sample. Even a modest amount of stray light causes apparent negative deviation from Beer's law, as can be seen in Fig. 4. For this reason, it is always wise to plot absorption versus concentration for a particular material over the concentration range of intended use. A linear curve is not a necessity for use, but sensitivity is reduced when the nonlinearity is large.

One way to provide a narrower bandwidth of light than a colored glass filter produces is to use the property of light interference. The wavelength λ of maximum transmission caused by constructive interference for normal incidence is given by the equation $\lambda = 2\,nb/m$, where n is the refrac-

tive index of the dielectric coating, b is the thickness, and m is the order number (*5*).

An interference filter can be used to isolate a narrow band of radiation, which is then used in the absorption measurement. Stable interference filters with orders to longer and shorter wavelengths blocked by colored glass are available in packages sealed against humidity from companies such as Ditric Optics, Inc. (Hudson, Massachusetts). The typical bandpass for an interference filter can range from 1 to 50 nm. The wavelength of an interference filter can be tuned to shorter wavelengths, usually about 1 bandpass, by turning it approximately 20° to the normal of the light axis (*6*). A different method, however, is usually selected to vary the wavelength in most instruments. A diffraction grating or prism that can be rotated to select various wavelengths desired for analysis is used. For a plane diffraction grating, the equation $m\lambda = b(\sin i + \sin e)$ is followed. The variable b is the spacing of lines on the grating in lines per millimeter, i is the angle between the incident light and the normal line to the grating, and n is the angle between the exiting light and the grating normal (*7*). Various orders m of light of wavelength λ can be expected to be produced from a grating instrument. Colored glasses or interference filters are used to block this unwanted radiation. For example, when a measurement is being made at 640 nm, light from 320 nm radiation is also being transmitted at the same exiting angle from the grating in the second order. Light of the latter wavelength needs to be blocked or a detector used that is not sensitive to that wavelength; otherwise it appears as stray light in the measurement.

Prisms depend on refraction in the prism material and do not have a problem with unwanted orders; the angular dispersion, however, is not linear with wavelength as it is with a diffraction grating. For a prism, light of higher frequency (shorter wavelength) is refracted or bent more than light of lower frequency (longer wavelength). Thus a prism instrument with a fixed slit offers a narrower bandpass at shorter wavelengths than at longer wavelengths.

The bandwidth for a particular grating or prism instrument can be changed by altering the slit width. In the case of the Spectronic 20, produced by Bausch & Lomb (Rochester, New York), the grating instrument provides a spectral bandwidth approximately 20 nm over the wavelength range from 340 to 950 nm (*8*). Most instruments can be assumed to have a triangular slit function. The total wavelength region isolated by the slit is called the spectral slit width; but the spectral band width is the function at $\frac{1}{2}$ the peak intensity. This turns out to be about $\frac{3}{4}$ the total radiant energy isolated by the slit. The definitions of these two terms are illustrated in Fig. 5. Some light of wider bandwidth than the instrument spectral band-

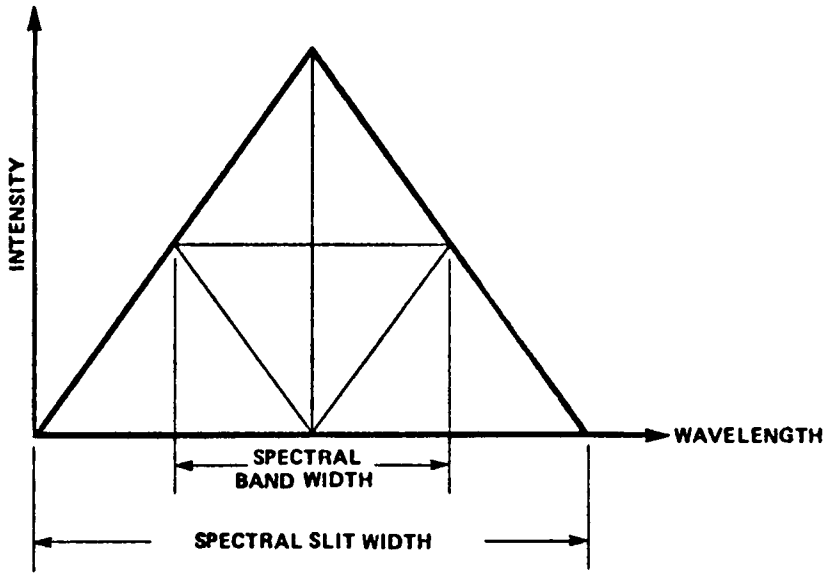

Fig. 5. Instrument slit-width definitions. Used with permission of Varian Instruments Group, Palo Alto, California.

width specification is being selected and can be estimated by using the triangular slit function assumption (*9*).

Some interesting advances have been made in the light sources used in absorption spectroscopy. The tungsten light has been replaced in many instruments with a tungsten–halide lamp. A halogen such as iodine is placed inside a quartz envelope, and as tungsten is vaporized away from the incandescent filament, it combines with the halogen and is decomposed and redeposited on the incandescent filament (*10*). The effect is twofold. First, the tungsten does not deposit on and blacken the interior of the lamp envelope. Thus the light output is more stable for a long period of time. Second, the redeposition of tungsten on the filament extends its life somewhat. The envelope of these lamps must be operated at high temperatures for the halogen cycle to continue. It is not recommended to operate at lower voltage and current than is necessary to sustain that cycle. The quartz envelope allows radiation of shorter wavelength than 320 nm to pass through the system. Thus the substitution of a tungsten lamp with a tungsten-halide quartz bulb can increase ultraviolet response for an instrument, which may cause stray light problems. Other light sources that can be used in the ultraviolet spectral region are the deuterium lamp, the mercury light, and the xenon discharge lamp. The

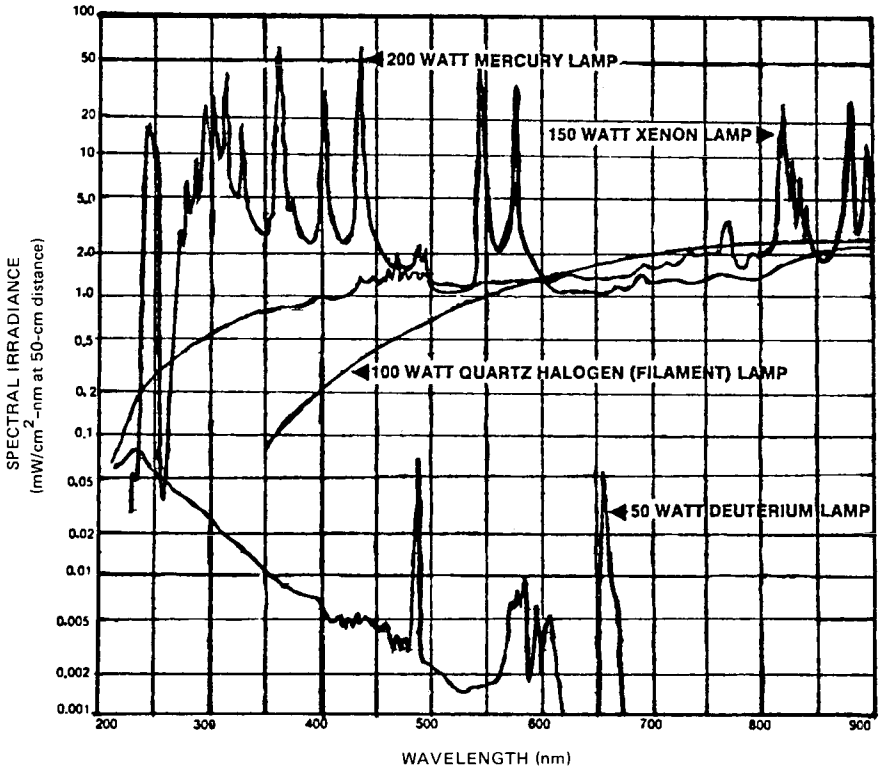

Fig. 6. Typical light-source spectra. Used with permission of Oriel Corporation, Stamford, Connecticut.

spectra, typical for these light sources, which are available from Oriel Corp. (Stamford, Connecticut) are shown in Fig. 6 compared with one another (*11*).

There have been improvements in the field of monochromators. One of these is the production of holographically produced gratings (*12*). These types of grating are produced from the interference patterns of lasers on photosensitive materials, which can then be used to produce gratings. One major advantage important to spectrophotometry of using these gratings is the reduction in stray or scattered light caused when conventional mechanically ruled gratings are employed. Thus, higher linear absorbancies can usually be measured with instruments using holographic gratings.

Some innovations have been made in the field of the sample cells. A good example is the incorporation of fiber optics to make a dip-and-read probe colorimeter available from Sybron–Brinkmann Instruments (Westbury, New York). The optical diagram for such an instrument is shown in

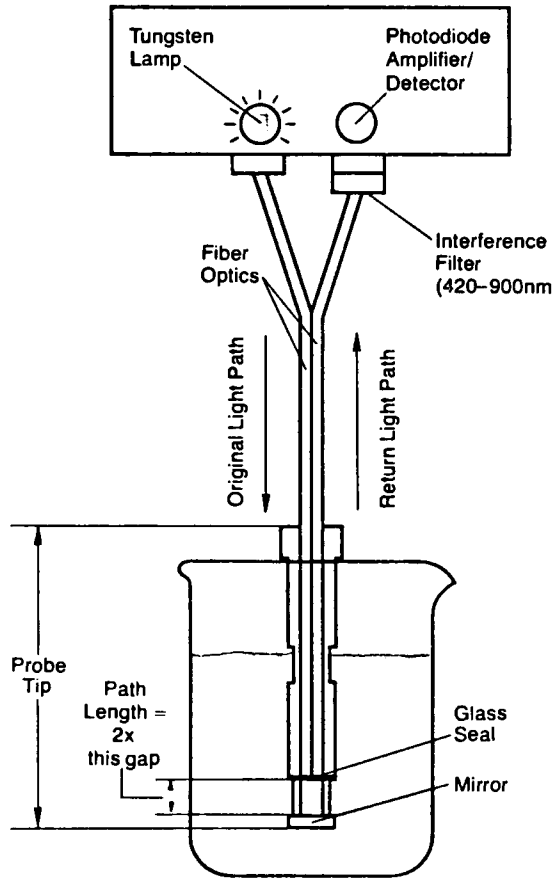

Fig. 7. Operational diagram of probe colorimeter. Used with permission of Sybron/ Brinkmann Instruments Co., Westbury, New York.

Fig. 7. Light from a pulsing tungsten lamp is directed through fiber optics into the solution and passes through the sample. It strikes a mirror and is reflected back once more through the sample, where it is picked up by returning fiber optics. The light then passes through a suitable interference filter to a detector and is read out to provide a measure of the concentration of material in the solution. The alternating current (AC) modulated light is phase shifted and the detector is AC coupled and gated to reject most of the ambient extraneous light. This allows measurements to be made without placing the sample into a darkened chamber (*13*).

An example of an instrument configured to do dual wavelength analysis is the bilirubinometer made by AO-Reichert Instruments (Buffalo, New

York). This instrument measures the absorption of the bilirubin in blood and compares it with a highly stable reference standard. The optical diagram for the instrument is shown in Fig. 8. The light is divided by a beam splitter, and each beam contains a bandpass filter and a photoresistor detector. One bandpass filter transmits at 461 nm, which is the wavelength for a maximum absorption of bilirubin in serum. The other wavelength is 551 nm and is selected because the absorption of hemoglobin is the same as at 461 nm. The instrument is arranged so that the difference between the response to the photocells is taken. Thus the system can correct for any hemoglobin in the sample. In a similar manner, a turbid sample will not greatly affect the instrument reading, because the material

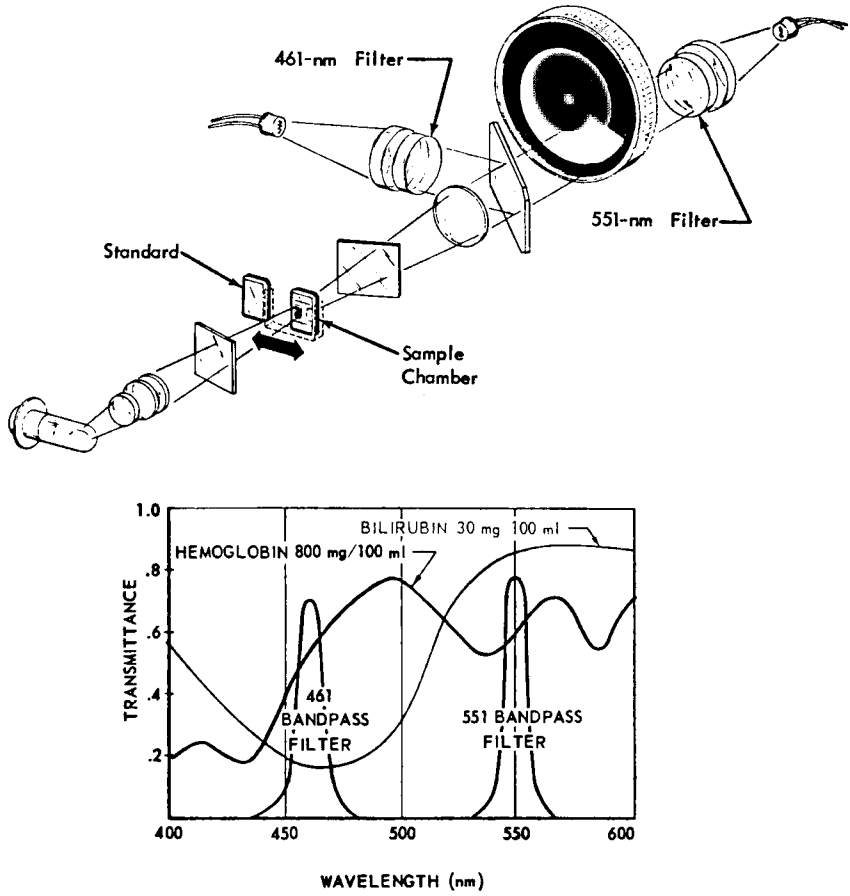

Fig. 8. Principles for bilirubinometer. Used with permission of AO-Reichert Scientific Instruments, Buffalo, New York.

producing the turbidity has approximately the same apparent absorption at wavelengths near each other. In operation, the instrument is first zeroed using a reference sample in the light path and an optical wedge that has a graduated scale unit. The sample is then moved into the light path and the scale knob readjusted to give the reading (*14*).

Another version of a specialized photometric system uses fiber optics and multiple wavelength measurements to determine the absorbance of a unique sample path on the patient. Using the pinna or upper part of the ear as the sample cell, this unit can determine oxygenated hemoglobin in blood. An example of this type of instrument is the ear oximeter made by Hewlett Packard (Waltham, Massachusetts). A block diagram of that unit is given in Fig. 9. To perform the measurements, eight wavelengths are selected and measured using a rotating filter wheel. Eight simultaneous equations having constants determined empirically from conventional blood-sample measurements are solved by the instrument central processor. The result is a percentage-oxygen saturation reading performed noninvasively on the patient (*15*).

Several advances have been made in photodiode array detectors, which are used in absorption spectroscopy. These detectors are a series of photodiodes stacked adjacent to one another, using the aerospace technology of integrated circuit miniaturization. Photodiode arrays of various dimensions are available from EG&G Reticon [Sunnyvale, California (*16*)]. One example of this type of instrument is made by Hewlett–Packard (Palo Alto, California) in their photodiode array spectrophotometers. These spectrophotometer systems employ holographic gratings that disperse the light onto photodiode arrays as shown in Fig. 10 (*17*).

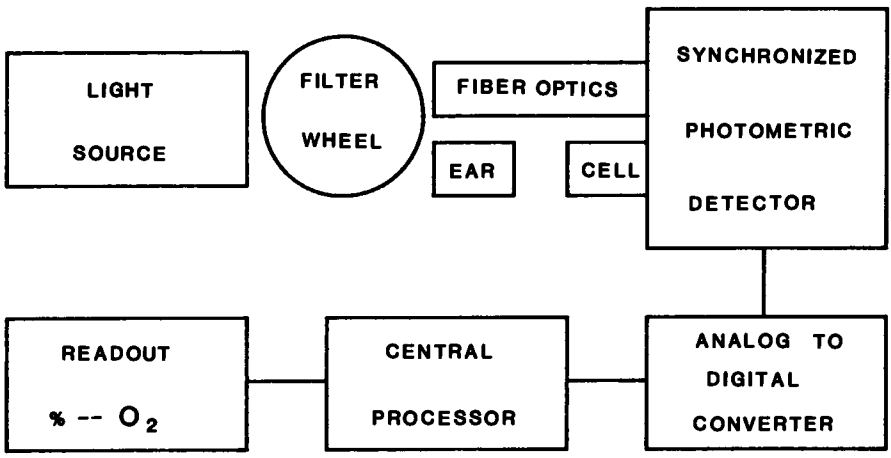

Fig. 9. Ear oximeter operational diagram.

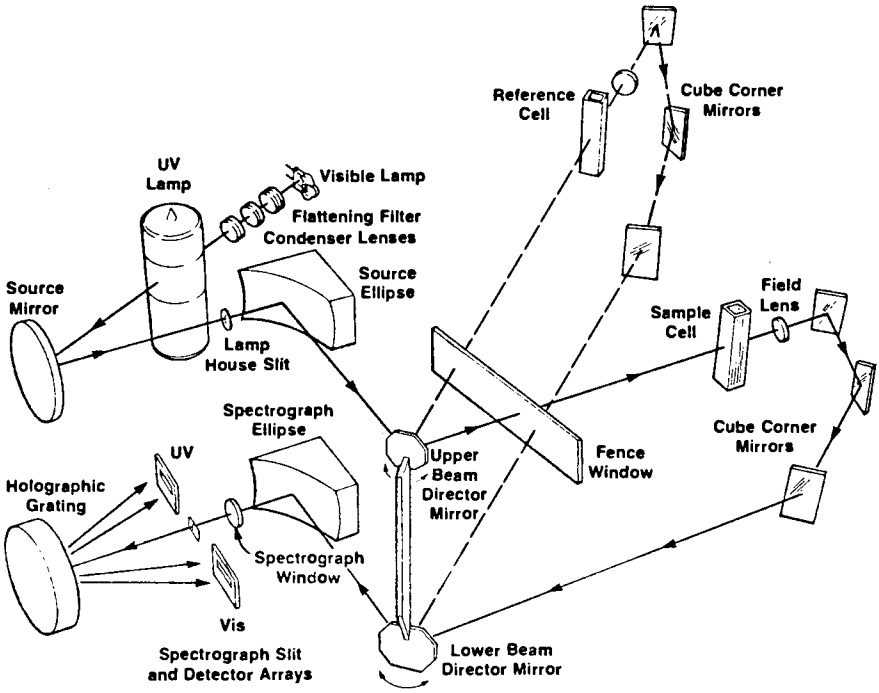

Fig. 10. Photodiode array spectrophotometer diagram. Reprinted from *American Laboratory*, **13**, No. 6, 62-71 (1981). Copyright 1981 by International Scientific Communications, Inc.

The instrument can take an entire spectrum in 1 sec and is able to integrate all wavelengths between 200 and 800 nm simultaneously. Thus the 1-sec integration spectrum compares favorably with a spectrum taking 500 times longer, or approximately 8 min to record on a conventional scanning spectrophotometer. Alternately, because random noise in the signal decreases as the square root of the amount of the observation time, an 8-min spectrum can be taken with a gain in signal-to-noise ratio >20 (*18*).

Availability of the entire absorption spectrum provides some particularly interesting advantages. Multiple wavelength absorption can be used to analyze or correct for multiple components, as is done in the ear oximeter. In addition, detection of unusual overlapping wavelengths can be made. Finally, corrections for light scattering in the sample can sometimes be accomplished.

Another novel sample cell and analysis system has resulted from the invention of a multichannel pipette. This unit consists of a disposable

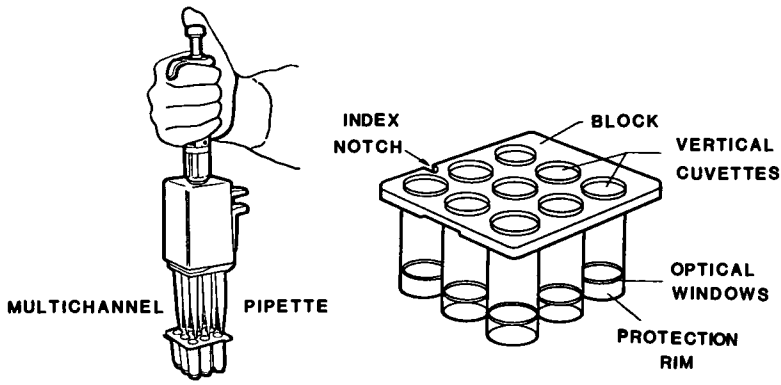

Fig. 11. Multichannel pipette and cuvette block. Reprinted from *American Laboratory*, **14**, No. 6, 106-111 (1982). Copyright 1982 by International Scientific Communications, Inc.

metering pipette having nine separate heads positioned in a 3 by 3 matrix. With this device, called the Finn Pipette (FP), reagents and samples can be handled simultaneously in groups of nine. A series of nine vertical curvettes, molded of plastic in the same matrix pattern (Fig. 11), receives the solutions of reagents.

There are some advantages to using a vertical spectrophotometric cell. Only one transparent optical-quality window needs to be near the bottom of the cell. Because the length of the light path is not fixed, inaccurate pipetting of nonabsorbing liquids will not change the absorbance. Evaporation of the solvents from samples for reagents during reactions does not affect the absorbance, and some inhomogeneity in the solution as a result of layering will not affect the results. The explanation of these effects can be expressed mathematically because the absorbance A is equal to a coefficient E times N, where N is the amount of the substance in the light path; N is equal to the total mass of absorbing substance and inversely proportional to the cross-sectional area a perpendicular to the light path $N = m/a$, where m equals the total mass of substance in solution. Thus, $A = Em/a$, and the absorbance is independent of the light path length. This means that only the amount of the sample is important, and the volume of the reagents can fluctuate without changing A.

The FP colorimeter instrument analyzer offers a system allowing nine samples to be analyzed simultaneously. A series of end-point and kinetic methods based on measurement of absorbance at one or more wavelengths has been developed for several clinical tests, including albumin, glucose, iron, high-density lipoprotein (HDL)-cholesterol, creatinine, alkaline phosphatase, and acid phosphatase (*19*).

Another unique sample cell configuration is available in the centrifugal

analyzers. In these devices, usually a disk of molded plastic is employed that rapidly mixes prescribed reagents and samples by centrifugal action. One such system, the COBAS-BIO made by Roche Analytical Instruments (Nutley, New Jersey) consists of a spectrophotometer system that places the sample, not perpendicular to the rotary axis but parallel to it (*20*).

In this case, the sample absorbance is measured in a cuvette lying longitudinal to the light beam. Only one cell surface is used as an optical window, as in the case of the vertical cell. The surface of the liquid provides the other interface. The Roche COBAS–BIO system utilizes xenon strobe-light emission as its light source. This source offers a very high output over a wide spectral range and is highly efficient in illumination per watt. The lamp is pulsed with different energies to alter the intensity. The entire centrifugal analyzer system is microprocessor controlled and has an optical diagram (Fig. 12). A mirror is used behind the light source to help collect and columnate the light, directing it through an entrance aperature to a holographically produced grating. The position of this grating is under computer control and can be selected for any particular wavelength region. Low stray light from a holographic grating allows the measurement of reasonably linear high absorbance values. A portion of the existing beam from the monochromator is split off and sent to a reference diode, and the remainder of the energy proceeds through the optics, sample, and cuvette. After passing through an order sorting filter,

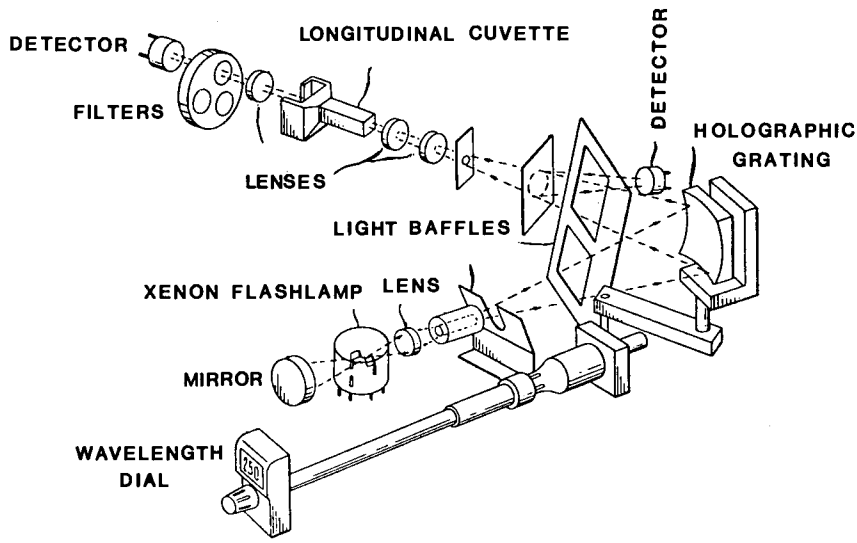

Fig. 12. Centrifugal analyzer, pictorial diagram.

the light is detected by a photodiode–amplifier combination, processed by an analog-to-digital converter, and read out digitally.

Some advantages of using a xenon flashlamp are that measurements can be performed during rotation of the sample disk and the entire spectral range can be covered with only one type of light source. The lamp power supply dissipates little power and is rather compact. Flash lamps have fairly long service life when operated within their specification range; thus the light source does not need frequent replacement. Small variations in intensity are compensated for by using the double-beam light system, where a small fraction of the energy is monitored by a reference diode (*21*).

Because the entire assembly—including the monochromator, sample cuvette rotor, and light source—is under computer control, absorbance data can be taken at several different wavelengths, ranging from 290 to 780 nm, to aid in reducing the effects of scattered light caused by particulate matter or lipemic serum. Because the path length of the measuring cell is not fixed, this type of geometric arrangement offers advantages similar to those found in the vertical cuvette described earlier. The system obviously can also be used for end-point reactions and for kinetic-type determinations such as initial reaction rates or enzyme kinetic mode. The centrifugal or rotary analyzer offers a degree of flexibility regarding the sample size in the microliter range. Tests can be run in a compact space using small quantities of sample and reagents. Twenty-five clinical samples are loaded into the sample disk, and a reagent module is inserted. The appropriate test (among 30) is selected. The instrument performs the sample and reagent pipetting, measures the absorbance at the proper wavelengths, and computes the answer using standards. A series of tests including lactate dehydrogenase (LDH), glucose, uric acid, calcium, blood urea nitrogen (BUN), albumin, and total protein is available for the unit (*22*).

B. Fluorescence Spectrometry

After molecules absorb radiant energy, some molecules undergo a process called fluorescence. Fluorescence is the emission of radiation at longer wavelengths than that with which the molecules were excited. For a low concentration of these molecules, the formula $F = P_0Kabc$ is valid, where F is the fluorescent intensity, P_0 is the power of light at the exciting wavelength, K is a constant, a is the absorptivity, b is the pathlength, and c is the concentration as it is in Beer's law. This equation is valid only for concentrations of molecules giving an absorbance $A = abc$ = about 0.01 (*23*). Inspecting this equation indicates why fluorescence is most useful at

lower concentrations. Because fluorescence is detected against a very dark background, the detector can be operated at high gain to detect very low concentrations of material. Finally, to a first approximation, the fluorescence intensity is directly proportional to the input power P_0. Thus a filter fluorometer with a reasonably wide bandpass and high light throughput, unlike a spectrofluorometer having a lower throughput from monochromators, can provide greater sensitivity because of the increase in P_0. The components necessary to perform fluorescence measurements are depicted in the block diagram of Fig. 13.

An example of such a system is the Fluorostat instrument made by Ames Division of Miles Laboratories (Elkhart, Indiana). This filter fluorometer with digital display, printer, and microprocessor is available in a compact instrument about 18″ wide. The system employs a pulsed-xenon flashlamp with a 405-nm wavelength excitation filter as the light source. The measurement cycle includes approximately 0.5 sec of measuring in the dark and then 0.5 sec of fluorescent measurements at 450 nm during excitation by the pulses from the xenon flashlamp. The filters are selected to maximally detect the single fluorophor for the competitive substrate binding method on which the tests are based. When the antibody unbound fluorogenic drug reagent (FDR) reacts with an enzyme, a fluorescent product will result. After drug containing a patient's serum is added, competition occurs for the antibody binding sites. Thus, some free FDR can react with enzyme to give fluorescence proportional to the patient's serum drug level. The fluorometer computer memory stores the standard curves for each reagent lot. Results are displayed on a fluorescent digital readout and can be recorded on printout paper. The system is designed for therapeutic drug monitoring. Assays include quinidine, gentamicin, phenobarbital, phenytoin, and theophylline, among others (*24*).

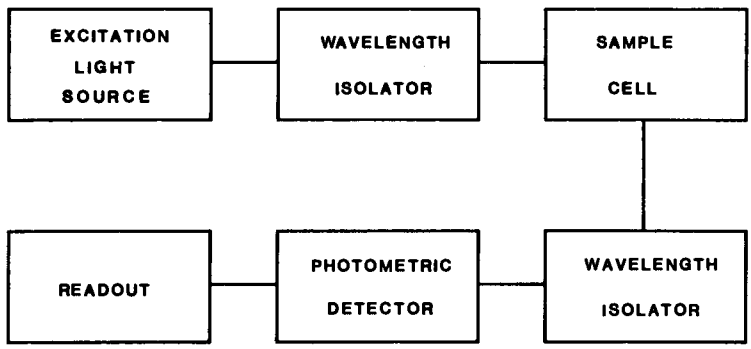

Fig. 13. Block diagram, fluorometer.

When a fluorophor is excited with plane polarized light as the molecule returns to the excited state it will emit polarized fluorescence. If the molecule does not undergo rotation during its excited state lifetime, the fluorescence will exhibit completely unchanged polarization. If the molecule is small enough to undergo rotation during the excited-state lifetime, the fluorescence will be less polarized. By measuring the change of molecular fluorescence polarization, an estimate of the size of molecules can be made because larger molecules rotate more slowly than smaller ones. The fluorescence depolarization ratio D is defined as

$$D = \frac{F_{\parallel} - F_{\perp}}{F_{\parallel} + F_{\perp}}$$

where $F_{\parallel}$ is the fluorescence intensity parallel to the exciting polarized light plane, and $F_{\perp}$ is the intensity perpendicular to it. If a fluorescein-labeled drug is used as a tracer and direct competitive binding immunoassay techniques are employed, both the patient's drug and tracer compete for binding sites on the antibody. The precise relationship between polarization and the concentration of drug in the patient's specimen can be calculated from the data of the fluorescence polarization instrument. The only additions in the previous block diagram necessary to implement this technique are a polarizer between the excitation interference filter and the sample and a method to measure the fluorescence perpendicular and parallel to the excitation plane. This is typically done with plastic polaroids, dichroic polarizers, or polarizing prisms.

Abbott, Diagnostics Division (Irving, Texas) markets a fluorescence polarization instrument. It uses a liquid-crystal polarizer to produce alternating perpendicular polarization of the exciting beam and a way to compensate for the inherent liquid crystal distortion. The instrument contains a carousel with sample cartridges and reagents that can accommodate 20 samples. Dilution of the sample is performed automatically by the robot pipette system.

The entire system is microprocessor controlled and features a hard-copy printout of the results. The light source is a 50-W tungsten–halide bulb with an excitation filter of 485 nm having a bandwidth of about 10 nm. The liquid-crystal polarizer produces polarized light alternating between horizontal and vertical orientation. By using the liquid-crystal polarizer, it is unnecessary to have any mechanically rotating elements. The green fluorescence emitted light at wavelengths around 525 nm is selected by the emission interference filter. An analyzing polarizer is oriented to give a larger signal when a more labeled drug is bound. The fluorescence is detected with a photomultiplier photometric detector and processed by the onboard microprocessor for readout.

A series of fluorescence polarization immunoassays is available, which includes digoxin, theophylline, gentamicin, carbamazepine, phenytoin, and lidocaine (*25*).

III. ATOMIC SPECTROSCOPY SYSTEMS

A. Atomic Absorption Principles and Components

To measure atomic absorption (AA), which is governed by the same Beer's-law expression $A = abc$, the components indicated in the instrument diagram of Fig. 14 are required.

These include a source of light energy of correct wavelength to be absorbed by the atoms of interest (an atomic line source), a sample reservoir having atoms in the ground atomic state, a wavelength isolator such as a monochromator, some suitable radiation detector, an optical chopper, and suitable electronics to process the signal to be presented on a readout device.

Many units use a hollow cathode lamp as the atomic line source for AA. The lamp is a sealed-off discharge tube with a flat quartz end window in which a gas, such as neon, is contained at low pressure. The gas is excited by a direct-current (DC) discharge of a few milliamps. The discharge sputters atoms from the thimble-shaped hollow cathode made or coated with the particular metal for which selected atomic emission radiation is desired. As a consequence of the atomic emission being produced in a low-pressure gas by sputtering, the line width is extremely narrow (*26*). This atomic-emission line source is directed through an external atom reservoir where the number of atoms in the sample is to be measured. One common atom reservoir is a laminar-flow premixed flame. Common flame

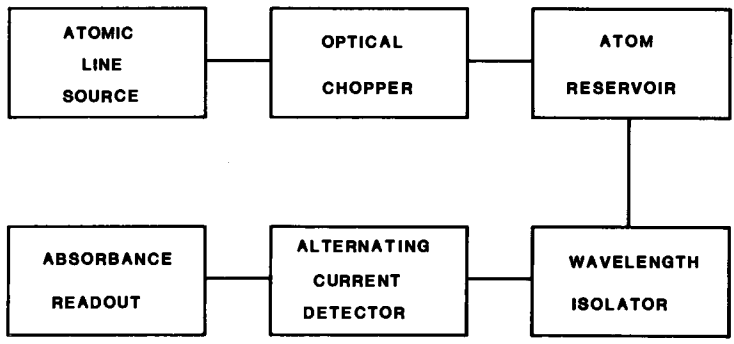

Fig. 14. Block diagram, absorption instrument

gases are acetylene and compressed air. The flames are formed on slot burners that shape the flame so that it extends over a 5- to 10-cm linear path. Because Beer's law is valid, the increase in pathlength b causes increased sensitivity. The sample is usually nebulized with a pneumatic system and combined with the premixed fuel gases. These emerge and burn in a relatively stable and structured flame a few millimeters above the surface of the burner. The combustion temperatures are sufficiently hot to disassociate most samples into atoms and to allow a significant fraction of them to be available in the ground electronic state suitable for AA. Rapidly flowing flame gases during combustion necessitate that the sample be continuously added to the flame. This allows the new atoms to be continually prepared for AA in the region through which the atomic line emission is directed. The Boltzmann equation defines the ratio of N^*, the number of excited state atoms, and N_0 the number of atoms in the ground state available for AA, to be $N^*/N_0 = ge^{-E/kT}$, where g is a quantum mechanical statistical number for the transition, E is the energy of the particular transition, k is the Boltzmann constant, and T is the absolute temperature of the atom reservoir (*27*).

In many instruments the spectral region around the absorption line of interest is isolated with a monochromator. This device utilizes a diffraction grating or prism and is suitable for selecting various wavelengths for analysis of different elements. In some simpler instruments, the wavelength isolator can be replaced with a narrow-band interference filter. At the output of the monochromator is a suitable photodetector. Most instruments use a photomultiplier tube, which combines a photodiode and an electron multiplier inside a vacuum tube. The unit provides detection and amplification of light as well and has a dynamic range of over 10^6. The amplification factor can be made to vary over a millionfold range by changing the high voltage applied to the photomultiplier.

Photomutipliers of various sensitivities, sizes, and types are available from Hamamatsu Corporation (Middlesex, New Jersey) and RCA (Lancaster, Pennsylvania), among others.

As a consequence of a phenomenon called *atomic emission* occurring in the flame, the same element being analyzed emits radiation at precisely the same wavelength at which the analysis is being performed. To separate the emission signal from the AA signal, the atomic line source is often optically chopped or pulsed periodically electronically. The continuous signal that arises from the atomic emission in the flame is subtracted from the total signal by a suitable alternating-current (AC) detection system. The result is the isolation of the absorption signal occurring in the flame atom reservoir. In some instances, an additional feature is incorporated in AA instruments. For a fraction of the measurement time, the signal from

the atomic line source is directed around the atom reservoir and through an optical system to the detector (*28*). This allows the electronics system to continuously adjust itself to compensate for any changes in the hollow-cathode light intensity. This type of optical arrangement is referred to as a double-beam system. It generally offers a more stable instrument at the cost of some ultimate sensitivity. A double-beam system is much more convenient to operate because the instrument does not need to be continuously zeroed to compensate for slow drift. An example of this type of AA instrument is illustrated in the optical diagram for a Perkin-Elmer (Norwalk, Connecticut) Model 370 AA instrument shown in Fig. 15 (*29*).

If magnesium is selected as an example element for analysis, the monochromator would be set to the atomic absorption line at 375.2 nm. A typical bandpass for the monochromator is about 0.5 nm. Because the measurement of magnesium is relatively sensitive by AA, a clinical sample such as blood serum is usually diluted to get into proper linear-absorption working range. This range can be established for a particular instrument and element by running a calibration curve using standards and examining it for linearity and reproducibility. A linear range for magnesium for AA instrumentation is typically up to approximately 10 μg/ml or parts per million (ppm) or (1 mg/dl) (*30*)].

There are other methods of producing atoms in the ground state that will undergo AA. A common method is to use the (flameless) ohmically heated miniature furnace. One example of such an atom reservoir device

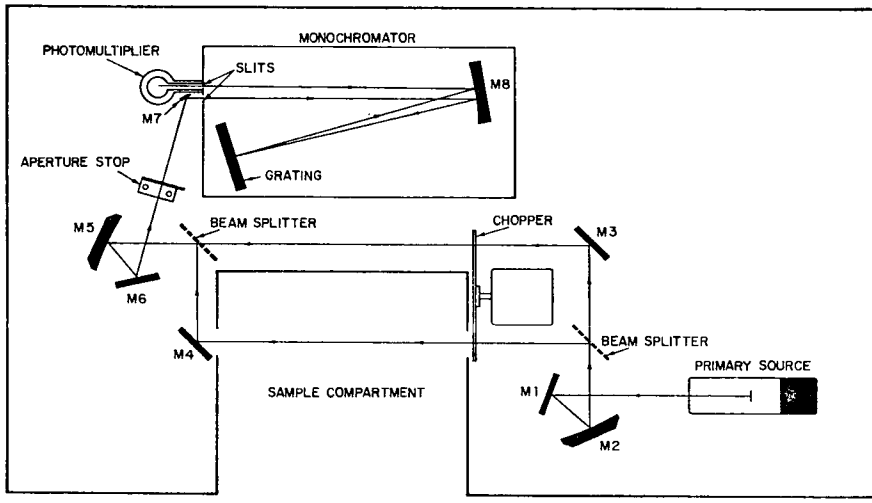

Fig. 15. Optical diagram, double-beam system. Used with permission of Perkin-Elmer Corp., Norwalk, Connecticut.

is the flameless AA unit made by the Instrumentation Laboratory, Inc. [Wilmington, Massachusetts (*31*)]. The furnace consists of a cylinder of very pure carbon approximately 5 mm in diameter and 30 mm in length. This unit is placed between two electrode contacts. It can be heated by passing a current of several hundred amperes through the carbon cylinder. To prevent rapid oxidation of the carbon furnace, this heating is done in an inert atmosphere such as argon or a reducing atmosphere of hydrogen gas. The Instrumentation Laboratory system uses quartz end windows and a larger metallic housing to contain the inert atmosphere. Another manufactured unit that has no end windows is produced by Varian Instrument Group (Palo Alto, California) and is pictured in Fig. 16. In this system, the gas simply flows over the carbon rods to prevent rapid oxidation (*32*).

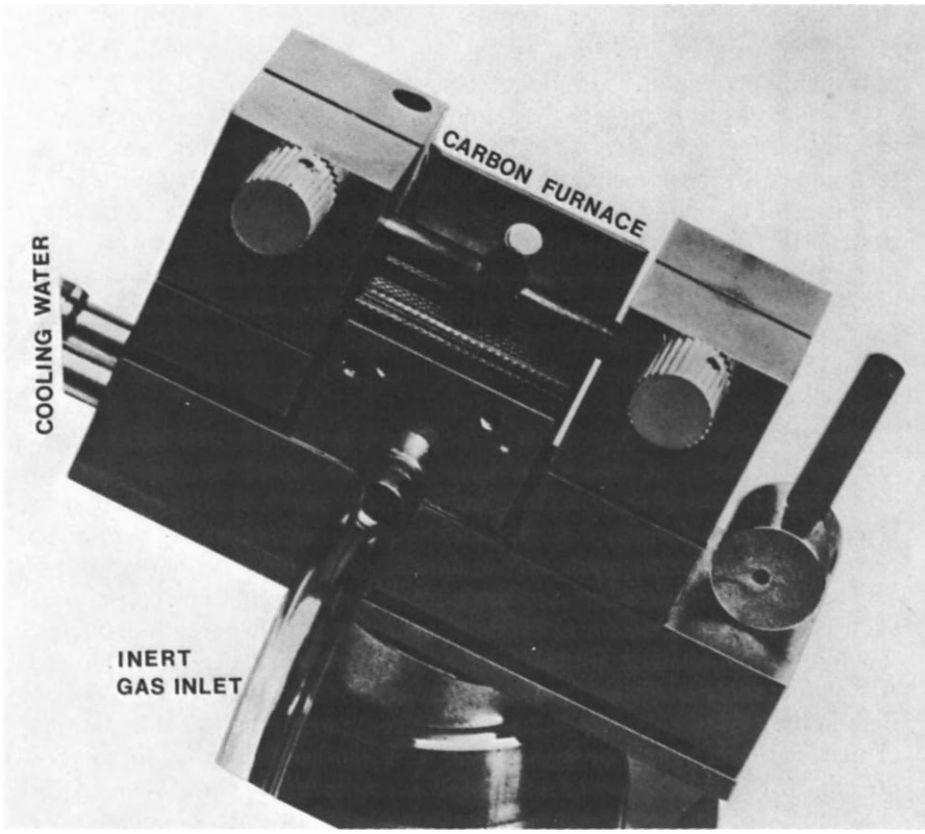

Fig. 16. Carbon-rod flameless atom reservoir. Used with permission of Varian Instruments Group, Palo Alto, California.

The liquid or solid sample is placed into the furnace cylinder through a small hole in the top with a microliter syringe or pipette. Inert gas flow is established around the carbon-furnace material and a small amount of current is passed through the carbon cylinder to dry the sample. After the sample is dried, the second stage of operation is performed by increasing the current level to a temperature high enough to ash the organic material present. This temperature and time must be carefully selected so that the element desired for analysis does not volatilize. The final analysis step occurs when a high current is sent through the carbon cell for a few seconds. This vaporizes the sample and forms an atomic vapor.

The AA furnaces can be heated to white-hot incandescent temperatures for a few seconds without damage. The atomic vapor resides in the measuring beam inside the cylinder for a much longer time than with a flame. Thus, several orders of magnitude of sensitivity are gained. For example, the absolute sensitivity (volume times concentration) for magnesium, calcium, zinc, copper, and iron, among others, is less than a nanogram for reasonably precise measurements using the flameless method.

In some cases, samples of organic material that give rise to spectroscopic interferences because of absorption can be digested by wet-ashing techniques. In these techniques, strong acids such as purified nitric acid or powerful oxidizing agents such as hydrogen peroxide, can be used to destroy the organic matrix before the sample is introduced into the furnace. By placing the sample on a small graphite platform inside the furnace, the temperature of the platform, heated primarily by radiation, lags behind the furnace tube wall. Vaporation of the sample is delayed and the result is an efficient decomposition of molecules and a reduction in matrix interferences (*33*).

For the element mercury, which is extremely volatile, another technique is used. The sample is chemically oxidized in a sealed container to destroy all the organic material and to oxidize the element of interest. The sample is then reacted rapidly with reagents, such as stannous chloride, to reduce the mercury to the elemental state. The reduced element of interest is entrained and carried from the sample by bubbling some carrier gas, such as air or nitrogen, through the sample. The test element is conducted to a long tube cell with or without end windows. This cell is placed in the AA path to provide a defined atom reservoir space. Again, the residence time in the absorption beam is sufficiently long that very small quantities of material can be measured. For mercury, the elemental vapor is produced. In the case of selenium and arsenic, hydrides are evolved by reacting the digested sample with sodium borohydride and directed into a heated absorption cell in the same fashion described. The heat from the flame-heated absorption tube decomposes the hydride to the free-atom form, which can undergo AA (*34*).

B. Background Correction in Atomic Absorption

Specialized instrumentation is used to aid in separating the interfering organic absorption or particulate light-scattering materials from AA. These techniques are particularly important for clinical flameless AA where organic vapor and salt concentrations may be present in varying amounts. One method used in several instruments today is a deuterium background corrector, which is diagrammed in Fig. 17 (*35*). The emission from a line source, such as a hollow cathode or an electrodeless discharge tube, is extremely narrow. It may be of the order of a few thousandths of a nanometer. Elemental AA can be separated from other interfering absorption if a broader wavelength source is alternately directed through the atom reservoir and the signals processed electronically. When a broadband or continuum source such as a deuterium lamp is directed through the optics, the bandwidth is limited by the monochromator, typically to about 1 nm. When the atomic line source is used, the bandwidth is about 0.001 nm or less. Atomic absorption and interfering absorption or scattering both occur when the line source is directed through the atom reservoir. Because organic material has a broadband absorption, as does scattering from salt particles or smoke when the continuum source is directed through the atom reservoir, the major portion of the signal results from the interferences. Only about 0.1% results from atomic absorption for equal absorbancies for the example chosen. By subtracting these two measurements, an AA signal can be corrected for organic background absorption or particle scattering (*35*). The deuterium background corrector is available for many commercial AA instruments (*36*).

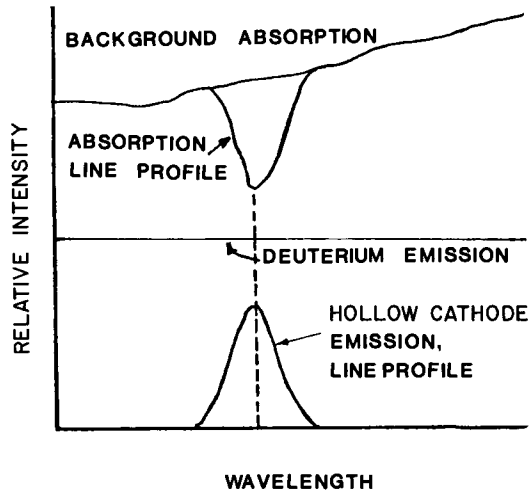

Fig. 17. Deuterium atomic-absorption background corrector system.

It is possible to split the absorption line for many elements into two different components (one π and two σ) by applying a strong magnetic field to the sample reservoir. This is called Zeeman splitting and is used for background correction. A polarized Zeeman system is shown in Fig. 18. In one implementation, a rotating polarizer is used in the incident beam of the hollow cathode lamp. The polarized components that are parallel (P$\|$) and perpendicular (P$\perp$) to the magnetic flux can be separately measured. The parallel beam is the only component that can be absorbed by the atomic vapor in the sample, and the perpendicular component is used to correct for light scattering and broadband molecular organic absorption. This is shown diagramatically in Fig. 18. Electronic subtraction produces the true absorption of the sample, using the polarized Zeeman effect. An instrument using this technique is available from Hitachi, NSA [Mountain View, California (*37*)].

Another background correction technique now available utilizes atomic line-broadening techniques for background correction. If a hollow cathode lamp is pulsed to high currents, the atomic line, which is normally very narrow, is broadened. A cloud of atoms is sputtered from the surface and remains in the local vicinity for a short period of time. Some of these atoms absorb light energy so that the output radiation from the hollow cathode lamp is a line-broadened source with a self-absorbed narrow center, as seen in Fig. 19. If this is directed through the atom reservoir, a large fraction of the energy lost will be from the organic species or particulate materials that are present. When the hollow cathode lamp is operated at low currents, a very narrow AA line emission is directed through the atom reservoir. This measures the atomic-absorbing species and the interference. Electronic subtraction yields the corrected atomic absorbance. This technique was developed by Hieftje and Smith and is now appearing on Instrumentation Laboratory atomic absorption systems (*38*).

C. Atomic Emission Analysis Systems

Some elements have sufficiently low excitation energies that they can easily be excited to atomic emission at relatively low temperatures. The intensity of the spectral line emission is given by

$$I = VAh\nu N_0 g e^{-E/kT}$$

where V is the volume of the source seen by the instrument, A is a transition probability, $h\nu$ is the energy of the transition, g is a statistical term, N_0 is the number of atoms in the ground electronic state, E is the excitation energy, k is the Boltzmann constant, and T is the absolute temperature. The clinically important elements sodium, potassium, and

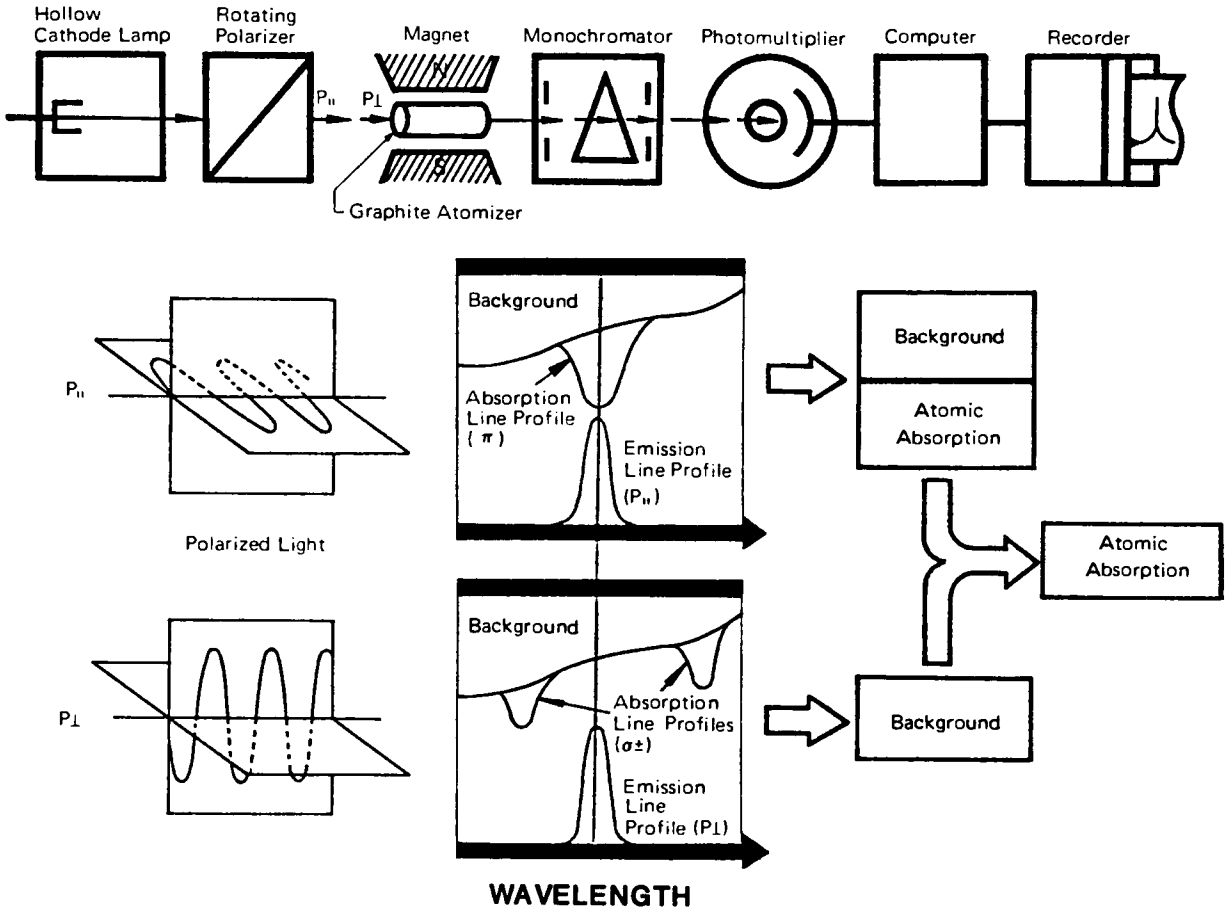

Fig. 18. Polarized Zeeman atomic-absorption background corrector. Used with permission of Hitachi, NSA, Mountain View, California.

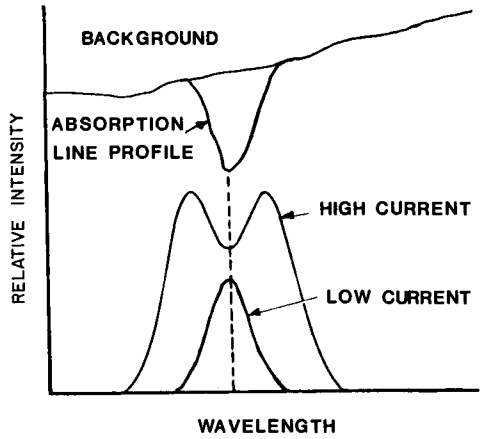

Fig. 19. Hollow cathode emission-line profiles, atomic line-width modulation atomic absorption background corrector. Reprinted from *American Laboratory* **14**, No. 11, 100–108 (1982). Copyright 1982 by International Scientific Communications, Inc.

lithium have sufficiently low excitation energies that atomic emission is produced in a propane or natural-gas flame in which air is used as the oxidant. For sodium, $E = 2.1$ eV for the line at 589 nm. This gives rise to the characteristic yellow-colored sodium flare in the flame when these samples are analyzed. Because there is a correlation between excitation energy E and wavelength λ of light, specifically, $\lambda f = C$ and $hf = E$, where h is the Planck's constant. For elements having wavelengths of emission longer than 400 nm, flame-excitation atomic emission is a satisfactory method of analysis. Atomic absorption instruments sometimes offer an atomic emission mode of detection which can be used for elements in this category.

The block diagram for the equipment necessary for atomic emission analysis is presented in Fig. 20. Calcium has its analysis line at 422.7 nm and can be analyzed with about equal sensitivity by flame photometry or atomic absorption for a flame at 3000°K, where the $N^*/N_0 = 4 \times 10^{-5}$. For zinc emission at 213.9 nm, the ratio is only 5×10^{-10} for the same flame (*39*). Coleman Instruments Division of Perkin Elmer (Oak Brook, Illinois) produces a flame photometric instrument for sodium, potassium, lithium, and calcium (*40*). To perform the analysis, the sample of interest is nebulized into the hot environment of the flame to produce atomic emission excitation. Several companies make a series of compact burners with the nebulizers concentrically located inside the burners. These total-sample consumption burners use turbulent flames with combustion gases such as hydrogen and oxygen, or acetylene and oxygen. The turbulent

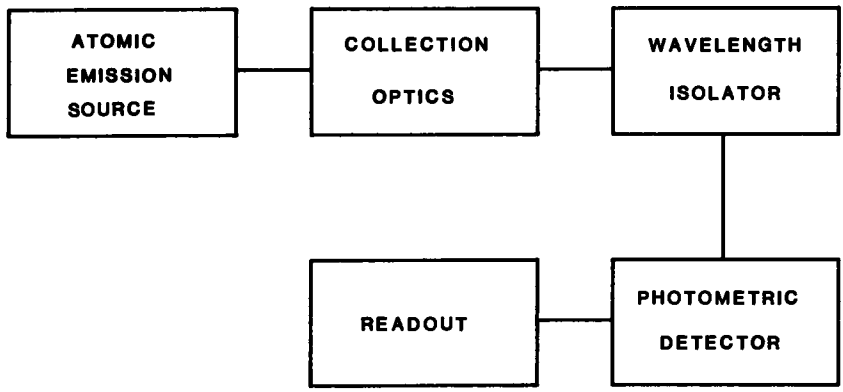

Fig. 20. Block diagram, atomic emission instrument.

flames, although extremely noisy audibly, still provide useful analyses. Laminar-flow premixed flames are suitable for flame atomic emission analyses of clinical samples. The nebulized droplets undergo a more uniform desolvation and excitation to yield atomic emission at a uniform height in the flame (*41*).

To achieve atomic emission excitation for elements with higher excitation energies, other systems having higher source temperatures T can be used. One of these is DC plasma excitation. One such plasma is sustained in argon gas and has a Y shape caused by the use of three electrodes. These electrodes help to positionally stabilize the DC discharge. If a nebulized sample is directed into close contact with the junction point of the plasma, strong atomic emission occurs that can be used for analytical purposes. Spectrometrics Division of Beckman Instruments (Fullerton, California) uses this technique in conjunction with an echelle monochromator system to achieve very good sensitivities. The echelle system allows a very high order of a specially shaped grating to be used so that wavelength separations several times greater than that of comparable focal-length monochromators are achieved. This translates directly into increased sensitivity in the DC plasma case where there is a significant amount of background emission from which the atomic emission line must be separated (*42*).

Inductively coupled plasma (ICP) excitation is the atomic emission analysis source that has achieved considerable popularity. In this technique, a series of concentric quartz tubes or other insulating material are used to form a plasma discharge where electrons are stripped away from their atoms. Argon or argon–nitrogen gases are commonly used as plasma material. Through the introduction of a strong tangential gas flow around

the perimeter and a strong flow directly up the center of the area in which the plasma is produced, a torroidal or doughnut-shaped discharge can be sustained. A multiple turn radio-frequency coil approximately 25 mm in diameter is used to inductively couple energy to the discharge. A sample can be introduced as an aerosol in the center of this doughnut-shaped plasma, where the atoms are stimulated to atomic emission as they travel through the extremely hot portion of the plasma, as is shown in Fig. 21. Because of the unique plasma torroidal geometry, the atoms emitting radiation detected by the instrument are from the hottest part of the ICP rather than from a cooler environment as they are from the outside of a conventional flame (*43*). This reduces self-absorption (atomic absorption resulting from cooler ground-state atoms between the emitting atoms and the detector) and provides an extremely long linear range for atomic emission signals. Some elements exhibit a 10^5 concentration range with a reasonably linear signal. This is quite different from the chemical flame excitation which exhibits a much shorter range of atomic emission before self-absorption occurs with a subsequent decrease in linearity. The ICP sources of this type are available from Plasma-Therm, Inc. (Kresson, New Jersey).

Extremely high electronic temperatures as great as 10,000 K have been measured in the ICP or electric flame. At this temperature, a much higher fraction of the atoms are in the excited state than with a chemical flame source. Thus, ICP atomic-emission sensitivities are comparable or superior to flame AA used for clinical analyses (*44*).

D. Atomic Emission Background Correction

Because the atomic emission signal originates from a plasma, there is considerable emission continuum background. For that reason, several systems are available to reduce this interference effect on the signal. In

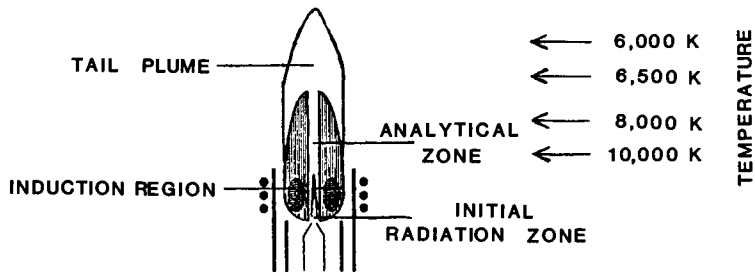

Fig. 21. Geometry and temperatures, inductively coupled plasma. Reprinted from *American Laboratory* **14,** No. 11, 142–147 (1982). Copyright 1982 by International Scientific Communications, Inc.

the simplest case a blank is run, and the background is simply subtracted. In a more sophisticated case, particularly when no suitable blank exists, the actual profile of the analytical line is measured as it is scanned across by the instrument. The background adjacent to the line on a single or on both sides can then be subtracted. The assumption is that the adjacent background is representative of background under the analytical line. This scanning or wavelength modulation technique allows the analyst to correct for such things as band emission or structured stray light from the diffraction grating, and so forth (*45*).

In a patented clinical multielement system, Hoffmann-La Roche Inc. (Nutley, New Jersey) employs a custom instrument that scans across the spectral line of interest about 50% of the time and collects data at the spectral line position for the other half of the time. It provides data for every analysis, which allows the technologist to determine whether the optical system is aligned properly and to correct for background. These types of data are shown in Fig. 22. The system permits a human to interact with the data graphically displayed by a computer. By viewing the data, the technologist can quickly determine the extent of spectral background or spectral interference. In most instances, a representative background can be selected to correct for significant interferences (*46*).

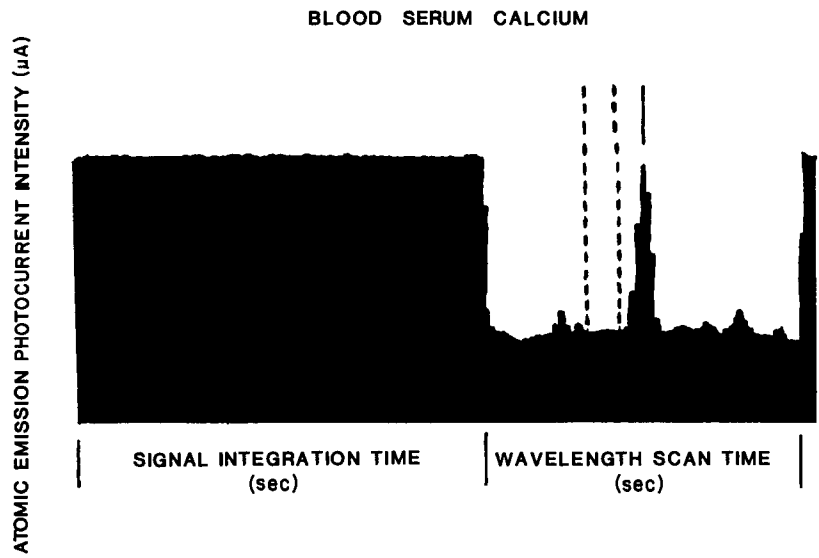

Fig. 22. Clinical multielement analyzer–inductively coupled plasma data presentation (blood serum calcium). The dashed lines are spectral background cursors; the peak in atomic emission intensity is at the optical alignment marker.

Some manufacturers provide a spectral background correction system that steps off the spectral line position on either or both sides of the spectral line at an arbitrary but small wavelength separation and subtracts the signal at those points from the spectral line signal plus background. One way of performing this technique is to change the angle of a galvanometer-driven refractor plate just behind the entrance slit of the spectrometer. This system is used by Jarrell-Ash Division, Fisher Scientific, an Allied Co. (Waltham, Massachusetts) in their direct-reading spectrometers for background correction (*47*).

If sufficient quantities of samples are available, the elements can be determined sequentially by scanning over each atomic emission line of interest. This is the method used by Perkin–Elmer (Norwalk, Connecticut) in their computer-controlled sequential-scan ICP that also incorporates an atomic absorption system in the same instrument (*48*).

E. Atomic Fluorescence Spectrometry

Atomic fluorescence is the result of optically excited atomic (emission) fluorescence of sensitive atomic resonance lines. The ICP has recently been used as an atomization cell of analyte atoms for atomic fluorescence. A series of hollow cathode lamps and interference filters surrounds the ICP produced atom reservoir. The lamps are AC excited and detected to produce and measure the atomic fluorescence, as is shown in Fig. 23. Baird Corporation (Bedford, Massachusetts) has a system that has shown good linearity of over four orders of magnitude. A particular advantage is that many spectral background interferences are circumvented because they are manifested in the DC portion of the signal. This system is being used for low-level analyses by atomic fluorescence for elements such as silver, copper, nickel, cadmium, lead, and zinc (*49*).

IV. ELECTROCHEMICAL TECHNIQUES AND INSTRUMENTATION

A. Potentiometric Measurements

Potentiometric measurement of materials in solution is based on the determination of a galvanic cell potential. Using oxidation and reduction of half-cell potentials, a mathematical expression referred to as the Nernst

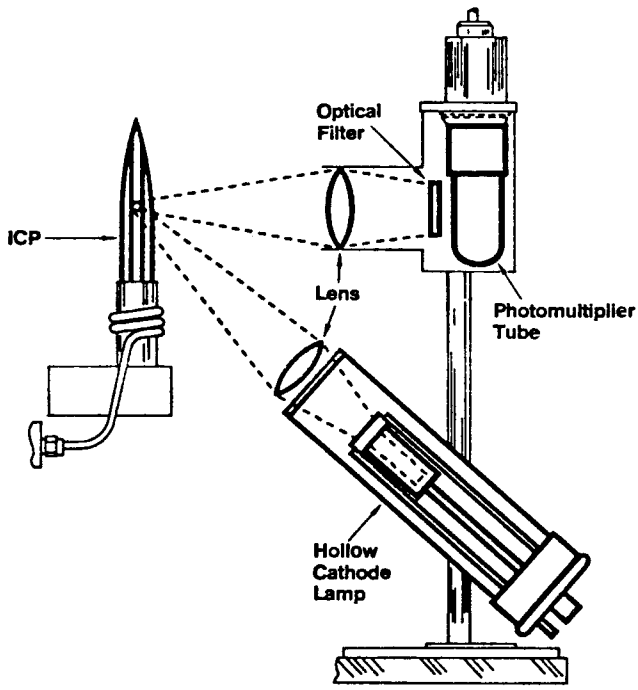

Fig. 23. Atomic fluorescence (ICP) system. Reprinted from *American Laboratory* **14**, No. 3, 167–176 (1982). Copyright 1982 by International Scientific Communications, Inc.

equation can be written. That expression is as follows for a typical silver–silver chloride reference electrode couple. The half cell reaction is

$$\mathrm{AgCl(s)} + e^- \longrightarrow \mathrm{Ag^+} + \mathrm{Cl^-} \qquad E^o = 0.222\ \mathrm{V}$$

$$E = E^o - \frac{RT}{nF} \ln \frac{[\mathrm{Ag^+}][\mathrm{Cl^-}]}{\mathrm{AgCl}}$$

where E is the cell potential, E^o the standard electrode potential, R is the gas constant, T the absolute temperature, n the number of electrons involved in the reaction, F the Faraday, and the activities or concentrations of the chemical species are used.

Another reference or standard electrode is the saturated calomel electrode (SCE). Polarographic and electrochemical potentials are often given with respect to it. However, in standard tables, the electrochemical potentials are given versus the standard hydrogen electrode (*50*). All standard electrodes can be related to each other.

One of the more useful electrodes used for potentiometric measurements is the hydrogen ion or pH electrode. Inside a thin glass membrane, which is responsive to hydrogen ions, is a solution of specified pH. The pH electrode potential is measured with respect to a separate reference electrode. When it is combined with a concentric reference electrode, the unit is called a combination pH electrode (*51*). A conventional measurement system is shown in Fig. 24. Electronics permit the measurement of the potential using a device having extremely high input impedance so that any current flow is negligible. Thus a potentiometric voltage can be determined. The Nernst expression has a temperature dependence that affects the slope of the signal. Some instruments use a separate temperature-measuring element such as a thermistor placed in the solution to correct for temperature-slope changes. The temperature-sensing element is incorporated into the circuitry so that it compensates for the temperature change when the pH is read out. Orion Research (Cambridge, Massachusetts) has recently utilized a technique of measuring the conductance of the glass electrode itself and using that to compensate for temperature fluctuation (*52*). Orion offers pH electrodes developed by J. W. Ross which replace the usual silver–silver chloride couple in the reference and pH half cells with a redox solution. The result is a faster-responding system with very low drift. By eliminating the solid junctions the electrode system can respond correctly to solution temperature changes in <30 sec; conventional pH electrodes may take 3 min or more to correctly respond to the same temperature changes. The Ross electrodes hold standardization even after repeated temperature cycling.

Sophisticated potentiometric instrument systems employ microprocessors for calibration. They take the values for two different pH buffer

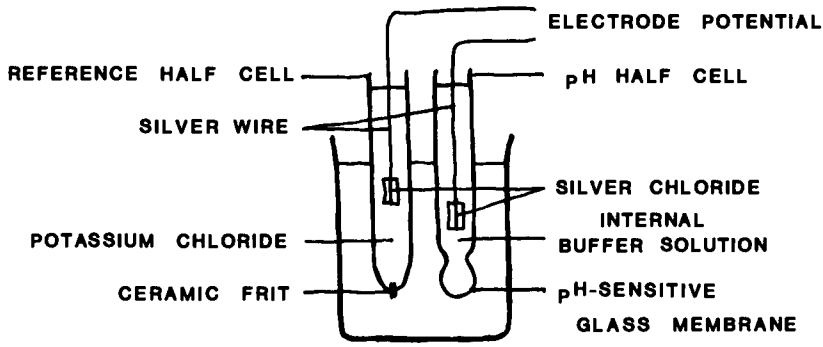

Fig. 24. Conventional glass pH-electrode system. Reprinted courtesy of Orion Research Incorporated, Cambridge, Massachusetts, U.S.A. "ORION" is a registered trademark of Orion Research Incorporated.

standards and change the slope or zero of the instrument automatically to produce an operating curve between those points (*53*). These electrodes and instruments are commonly used to measure the pH of blood and other body fluids.

The availability of membranes that have selectivity for various ions makes it possible for the devices to be ion selective. The "specific ion" electrode or ion-selective electrode concept has been developed for several clinically important quantities. Three of those electrodes are useful for chloride, sodium, and potassium ion determinations. The chloride ion electrode utilizes a crystal membrane of silver chloride that is highly water insoluble. Inside the electrode, a fixed concentration of silver nitrate solution is placed, along with a silver wire contact (*54*). It is important to note that other anions (negatively charged ions) that form insoluble compounds with silver can also produce a signal resulting in an interference. Among these are sulfur, iodine, and bromine ions. In addition, the OH^- (hydroxyl ion) can also interfere. When these ions are present, it is very important to follow procedures that provide a way of fixing or minimizing their effect. For example, a pH buffer can be used to reduce OH^- effects. As a general rule, ion-selective electrodes can be used for quantities of materials present in solution at millimolar or greater concentration. Accurate blood serum results can be achieved for the determination of chloride and sodium ion present at approximately a 0.15 *M* (150 m*M*) concentration and for potassium present at a 5 m*M* (mEq/liter) level. Ion-selective electrodes provide great convenience in reducing sample preparation significantly and are available for a considerable variety of anion and cation determinations. They measure free or ionized concentrations that may differ from the total elemental concentration. The suitability of using a particular ion selective electrode depends on having a membrane or crystal with sufficient sensing capability that has a selectivity in the presence of other ions in the sample. Electrodes exist for the following ions which can be clinically important: calcium, copper, cadmium, lead, bromide, fluoride, iodide, cyanide, and nitrate (*55*).

B. Amperometric Measurements

In some cases it is possible to force an oxidation or reduction at an electrode at a fixed potential and to measure a current related to the species of importance. This technique is called voltammetry or polarography (*56*). Illustrating one clinically useful determination using this technique is the measurement of oxygen in solution using a polarized electrode. This is an electrode that is current limited by the diffusion of the chemical species to its electrode surface. A plot of the current versus the

potential measured against a reference electrode (SCE) shows two different oxygen waves caused by the two-step reduction of oxygen in solution. The equation of the first wave is

$$O_2 + 2e^- + H_2O \longrightarrow 2H_2O_2$$

and for the second reduction wave

$$H_2O_2 + 2\,e^- \longrightarrow 2\,OH^-$$

In polarographic determinations of other materials, oxygen is usually displaced by bubbling nitrogen through the solution or chemically degraded to prevent its interference with other reduction reactions of importance.

The Clark electrode has been commonly used to measure the amount of oxygen in biological specimens. It usually has lead as its anode electrode, which is degraded and finally falls apart. That reaction is

$$H_2O + Pb^\circ \longrightarrow PbO + 2\,H^+ + 2\,e^-$$

At the noble-metal cathode, such as gold, a reaction occurs, which consumes hydrogen ion generated at the anode and consumes oxygen under diffusion conditions so that it can be measured. That reaction is

$$\tfrac{1}{2}\,O_2 + 2\,H^+ + 2\,e^- \longrightarrow H_2O$$

The Clark oxygen electrode depends on the quality of the membrane to continually transport the oxygen across it to a suitable solution such as potassium chloride in which the oxygen is then measured. The membrane often degrades with age and causes a continuous change in the sensitivity of the electrode as it deteriorates. Leeds & Northrup Company (North Wales, Pennsylvania) has developed an electrode that polarographically measures oxygen without a net consumption of oxygen at the electrode surface. The oxygen that diffuses across the permeable membrane is measured by a reduction reaction at the cathode that is the same as the Clark electrode. An equivalent amount of oxygen is generated at a multiplicity of anodes by the reaction

$$H_2O \longrightarrow \tfrac{1}{2}\,O_2 + 2\,H^+ + 2\,e^-$$

yielding no net oxygen consumption. Consequently, the membrane is made more rugged and lasts for a long time, maintaining the sensitivity of the electrode constant (*57*).

Systems using these types of electrodes have been developed for measuring the concentration of oxygen in blood through the skin. This measurement is performed by perfusing the oxygen to the capillaries by heating the skin at a localized spot. Heating dilates the capillaries and allows oxygen-rich "arterial" blood flow to be promoted at that spot. The electrode membrane which is directly attached to the skin and sealed around

the edges is in contact with the oxygen from the skin. The oxygen passes through the skin and the membrane and into the detector. With suitable calibration, the instrument can be used to monitor oxygen concentration proportional to that in blood. This is particularly useful for monitoring high-risk newborns (*58*).

It is possible to use polarography or amperometry techniques to measure the concentrations of other materials in biological samples such as copper, lead, cadmium, and zinc. One measurement instrument is a DC polarograph using a dropping mercury cathode. The current from such an instrument is described by the Ilkovic equation:

$$i = 607 \cdot n \cdot CD^{1/2} \cdot m^{2/3}t^{1/6}$$

where i is the average current in microamps, n the number of electrons involved in the redox step, C the concentration in millimoles per liter, D the diffusion coefficient in centimeters per second, m the mass flow of mercury in milligrams per second, and t the drop time in seconds. Reducing current is measured as the potential with respect to a standard electrode (usually SCE) and is shown increasing in a reducing direction to the right in polarographer's convention. This is sketched in Fig. 25, which describes the various portions of the polarographic wave (*59*).

One way of increasing the sensitivity of the measurement is the use of AC polarography. If an AC polarogram is superimposed with the DC polarogram, the maximum rate current for the AC polarogram occurs at the point of maximum rate of change of the DC polarogram, which is at approximately the half-wave potential. This signal is essentially the result of differentiating the DC polarogram. Use of AC techniques and phase measurements make it possible to use selective filtration to increase the sensitivity of the instrumentation to capture this signal.

The most sophisticated instrument technique of this type is pulse polarography. It allows a stepped potential to be applied over a short period of time as the major potential is being changed gradually. The sensitivity of the measurement is enhanced by making it after the capacitance double layer of ions has been charged. Instrumentation of this kind is available from PAR [(Princeton Applied Research) Princeton, New Jersey (*60*)].

C. Anodic Stripping Voltammetry

When sample sizes are greatly limited, it may be necessary to resort to still another electrochemical technique to increase the sensitivity. In that technique, called anodic stripping, all the reducible ions in solution are plated into an electrode. The electrode is usually either a hanging drop of mercury or a carbon electrode impregnated with mercury. Mercury is

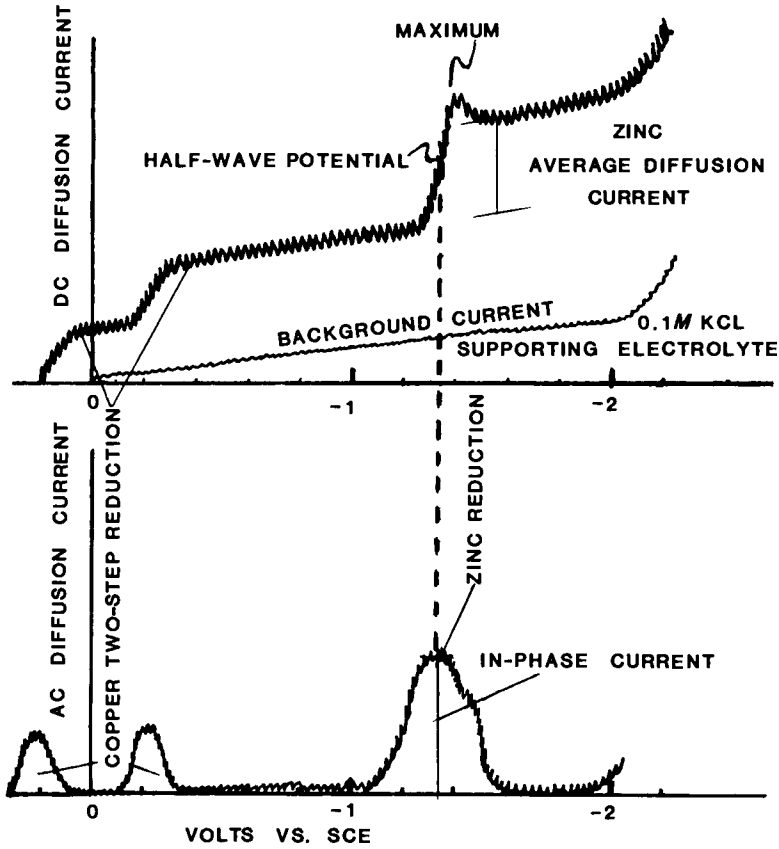

Fig. 25. Idealized DC and AC polarograms.

chosen because the hydrogen overvoltage potential is rather large. This means that hydrogen gas from the reduction of hydrogen ions in solution does not interfere until a potential of greater than about negative two volts measured against a SCE is reached. A requirement of polarographic-type techniques is that the ions be reducible. In the case of anodic stripping, the reduction–oxidation should be reversible as well. This means the material should be reduced into mercury and oxidized out of the mercury amalgam with equal ease. Anodic stripping is carried out in the following fashion. After chemical digestion of the sample to put all the ions of interest into the most oxidized state, the solution is deaerated and stirred while the potential between the electrodes is kept at a high reducing value. Four reducible and reversible elements successfully measured in blood by anodic stripping are copper, lead, cadmium, and zinc. These

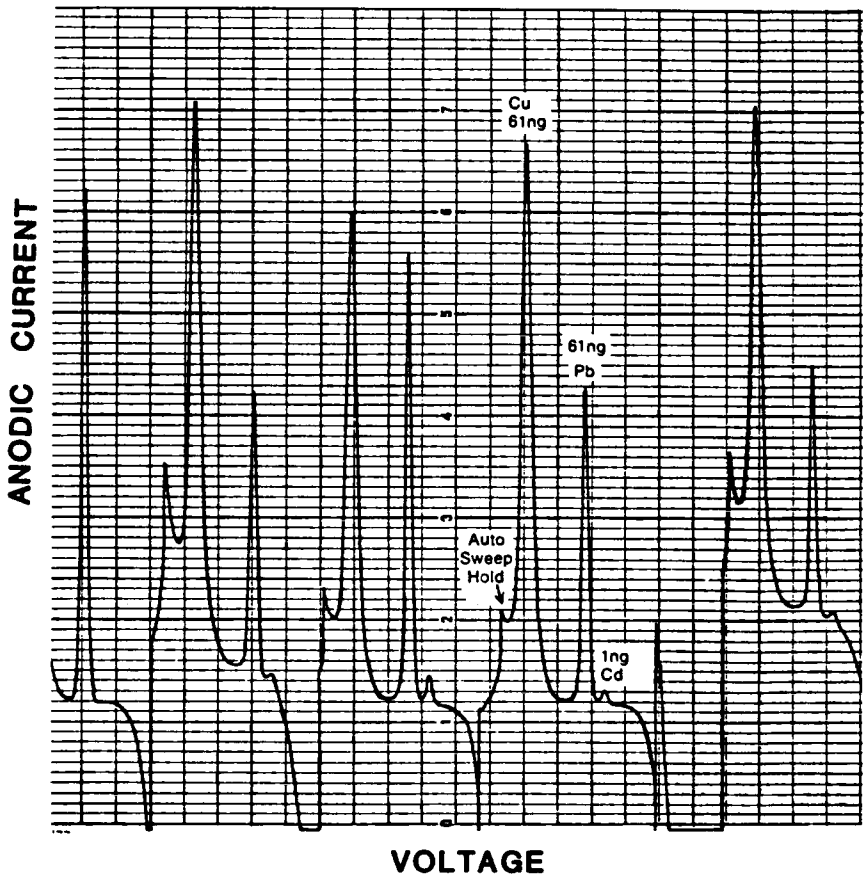

Fig. 26. Anodic stripping voltammetry of 0.1-ml blood sample (30-min plating time at −1.0 V). Used with permission of ESA, Bedford, Massachusetts.

elements are reduced and collected into the mercury drop for a considerable time. The elements are then oxidized quickly back into solution by sweeping the potential in the anodic direction and measuring the current as each ion is stripped out of the mercury electrode. The reducing process is a plating step or a concentrating process. Thus, small quantities of cations can be measured in a few drops of whole blood, as shown in Fig. 26. This is particularly useful for lead screening and has been commercialized by a company called ESA, Inc. [Environmental Sciences Associates, Burlington, Massachusetts (*61*)].

A general diagram of the type of instrumentation used for voltammetric measurements is presented in the Fig. 27. This type of instrument can be

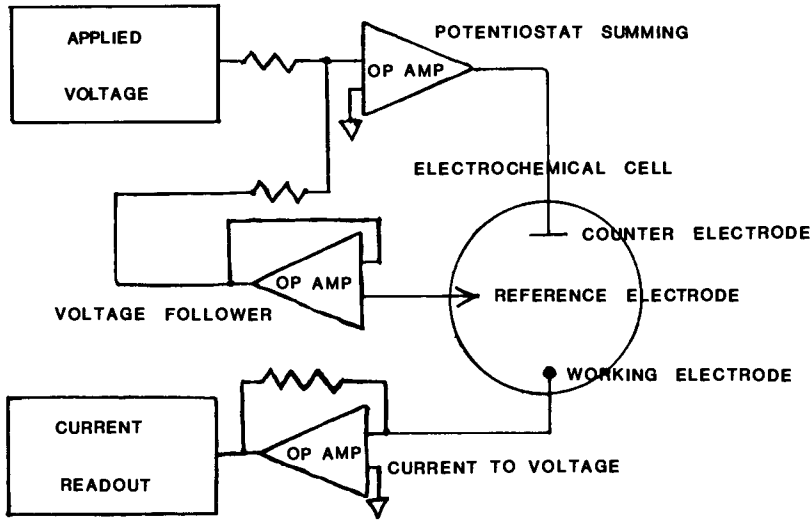

Fig. 27. Basic electrochemistry instrumentation system.

easily assembled from operational amplifiers (OA). Using this potentiostat design, measurements in high-resistance solutions can be made, because the potential—directly at the working electrode—is controlled. This system can be configured for DC polarography, AC polarography, differential pulse polarography, or anodic stripping by changing the waveform of the applied voltage and using suitable timing in the current readout circuitry (*62*).

D. Coulometric Measurements

Still another electrochemical technique can be employed to measure the concentration of materials such as chloride ion in serum, plasma, and cerebrospinal fluid. This technique is called coulometry. Faraday's law of electrolysis can be stated: the current

$$i = \frac{Q \times m}{nF},$$

where w is the weight of the substance of formula weight M oxidized or reduced by Q coulombs of electrical charge. The number of electrons involved in the reaction n and the Faraday F = 96,500 coulombs/equivalent complete the equation. If a particular potential can be selected so that only the ionic species is reacting, the integral of the current can be measured. This is proportional to the amount of charge required to reduce or

oxidize the species. Since $QT = i$ [where Q is the charge in coulombs, T is time in seconds, and i is current in amperes (*63*)].

The Cotlove Chloridometer, Buchler Instrument Division, Nuclear Chicago Corp., (Fort Lee, New Jersey) uses this technique to measure chloride concentration in biological fluids by reacting chloride ions with silver ions generated by constant current. A constant current is forced into solution from the silver wire anode generating silver ions, which reacts with the chloride ions forming an insoluble precipitate. The time required for a constant current to generate sufficient silver ions to combine with all the chloride in solution is a measure of the chloride concentration. When the chloride ion species is depleted, the current flow between two silver wire electrodes with about 100 mV of potential impressed across them increases rapidly, signaling the end point. This measurement can be termed constant current coulometric titration with an amperometric end point. It can determine chloride ion in 0.1–0.01 ml of serum. After dilution with a suitable fluid containing dilute acetic and nitric acids, samples of serum, plasma, cerebrospinal fluid, and urine can also be titrated with this instrument (*64*).

V. SEPARATION INSTRUMENT TECHNIQUES

A. Physical Separation Properties

In the analysis of clinical specimens, it is often desirable to separate the various chemical components into more manageable fractions. This separation can occur as a result of different densities of materials, such as the cellular components of human blood, which are commonly separated by centrifugation. If the separation occurs as a result of other physical chemistry properties, such as solubility differences as a result of pH changes, the separation is called an extraction. Higher degrees of separation can be made to occur by using a technique called chromatography. In this technique, mobile phase material containing the sample is passed through a stationary phase. As the mobile phase moves along the stationary phase, components of the sample having different affinities or other physical properties are retained in varying degrees on the stationary support. As the mobile phase material elutes from the end of the stationary material or column, different fractions containing separate components of the original sample can be detected or isolated.

A very practical graphic definition of resolution applicable to all chromatography is presented in Fig. 28. The height equivalent to a theoretical plate (HETP) can be estimated from the equation $\text{HETP} = y^2/16X$, where

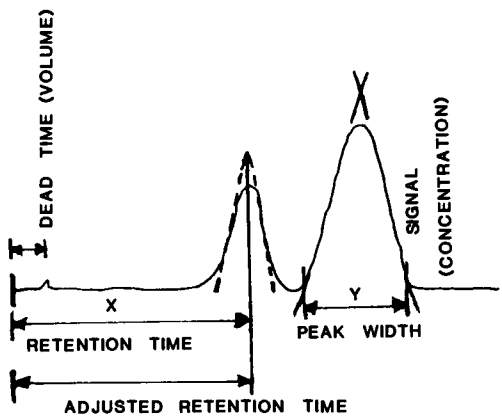

Fig. 28. Chromatographic definitions.

y is the peak width and X is the retention time defined in the figure. Using this formula and data from the chromatographic readout, separation conditions can be varied to achieve the minimum HETP in order to maximize resolution.

B. Paper Chromatography

One example of this separation technique is paper chromatography of amino acids derived from hydrolyzed protein. The hydrolysate is applied in small volume as a spot near the bottom of a piece of filter paper. The edge of the paper below the spot is placed in a suitable solution reservoir. The solvent mobile phase moves up the paper by capillary action. Since the amino acids in the mobile phase have different affinities for the cellulose material of the paper, they are carried along at different rates. After the paper is developed, the identity of many of the amino acids can be determined by their relative fraction, which is the ratio of the distance they traveled to the position of the solvent front. After drying, a two-dimensional separation can be achieved by sending a second solvent through the paper at right angles to the first solvent migration direction. Amino acids themselves are usually developed or made visible with suitable chemical reagents such as ninhydrin, which forms a red–purple spot when it reacts with a terminal amino group.

C. Thin-Layer Chromatography

A second technique that has almost replaced paper chromatography is thin-layer chromatography (TLC). In this technique, a stationary phase

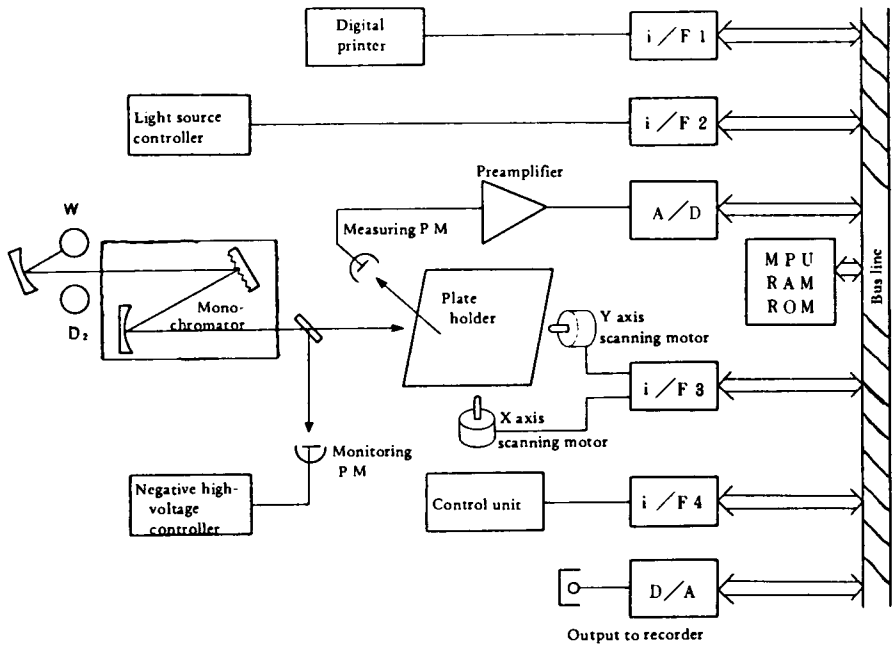

Fig. 29. Thin-layer chromatographic photometric reader diagram. Used with permission of Shimadza Scientific Instruments, Inc., Columbia, Maryland.

such as silica gel or alumina is coated with a suitable binder onto thin pieces of impervious support material, such as glass or plastic. The affinity between substrate material and sample components is generally higher than with paper, and different substrate materials can be attached to the support. Thus, TLC is more versatile than paper chromatography. The sample is spotted as a tiny drop near the bottom of the TLC plate and the solvent is allowed to migrate from that point toward the other end of the support. After the plate is developed in the solvent, the individual spots can be identified visually by reaction with certain chemicals such as ninhydrin, sulfuric acid, or other suitable visualizing materials (*65*). Thin-layer chromatography is often used to provide qualitative information and to estimate the quantity of various drugs found in the urine. Because most of the drugs absorb light radiation, it is possible to identify certain spots on the plates by examining the absorbance or reflectance on the plates with ultraviolet illumination. Shimadza Scientific Instruments, Inc. (Columbia, Maryland) offers an automatic TLC scanning instrument. The diagram of this TLC spectrophotometric reader is shown in Fig. 29. The signal processing, scanning stage control, and other components are all controlled by a microprocessor. The scanning stage is driven in a zigzag

scan by pulse scanning motors both in the X and Y axis directions. With every step, the signal is measured and converted to a digital signal; then the digital signal undergoes a curve correction, background correction, peak detection, and quantitative computation.

A fluorescence-measuring modification is available for the TLC scanner in which a mercury vapor light source is used, and a fluorescence emission filter is placed in front of the measuring photomultiplier (PM). A monitoring PM tube corrects for source intensity change in both the reflectance and fluorescence modes. If fluorescent compounds are present, they show up as bright spots. Compounds that absorb but are not fluorescent show up as dark spots against the fluorescence of the TLC plate substrate. The relative fraction is used to identify the particular drug from its position on the TLC plate. The wavelength of maximum absorbance can be used to aid identification as well. The amount of drug is estimated from the intensity of the absorption or fluorescent spot (*66*).

D. Liquid Extraction Separations

In many instances, to prepare the sample before different chromatography techniques are used, a simple extraction is performed. The most common type of manual extraction is provided by fixing the pH and shaking the aqueous material together with an immiscible nonaqueous solvent, such as ether or chloroform, in a separatory flask. The materials not ionically charged can then be separated into the nonaqueous phase. A separation of samples into acid extractables and base extractables is often made before other chromatography is begun.

A completely automated solvent extraction can be performed with systems such as the Technicon Auto-Analyzer. The sample is separated by air bubbles, and the segmented sample is joined with an immiscible extraction solvent. As material flows down the stream, new interfacial surfaces occur and allow the extraction to proceed without manual agitation. Separation of the desired phase from the waste material is accomplished with a phase separator containing a hydrophobic insert. The insert is preferentially wetted by the nonaqueous solvent, thereby causing the phases to be properly directed in a separator much like the standard debubbler. With the proper connections of tubing, a solvent extraction can be carried out automatically as shown in Fig. 30 (*67*).

E. Counter Current Chromatography

Counter current chromatography (CCC) is a method that uses liquid–liquid partition chromatography without solid support. Instead of a one-

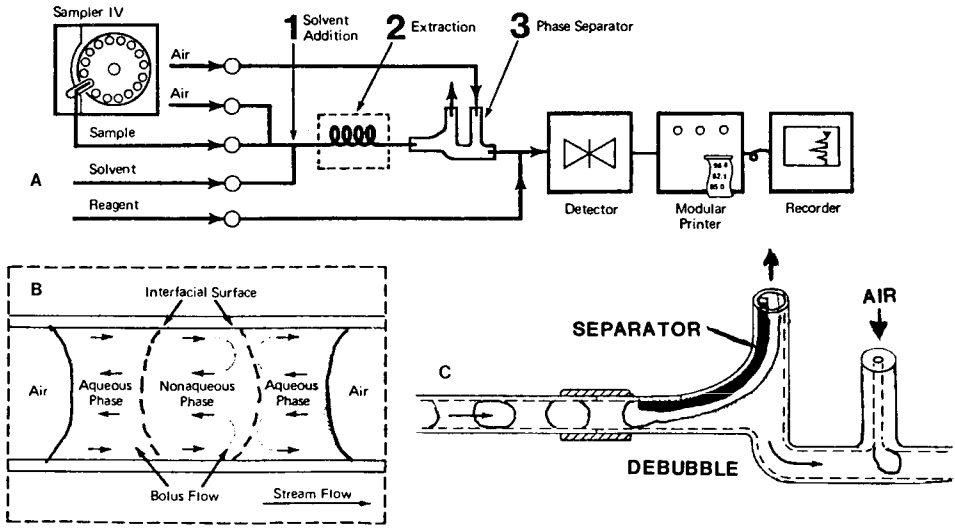

Fig. 30. A. Automated solvent-extraction system. B. Phase-mixing diagram. C. Phase-separation component. Used with permission of Technicon Industrial Systems, Tarrytown, New York.

stage liquid extraction, CCC can offer a much larger number of effective extractions. It is applicable from small ions and molecules to macromolecules and particles. This CCC is carried out by carefully maintaining the dynamic flow behavior of two immiscible solvents. A large volume of the stationary phase is retained usually in a coil while the mobile phase is continuously eluted. Again, the mixing that occurs within each solvent phase is a result of the flowing stream. In some instances, motion is imparted to the coils to take advantage of centrifugal forces to maintain the desired separations of solute and mobile phases. Large quantities of material can be separated, unlike many other chromatographic methods (*68*).

F. Gas-Liquid Chromatography

One of the chromatographic techniques that has attained a high degree of instrumental sophistication is gas chromatography (GC). In GC or gas–liquid chromatography (GLC), a small sample is volatilized and allowed to flow through a mobile phase being carried along with a carrier gas. The liquid phase is normally held in a column containing solid substrate or even the tubing wall itself. Different components in the samples can be eluted at different times and detected with a series of detectors at the end of the effluent stream.

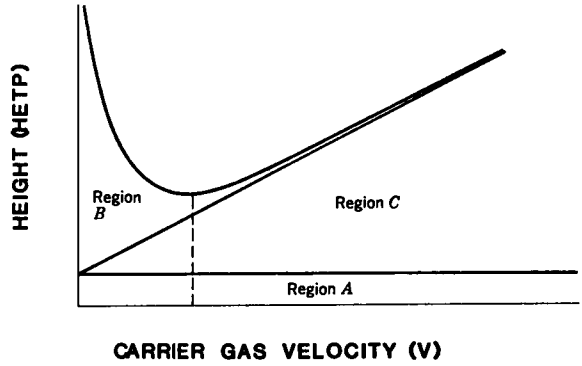

Fig. 31. Van Deemter equation plot. From "Instrumental Methods of Chemical Analysis," G. W. Ewing, McGraw Hill, New York (3rd ed.) with permission.

The equation which governs the efficiency of separation is referred to as the Van Deemter equation and has the following form:

$$H = A + \frac{B}{V} + CV,$$

where V is the carrier-gas velocity. A plot of plate height versus carrier gas velocity in Fig. 31 shows that there is an optimum velocity of carrier gas corresponding to a minimum height equivalent to a theoretical plate (HETP). The A term results from eddy or multipath geometry effects and is constant with changing velocity. When the gas flow is too low, longitudinal diffusion effects in the B term degrade column performance. When it is too high, mass transfer in the mobile and stationary phase of the C term predominates and can reduce column efficiency (*69*). The areas of the curve affected by the various terms are shown in the figure.

A block diagram showing the essential components of a gas chromatographic system is given in Fig. 32. There are several components necessary for gas chromatography. A source of carrier gas, such as helium or nitrogen, is needed. After suitable pressure and flow regulation, the gas goes through a heated injector port. In that area, thermal heat of sufficient magnitude is provided to vaporize the sample if it is in liquid or solid phase. The sample material is entrained in the carrier gas and flows onto the glass coil or metal tubing in which the column substrate is packed. The carrier gas containing the vaporized sample proceeds through the separation column, which is placed in an oven. The sample components having different affinities for the nonmobile phase proceed at differing rates and then separate. A signal related to a physical property of the sample or a bulk property of the gas is sensed in a detector. Finally, after suitable electronic signal processing, the signal is read out (*70*).

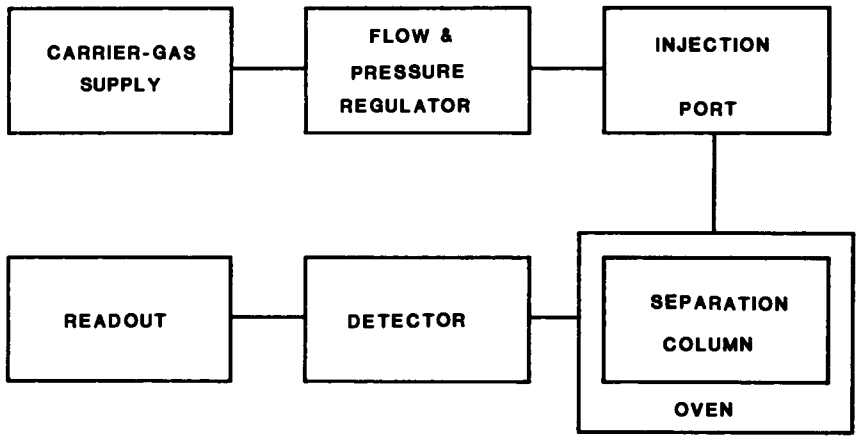

Fig. 32. Block diagram, gas chromatograph.

G. Detectors for Gas–Liquid Chromatography

The thermal conductivity detector is fairly universal but not very sensitive in its response which measures the bulk property of the carrier gas. If a gas such as helium is used, the amount of heat conducted away when an organic component is present is different from than when a pure gas is present. If a thermistor or hot wire electrode is put in a Wheatstone bridge-measuring circuit, an imbalance current can be read out on a device such as a chart recorder as a function of time to produce a chromatogram. This chromatogram can be related to various components by running pure standards and measuring the retention time, which is the time from injection until the time the material emerges from the column. The quantity of material can be estimated from the height of a peak after calibration. Gas chromatographic instrumentation offers several refinements to help eliminate instrument problems. One technique is to employ two columns packed with the same material and to use one of them as a reference. This column should have the same plumbing, pathlength, and packing material as the analytical column. It compensates for such things as column bleed-off or subtle changes in carrier gas flow or composition. Both the analytical and the reference columns are placed in an oven. This allows the chromatogram to be run either isothermally (at a constant temperature) or to be temperature programmed. In programmed mode, the oven temperature is raised along a specific profile with time. This facilitates the removal of less volatile components in the sample and speeds up the complete chromatogram. When this mode is used, it is particularly valuable to use a reference column to compensate for base-

line drift. Matched or twin detectors are normally used and their outputs placed in a Wheatstone bridge circuit so that the reference and the sample are compared in the same circuit. The detection limit is about 10^{-8} g of sample and the dynamic range about 10^5 (*71*).

Another quite common detector for the gas chromatographic system is the flame-ionization detector (FID). In this detector, the carrier gas is mixed with air and hydrogen and allowed to flow into a small flame. This flame produces ions which can be collected by two electrodes mounted above the flame. A voltage of several hundred volts is applied to the electrodes to attract and collect the ions. This ion current produces a signal related to the sample, so that it is often referred to as a carbon-counting detector. This detection does not require precise temperature control. It is also insensitive to water vapor. The FID itself is linear over a wide dynamic range region and is sensitive to most organic compounds. The sensitivity limit is about 10^{-11} g of sample weight and the linear range is 10^7 (*72*).

Another type of detector for gas chromatography is the electron capture (EC) detector. In this detector, a beta-emitting material such as nickel-63 provides a standing electron current between two electrodes, which have a collection voltage applied. When material, such as those containing electronegative atoms such as the halogens (chlorine, etc.), proceeds through this environment, electrons are captured and the standing current caused by the ionized nitrogen carrier gas decreases. With an EC detector, compounds having an affinity for free electrons, such as chlorinated pesticides or halogenated anaesthetics, are detected. The response is nonlinear (*73*), but by using feedback techniques a linear range of 10^2 can be established. Materials can be detected at extremely low levels and the detection limit is 10^{-13} g.

There are several other detectors used for specialized applications that have been used to good advantage on the output of gas chromatographs. These are detectors manifesting some degree of selectivity for sample detection. A phosphorus–nitrogen detector uses a tablet of alkali metal salt vaporized very slowly by a temperature-controlled heater system to sensitize the flame. When compounds containing phosphorus or nitrogen are present, they combine to produce an increased flame ionization-detector ion current. The detector can be made linear to nitrogen over a 10^5 range with a selection ratio for $N/C = 10^4$ and $P/C = 10^5$ for most operating conditions (*74*).

Gas chromatography is commonly used to determine the level of drugs in body fluids such as serum or urine. In many instances, it is necessary to react the drugs with other chemical reagents to form derivatives, such as volatile esters, to enhance the volatility of the analysis compounds or to

separate them by extraction. An example of the GC–FID screening of urine after a charcoal extraction for several abused drugs is shown in Fig. 33 for two columns with different packing (*75*).

In some instances, atomic emission techniques have been employed to measure the atoms or ions present in the hydrogen flame of the FID. DC arc or ICP atomic emission can be used if the GC output is directed into the sample inlets of these atomic emission sources.

Another photometric detector either for gas chromatography or other liquid chromatographies uses the chemiluminescent reaction between the nitrosyl radical and ozone to form an electronically excited nitrogen dioxide. This detector is particularly suited for nitrosamine analysis. Using the proper interference filter, the near-infrared emission can be detected. The system usually pyrolizes the products in the sample, then condenses and traps compounds that might provide interference. The reaction itself is

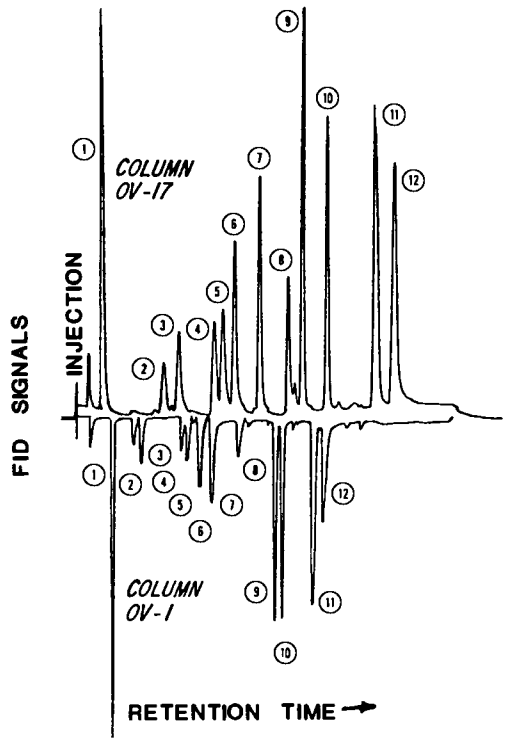

Fig. 33. Gas Chromatographic–flame ionization detector dual-column analysis. 1 amphetamine. 2 standard. 3 methyprylon. 4 amobarbital. 5 pentobarbital. 6 secobarbital. 7 glutethimide. 8 phenobarbital. 9 methadone. 10 cocaine. 11 codeine. 12 morphine. Used with permission of Perkin-Elmer Corp., Norwalk, Connecticut.

performed in a cell into which ozone is admitted. The detector response is fairly linear over a range of about 10^5 and can detect material at a level of 10^{-12} g (*76*).

H. Ion Exchange or Ion Chromatography

Because only about 15–20% of all organic compounds are volatile enough to be used in GLC, there is a great need for separation techniques to operate in the liquid phase. Separation of ions in solution can be effected by using column materials of highly polymerized chemical compounds containing large numbers of acidic or basic groups. Depending on the pH of the eluent and on the affinity of the sample cations (positively charged ions) or anions (negatively charged ions), a separation or binding can occur on the column. One example is a strongly acidic cation exchanger made of sulfonated polystryrene. It can be used to fractionate cations to provide inorganic ionic separation. In addition, compounds such as peptides and amino acids can be separated. Other types of column materials for ion exchange separation are weakly acidic cation exchangers, strongly basic anion exchangers, and weakly basic anion exchangers (*77*).

The technique known as ion chromatography has provided a useful method for the determination of aqueous ions such as HF, H_2SO_4, an HNO_3 in a variety of matrices. The anions are eluted from the exchange column usually in the sodium form in a sodium hydrogen carbonate elution solution. A stripper or suppressor column is then placed downstream of the separator column so that the ions are eluted in hydrogen form, which is much more conductive. These anions can then be easily detected in a conductivity cell. This technique has been developed and is available as an instrument system from, companies such as Dionex Corporation (Sunnyvale, California). A chromatogram of nine common anions separated by ion chromatography is shown in Fig. 34. This technique separates ion species at submicrogram per milliliter concentrations (*77*).

I. Gel Permeation Chromatography

Another method of carrying out chromatographic separations is based on the molecular size exclusion principle, which is sometimes called gel permeation chromatography. In this technique, polysaccharides are cross-linked to varying degrees. This forms void spaces that can be selected so that compounds of particular molecular weight diffuse into them. Compounds of larger molecular weight are excluded and are eluted from the gel permeation columns most quickly, usually in the void vol-

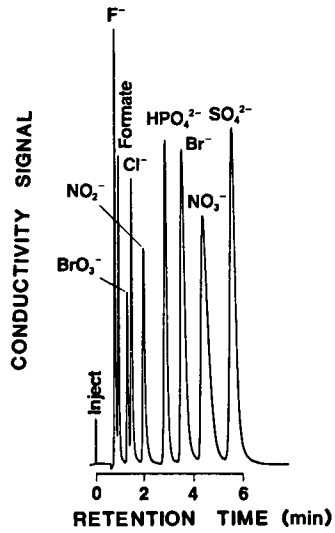

Fig. 34. Ion chromatographic separation. Concentrations (in ppm): F^-, 3; formate, 8; BrO_3^-, 10; Cl^-, 4; NO_2^-, 10; HPO_4^{2-}, 30; Br^-, 30; NO_3^-, 30; SO_4^{2-}, 25. Reprinted from *American Laboratory* **14,** No. 2, 98–104 (1982). Copyright 1982 by International Scientific Communications, Inc.

ume. Separations based on molecular weight or size differences can be carried out with the largest molecular weight fractions eluted first. Care should be used to select the proper size separating-type form to perform the fractionation most efficiently. Materials for this type of separation are available from Pharmacia Fine Chemicals, Piscataway, New Jersey among others.

J. Adsorption-Partition High Pressure Liquid Chromatography (HPLC)

Many advances have been made in adsorption chromatography. In this technique, organic materials are separated because of differences in adsorption on solid materials such as alumina. The attraction forces probably result from dipole interactions or other weak chemical forces such as hydrogen bonding. A typical chromatographic separation is carried out by using an active adsorbant, such as alumina or silica gel, and using different solvents to elute the materials selectively. Normally, nonpolar solvents such as petroleum ether are used to start the fractionation, and the solvent composition is slowly changed to solvents such as acetone, chloroform, alcohols, and, sometimes, water. One early problem with adsorption chromatography was that the solvent was fed with only gravity pres-

sure from a reservoir. By increasing the solvent pressure, there is an increase in the rate of effluent flow, which greatly shortens the chromatographic separation time. This variation of the technique is usually termed HPLC for high-pressure or high-performance liquid chromatography.

A more complex equation bearing a close resemblance to the gas chromatographic HETP equation can be derived for liquid chromatography. It includes terms for longitudinal diffusion, which is the major contributor at low flow rates. At intermediate velocities, a convective mixing term is important and, at high flow rates, mobile phase mass transfer resistance is large. Since a reasonable analysis time is desirable, a reduction in the resistance to mass transfer in the stationary phase is usually the most important term (*78*). Figure 35 shows a block diagram of the HPLC systems available today.

To perform HPLC, a high-pressure solvent pump is necessary. After the solvent has been degassed by applying a vacuum, it can be fed into the analysis system with a high-pressure pump. Pumps today can deliver pressures of more than 5000 lb./sq. in. and virtually pulseless flow. Most pumps employ multiple pistons and gear-drive geometry to achieve rapid and even flow rates (*79*). Downstream of the pump, a sample injection system is required. One method commonly used is sample loop injection. The sample is placed in a small loop of metal tubing and then switched into the sample stream with a series of valves. This eliminates the need to inject material directly into a high-pressure area.

K. High Pressure Liquid Chromatography Columns

Columns have become smaller in diameter as the pressures available have increased. The stationary phase is often contained in stainless steel or plastic tubing. The small surface volume of the metal columns allows them to be operated at a very high pressure. A detector, usually a photometric one, is employed at the separating column output, and the signal is displayed on a suitable readout device.

One big advance in liquid chromatography is the reverse phase column. In this column, hydrophobic material is bonded to a stationary substrate. This allows the use of polar solvents, such as water, as the eluting agent. Thus, components in aqueous solutions can be separated directly in a so-called reverse phase mode. Instruments are available that allow the separation to be run isocratically with the same solvent composition or by programming various mixtures of the solvent. This changing elution composition can be carried out by reproducibly mixing the solvents from two or more reservoirs.

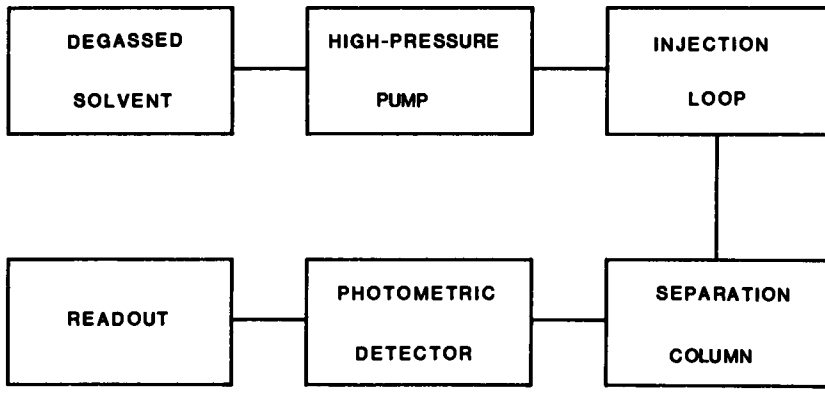

Fig. 35. High-pressure/performance liquid chromatographic instrument diagram.

The columns themselves are different in size, depending on the application. The smallest column or microbore columns give very high absolute sensitivity because small quantities of materials can still be separated. A typical conventional column may be approximately 3–5 mm in diameter and is often packed into stainless steel tubing. Many columns are typically 20 or 30 cm in length.

Waters Associates (Milford, Massachusetts) has recently announced a radially compressed column that offers some interesting advantages. This column is approximately 5–10 ml in diameter but considerably shorter in length, typically 10–15 cm. By packing the separating material in a tough plastic cylinder and slipping it into a specialized holder, the column itself can be squeezed or radially compressed before use. A series of plates with narrow spiral orifices in them distribute the sample uniformly over the entire surface of the column. Radial compression forces the plastic column wall into intimate contact with the packing material at the column wall. This reduces void spaces and prevents packing material movement during the separation. The result is an HPLC column that performs very well and that achieves higher flow rate because of reduced back pressure resulting in faster analyses. A standard steel column and a radially compressed column are described pictorially, and typical chromatograms that include lidocaine and similar compounds are shown for comparison in Fig. 36. The radial compressed column offers the advantage of rapid switching from one elution solvent composition to another. Larger amounts of material can be separated at a lower pressure with a higher speed of analysis as well. The manufacturer also claims extended life for the column and reduced cost (*80*).

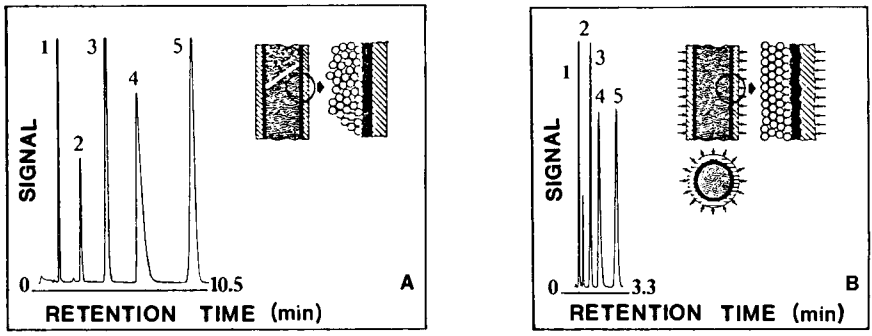

Fig. 36. Rigid-wall (A) versus flexible-wall (B) high-pressure/performance liquid chromatographic columns. A. Steel Column. Type: μ Bondpak C_{18} column; flow rate: 2 ml/min; pressure: 2000 psi. B. Radial-PAK Cartridge. Type: Radial-PAK μ Bondpak C_{18} cartridge; flow rate: 10 ml/min; pressure: 2000 psi. (1, procainamide; 2, benzocaine; 3, lidocaine; 4, tetracaine; 5, editocaine.) Used with permission of Waters Associates, Milford, Massachusetts.

L. High Pressure Liquid Chromatography Detectors

At the output of the chromatographic column is a detector that is often connected to a chart recorder to provide a readout. Most of the detector concepts have been previously discussed. Several types used in HPLC are covered in more detail in other references (*81*). One very popular type of detector is the photometric absorption detector. It utilizes an ultraviolet wavelength, often the mercury atomic emission line at 254 nm, to measure changes in absorption when organic materials are present in the solvent stream. Photometric detectors are usually so sensitive that few problems in detection result, because they measure a property of the solute. Liquid chromatographic detectors of high sensitivity have enabled HPLC to be developed by facilitating the evaluation of high-efficiency packings (*82*).

Multiple-wavelength absorbance detectors using photodiode-array detector techniques are now coming into use for HPLC analysis. By comparing normalized spectra taken on the upslope, apex, and downslope for each peak the question of HPLC peak purity can be answered while the peak is eluting (*83*). These detectors can record a spectrum in as little as 10^{-2} seconds.

There are several additional detectors being used with HPLC. A number of organic substances produce strong fluorescence, and several companies provide fluorescence detectors that can be utilized at the output of

an HPLC system. An example is the Fluoromonitor III-LC detector made by Laboratory Data Control Division, Milton Roy Co. (Riviera Beach, Florida).

Another type of detector is the refractive index (RI) unit, which provides a signal when the solvent composition changes because of the presence of an eluting substance. The RI detectors are not very sensitive, because the bulk property of the eluting solvent does not change much when a small concentration of sample component is present.

Atomic absorption can be used at the output of an HPLC system to determine metal ion content in the sample. The chromatographic effluent can be connected directly to the AA sample aspirator.

It is also possible to connect the output of the HPLC system directly into an inductively coupled plasma unit where elemental determinations of the components of each peak can be made directly by atomic emission analysis (*84*).

Conductivity detectors can be used where the eluents themselves will provide some sort of conductive signal. Dielectric detectors measuring either the induction of dipoles or orientation effects can sometimes be used as well.

Electrochemical cell detectors can be employed if the effluent contains organic materials that are oxidizable or reducible when an electric potential is applied. The test electrodes are often made of porous graphite having a large surface contact area; the eluent flows through them. The reference and counter electrodes are placed in proximity to the graphite to reduce high solution resistance usually found in nonaqueous eluents (*85*).

VI. IMPACT OF MICROPROCESSORS ON CLINICAL INSTRUMENTATION

The microprocessor has allowed the distribution of intelligence to the instruments themselves. Formerly, instruments were connected directly to a separate computer, where data collection and computations were carried out. Microprocessor hardware costs have dropped lower as a larger scale of integration of circuits has been established. The programming or software has become more "user friendly" as memory space and digital processing speed has been increased.

A frequent application today for microprocessors in the clinical laboratories is the instrument control task. The microprocessor inside the instrument remembers a specific set of conditions for the instrument and

will, on command, perform routine program tasks with a high degree of precision.

A typical example is the setup of an AA system as exemplified by an Instrumentation Laboratory unit. Method parameters can be stored in the computer itself and, upon command, the instrument will display the conditions necessary for the test itself on a television console. In many instances, the instrument will find the appropriate wavelength by scanning the monochromater under microprocessor control to the selected analytical wavelength. After the unit finds the line maximum, it then zeros the instrument. Sensors allow the microprocessor to check the setup conditions against the values in memory for the analysis selected (*86*).

A somewhat more traditional application is the collection of data by the microprocessor. Microprocessor technology has become so inexpensive that a separate microprocessor is often dedicated to data collection. The microprocessor runs in a looplike condition and utilizes a program that can store, integrate, subtract, normalize, and so forth. The microprocessor can perform very sophisticated mathematical operations and displays the data in the most useful form for the user. Using an absorption photometric example, the microprocessor can collect intensity information from the sample channel, subtract the background, ratio that with the reference channel, take the logarithm, and store the absorbance values that are proportional to concentration in memory to be read upon command. Changes in data collection, such as changes in integration time, now become a matter of merely changing the software and not the mechanical or electronic components. The microprocessor can be used to diagnose the problems in an instrument and indicate which module is defective.

Finally, the intelligence available in the microprocessors now allows local instrument data analysis tasks to be performed as well. Points for different determinations can be computed from different types of fit to standard curves. For example, if several standards are read into the computer memory a curve-fitting routine such as least-squares analysis or point-to-point connection can be employed and the data stored in the microprocessor memory. When the appropriate value for the sample is determined, the correct concentration reading can be computed using the onboard microprocessor. Some systems actually plot the standards and the computer-generated curve on a cathode ray tube (CRT) or printer during the analysis for the convenience of the operator. In addition, the derivative analysis of spectra is facilitated with digital microprocessors because various smoothing functions can be used to selectively eliminate noise. Enhanced resolution and discrimination of sharp spectral features are available if the first or second derivative of a spectrum is studied (*87*).

VII. LABORATORY AUTOMATION AND ROBOTICS

Microprocessor control of the sample as it approaches the instrumentation is now beginning to be used in the laboratory. In many instances, systems already use automation to process samples placed in a rack or carousel to speed the analysis. If the sample device that presents the sample to the instrument for analysis is replaced by a completely programmable device, laboratory robotics has been introduced.

Several different types of robots can be employed. The simplest of these is a pick-and-place unit. This type of laboratory robot will pick up the material from a fixed location and place it in another location. This finds utility in the clinical laboratory by picking up a sample from a carousel, presenting it to an aspiration tube for an instrument to utilize, and then returning the empty container after an established time to its original position or to a reject position such as a waste can. Robots of this type are also utilized as automatic pipetting and diluting systems on instruments such as centrifugal analyzers, and the like.

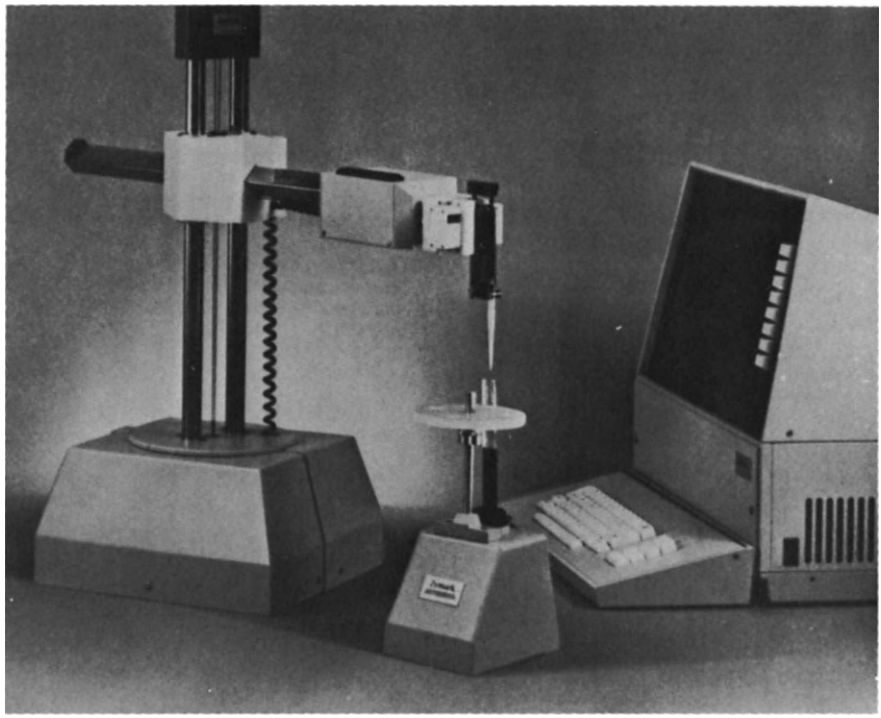

Fig. 37. Laboratory automation robot. Used with permission of Zymark Corp., Hopkinton, Massachusetts.

A system more versatile for laboratory analysis is exemplified by the servo-controlled robot made by Zymark (Hopkington, Massachusetts). This Zymate unit, shown in Fig. 37, consists of an electrically operated servo-robot having voltage feedback capability so that its position in space can be defined within certain limits. A microprocessor is connected to this unit, which can be programmed to execute certain basic steps. Because of the additional intelligence of the system, more-complex procedures can be carried out. One example is the computation of the next position for a series of test tubes in a rack. Again, the microprocessor allows the robot to position samples under reagent-pump outlets of various kinds and to mix certain reagents. It can pour and separate samples as well. After preparation, the final sample can be presented to an instrument for analysis (*88*).

A third type of robot is the most expensive and has more sophistication in its position-determining capability. In these cases, a television camera or mosaic-type solid-state detector is utilized along with decision-making software to indicate to the robot the position it has in relationship to other important devices nearby. A more difficult task of aligning something or finding the top of the test tube and placing a closure on it can be performed with this more intelligently controlled unit. The large expense of this most versatile type of robot is more difficult to justify for the clinical laboratory.

VIII. SUMMARY

The revolution in electronics (the third wave) has allowed instruments to become extremely sophisticated. Many instruments in the clinical laboratory already possess high degrees of data collection and data analysis capabilities. These instruments receive instrument control parameters from the microprocessors, which allow easy changes of many clinical chemistry parameters. Thus, microprocessor-controlled instruments can be easily reprogrammed to use new test chemistries after they are developed. Certain protocols stored in the computer memory are performed without omitting a particular step—as a human might. Future instruments will be able to troubleshoot themselves and indicate faulty components for repair, perhaps over telephone lines to the factory. The age of computer-aided chemistry is here (*89*); the computers will be used to automate the traditional steps, thus creating the time necessary for the clinical chemist to expand toward higher-valued interpretation tasks. Intelligent instruments will be able to communicate their data to stations or laboratory information systems for the clinical chemist to analyze and select for

the most informative communication format or to report to the requesters of tests. It will obviously still be essential for someone to program the instrument correctly for the first operation.

Because the microprocessor has control of some instrument parameters, it should be possible to use simplex optimization techniques in the future to ensure the best instrument operating conditions (*90*). Simplex is a method of optimization that utilizes a hill-climbing algorithm that moves to optimal points on the *n*-dimensional response surface.

In times past, the introduction of automated sampling steps has increased the precision for various types of instrumental analyses. Robotics allow the automation of complex steps in analysis procedures. These analysis steps can be altered in only a few minutes by a change in programming. Thus, robotics will extend the precision of automation to complex operations done infrequently.

REFERENCES

1. G. W. Ewing, "Instrumental Methods of Chemical Analysis," 3rd ed., pp. 58–60. McGraw-Hill, New York, 1969.
2. "Lovibond 2000 Technical Data Sheet." Tintometer Co., Williamsburg, Virginia.
3. G. W. Ewing, "Instrumental Methods of Chemical Analysis," 3rd ed., p. 49. McGraw-Hill, New York, 1969.
4. "Unimeter 300 Operation and Service Manual." Bio-Dynamics, Division of Boehringer Mannheim Diagnostics, Inc. Indianapolis, Indianna, 1972.
5. H. H. Willard, L. L. Merritt, Jr., and J. A. Dean, "Instrumental Methods of Analysis," 5th ed., pp. 56–57. Van Nostrand, New York, 1974.
6. Oriel Corp. of America, Stamford, Connecticut, 1975 Catalog, p. 129.
7. H. H. Willard, L. L. Merritt, Jr., and J. A. Dean, "Instrumental Methods of Analysis," 5th ed., pp. 58–60. Van Nostrand, New York, 1974.
8. Spectronic 20 Specification Sheet, Bausch & Lomb, Rochester, New York.
9. "Optimum Parameters for Spectrophotometry," p. 10. Varian Instruments, Palo Alto, California.
10. General Electric, Miniature Lamp Products, Halogen-Cycle Lamps, Data Sheet, Cleveland, Ohio.
11. Oriel Corp. of America, Stamford, Connecticut, 1975 Catalog, p. 74.
12. Instruments SA, Inc., Jobin Yvon Optical Systems, Diffraction Gratings Handbook, Metuchen, New Jersey.
13. "Dipping Probe Colorimeter Data Sheet," p. 4. Sybron/Brinkmann Instrument Co., Westbury, New York.
14. AO (American Optical) Reichert Scientific Instruments, Buffalo, New York, "Performance Principles of the AO, No. 10200 Bilirubinometer."
15. E. B. Merrick and T. J. Hayes, "Continuous, Non-Invasive Measurements of Arterial Blood Oxygen Levels." Hewlett-Packard, Waltham Division, Waltham, Massachusetts, 1976.
16. EG&G Reticon Corp., Sunnyvale, California, Solid-State Scanners, Product Information Sheets.

17. B. G. Willis, D. A. Fustier, and E. J. Bonelli, *Am. Lab.*, **13,** No. 6, 62–71 (1981).
18. H. V. Malmstadt, C. G. Enke, S. R. Crouch, and G. Horlick, "Instrumentation for Scientists, Moldule 4," p. 112. Benjamin, Reading, Massachusetts, 1974.
19. O. Suovaniemi and J. Jarnefelt, *Am. Lab.*, **14,** No. 6, 106–111 (1982).
20. H. G. Eisenwiemer and M. Keller, *Clin. Chem.* **25,** 117–121 (1979).
21. R. Farkas, M. Moulin, and G. Revillet, U.S. Patent 4,241,998. Hoffmann-La Roche, Nutley, New Jersey, 1980.
22. Roche Analytical Instruments, Hoffmann-La Roche, Nutley, New Jersey, COBAS-BIO, Biochallenge No. 1.
23. G. W. Ewing, "Instrumental Methods of Chemical Analysis," 3rd ed., p. 107. McGraw-Hill, New York, 1969.
24. Ames Division of Miles Laboratories, Elkhart, Indiana, Product Profile Sheets.
25. S. R. Popelka, D. M. Miller, J. T. Holen, and D. M. Kelso, *Clin. Chem.* **27,** 1198–1201 (1981).
26. H. H. Willard, L. L. Merritt, Jr., J. A. Dean, and F. A. Settle, Jr., "Instrumental Methods of Analysis," 6th ed., pp. 134–143. Wadsworth Pub. Co., Belmont, California, 1981.
27. H. H. Willard, L. L. Merritt, Jr., J. A. Dean, and F. A. Settle, Jr., "Instrumental Methods of Analysis," 6th ed., p. 140. Wadsworth Pub. Co., Belmont, California, 1981.
28. Instrumentation Laboratory, Lexington, Massachusetts, "How to Choose an AA Spectrophotometer."
29. Perkin-Elmer Corp., Norwalk, Connecticut, Model 370, Atomic Absorption Spectrophotometer Manual.
30. Perkin-Elmer Corp., Norwalk, Connecticut, Standards Procedure Manual, 1973.
31. Instrumentation Laboratory Inc., Lexington, Massachusetts, Flameless Atomic Absorption Furnace, Model 556, Data Sheet.
32. Varian Techtron, Palo Alto, California, Carbon Rod Atomizer, Model 63 Data Sheet.
33. S. R. Kortyohann and M. L. Kaiser, *Anal. Chem.* **54,** No. 14, 1515A–1524A (1982).
34. H. L. Kahn, L. C. Cristiana, G. R. Dulude, and J. J. Sotera, *Am. Lab.*, **13,** No. 11, 136 (1981).
35. S. R. Koirtyohann and E. E. Pickett, *Anal. Chem.* **37,** 601 (1965).
36. Perkin-Elmer Corp., Norwalk, Connecticut, Model 370 Operations Manual.
37. Hitachi, NSA, Mountain View, California, "What's Polarized Zeeman Effect AAS?," p. 5.
38. J. J. Sotera and H. L. Kahn, *Am. Lab.*, **14,** No. 11, 100–108 (1982).
39. H. H. Willard, L. L. Merritt, Jr., and J. A. Dean, 5th., p. 368. Van Nostrand, New York, 1974.
40. Perkin-Elmer Corp., Coleman Instruments Division, Oak Brook, Illinois, Model 51 Flame Photometer, Clinical Procedures for Flame Photometry of Sodium, Potassium, Lithium, plus Calcium.
41. G. M. Hieftje and H. V. Malmstadt, *Anal. Chem.*, **40,** No. 12, 1860–1867 (1968).
42. A. T. Zander, *Ind. Res. Dev.* **24,** No. 3, 142–147 (1982).
43. L. N. Faires, *Am. Lab.*, **14,** No. 11, 16–22 (1982).
44. R. N. Kniseley, V. A. Fassel, and C. C. Butler, *Clin. Chem.* **19,** 807–812 (1973).
45. H. R. Sobel and R. L. Dahiquist, *Am. Lab.* **13,** No. 11, 152–157 (1981).
46. M. L. Franklin, U.S. Patent No. 4,346,998. Hoffmann-La Roche, Nutley, New Jersey, 1982.
47. Jarrell-Ash Division of Fisher Scientific, Waltham, Massachusetts, Atom Comp, 750 Instruction Manual.
48. T. J. Hanson and R. D. Ediger, *Am. Lab.*, **12,** No. 3, 116–123 (1980).
49. D. R. Demers, D. A. Busch, and C. D. Allemand, *Am. Lab.*, **14,** No. 3, 167–176 (1982).

50. G. W. Ewing, "Instrumental Methods of Chemical Analysis," 3rd ed., pp. 242–243. McGraw-Hill, New York, 1969.
51. G. W. Ewing, "Instrumental Methods of Chemical Analysis," 3rd ed., p. 225. McGraw-Hill, New York, 1969.
52. Orion Research, Cambridge, Massachusetts, Model 611, pH meter Specification Sheet.
53. Orion Research, Cambridge, Massachusetts, Model 881, pH meter Data Sheet.
54. G. W. Ewing, "Instrumental Methods of Chemical Analysis," 3rd ed., pp. 258–259. McGraw-Hill, New York, 1969.
55. Orion Research, Cambridge, Massachusetts, Analytical Methods Guide.
56. H. H. Willard, L. L. Merritt, J. A. Dean, and F. A. Settle, Jr., "Instrumental Methods of Analysis," 6th ed., pp. 691–720. Wadsworth Pub. Co., Belmont, California, 1981.
57. D. N. Phelan, R. M. Taylor, and S. Fricke, *Am. Lab.*, **14,** No. 7, 65–72 (1982).
58. Roche Medical Electronics, Hoffmann-La Roche, Inc., Cranbury, New Jersey, Oxygen Monitor 5300 Instruction Manual.
59. H. H. Willard, L. L. Merritt, J. A. Dean, and F. A. Settle, Jr., "Instrumental Methods of Analysis," 6th ed., p. 711. Wadsworth Pub. Co., Belmont, California, 1981.
60. PAR (Princeton Applied Research) Corp., Princeton, New Jersey, Application Notes.
61. ESA (Environmental Sciences Associates), Burlington, Massachusetts, Application Notes.
62. J. B. Flato, *Anal. Chem.* **44,** 75A–87A (1972).
63. G. W. Ewing, "Instrumental Methods of Chemical Analysis," 3rd ed., p. 323. McGraw-Hill, New York, 1969.
64. J. D. Bauer, P. G. Ackermann, and G. Toro, "Clinical Laboratory Methods," 8th ed., p. 423. Mosby, St. Louis, Missouri, 1979.
65. J. D. Bauer, P. G. Ackerman, and G. Toro, "Clinical Laboratory Methods," 8th ed., pp. 364–368. Mosby, St. Louis, Missouri, 1979.
66. Shimadza Scientific Instruments, Columbia, Maryland, Thin-Layer Chromatographic Scanner Instruction Manual, p. 11.
67. Technical Instruments Corp., Tarrytown, New York, Product Information, Fully Automated Solvent Extraction.
68. Y. Ito, *Ind. Res. Dev.*, **24,** No. 7, 108 (1982).
69. L. R. Snyder and J. J. Kirkland, "Introduction to Modern Liquid Chromatography," 2nd ed., p. 28. Wiley, New York, 1979.
70. H. H. Willard, L. L. Merritt, Jr., J. A. Dean, and F. A. Settle, Jr., "Instrumental Methods of Analysis," 6th ed., p. 477. Wadsworth Pub. Co., Belmont, California, 1981.
71. H. H. Willard, L. L. Merritt, Jr., J. A. Dean, and F. A. Settle, Jr., "Instrumental Methods of Analysis," 6th ed., pp. 465–468. Wadsworth Pub. Co., Belmont, California, 1981.
72. H. H. Willard, L. L. Merritt, Jr., J. A. Dean, and F. A. Settle, Jr., "Instrumental Methods of Analysis," 6th ed., pp. 468–471. Wadsworth Pub. Co., Belmont, California, 1981.
73. H. H. Willard, L. L. Merritt, Jr., J. A. Dean, and F. A. Settle, Jr., "Instrumental Methods of Analysis," 6th ed., pp. 473–475. Wadsworth Pub. Co., Belmont, California, 1981.
74. B. J. Ehrlich, *Ind. Res. Dev.*, **22,** No. 4, 107–110 (1980).
75. R. F. Adams, *Clin. Chem. Newsl.* **4/1,** 22 (1972).
76. I. S. Krull and M. H. Wolf, *Am. Lab.*, **10,** No. 5, 84–91 (1979).
77. E. L. Johnson, *Am. Lab.*, **14,** No. 2, 98–104 (1982).
78. L. R. Snyder and J. Kirkland, "Introduction to Modern Liquid Chromatography," 2nd ed., Chapter 3. Wiley (Interscience), New York, 1979.
79. DuPont, Analytical Instrument Division, Wilmington, Delaware, Product Booklet.

80. Waters Associates, Milford, Massachusetts, 2-Module Data Sheet.
81. R. P. W. Scott, "Liquid Chromatography Detectors." Elsevier, New York, 1977.
82. K. A. Klotter, *Am. Lab.*, **13,** No. 6, 126 (1981).
83. Hewlett Packard Co., Analytical Group, Palo Alto, California, High-Speed Spectrophotometric Detector Data.
84. I. S. Krull and S. Jordan, *Am. Lab.*, **12,** No. 10, 1–33 (1980).
85. W. A. McKinley, D. J. Popovich, and T. Layne, *Am. Lab.*, **12,** No. 8, 37–47 (1980).
86. Instrumentation Laboratory, Inc., Lexington, Massachusetts, Atomic Absorption Instruments Data Sheet.
87. J. E. Cahill and F. G. Padera, *Am. Lab.*, **12,** No. 4, 101 (1980).
88. Zymark Corp., Hopkinton, Massachusetts, Product Information Sheet, No. 103.
89. G. V. Peterson, *Am. Lab.*, **14,** No. 3, 136 (1982).
90. S. N. Deming, S. L. Morgan, and M. R. Willcott, *Am. Lab.*, **7,** No. 10, 13 (1976).

6

Hemoglobin Analysis and Hemoglobinopathies

RANDHIR S. SANDHU AND STEVEN J. FISCHMAN

CLINICAL BIOCHEMISTRY
Contemporary Theories and Techniques, Vol. 3

ISBN 0-12-657103-1

I. GENERAL REVIEW OF HEMOGLOBIN

A. Definition and Functions

Hemoglobin (Hb), a conjugated protein produced in erythrocytes, functions as a main vehicle for oxygen transport in the body. It also plays an important role as a carrier for carbon dioxide and hydrogen ion.

B. Structure

Extensive studies of hemoglobin have provided precise information about its structure and have helped in the understanding of other proteins of the body. The hemoglobin molecule shows four basic structural formations of the protein. Its primary structure gives number, kind, and sequence of amino acids in polypeptide chains (Table I). The secondary

TABLE I

Primary and Secondary Structure of Hemoglobin Polypeptide Chains[a,b]

	Amino acid sequence					
Helix #	#	α	β	γ	δ	#
NA1	1	Val	Val	Gly	Val	1
NA2	2	Leu	His	His	His	2
NA3			Leu	Phe	Leu	3
A1	3	Ser	Thr	Thr	Thr	4
A2	4	Pro	Pro	Glu	Pro	5
A3	5	Ala	Glu	Glu	Glu	6
A4	6	Asp	Glu	Asp	Glu	7
A5	7	Lys	Lys	Lys	Lys	8
A6	8	Thr	Ser	Ala	Thr	9
A7	9	Asn	Ala	Thr	Ala	10
A8	10	Val	Val	Ile	Val	11
A9	11	Lys	Thr	Thr	Asn	12
A10	12	Ala	Ala	Ser	Ala	13
A11	13	Ala	Leu	Leu	Leu	14
A12	14	Try	Try	Try	Try	15
A13	15	Gly	Gly	Gly	Gly	16
A14	16	Lys	Lys	Lys	Lys	17
A15	17	Val	Val	Val	Val	18
A16	18	Gly				
AB1	19	Ala				

TABLE I (*continued*)

Helix #	Amino acid sequence					
	#	α	β	γ	δ	#
B1	20	His	Asn	Asn	Asn	19
B2	21	Ala	Val	Val	Val	20
B3	22	Gly	Asp	Glu	Asp	21
B4	23	Glu	Glu	Asp	Ala	22
B5	24	Tyr	Val	Ala	Val	23
B6	25	Gly	Gly	Gly	Gly	24
B7	26	Ala	Gly	Gly	Gly	25
B8	27	Glu	Glu	Glu	Glu	26
B9	28	Ala	Ala	Thr	Ala	27
B10	29	Leu	Leu	Leu	Leu	28
B11	30	Glu	Gly	Gly	Gly	29
B12	31	Arg	Arg	Arg	Arg	30
B13	32	Met	Leu	Leu	Leu	31
B14	33	Phe	Leu	Leu	Leu	32
B15	34	Leu	Val	Val	Val	33
B16	35	Ser	Val	Val	Val	34
C1	36	Phe	Tyr	Tyr	Tyr	35
C2	37	Pro	Pro	Pro	Pro	36
C3	38	Thr	Try	Try	Try	37
C4	39	Thr	Thr	Thr	Thr	38
C5	40	Lys	Gln	Gln	Gln	39
C6	41	Thr	Arg	Arg	Arg	40
C7	42	Tyr	Phe	Phe	Phe	41
CD1	43	Phe	Phe	Phe	Phe	42
CD2	44	Pro	Glu	Asp	Glu	43
CD3	45	His	Ser	Ser	Ser	44
CD4	46	Phe	Phe	Phe	Phe	45
CD5	47	Asp	Gly	Gly	Gly	46
CD6	48	Leu	Asp	Asn	Asp	47
CD7	49	Ser	Leu	Leu	Leu	48
CD8			Ser	Ser	Ser	49
D1	50	His	Thr	Ser	Ser	50
D2	51	Gly	Pro	Ala	Pro	51
D3			Asp	Ser	Asp	52
D4			Ala	Ala	Ala	53
D5			Val	Ile	Val	54
D6			Met	Met	Met	55
D7			Gly	Gly	Gly	56
E1	52	Ser	Asn	Asn	Asn	57
E2	53	Ala	Pro	Pro	Pro	58
E3	54	Gln	Lys	Lys	Lys	59
E4	55	Val	Val	Val	Val	60
E5	56	Lys	Lys	Lys	Lys	61
E6	57	Gly	Ala	Ala	Ala	62
E7	58	His	His	His	His	63

(*continued*)

TABLE I (*continued*)

Helix #	Amino acid sequence					
	#	α	β	γ	δ	#
E8	59	Gly	Gly	Gly	Gly	64
E9	60	Lys	Lys	Lys	Lys	65
E10	61	Lys	Lys	Lys	Lys	66
E11	62	Val	Val	Val	Val	67
E12	63	Ala	Leu	Leu	Leu	68
E13	64	Asp	Gly	Thr	Gly	69
E14	65	Ala	Ala	Ser	Ala	70
E15	66	Leu	Phe	Leu	Phe	71
E16	67	Thr	Ser	Gly	Ser	72
E17	68	Asn	Asp	Asp	Asp	73
E18	69	Ala	Gly	Ala	Gly	74
E19	70	Val	Leu	Ile	Leu	75
E20	71	Ala	Ala	Lys	Ala	76
EF1	72	His	His	His	His	77
EF2	73	Val	Leu	Leu	Leu	78
EF3	74	Asp	Asp	Asp	Asp	79
EF4	75	Asp	Asn	Asp	Asn	80
EF5	76	Met	Leu	Leu	Leu	81
EF6	77	Pro	Lys	Lys	Lys	82
EF7	78	Asn	Gly	Gly	Gly	83
EF8	79	Ala	Thr	Thr	Thr	84
F1	80	Leu	Phe	Phe	Phe	85
F2	81	Ser	Ala	Ala	Ser	86
F3	82	Ala	Thr	Gln	Gln	87
F4	83	Leu	Leu	Leu	Leu	88
F5	84	Ser	Ser	Ser	Ser	89
F6	85	Asp	Glu	Glu	Glu	90
F7	86	Leu	Leu	Leu	Leu	91
F8	87	His	His	His	His	92
F9	88	Ala	Cys	Cys	Cys	93
FG1	89	His	Asp	Asp	Asp	94
FG2	90	Lys	Lys	Lys	Lys	95
FG3	91	Leu	Leu	Leu	Leu	96
FG4	92	Arg	His	His	His	97
FG5	93	Val	Val	Val	Val	98
G1	94	Asp	Asp	Asp	Asp	99
G2	95	Pro	Pro	Pro	Pro	100
G3	96	Val	Glu	Glu	Glu	101
G4	97	Asn	Asn	Asn	Asn	102
G5	98	Phe	Phe	Phe	Phe	103
G6	99	Lys	Arg	Lys	Arg	104
G7	100	Leu	Leu	Leu	Leu	105
G8	101	Leu	Leu	Leu	Leu	106
G9	102	Ser	Gly	Gly	Gly	107
G10	103	His	Asn	Asn	Asn	108

TABLE I (*continued*)

Helix #	Amino acid sequence					
	#	α	β	γ	δ	#
G11	104	Cys	Val	Val	Val	109
G12	105	Leu	Leu	Leu	Leu	110
G13	106	Leu	Val	Val	Val	111
G14	107	Val	Cys	Thr	Cys	112
G15	108	Thr	Val	Val	Val	113
G16	109	Leu	Leu	Leu	Leu	114
G17	110	Ala	Ala	Ala	Ala	115
G18	111	Ala	His	Ile	Arg	116
G19	112	His	His	His	Asn	117
GH1	113	Leu	Phe	Phe	Phe	118
GH2	114	Pro	Gly	Gly	Gly	119
GH3	115	Ala	Lys	Lys	Lys	120
GH4	116	Glu	Glu	Glu	Glu	121
GH5	117	Phe	Phe	Phe	Phe	122
H1	118	Thr	Thr	Thr	Thr	123
H2	119	Pro	Pro	Pro	Pro	124
H3	120	Ala	Pro	Glu	Gln	125
H4	121	Val	Val	Val	Met	126
H5	122	His	Gln	Gln	Gln	127
H6	123	Ala	Ala	Ala	Ala	128
H7	124	Ser	Ala	Ser	Ala	129
H8	125	Leu	Tyr	Try	Tyr	130
H9	126	Asp	Gln	Gln	Gln	131
H10	127	Lys	Lys	Lys	Lys	132
H11	128	Phe	Val	Met	Val	133
H12	129	Leu	Val	Val	Val	134
H13	130	Ala	Ala	Thr	Ala	135
H14	131	Ser	Gly	Gly[c]	Gly[c]	136
H15	132	Val	Val	Val	Val	137
H16	133	Ser	Ala	Ala	Ala	138
H17	134	Thr	Asn	Ser	Asn	139
H18	135	Val	Ala	Ala	Ala	140
H19	136	Leu	Leu	Leu	Leu	141
H20	137	Thr	Ala	Ser	Ala	142
H21	138	Ser	His	Ser	His	143
HC1	139	Lys	Lys	Arg	Lys	144
HC2	140	Tyr	Tyr	Tyr	Tyr	145
HC3	141	Arg	His	His	His	146

[a] Amino acids are indicated by a standard three letter code.

[b] Reprinted by permission from Witrobe, M. M., Lee, G. R., Boggs, D. R., Bithell, T. C., Athens, J. W., Foerster, J. (1981). "Clinical Hematology." Lea and Febiger, pp. 91–92.

[c] Ala at H 14 (136) in the γ and δ chains may be normal variants.

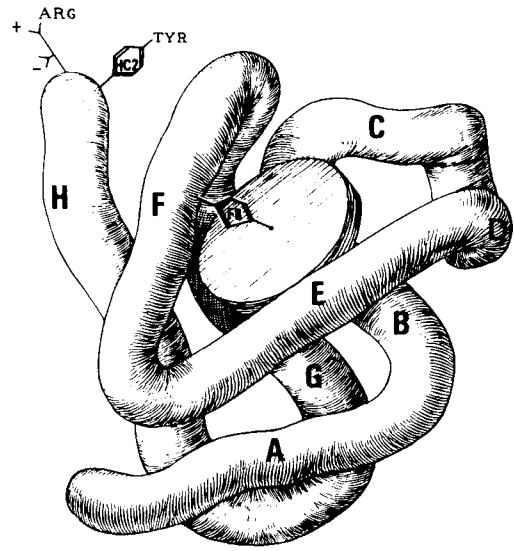

Fig. 1. The amino acid chain is coiled up into a series of helices (A–H), which have been twisted into a globular tertiary structure. Reprinted by permission from Charache, S. (1974) In "Clinics of Hematology" (D. J. Weatherall, Ed.), p. 359. Saunders, Philadelphia.

structure is a regular α-helical coiling of the polypeptide chains stabilized by hydrogen bonds between the CO and NH groups of adjacent coils (*61b*). Tertiary structure describes the folding of the coiled polypeptide chain to form a three-dimensional unit (Fig. 1). The quarternary structure refers to the relative position of the four polypeptide chains and their contact points that form a single molecule.

A molecule of hemoglobin is ellipsoidal with a molecular weight near 64,500, of which approximately 97% is contributed by the polypeptide chains of globin. The hemoglobin tetramer consists of two pairs of polypeptide chains and four prosthetic heme groups (Fig. 2). The heme groups are all identical disk-like molecules of protoporphyrin, containing a ferrous atom in the center (Fig. 3). The polypeptide chains are not identical, but are two matched pairs held together by noncovalent interaction. Each heme group is attached in a nonpolar fold of one of the polypeptide chains, where it reversibly combines with oxygen without eliciting a change in the valency of iron.

The secondary structure of the various globin chains shows more similarities compared to the primary structure. All the chains consist of eight helical portions A–H (*63a–f*), interspersed by nonhelical segments named

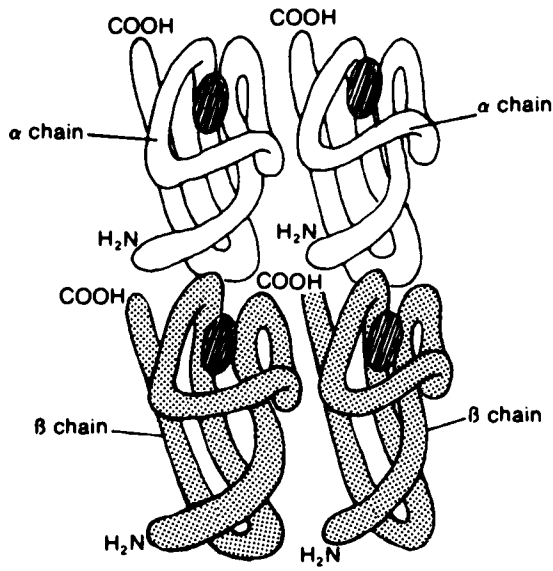

Fig. 2. Hemoglobin Tetramer of HbA. Reprinted with permission from Walton, D. (1983). In "American Clinical Products Review", (International Scientific Communication, Inc.) May/June, p. 58.

by the letters of the preceding and succeeding helicals. Example: The D helix in β-, γ-, and δ-chains contains seven amino acids, whereas the α-chain consists of only two amino acids. All other helices in the four normal chains (α, β, γ, and δ) are of similar length (Table I).

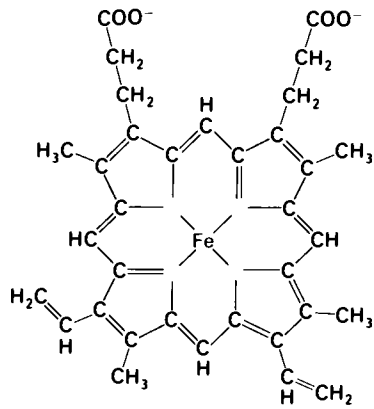

Fig. 3. Heme consisting of an organic part (Fe-protoporphyrin IX) and an iron atom. Reprinted by permission from Stryer, L. (1981). "Biochemistry" W. H. Freeman and Co., 2nd Edition, p. 44.

There are two systems to designate a given amino acid in a polypeptide chain.

1. The sequential system, where the N-terminal amino acid is assigned Number 1, and each succeeding amino acid receives the next higher number until the C terminal is reached.
2. The helical system where each amino acid is named by a letter and a number that indicates the helix and its position in the helix. An amino acid position in the nonhelical part is designated by two letters and a number.

The helical system is more useful because it illustrates homology between chains and has more structural significance. For example, heme attaches to histidine, which is amino acid No. 87 in the α-chain and No. 92 in β-, γ-, and δ-chains; the helical designation for this histidine is F8 in all the normal chains (*88*).

C. Normal Polypeptide Chains

There are six types of normal globin chains designated as alpha (α), beta (β), gamma (γ), delta (δ), epsilon (ε), and zeta (ζ). These globin chains differ from one another in the number and sequence of the amino acids. The α-chain consists of 141 amino acids, whereas β-, γ-, and δ-chains maintain a sequence of 146 amino acids. The amino acid sequence in ε- and ζ-chains is not clearly understood. The relative amount of these chains during different stages of human development are shown in Fig. 4.

D. Normal Hemoglobins

There are six normal hemoglobins and these differ from each other in their globin structure. In the first three months of fetal life, the three embryonic hemoglobins found are Hb Gower I, Hb Gower II, and Hb Portland (*12, 35a, 42*). These hemoglobins are designated by the subunit formulas $\zeta_2\varepsilon_2$, $\alpha_2\varepsilon_2$, and $\zeta_2\gamma_2$, respectively.

The three adult normal hemoglobins are adult hemoglobin (HbA)*, a minor adult component (HbA_2), and fetal hemoglobin (HbF). These hemoglobins also start in fetal life but continue in different proportions after birth. In HbA, two α-chains combine with two β-chains: In HbA_2 two α-chains combine with two δ-chains, and in HbF two α-chains combine with two γ-chains; their subunit formulae are $\alpha_2\beta_2$, $\alpha_2\delta_2$, and $\alpha_2\gamma_2$, respectively.

* Logically, HbA should be designated HbA_1, but the convention is to simply call this HbA.

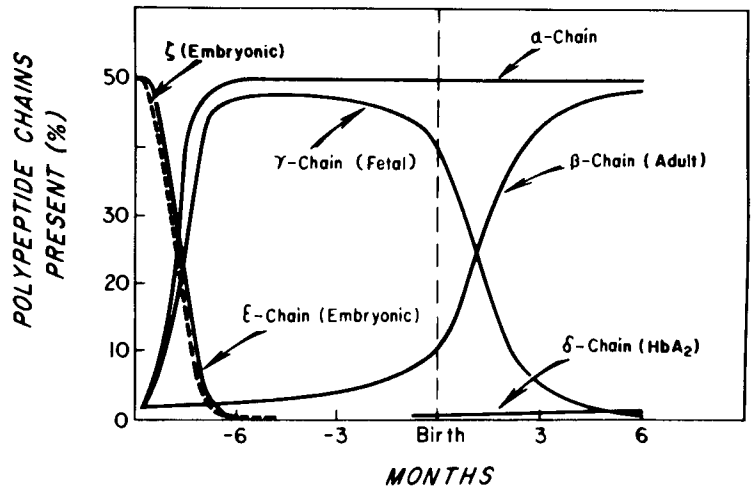

Fig. 4. Relative proportions of polypeptide chains of hemoglobin present during fetal and neonatal life. Reprinted by permission from Bunn, H. F., Forget, B. G., and Ranney, H. M. (1977). "Human Hemoglobins," Saunders, Philadelphia.

Hemoglobin F is the predominent hemoglobin in fetal life and early infancy. After birth, its level starts decreasing and is less than 2% in the normal adult.

HbA is the major normal adult hemoglobin, and it constitutes approximately 95–97% of the total hemoglobin. Hemoglobin A complexes with a number of small molecules such as glutathione, fructose 1,6-diphosphate, glucose 6-phosphate, and hexoses to form minor components designated HbA_3, HbA_1a, HbA_1b, and HbA_1c.

Usually, these complexes take place at the N-terminal of β-chains. Hemoglobin A_1,c, which is formed by nonenzymatic glycosylation of both β-chains (*77*) in HbA, has attracted more interest because of its higher level in erythrocytes of diabetic patients (*23, 50*). Hemoglobin A_2 is found in traces in the fetus but increases after birth and reaches 3–5% in the adult.

E. Genetic Control of Hemoglobin Synthesis

The polypeptide chain synthesis is controlled by structural genetic loci. The actual synthesis of globin chains takes place at the ribosomal level in the cytoplasma of erythrocytes. The information for the synthesis is carried from the structural gene at the chromosome to the ribosomes by mRNA.

In normal caucasian and oriental populations, there are two pairs of identical α-genes, but in blacks and melanesians the number varies from one to two pairs (*8*). Normal γ-chains are of two types; one having glycine and the other having alanine at position 136 (*70a*). The two different pairs of genes are designated as *Gr* and *Ar* to code for glycine and alanine, respectively.

There is only one pair of each gene coding for β-, δ-, γ-, ε-, and ζ-chains. The β-, δ-, ε-, and γ-genes are located on chromosome No. 11 (*68*); α- and ζ-genes are on a separate chromosome, No. 16 (*22a, 22b*).

The incorporation of various amino acids in the polypeptide chain and its termination is determined by trinucleotide base codon (triplet codon) of the gene (*58a*) (e.g., UUU, CCU, and AAA) specify for the incorporation of phenylalanine, proline, and lysine, respectively; whereas UAA is a signal for polypeptide chain termination. An amino acid can be incorpo-

TABLE II

The Genetic Code[a,b]

First Position (5′ end)	Second Position				Third Position (3′ end)
	U	C	A	G	
U	Phe	Ser	Tyr	Cys	U
	Phe	Ser	Tyr	Cys	C
	Leu	Ser	Stop	Stop	A
	Leu	Ser	Stop	Trp	G
C	Leu	Pro	His	Arg	U
	Leu	Pro	His	Arg	C
	Leu	Pro	Gln	Arg	A
	Leu	Pro	Gln	Arg	G
A	Ile	Thr	Asn	Ser	U
	Ile	Thr	Asn	Ser	C
	Ile	Thr	Lys	Arg	A
	Met	Thr	Lys	Arg	G
G	Val	Ala	Asp	Gly	U
	Val	Ala	Asp	Gly	C
	Val	Ala	Glu	Gly	A
	Val	Ala	Glu	Gly	G

[a] Given the position of the bases in a codon, it is possible to find the corresponding amino acids.

[b] Reprinted by permission from Stryer, L. (1981). "Biochemistry." W. H. Freeman, 2nd ed., p. 629.

rated in the normal and mutant peptide chain by more than one codon. For example, lysine can be coded for by AAA or AAG (Table II).

F. Nomenclature

Initially, hemoglobin variants were designated by letters of the alphabet (e.g., HbA and HbF). This designation was usually based on electrophoretic migration. With the increase in the number of variants, it became apparent that this system would not provide enough designations to accomodate new variants. Furthermore, structurally different hemoglobins, but with same net charge on the molecule, occasionally were assigned the same designation. In 1960, it was decided to leave the letters R and T, through Z unassigned (*29b*). Currently, new abnormal hemoglobins are given geographical names, and letter designations are also added to indicate their electrophoretic migration. For example, HbG Copenhagen indicates that the new hemoglobin was discovered in Copenhagen and has the mobility of hemoglobin G. When the site of biochemical aberration, abnormality of the peptide chain, and number of altered amino acids are known, the designation can be more precise. For example, HbS is expressed as $\alpha_2\beta_2{}^6\text{Glu} \rightarrow \text{Val}$ or $\alpha_2\beta_2{}^6(\text{A3})\text{Glu} \rightarrow \text{Val}$, indicating the substitution of valine for glutamic acid at the No. 6 position from N-terminal, third amino acid in the A helix, in the β-chain.

II. HEMOGLOBINOPATHIES

A. Definition and General Description

Hemoglobinopathies are inherited hematological disorders caused by molecular lesion in hemoglobin synthesis. A molecular lesion results in the synthesis of abnormal hemoglobin molecules (variant Hb), deficient production of normal hemoglobin, or failure of the normal switch from HbF to HbA.

The deficient production of normal hemoglobin is caused by the decreased rate or absence of synthesis of one or more polypeptide chains. This imbalance in the chain synthesis gives rise to the thalassemia syndromes.

The failure to switch from HbF to HbA results in persistently high levels of HbF beyond the neonatal period. This condition is called hereditary persistance of fetal hemoglobin (HPFH).

These changes show diverse and complex clinical and laboratory abnormalities and often, but not always, are accompanied by anemia. The

variant hemoglobins are formed from an abnormal structure of one or more of the polypeptide chains. A substitution of simple amino acids at a specific site, an addition or deletion of one or more amino acids, or combination of different pieces of two normal chains (Hb Lepore) results in the production of abnormal polypeptide chains (*8*).

Each amino acid incorporated in the polypeptide chain has a corresponding triplet codon of the gene (e.g., GUG for valine and GAG for glutamic acid). A single base substitution in the corresponding triplet usually results in the incorporation of a different amino acid in the polypeptide chain. For example, formation of HbS, an abnormal variant, is caused by a change of codon GAG to GUG leading to the substitution of glutamic acid by valine at Position 6 of the β-chain.

The mutation-related clinical manifestations occur because substitution affects hemefold and $\alpha_1\beta_1$ interfaces and disrupts the α-helical conformation, causing deoxyhemoglobin to form rigid fibers that affect the equilibrium between relaxed (R) and tense (T) conformation required in oxygen transport by the hemoglobin molecule (*63d*). The quarternary structure of oxyhemoglobin is termed the R form and that of deoxyhemoglobin the T form (*80*).

These hemoglobin disorders are inherited as autosomal, recessive, or dominant traits. When abnormal hemoglobin produces symptoms in the heterozygous state, as in unstable hemoglobin disease, the inheritance of the "disease" is an autosomal dominant trait. If only the homozygous state is symptomatic, as in sickle cell anemia, a recessive inheritance is present. The heterozygous state, without symptoms, is called the "trait." When normal hemoglobin with its variant or only variant hemoglobin are present, the phenotype is designated by listing the hemoglobins in order of their decreasing concentration. For example, the phenotype for sickle cell trait is AS, for sickle cell disease SS, and for sickle cell HbD disease is SD.

B. Classification and Description

The hemoglobinopathies, though different in etiology and clinical significance, can be classified into four main groups with overlapping characteristics (Table III).

1. Structural Abnormalities of the Globin Chain

There are approximately 380 known hemoglobin variants, out of which close to 300 differ from normal in substitution of a single amino acid for another. The location of the amino acid substitution is important in determining its effect on the function of hemoglobin. There are many known

TABLE III

Classification of Hemoglobinopathies by Cause

	Subunit Formula
I. Structural Abnormality of the Globin Chain	
A. Hemoglobins not readily precipitated with erythrocytes showing altered membrane characteristics	
1. β-chain abnormality	
HbS	$\alpha_2\beta_2^{6}$Glu → Val
HbC	$\alpha_2\beta_2^{6}$Glu → Lys
HbD Punjab	$\alpha_2\beta_2^{121}$Glu → Gln
HbE	$\alpha_2\beta_2^{26}$Glu → Lys
HbO Arab	$\alpha_2\beta_2^{121}$Glu → Lys
HbC Harlem[a]	$\alpha_2\beta_2^{6,73}$Glu → Val, Asp → Asn
Hb Korle Bu	$\alpha_2\beta_2^{73}$Asp → Asn
2. α-chain abnormality	
HbG Philadelphia	α_2^{68}Asn → Aspβ_2
HbJ Singapore[a]	$\alpha_2^{78,79}$Asn → Asp, Aln → Glyβ_2
3. δ-chain abnormality	
HbA_2 New York	$\alpha_2\delta_2^{12}$Asn → Lys
4. γ-chain abnormality	
HbF Kuala Lumpur	$\alpha_2\gamma_2^{22}$Asp → Gly
B. Interaction between two structurally abnormal globin chain	
1. β-chain abnormality	
HbSC	
HbSD	
HbSE	
2. Both β- and α-chain abnormality	
HbG Philadelphia-S	
C. Hemoglobins readily susceptible to precipitation with Heinz-body formation (unstable hemoglobins)	
1. β-chain abnormality	
Hb Freiburg	$\alpha_2\beta_2^{23}$Val → O
Hb Zurich	$\alpha_2\beta_2^{63}$His → Arg
Hb Koln	$\alpha_2\beta_2^{98}$Val → Met
Hb Hammersmith	$\alpha_2\beta_2^{42}$Phe → Ser
Hb Gun Hill	$\alpha_2\beta_2^{91-95}$Leu–His–Cys– or $^{92-96}$Asp–Lys → O or $^{93-97}$
2. α-chain abnormality	
Hb Ann Arbor	α_2^{80}Leu → Argβ_2
D. Associated with abnormal oxygen transport	
1. Increased oxygen affinity	
a. β-chain abnormality	
Hb Kempsey	$\alpha_2\beta_2^{98}$Val → Met
Hb Travis[a]	$\alpha_2\beta_2^{6,142}$Glu → Val, Ala → Val
b. α-chain abnormality	
Hb Chesapeake	α_2^{92}Arg → Leuβ_2
Hb J Capetown	α_2^{92}Arg → Glnβ_2

(*continued*)

TABLE II (*continued*)

2. Decreased oxygen affinity	
a. β-chain abnormality	
Hb Beth Israel	$\alpha_2\beta_2^{102}$Asn → Ser
Hb Kansas	$\alpha_2\beta_2^{102}$Asn → Thr
b. α-chain abnormality	
Hb Titusville	α_2^{94}Asp → Asnβ_2
3. Methemoglobin	
a. β-chain abnormality	
Hb Milwaukee	$\alpha_2\beta_2^{67}$Val → Glu
b. α-chain abnormality	
HbM Boston	α_2^{58}His → Tyrβ_2
E. Resulting from alteration of globin chain length	
1. Increased	
a. β-chain	
Hb Tak	$\alpha_2\beta_2^{146}$ + 11 additional residues $_{147}$ (Thr-Lys-Leu-Ala-Phe-Leu-Leu-Ser-Asn-Phe-Tyr)
b. α-chain	
Hb Constant Spring	α_2^{141} + 31 additional residues $_{142}$ (Gln-Ala-Gly-Ala-Ser-Val-Ala-Val-Pro-Pro-Ala-Arg-Trp-Ala-Ser-Gln-Arg-Ala-Leu-Leu-Pro-Ser-Leu-His-Arg-Pro-Phe-Leu-Val-Phe-Glu)β_2
2. Decreased	
a. β-chain	
Hb Vicksburg	$\alpha_2\beta_2^{75}$Leu → O
Hb Frieburg	$\alpha_2\beta_2^{23}$Val → O
Hb Gun Hill	$\alpha_2\beta_2^{91\text{–}95,92\text{–}96}$Leu-His-Cys-Asp-Lys → O or $_{93\text{–}97}$
Hb Leiden	$\alpha_2\beta_2^{6 \text{ or } 7}$Glu → O
Hb Leslie	$\alpha_2\beta_2^{131}$Gln → O
b. α-chain	
Hb Koellicker	α_2^{141}Arg → Oβ_2
F. Resulting from fusion of globin chains	
1. Lepore syndrome	Fusion Site
Hb Lepore Baltimore	$\alpha_2(\delta - \beta)_2\ \delta_{50}$ and β_{86}
2. Anti-Lepore syndrome	
Hb Miyada	$\alpha_2(\beta - \delta)_2\ \beta_{12}$ and δ_{22}
II. Abnormal Rate (Decrease of Absence) of Globin Chain Synthesis	
A. β-thalassemia	
1. Homozygous β^+-thal[b]	
2. Homozygous β°-thal[c]	
3. Heterozygous β^+-thal	
4. Heterozygous β°-thal	
B. $\delta\beta$-thalassemia	
1. Homozygous $\delta\beta$-thal	
2. Heterozygous $\delta\beta$-thal	
3. Double heterozygous $\delta\beta$-β thal	

TABLE II (*continued*)

C. α-thalassemia
   1. Homozygous α-thal
   2. Heterozygous α-thal
   3. Homozygous mild α-thal
   4. Heterozygous mild α-thal

D. δ-thalassemia
   1. Homozygous δ-thal
   2. Heterozygous δ-thal

III. Interaction Both of Abnormal Structure and Abnormal Rate (Decrease or Absence) of Globin Chain Synthesis

A. Abnormal variant and β-thalassemia
   1. HbS–β-thal

B. Abnormal variant and α-thalassemia
   1. HbI–α-thal

C. Abnormal variant and $\delta\beta$-thalassemia
   1. HbS–$\delta\beta$-thal

IV. Hereditary Persistance of Fetal Homoglobin (HPFH)

A. HPFH

B. Heterozygous HPFH with another hemoglobinopathy
   1. HPFH–HbS
   2. HPFH–β-thal
   3. HPFH–α-thal

[a] Two-point abnormality on the same beta chain.
[b] thal$^+$ (diminished production of beta chain).
[c] thal$^\circ$ (complete absence of beta chain synthesis).

substitutions that do not affect hemoglobin function and are of no clinical consequences. On the other hand, some substitutions demonstrate deleterious effects because they alter the stability and oxygen affinity of the hemoglobin molecule. Approximately 60% of the known variants are the result of β-chain abnormalities; 32% have α-chain involvement. A relatively small percentage is reported for γ-chain (5%) and δ-chain (3%) variants (*39*).

a. Hemoglobins Not Readily Precipitated with Erythrocytes Showing Altered Membrane Characteristics. The well-known and extensively studied variant HbS (which differs from normal HbA in substitution of valine for glutamic acid at the six position of the β-chain) causes the hemoglobinopathy (sickle cell disease) having enormous clinical significance and wide geographical distribution. Black Africans and their descendants in the new world show the highest incidence of HbS. In the United States, approximately 8% of blacks carry the sickle cell gene, with

a 1 in 625 chance of sickle cell disease occurring at birth (*10, 54, 62*). The sickling disorder was first recorded in 1910 by Herrick, a Chicago cardiologist (*34*). He observed the tendency of the erythrocytes to assume a slender sickle-like shape in the blood of a black medical student suffering from anemia. The occurence of the sickling phenomena in apparently healthy nonanemic patients was reported by Emanel in 1917 (*28*).

Pauling *et al.* (*61a*), showed that hemoglobin in sickle cell anemia (HbS) migrates differently from normal adult hemoglobin (HbA) when subjected to electrophoresis. This finding was the original basis for his seminal concept of molecular disease. It was Ingram (*37*) who explained that the electrophoretic separation of HbS from normal HbA was caused by the substitution of valine for glutamic acid at the six position of the β-chain. The normal glutamic acid residue carrying a negative charge is replaced by valine, which has no charge. (If a substitution results in a change in a net electrical charge of the molecule, as in the case of HbS, it will also affect the electrophoretic ability of the hemoglobin variants as compared to HbA). The S hemoglobin has been found to be less soluble in its deoxygenated or "reduced" form; this in turn causes an increase in viscosity of the whole blood, which leads to stasis and obstruction of blood flow in the capillaries, terminal arterioles, and veins. Sickling will occur at low oxygen tension, especially with low pH of the blood. In homozygous state clinical symptoms are quite variable, with acute episodes consisting of recurrent attacks of fever, pain in the arms, legs, or abdomen, and jaundice. The heterozygous state is usually asymptomatic but with the possibility of sickling occurring with low P_{O_2} (*17*), with severe crisis resulting from hypoxia and strenuous exercise (*89*). Sickle cell disease has been reported in South Arabian and Israeli Arabs, producing benign-to-mild clinical conditions (*67*).

An unusual variant of the HbS structure is HbC Harlem, one of the few abnormal hemoglobin with two amino acid substitutions on the same chain. It was first identified by the letter *C* to indicate that is had the same electrophoretic mobility at pH 8.6 as does HbC. It has since been shown to have the same substitution at $\beta 6$ as does HbS, and in addition it has a second amino acid substitution ($\beta 73$ Asp $\rightarrow$ Asn) on the same β-chain. The first substitution accounts for the ability of HbC Harlem to sickle, and the second accounts for the additional charge difference resulting in the same migration pattern as HbC. The second amino acid substitution $\beta 73$ Asp $\rightarrow$ Asn (Hb Korle Bu) is one of several hemoglobins that retards sickling. Hemoglobin C Harlem will sickle because of the first amino acid substitution but with less ease than HbS alone, because of the second amino acid substitution (Hb Korle Bu) and its retarding capabilities.

Unlike HbC Harlem, HbC has only one amino acid substitution ($\alpha_2\beta_2^6$

Glu → Lys), which is different from HbS in the substitution of lysine for glutamic acid at the β6 position. Hemoglobin C disease is most prevalent in West Africans, considered rare in the United States with the incidence running about 0.2% in blacks (*69d*). Hemoglobin-C disease is clinically characterized by a mild-to-moderate anemia while the trait is asymptomatic (*74*).

Hemoglobin D is found predominantly in India, where HbD Punjab (Los Angeles) is the most common D variant. Both HbD disease and trait exist but are virtually asymptomatic (*15b*). A variable number of codocytes (target cells) are present in the peripheral blood smear. The electrophoretic migration pattern of HbD at pH 8.6 is identical to that of HbS.

Hemoglobin E is the most prevalent hemoglobin variant worldwide. More than 80% of the persons affected live in Southeast Asia. Late in the 1970s, resettlement of Indochinese brought the variant to North America. Hemoglobin E disease is characterized by mild anemia with an abundance of target-cell formation. The trait is asymptomatic with no hematologic abnormalities.

Hemoglobin O Arab was first identified in an Arabic family, and it has since been detected in Jamaicans, Sudanese, and American blacks. The disease state is characterized by mild hemolytic anemia, and the trait lacks clinical and hemological findings. Major structural abnormal hemoglobins with some of their major laboratory findings are listed in Table IV.

b. Interaction between Two Structurally Abnormal Globin Chains. The interaction between two structurally abnormal hemoglobins (double heterozygous states) is produced by having a different abnormal globin chain inherited from each parent. Hemoglobin SC and SD are the most common, and all demonstrate the sickling phenomenon because of the inheritance of HbS (Table IV lists clinical findings HbSC and HbSD diseases). The incidence of HbSC disease is fairly high among blacks with 1 birth out of 833 having HbSC disease (*54*). Hemoglobin SC disease is intermediate in severity between sickle trait (HbAS) and sickle cell anemia (HbSS). Anemia varies from moderate to mild, with numerous target cell formation. Intraerythrocytic crystals are predominant in individuals with coexisting HbS and HbC. Hemoglobin D Punjab and HbO Arab interact mostly with HbS, but because of the low frequency of HbD, HbO, HbSD Punjab, and HbSO Arab, they are not as common as the other double heterozygous states. Hemoglobin SD Punjab disease is more severe than other double heterozygous conditions. Hemoglobin SO Arab has been reported in the United States and can give rise to moderately severe hemolytic anemia. Other double heterozygous hemoglobinopathies, such as HbSJ Baltimore, HbSK, HbSE, HbSG Philadelphia, and

TABLE IV

Hematologic Findings of Hemoglobinopathies Caused by Structural Changes of the Globin Chain[a]

Hemoglobin Disorders (phenotype)	Erythrocytes (mil/μl)[b]	Hemoglobin (g/dl)[c]	RBCs		Reticulocytes (%)	Target Cells (%)	Hemoglobin (%)
			MCV (μ^3)[d]	MCHC (%)[e]			
Normal (A/A)	4.2–6.2	12–18	82–92	32–36	0.5–1.5	0	A,97 A_2,2–3 F,0–2
Sickle cell anemia (S/S)	1.5–4.0	2–11	Normal	Normal	5–30	Some	S,80–100 A_2,3–4 F,0–20
Sickle cell trait (A/S)	Normal	Normal	Normal	Normal	Normal	0	A,50 A_2,3–4 S,22–48 F,0–2
HbC disease (C/C)	3.1–5.0	7.0–14.5	Normal	Increased	1–12	20–100	C,97–100 F,0–3
HbC trait (A/C)	Normal	Normal	Normal	Normal	Normal	0–40	A,50–70 C,30–50 F,0–3

HbD disease (D/D)	5.5–7.1	12–13	Decreased	Normal	1.0–1.5	50–80	D,98–100 F,0–2
HbD trait (A/D)	Normal	Normal	Normal	Normal	Normal	0	A,50 D,50 F,0–2
HbE disease (E/E)	Normal–increased	Decreased (10–normal)	Decreased (60–65)	Normal	1–12	25–60	E,92–98 F,0–3
HbE trait (A/E)	Increased	Slightly decreased–normal	Slightly decreased–normal	Normal	Normal	0–4	A,55–70 E,30–45 F,0–2
HbS–C disease	2.5–5.5	8–18	Normal	Normal	0.2–10	5–85	S,37–67 C,30–60 F,0–8
HbS–D disease	2.5–4.0	7–12	Normal	Normal	7–13	2	S,25–77 D,23–75 F,trace

[a] Reprinted by permission from Dacie, J. V. (1960). "The Hemolytic Anemias, Congenital and Acquired." 2nd ed., Part 1. San Diego: Grune & Stratton (modified).
[b] Million per microliter.
[c] Grams per deciliter.
[d] Mean corpuscular volume in cubic microns.
[e] Percentage.

HbSO Arab, show rare occurrence. Hemoglobin O and HbC demonstrate identical electrophoretic migration at pH 8.6 and can result in misdiagnosis of the HbSO Arab state for that of HbSC disease.

c. Unstable Hemoglobins. A number of variants called *unstable hemoglobins* are readily denatured and precipitated *in vivo* and *in vitro* by mild temperature changes (50–60°C) and exposure to chemicals. The instability of these hemoglobins is mainly caused by easy dissociation of heme from the globin, which are generally caused by the replacement of (1) amino acid in contact with heme, (2) a nonpolar by a polar residue, (3) a helically situated amino acid by the amino acid proline, which doesn't support a helix, and (4) subunit contacts that weaken the bonds between the contact (*19*). Some of the hemoglobinopathies caused by these variants are asymptomatic, but others show severe hemolytic anemia with heinz-body formation. The heinz-body formation causes an increase in the cell membrane permeability, leading to lysis and shortening of red cell life and, finally, anemia. The inheritance of these variants is autosomal dominant, and only the heterozygous cases are known. Currently about 80 unstable hemoglobin variants have been identified, with the majority being caused by single amino acid substitutions in the β-chain. The homozygous state for these hemoglobins is probably incompatible with life. Heterozygotes for Hb Zurich manifest heinz bodies, hemolysis, and anemia after administration of sulfonamides or primaquine and related oxquinolones. Hemoglobin Hammersmith shows continuous and marked red cell breakdown. Hemoglobin Frieburg is unstable and has additional properties of methemoglobin, which makes the affected individuals both cyanotic and anemic. Hemoglobin Gun Hill is unique because seven amino acids are deleted from the β-chain. Abnormal chains in Hb Gun Hill cannot bind to heme groups and thus reduce the oxygen-carrying capacity of the hemoglobin molecule.

d. Hemoglobins with Abnormal Oxygen Transport. Structural abnormal hemoglobins that manifest hemoglobinopathies with abnormal oxygen transport fall into three subgroups (1) increased oxygen affinity, (2) decreased oxygen affinity, and (3) congenital methemoglobinemia.

There are five types of amino acid substitution that disturb the oxygen affinity of hemoglobin: (1) those involving the pocket in the globin chain into which heme is wedged, (2) those that alter the Bohr effect, (3) those involving 2,3-diphosphoglycerate (DPG) binding site, (4) those shifting the equilibrium between oxy and deoxy tertiary structures in individual globin chains, and (5) those shifting the equilibrium between quartenary in the molecules as a whole (*19*).

A large number of abnormal hemoglobins with increased oxygen affinity have been reported. In most of these variants the amino acid substitution that influences the hemoglobin function tends to be in the area of the F and G helices and impairs the α_1 and β_2 contact area (e.g., Hb Chesapeake and HbJ Capetown). Only a few variants have substitutions occurring at the site near the carboxy terminus, which will impair the binding of 2,3-DPG, (e.g., Hb Little Rock). Both conditions virtually lock heme–heme interaction, with the oxygen dissociation curve becoming hyperbolic rather than sigmoidal. This results in a decrease in the P_{50}. The affinity of hemoglobin for oxygen is conventionally expressed as P_{50}—that is, the oxygen partial pressure, at the physiological temperature and pH, at which 50% of the hemoglobin is saturated with oxygen. A high P_{50} indicates low affinity for oxygen, and a low P_{50} indicates high affinity for oxygen, indicating the impairment of the release of oxygen to the tissue (*85*). This abnormal release causes a relatively hypoxic condition at any given P_{O_2}, resulting in an increased erythropoietin production and polycythemia (*78c, 79*). This is an autosomal dominant disorder exhibited in the heterozygous state. The affected individuals are usually asymptomatic. With normal individuals the P_{50} value is about 26 mm Hg. With these disorders a range of 12–18 mm Hg should be expected. With the exception of HbJ Capetown, all cases of increased oxygen affinity show erythrocytosis.

Decreased oxygen affinity is caused either by a reduction in the percentage saturation of hemoglobin occuring at various values for P_{O_2}, diminished capacity of hemoglobin to reversibly combine with oxygen at any value of P_{O_2}, or both depending on the amino acid substitution in the globin chains. Usually, the affected crevice of the globin chain is the region of the E–F helices, where the heme prosthetic group is located. The oxygen dissociation curve will shift to the right, and the P_{50} valve will be increased. Usually, affected individuals are cyanotic, but they are generally otherwise asymptomatic. Only the heterozygous condition has been observed. It is believed, because of the critical need of fetal tissue for oxygen during the gestation period, that the homozygous (disease) state is lethal *in utero*.

In methemoglobinemia the amino acid substitution of HbM is such that the hemoglobin iron remains in the ferric state [Fe^{3+} (e.g., Hb Kansas)], forming a stable bond with tyrosine rather than the usual histidine. In this conformation the molecule is stabilized but is a poor carrier of oxygen. Cyanosis from birth is seen in HbM disease caused by α-chain abnormalities but does not appear for 2–4 months if the hemoglobin variant has β-chain involvement (because of normally low level of β-Chain production and predominance of γ-chains in the initial months after birth).

e. Hemoglobinopathies Resulting from Alteration of Globin Chain Length

i. INCREASED GLOBIN CHAIN

Alteration of the globin chain length occurs from an addition or deletion of one or more amino acids. One of the most common extended chain variants is hemoglobin Constant-Spring (HbCS), which contains 31 additional amino acids residues at the C-terminal of the α-chain (*16*). This alteration is the result of a change in the normal termination codon of UAA to CAA because of a single base exchange. The CAA codes for glutamine, thus missing the normal termination signal, and the globin-chain elongation continues. It is only after the addition of an extra 31 amino acids that the recognition of a termination codon stops the further elongation of the globin chain. Synthesis of HbCS is slow and tends to give thalassemia-like symptoms. Other chain terminations codon errors occur for α- as well as β-chains (*11*).

ii. DECREASED GLOBIN CHAIN

About a dozen abnormal hemoglobins with 1 to 5 amino acids deletions have been designated. Some of these variants are unstable and may also demonstrate abnormal oxygen affinity: Hb Gun Hill (5 amino acids deleted, unstable), Hb Leiden (unstable, increased O_2 affinity), Hb Leslie (unstable), Hb Freiburg (increased O_2 affinity and unstable).

f. Lepore Hemoglobins. Fusion hemoglobins (e.g., Hb Lepore) are formed from the combination of two α-chains and two abnormal δ-chains produced as "hybrids" between parts from two different chains (*36f*). These abnormal chains or hybrids are considered to be the product of fusion genes arising from the crossover between misaligned genes on different chromosomes. Hemoglobin Lepore was first described by Gerald and Diamond (*29a*) and derived its name from the first family in which it was recognized. Like β-thalassemias, the hemoglobin Lepore syndromes are characterized by decreased synthesis of the major non-α-chain; but unlike other thalassemias, a structurally abnormal hemoglobin is synthesized. Hemoglobin Lepore is the product of $\delta\beta$ fusion genes and comprises a pair of normal α-chains and two abnormal chains, consisting of the N-terminal residues of a δ-chain and the C-terminal residues of a β-chain. The simplest explanation for Hb Lepore is that its structural genes are generated by nonhomologous crossover involving the β and δ structural genes (Fig. 5). At least three different variants of hemoglobin Lepore have been described, based on the amount of δ- and β-chains they contain (*87b*). Clinically, homozygous conditions resemble patients with homozy-

gous β-thalassemia, having severe transfusion dependent anemia; the heterozygous state is similar to β-thalassemia trait. Major properties both of homozygous and heterozygous states of Hb Lepore are also listed in Table V.

The "left-over" portions of the structural genes during the formation of $\delta\beta$ fusion gene fuse to form an "anti-Lepore" structural gene that controls the formation of anti-Lepore hemoglobins (Fig. 5) with a β-N-terminal and a δ-C-terminal (e.g., HbMiyada). A hybrid of γ-, β-chains (HbKenya) has also been recorded.

2. *Abnormal Rate (decrease or absence) of Globin Chain Synthesis (thalassemia)*

Thalassemia is a heterogeneous group of hereditary hemolytic anemias, which, unlike the hemoglobinopathies associated with structural hemoglobin variants, is characterized by the reduction or absence of synthesis of one or more of the globin chains (*4a, 4b*).

The abnormal synthesis of the globin chains may be caused by gene deletion, as in the α-thalassemia syndrome, or because of a defect in the transcription or metabolism of globin mRNA, as in the case of β-thalassemia (*6*). Imbalance between α- and non-α-globin chain production results in accumulation of globin chains. Excessive accumulation of unused globin chains will result in the formation of unstable tetramers, such as β_4 (HbH) and γ_4 (Hb Bart's). These unstable tetramers denature spontaneously and cause erythrocyte membrane abnormalities leading to premature destruction of the cells. Thalassemia was first recognized by Cooley in 1925 (*18*). He described the syndrome among children of Italian de-

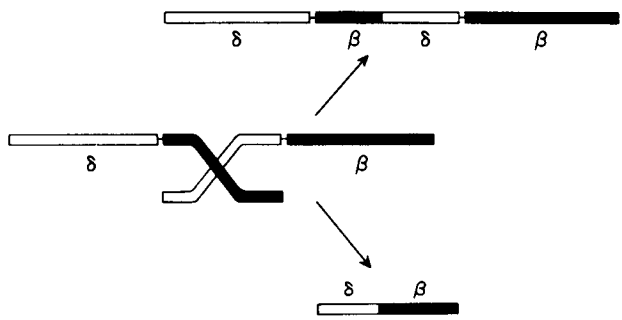

Fig. 5. Production of Lepore ($\delta\beta$) and anti-Lepore ($\beta\delta$) fusion genes during meiosis by the postulated mechanism of unequal, nonhomologous cross over. Reprinted by permission from Stamatoyannopoulous, G. and Nute, P. E. (1974). In "Clinics in Haematology" (Weatherall, D. J. Ed.), Vol. 3, p. 268. Saunders, Philadelphia.

TABLE V

β-Thalassemias[a]

Condition	Parental Genotypes	Hemoglobin Pattern[b]	Severity	β-mRNA	Genes
Homozygous states					
β^+-thalassemia	Both β^+/β	↓HbA; ↑HbF; variable HbA_2	Variable; usually Cooley's anemia	Marked deficiency of β-mRNA	β-genes present
β°-thalassemia	Both β°/β	O HbA; variable HbA_2; residual HbF	Cooley's anemia	(i) absent, β-mRNA (ii) mutant, nonfunctional β-mRNA (present in rare oriental cases)	β-genes present
$\delta\beta^\circ$-thalassemia	Both $\delta\beta^\circ/\delta\beta$	O HbA; O HbA_2; 100% HbF	Thalassemia intermedia	δ- and β-mRNAs absent	β-genes deleted; probable δ-gene deletion
Hb Lepore	Both Hb Lepore	O HbA, O HbA_2; 75% HbF; 25% Hb Lepore	Cooley's anemia	β-like mRNA present in reduced amounts (absent normal β- and δ-mRNA)	β-δ-fusion genes present; no normal β- and δ-genes
Heterozygous states					
β^+-thalassemia	β^+/β, normal	↑HbA_2; slight ↑HbF	Thalassemia minor	Deficient β-mRNA	β-genes present
β°-thalassemia	β°/β, normal	↑HbA_2; slight ↑HbF	Thalassemia minor	Deficient β-mRNA or rarely nonfunctional β-mRNA present	β-genes present
$\delta\beta^\circ$-thalassemia	$\delta\beta^\circ/\delta\beta$, normal	5–20% HbF	Thalassemia minor	Presumed deficiency of β- and δ-mRNA	β- and probable δ-gene deletion on one homologous chromosome
Hb Lepore	Hb Lepore/β_2, normal	↑HbF; ↓HbA_2; 5–15% Hb Lepore	Thalassemia minor	β- like mRNA present; deficiency of normal β- and δ-mRNA	Hb Lepore gene replaces normal β- and δ-genes on one chromosome

[a] Reprinted by permission from Orkin S. H., and Nathan, D. G. (1976). "The Thalassemias," *N. Engl. J. Med.* **295,** 713.

scent, characterized by profound anemia, splenomegaly, and bony deformities. The clinical severity of thalassemia syndromes varies considerably, ranging from completely asymptomatic to conditions incompatible with life. The thalassemias are more prevalent in certain regions of the world, such as the Mediterranean countries, Southeast Asia, the Philippines, the Hawaiian Islands, and China. The thalassemias are classified according to the globin chain involved. The two major types are α- and β-thalassemia. α-Thalassemia refers to disorders associated with deficiencies in α-chain production, and, similarly, β-thalassemia is associated with deficiencies in β-chain synthesis, and so on. Thalassemias are further divided into several subtypes based on their genetic makeup. The β-thalassemias have been classified into four clinical groups. There is little correlation between the clinical classification and the genetic type of thalassemia.

1. Thalassemia major—severe anemia usually beginning in infancy.
2. Thalassemia intermedia—less-severe anemia.
3. Thalassemia minor—little or no anemia but with morphological abnormalities of the red blood cells.
4. Thalassemia minima—normal morphology but with the presence of the thalassemia gene.

a. β-Thalassemia

I. HOMOZYGOUS

β-Thalassemia (β-Thal) also known as Cooley's anemia and Thalassemia major is considered a serious disease and is usually fatal in children. β-Thalassemia is characterized in two forms. The first being β^+-thalassemia (diminished production of β-chain synthesis); the second is known as β°-thalassemia (the complete absence of β-chain synthesis). The hemoglobin type will vary based on these two types of homozygous β-thalassemia. In the more common homozygous β^+-thalassemia, also known as the high F type, HbF is markedly elevated, HbA_2 levels are variable, and HbA is decreased. In the less common homozygous β°-thalassemia, also known as the high A_2 type, the HbF is low and the HbA_2 level is high. Because there is an absence of β-chain synthesis, there is no HbA.

In most β-thalassemias the structural genes are present, but the mRNA either is not produced efficiently or is degraded rapidly (*6*), resulting either in β° (absence of β-mRNA) or β^+-thalassemia (marked deficiency of β-mRNA). Patients with β-thalassemia show severe hypochromic anemia with an increase in target-cell formation (Table VI).

TABLE VI

Clinical and Hematologic Features of the β-Thalassemia Syndromes[a]

Feature	Syndrome			
	Major	Intermedia	Minor	Minima
Splenomegaly	++++	++,+++	+,0	0
Jaundice	+++	++,+	0	0
Anemia (Hb in g/dl)	7	7–10	10	normal (12–18)
Hypochromia	++++	+++	++	+
Microcytosis	+++	++	++	0
Target cells	10–35%	5–20%	0–10%	0–5%
Basophilic stippling	++	+	+	0,+
Reticulocytes	5–15%	3–10%	2–5%	1–2%
Nucleated red cells	+++	+,0	0	0

[a] Reprinted by permission from: Witrobe, M. M., Lee, G. R., Boggs, D. R., Bithell, T. C., Athens, J. W., Foersler, J. (1981). "Clinical Chemistry," 8th Ed., p. 880. Lea & Febiger, Philadelphia. (modified)

ii. HETEROZYGOUS

β-Thalassemia is also known as Cooley's trait and thalassemia minor. The clinical picture ranges from thalassemia intermedia to thalassemia minor. In the more-severe cases, pallor and splenomegaly may be present. In some cases with unusually severe clinical and hematologic findings, β-thalassemia may be classified in the category of "Inclusion-body β-thalassemia trait" (*78d*). An excess of α-chain production in those cases produces numerous inclusion in the normoblasts. In mild cases there are no signs or symptoms, and β-thalassemia often require family studies to confirm the trait existence.

b. δβ-Thalassemia. δβ-Thalassemia was originally called HbF thalassemia or β-thalassemia Type 2. It is thought to differ from β-thalassemia in that it represents defective synthesis both of δ- and β-chains (*78b*, *87b*, *90*).

The basic defect is the deletion of the γ and β structural genes (*60*). Homozygous individuals clinically display thalassemia intermedia conditions because γ-chain synthesis fails to compensate for the deficiency in δ- and β-chain synthesis. The heterozygous condition displays thalassemia minor symptoms. Several properties of the β- and δβ-thalassemia syndromes are listed in Table V.

c. α-Thalassemia. α-Thalassemias (α-thal) are hereditary anemias characterized by partial or complete absence of α-globin synthesis. Although some nondeletion defects have been reported, the major cause of α-thalassemia is gene deletion. These syndromes have been extensively studied in Asian, Mediterranean, and African populations. Because there are four copies (two pairs) of the globin gene in normal individuals, four types of α-thal can result, depending on the number of globin genes deleted. These are homozygous α-thalassemia, HbH disease, heterozygous α-thalassemia (thalassemia minor), and the "silent carrier."

In homozygous α-thalassemia (disease), also known as lethal Hb Bart's or hydrops fetalis with Hb Barts, there is a complete lack of normal α-globin genes and no production of α-globin chains for production of HbA, HbA_2, or HbF. Instead, free γ-chains form tetramer γ_4 (Hb Bart's), and some of the free β-chains form variable amounts of tetramer β_4 (HbH), and a few of the γ-chains combine with the embryonic ε-chain to form Hb Portland. Hemoglobin Bart's, because of its high oxygen affinity, is considered not very useful to deliver oxygen to the tissues. Affected infants are usually stillborn, hydropic, and have marked hepatomegaly in the absence of isoimmune hemolytic disease. This condition is a major cause of stillborn births in Southeast Asia because of the high frequency of α-thalassemia trait. In people of African origin, hydrops fetalis has never been reported. Lethal Hb Bart's is one of the non-S hemoglobinopathies characterized by sickling.

Hemoglobin H disease arises when only one normal globin gene is present. Patients with this disease have the characteristics of chronic hemolytic anemia, which varies from mild to severe but is considered much less serious than Hb Bart's disease. The average hemoglobin level is 8.6 g/dl. Hypochromia is striking, and microcytosis, target cells, fragmented red cells, and basophilic stippling are always present. Blood mixed with brilliant crystal blue at room temperature will cause precipitation of HbH, which is seen as numerous small inclusions.

Thalassemia minor results when two out of four α-globin genes are lacking; there may be little or no anemia, with slight thalassemic red cell morphology. The silent-carrier condition indicates the absence of one α-globin gene and with no other apparent clinical abnormalities. The major properties of α-thalassemia syndrome are listed in Table VII.

d. δ-Thalassemia. In homozygous δ-thalassemia there is complete suppression of δ-chains and, consequently, no HbA_2 is formed. In the heterozygous state, HbA_2 is present but in small quantities. Neither condition is accompanied by anemia, and both are completely harmless.

TABLE VII

α-Thalassemia[a]

Condition	Parental genotypes	Hemoglobin pattern	Severity	α-mRNA	Genes
Homozygous state					
α-Thalassemia (hydrops fetalis) with Hb Bart's)	Both α-thalassemia trait (homozygous α-thal[1])	80% Hb Bart's syndrome, HbH, and, Hb Portland	Lethal (death *in utero*)	α-mRNA absent	All α-genes deleted
HbH disease	(i) α-Thalassemia trait; silent carrier	4–30% HbH in adults; approximately 25% Hb Bart's in cord blood; 2–3% HbCS when HbCS gene is present	Variable, usually thalassemia intermedia (chronic hemolytic anemia)	Marked deficiency of α-mRNA	(i) 3 or 4 α- genes deleted
	(ii) α-Thalassemia trait; HbCS heterozygote				(ii) 2 of 4 deleted; 1 normal; 1 HbCS gene
Heterozygous state					
α-Thalassemia (α-thal trait)	α-Thalassemia trait normal	Approximately 5% Hb Bart's in cord blood	Mild, very mild in blacks (little or no anemia	Presumed deficiency in α-mRNA	1 of 4 α-genes deleted; 1 of 3 deleted in blacks
Silent carrier	Silent carrier; normal	Approximately 1–2% Hb Bart's in cord blood	No clinical or hematologic abnormalities	Presumed slight deficiency of α-mRNA	1 of 4 α-genes deleted
Heterozygous Hb Constant Spring (CS)	HbCS heterozygote; normal	Approximately 1% HbCS	No clinical or hematologic abnormalities	Presumed deficiency of α-mRNA	3 of 4 α-genes present; 1 HbCS gene

[a] Reprinted by permission from Orkin S. H., and Nathan, D. G. (1976). "The Thalassemias," *N. Engl. J. Med.* **295,** 712.

3. Interaction Both of Abnormal Structure and Abnormal Production Rate of Globin Synthesis

These hemoglobin variants are a combination of the two previous groups discussed—structural abnormalities and thalassemias. The most common variant of this group is HbS β-thalassemia (microdreparocytic disease). This disease is found mostly in persons of Mediterranean ancestry and there is a 1 : 1667 possibility of its occurring in American blacks. Red cell morphology usually appears as a combination of both variant characteristics. Other variants such as HbC β-thalassemia can exist either as severe or mild conditions. In blacks the combination is usually asymptomatic (*87a*), whereas in Italians, Turks, and Algerians cases are severe (*64*). Hemoglobin E-β-thalassemia has been found in Thailand, an area with a high frequency of HbE (*86*); cases show a moderately severe anemia. Other rare variants reported with β-thalassemia have been hemoglobins D, G, and J. Heterozygous δβ-thalassemia sickle cell is not common (*78a*) but is interesting because it confirms the difference between β-thalassemia and δβ-thalassemia. Usually, mild anemia with splenomegaly is present. α-Thalassemia has been found in association with hemoglobins Q, I, S, O, Constant Spring, and New York. Hemoglobin Q-α-thalassemia is common in orientals (*47*). The condition resembles HbH disease with moderate anemia.

4. Hereditary Persistence of Fetal Hemoglobin

This term (HPFH) describes a group of genetic disorders producing a completely benign condition characterized by the persistence of large percentages of fetal hemoglobin synthesis into adult life. These disorders are considered variants of thalassemia in which usually a complete deletion of the entire δ and β globin gene occurs. As in δβ-thalassemia there is a complete absence of δ- and β-globin synthesis in HPFH; but unlike δβ-thalassemia, HPFH is compensated and characterized by an increase in γ-chain production sufficient to almost balance α-chain synthesis. Three types of HPFH have been described: (1) Negro, (2) Greek, and (3) Swiss. In all three there is usually no clinical abnormalities.

HPFH can combine with structural hemoglobin variants or thalassemia, which usually results in a benign combination with few hematologic abnormalities (*14*). One of the more common variants for this subgroup is HbS-HPFH, first described by Weatherall and Clegg (1966). There is usually no anemia, and the slide shows some anisocytosis, poikilocytosis, and a variable number of target cells. Sickling will occur at low P_{O_2} (*87b*).

III. TECHNIQUES USED IN EVALUATION OF HEMOGLOBINOPATHIES

A. Electrophoresis: General Description

Electrophoresis is the single-most valuable procedure in the identification of hemoglobin abnormalities. This technique separates charged particles by inducing their migration in an electrical field.

The "classical" Tiselius technique of moving boundary electrophoresis used by Pauling *et al.* (*61a*) in the separation and detection of HbS from HbA is now rarely used. It has been replaced by the simpler, less expensive, and time-saving technique of zone electrophoresis.

In zone electrophoresis, charged molecules move as separate zones on a support medium impregnated with a suitable buffer. The mobility of molecules primarily depends on the magnitude of net charge and the strength of field current. The net charge of the molecule varies with the pH of the surrounding medium. For example, ampholytes such as hemoglobins and other proteins at the isoelectric point (PI) have zero charge and thus no mobility. At pH below PI there is a net positive charge with migration toward the cathode; at pH above PI there is a net negative charge and migration toward the anode. The mobility of ampholyte is also influenced by the nature of the buffer, support medium, ionic strength, and size and shape of the molecules.

Hemoglobin variants arise as a result of a change in the amino acid composition of the globin chain, which affects the electrical charge and mobility of the molecule. But in many variants there is no change in the net charge, resulting in no change in mobility of the molecule. Hemoglobin variants producing no net charge with several media will require techniques other than electrophoresis for determination. The variants with changed mobilities can be identified by electrophoresis using supporting media (e.g., filter paper, starch block, and cellulose acetate) under variable conditions, such as pH and ionic strength of the buffer. Zone electrophoresis is usually performed at alkaline pH, at which most proteins are negatively charged and are weakly absorbed to a negatively charged support medium. Four electrophoretic techniques are currently used for identifying and quantitating hemoglobin variants: (1) alkaline electrophoresis, (2) acid citrate agar electrophoresis, (3) globin chain electrophoresis, and (4) isoelectric focusing (IEF).

1. Alkaline Electrophoresis

Alkaline electrophoresis (pH 8.4–8.8) is based on the hemoglobin variants acquiring a negative charge with an alkaline pH support medium

causing them to migrate toward the anode. Several support media have been used for alkaline hemoglobin electrophoresis, such as filter paper (*15a*), agar gel, acrylamide gel, starch block, starch gel (*35c, 81*), and cellulose acetate (*33, 38, 49, 66*). None of these media are both practical and adequate for all separations (*45*). Filter-paper electrophoresis with barbital buffer was the first popular supporting medium for zone electrophoresis; it was considered simple but did not show HbA_2 separation. Separation of HbA_2 was achieved on filter paper by changing to TEB (TRIS–EDTA–Borate) buffer but in so doing poorer resolution of HbS from HbC and HbE occurred. Filter-paper electrophoresis is rarely used since the development of superior media, which has prevented the blurring of zones caused by the absorption or trailing of hemoglobins. The new media have also provided faster separation times of the hemoglobin variants. Starch gel and starch block are better suited for quantitation of HbA_2, along with good separation of many of the other hemoglobin variants, but require more time and effort for separation. Hemoglobin F determination is best performed on agar gel in alkaline pH. Electrophoresis in alkaline pH on cellulose acetate—because of its simplicity, permanency, and low cost and because it is rapid and reproducible in separating hemoglobin variants (Fig. 6)—is the method of choice for initial screening (*9*) and is recommended by the National Sickle Cell Anemia Program for detecting HbS and other abnormal hemoglobins.

2. *Acid Citrate Agar Electrophoresis*

Acid citrate agar electrophoresis [pH 6.0–6.2 (*52, 69b, 82*)] provides good separation along with confirmation of hemoglobin variants that tend to migrate together on cellulose acetate in alkaline conditions because of similar net charges [e.g., HbS from HbD and HbG; HbC from HbE and O-Arab (Fig. 6b)]. Originally called "electrochromatography," the procedure involves applying an electrical current to the citrate agar support medium under acid conditions. The hemoglobin molecules become positively charged migrating toward the cathode. The migration of the hemoglobin on acid citrate agar not only depends on the charge on the hemoglobin but also on the adsorption and solubility of the hemoglobin in the agar medium. Following the cellulose acetate method, citrate agar electrophoresis has been considered the single-most useful method for screening purposes and has been highly recommended for confirmation of abnormal variants appearing on cellulose acetate.

3. *Globin Chain Electrophoresis*

Globin chain electrophoresis on cellulose acetate is used to determine the location of chain abnormalities of hemoglobin variants and can be

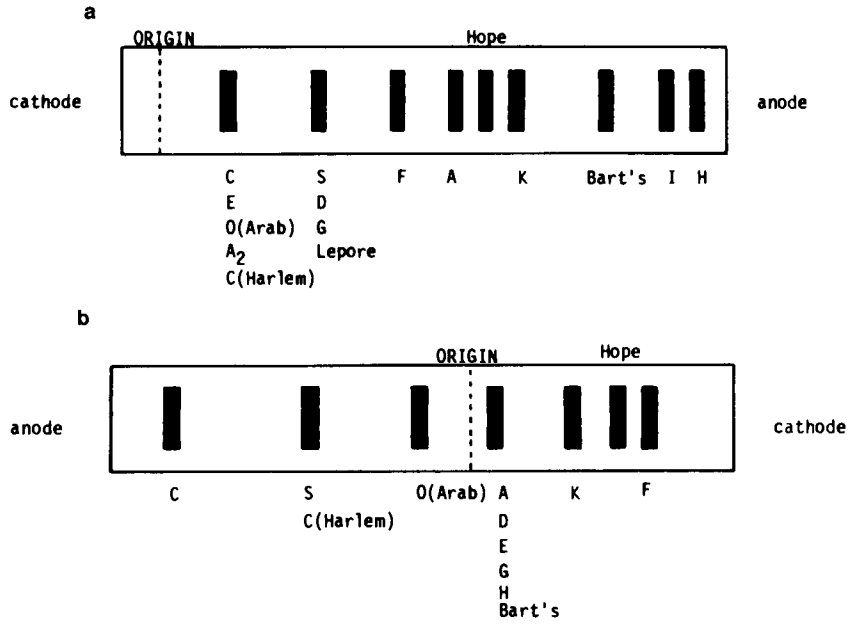

Fig. 6. a. Relative mobilities of some hemoglobins on cellulose acetate (pH 8.4). b. Relative mobilities of some hemoglobins on citrate agar (pH 6.0).

performed both at acid and alkaline conditions [pH 6 and 9, respectively (*38*)]. Erythrocyte hemolysates are prepared using large amounts of urea and 2-mercaptoethanol. The 2-mercaptoethanol liberates heme from the globin, and the urea separates the α- and non-α-chains (*69c*). The heme groups will migrate off the strip; globin chains will migrate at a characteristic rate, which varies according to the pH at which the test is performed. The separation of globin chains requires only a few hours and minimal preparation. With this method, not only can the chain abnormality be located, but the hemoglobin variants can be characterized according to their electrical charge—when both alkaline and acidic conditions are performed. Globin chain electrophoresis is particularly useful in separating HbD from HbG and in confirming hemoglobin C, E, and O Arab (Fig. 7). In the less common hemoglobin variants, reference standards are required to be run simultaneously for identification.

4. Isoelectric Focusing or Electrofocusing

Isoelectric focusing, a steady-state spearation technique based on differences in isoelectric point, is probably the most effective electrophoretic procedure for separating proteins (*46*). Once all proteins are focused,

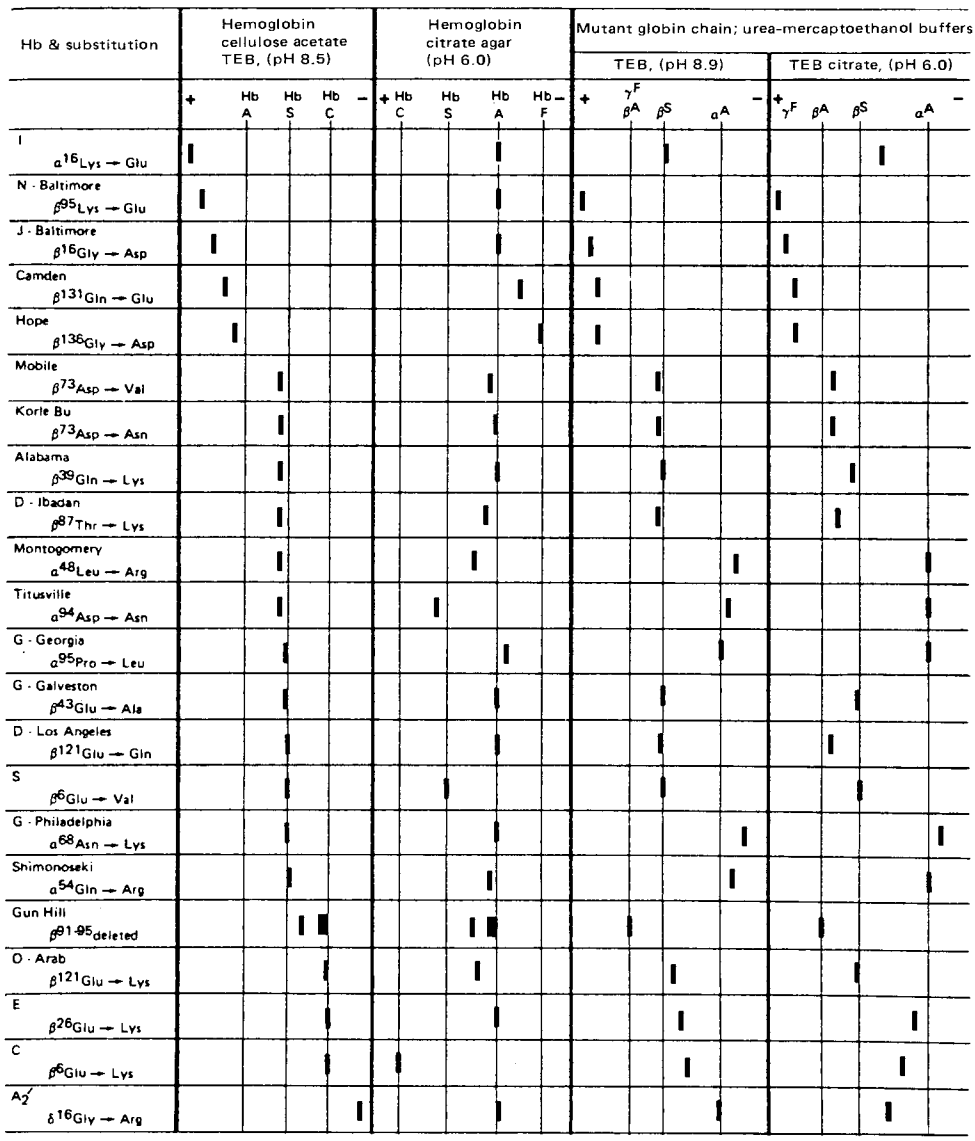

Fig. 7. Comparative electrophoretic mobilities of 22 mutant hemoglobins and their globin chains (TEB, tris EDTA-borate buffer). Reprinted with permission from Schneider, R. G., Hightower, B., Hosty, T. S., *et al.*, (1976). *Blood* **48,** 635.

separation is complete; whereas in electrophoresis, separation continues until the electric current is stopped. With longer runs, separation is counteracted by diffusion.

Electrophoretic separation depends on pH, type of matrix, and degree of cross-linkage; whereas in IEF, optimum resolution only requires selection of proper pH. Isoelectric focusing gives a much better resolution between proteins, with the same or similar migration by electrophoresis, and it also allows the detection of minor sample components. It is a method of great value both for analysis and purification of proteins. Rilbe (*65*) reported the resolving power of IEF in gels greater than 0.01 pH units. Allen *et al.* (*2*), using thin-layer polyacrylamide gel and 24-cm long electrodes, resolved proteins that differed in PI by only 0.0025 pH units. A separation can be completed in 1.5–3 hr, depending on the current and pH range used.

Hemoglobin variants separated by IEF using polyacrylamide gel tubes were first achieved by Drysdale *et al.* (*25*). The procedure was considered inadequate for large-scale screening and difficult for comparing several samples. The use of thin-layer gels (*5a, 41, 53, 83*), high voltage (*75*), and reduced volume of ampholytes made the IEF technique faster, less expensive, and thus more attractive for routine analysis and large-scale screening. Human hemoglobin require uniform pH gradient between pH 6.5 and 7.5 (*5b*). The carrier ampholytes are now commercially available, and the thin-layer isoelectric focusing (TLIF) equipment is relatively simple and requires a cooling system and constant power supply. Thin-layer isoelectric focusing is a convenient procedure for screening abnormal hemoglobins and for studying minor normal and abnormal fractions. The low concentration of hemoglobins A, S and C in cord blood is conveniently studied by TLIF. No other system presently available offers such a possibility. It also gives a clear separation between HbF and HbA. A mixture of four hemoglobin variants (HbJ Baltimore, HbJ Calabria, HbFannin–Lubock, and Hb Hope) with the same type of substitution (Gly → Asp) and HbA separate into five bands on TLIF, whereas only two bands are seen on cellulose acetate electrophoresis. Hemoglobins S, C-Harlem, E, and O-Arab are not well separated from each other owing to the same PI. For rare hemoglobin variants, simultaneously running a reference standard for proper identification is very important. Isoelectric focusing shows great promise in prenatal diagnosis of hemoglobinopathies and identification of β-thalassemia heterozygosity and homozygosity (*30*).

B. Chromatography

Chromatography procedures are very valuable for the separation and identification of compounds. The ion-exchange chromatographic tech-

niques have been applied in the isolation, identification, and quantitation of hemoglobin variants and globin chains (*5b, 36d, 70d*). In ion-exchange chromatography, hemoglobins in alkaline buffer take on a net negative charge. These negatively charged hemoglobins are attracted to the positively charged anion exchange resin and bind to it. Following binding, the hemoglobins are selectively removed from the resin by altering the pH of elution buffer. Hemoglobin A_2 and other positively charged hemoglobins (C, E, and O) are among the first to be eluted (*45*). The fractions are then collected and measured spectrophotometrically. The most commonly used resins for hemoglobin column chromatography are diethylamine (DEAE)-sephadex (*36b, 36c*), DEAE-cellulose (*36a*), carboxymethyl (CM)-cellulose (*70c*), CM-sephadex (*24*), and Amberlite IRC-50 (*36e*). In ion-exchange chromatography, accurate control of pH, molarities of buffers, and temperature are very important for the procedure to give reproducible results. In the clinical laboratory the use of microcolumn chromatography for isolation and quantitation of hemoglobins has been gaining popularity, and microcolumns for determination of some hemoglobins (A_2 and glycosylated hemoglobins) are now commercially available. Adaptation of microcolumn ion-exchange chromatography to screening for HbA_2 on DEAE-cellulose was first presented by Efremov *et al.* (*26*). Further improvements in techniques resulted in methods less sensitive to minor changes in pH (of the developer and ion exchangers), along with no interference from HbS (*69a*). Recently, a test tube method for HbA_2 determination, requiring no column and using DEAE-cellulose, has been reported (*36g*). Two new chromatographic procedures for determination of HbA_2 in the presence of HbC have been proposed by Abraham *et al.* (*1*). Previously, a microchromatographic method for identification of hemoglobins AS, AC, SS, SC, and CC conditions at birth from cord blood was reported by Schroeder *et al.* (*70b*). Measurement of Hb Bart's is helpful in identification of α-thalassemia and is reported to be best quantitated by microcolumn chromatography using the ion-exchanger CM-cellulose (*33*). Determination of HbF by ion-exchange chromatography has not gained overall acceptance, because this procedure is unable to completely separate HbA from HbF. But chromatography still remains a preferred method for determining HbF at higher levels (40%) where the overlap between HbA and HbF is quantitatively of no significance.

C. Alkaline Denaturation Test

The alkaline denaturation test (*73*) is based on the fact that fetal hemoglobin resists alkaline denaturation and adult hemoglobin does not. A hemolysate normally composed of normal HbA and fetal HbF is first

made alkaline using KOH or NaOH and then neutralized. The denatured adult hemoglobin is then precipitated out by using ammonium sulfate. The filtrate will then contain only the alkaline resistant HbF, which is measured spectrophotometrically and expressed as a percentage of the total hemoglobin.

D. Acid Elution Test

The acid elution test, first developed by Kleihauer and Betke (*44, 58b*), has since been modified by Shepard (*72*). Blood smears are immersed in a buffered acid solution (Citric acid phosphate buffer, pH 3.3). Where HbA and other adult hemoglobins are eluted from the red blood cells, only HbF remains in the fixed red cells. The slides are then stained with eosin. Upon examination of slides, cells containing only adult hemoglobin appear as ghost cells, and those containing HbF are stained with a blue intensity proportional to their HbF content.

This staining method aids in the clinical diagnosis of elevated HbF because of HPFH from that of thalassemia and other hemoglobinopathies. If the HbF elevation is not caused by thalassemia or a hemoglobinopathy other than HPFH, the slide will stain heterogenously (unevenly), with some cells containing little-to-none of HbF; others will contain considerable amounts. If HPFH is the cause of the high HbF, the slide will stain homogenously (evenly) and contain the same amount of HbF in each fixed cell. The acid elution test has also been useful in determining the degree of fetal-maternal hemorrhage.

E. Immunological Techniques

The development of monospecific antisera (*31a*) has resulted in the development of very sensitive immunologic techniques for detection and quantitation of normal and abnormal hemoglobin variants. Now, with radioimmunoassay (RIA) or enzyme-linked immunosorbent assay (ELISA) (*48*) techniques, it is possible to measure hemoglobins in nanogram quantities. It has been possible to detect HbS by RIA in amniotic fluid of pregnant woman heterozygous for HbS as early as 16 weeks gestation (*31b*). At present, the availability of antisera is limited to research laboratories; otherwise the time seems to be right for its adoption in clinical laboratories.

F. Solubility Test

In 1953, Itanto (*40*) reported a sensitive technique for measuring the solubility of deoxygenated hemoglobin in a phosphate buffer and noted

that HbS is virtually insoluble in this system. Since this time, several commercial kits have been placed on the market. The principle of this solubility test (dithionite tube test) is as follows. Hemoglobin S is reduced by dithionite and is insoluble in the concentrated inorganic buffer used; most of the other hemoglobins remain soluble (*14*). The polymers of reduced HbS obstruct the light rays from passing through the solution. A cloudy solution indicates an insoluble hemoglobin, which in most cases is HbS. Non-S sickling hemoglobins will also give a positive result (e.g., HbC Harlem, Hb Bart's). Normal hemoglobin will appear transparent. Positive tests are usually confirmed by hemoglobin electrophoresis (previously described) or by the metabisulfite test. In the metabisulfite microscopic test, metabisulfite reduces the oxygen tension of the erythrocytes. Under low oxygen tension, erythrocytes containing HbS will form the characteristic sickle shapes. False positives will also be seen (e.g., HbC Harlem, HbI) with this method, making conformation of HbS by electrophoresis the method of choice for both procedures. Both screening tests (dithionite tube test and metabisulfite microscopic slide test) will not differentiate sickle cell anemia from sickle cell trait or other HbS syndromes.

G. Heat Denaturation Test

The heat denaturation (instability) test is used in the determination of unstable hemoglobins. The unstable hemoglobins precipitate more rapidly when incubated at 60°C then will normal hemoglobins (*20*). The heat denaturation instability test was first applied to blood from patients with HbH disease (*7*) and then later to patients with Heinz-body anemia (*32a, 32b*). The main conditions controlling the stability of those hemoglobin variants are (1) bonding within the globin subunit, (2) bonding of the globin to the heme, and (3) bonding between hemoglobin. Decreased stability may result from any of these alterations. The unstable hemoglobins are precipitated, whereas normal hemoglobins show little or no precipitate. The precipitate is removed by centrifugation, and the supernatant is read at 523 nm. The percentage of heat precipitate hemoglobin is calculated from the amount originally present and the amount remaining in the supernatant fluid.

H. Isopropanol Precipitation Test

The isopropanol precipitation test is also used in the determination of unstable hemoglobin. This test incorporates the use of a nonpolar solvent (isopropanol-tris buffer, pH 7.4) that weakens the internal bonding forces of unstable hemoglobin more readily than the bonding forces of normal

hemoglobins. This decrease in their stability results in the precipitation of the unstable hemoglobin more rapidly than in normal hemoglobins when incubated (*13*). The mixture of hemolysate with the solvent is incubated at 37°C for various time periods. The amount of denatured hemoglobin (%) is estimated visually or quantitated spectrophotometrically at 540 nm.

Red blood cells containing unstable hemoglobin will form Heinz bodies, which are not seen in presplenectomy peripheral-blood smears when stained with "wright stains." To induce Heinz-body formation, whole blood should be mixed either with cresyl blue or methylene blue stain and incubated for approximately 10 min. Smears prepared from this mixture will show few Heinz bodies staining deep purple and usually attaching to the cell membrane. Postsplenectomy samples show numerous Heinz-body formations (approximately 50% of the cells) taking the form of a large solitary inclusion in the center of the cell, and free from the cell membrane. The generation of Heinz-body formation depends on the degree of instability of Hb.

I. Osmotic Fragility Test

The osmotic fragility test measures the resistance of erythrocytes to hemolysis by osmotic stress (*27*). The test is prepared by placing erythrocytes in decreasing strengths of hypotonic saline solutions and measuring the degree of hemolysis. A conventional curve—percentage of hemolysis on the vertical axis against decreasing saline concentrations on the horizontal axis—is plotted (*21*). Usually, a symmetrical curve, sigmoidal in shape, is obtained in most subjects. Increased osmotic fragility is indicated by a shift of the curve to the left. Decreased osmotic fragility (osmotic resistance) is signified by a curve shift to the right. Increased osmotic fragility is observed in conditions associated with spherocytosis. Decreased osmotic fragility is observed in thalassemia, sickle cell anemia, and other disorders in which many target cells are observed.

J. Peptide Fingerprinting Technique

The peptide fingerprinting technique, developed by Ingram 1958 (*37*), is based on the enzymatic digestion using trypsin (trypsin splits the polypeptide chain only at those points where the basic amino acids, lysine and arginine, occur), which will split the polypeptide chains into peptides giving rise to 14 tryptic peptides of the α-chain and 15 peptides from the β-globin chain. The peptides are then separated by performing two-dimensional electrophoresis, filter-paper electrophoresis in one direction, followed by chromatography at a right angle.

The resulting peptide map, or "fingerprints," are compared to known

fingerprint patterns that will show the different peptide positions, enabling the location of the abnormal peptide. The abnormal peptides can then be eluted by column chromatography and its amino acid content determined. Using this technique Ingram first identified the polypeptide difference between normal HbA and sickle cell HbS, with the difference occurring at the first tryptic split in the β-chain. Discussion of the current techniques for identification of variant hemoglobins is provided by Huismann (*36d*).

K. Hybridization Technique

The hybridization technique involves the spontaneous dissociation and recombination of the hemoglobin molecule. The hemoglobin molecule is first dissociated into subunits half the size of the original molecule. This can be achieved either by making the hemoglobin solution acidic (pH <5.0) or alkaline (pH 11.0–11.6). The hemoglobin subunit will then recombine when the pH of the solution is brought back to neutral pH, completing the hybridization sequence. An example of this procedure is discussed by Vinograd (*84*). He feels the probable dissociation of the whole molecules ($\alpha_2\beta_2$) is divided into two asymmetric half molecules ($\alpha\beta$,$\alpha\beta$) and finally into individual chains (α and β). The individual chains then can be recombined (when neutralized) to form new symmetric half molecules and, finally, the whole molecule. When the recombination of normal hemoglobin takes place with different types of hemoglobins (e.g., canine), the resulting hemoglobin is called hybrid. Hybrid hemoglobins will show different physical properties, such as electrophoretic mobility, and therefore will be identifiable as being different from the original hemoglobin molecule. We have seen that canine hemoglobin is excellent for hybridization studies because dissociation of canine hemoglobin molecules will give different α- and β-subunits. The hybridization of human hemoglobin ($\alpha_2^{A}\beta_2^{A}$) with canine hemoglobin ($\alpha_2^{can}\beta_2^{can}$) will yield two new hybrid molecules ($\alpha_2^{A}\beta_2^{can}$ and $\alpha_2^{can}\beta_2^{A}$). Upon examination of the two hybrid hemoglobins, it can be determined whether the abnormality of the hemoglobin molecule resides in the α- or the β-chain (*35b*). This technique can also be used to prove the identity or difference between two hemoglobins. If the hemoglobins are the same, the recombination will not form a hybrid. If the chain combination is different, a new hybrid will be formed, which can be compared to known standards for identification.

L. Globin Chain Synthesis

Analysis of globin chain synthesis is useful in characterization and prenatal diagnosis of the thalassemias. Peripheral blood containing reticulocytes is incubated with radioactive amino acids (usually, [^{14}C] leucine or

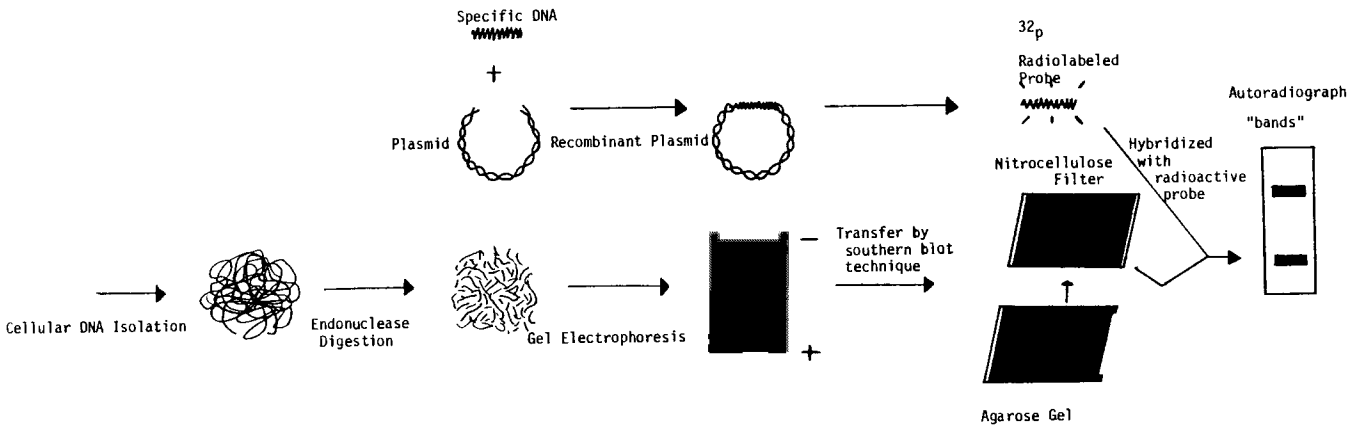

Fig. 8. Gene mapping. Reprinted by permission from Antonarakis, S. T., Phillips, J. A., Kazazian, H. H. (1982). *J. Pediatr.* **100,** 847. (modified)

[^{3}H] leucine). For prenatal analysis fetal reticulocytes are obtained by fetoscopy or placental aspiration, an invasive procedure requiring an experienced obstetrician (*59*). Newly synthesized and labeled globin chains are separated by chromatography on a CM-cellulose column in urea, and their specific activity is measured. In case of α- and β-chains, results are reported as a ratio of β-chain activity to α-chain activity. In normal subjects the ratio is close to 1.0 (*56, 71*). Prenatal diagnosis is very helpful in identification of homozygous β-thalassemia; it is not considered encouraging for the heterozygous state.

M. Gene Mapping

For the last few years, a new approach using restriction endonucleases (*43, 59*) has been used for prenatal detection of sickle cell anemia and α-homozygous and β-thalassemias. This new technique uses fetal fibroblasts obtained routinely and safely by amniocentesis (*57*), whereas sampling of fetal blood used in globin chain synthesis analysis is difficult and risky (*3*).

In this relatively new technique, restriction endonucleases are used to digest cellular DNA at specific sites. The DNA fragments generated are separated according to size by electrophoresis on agarose gel and then transferred (blotted) to nitrocellulose filters and immobilized as single strands of DNA. This procedure was described by Southern and is referred as Southern blot technique (*76*). The gene identification in bands is achieved by autoradiography after hybridization with a specific marker, ^{32}P-labeled globin complementary DNA probe, which is produced by recombinant DNA cloning techniques (*51*). The steps involved in gene mapping are illustrated in Fig. 8.

IV. CONCLUSION

The hematological disorders caused by molecular lesion in hemoglobin synthesis may result in asymptomatic–trivial-to-severe or lethal clinical conditions. The identification of these disorders is important both for clinical diagnosis and genetic counseling and presents a real challenge for the clinical laboratory.

A patient presenting unexplained hematological abnormalities—such as anemia refractory to treatment hemolysis, cyanosis, polycythemia, bizarre cell morphology, Heinz-body formation and abnormal red cell indices, spontaneous abortion, or hydrops fetalis—should be screened for hemoglobinopathies.

Many procedures are available for detecting hemoglobin disorders, but the importance of clinical hematologic and genetic information can never be overstated. Combined data from cellulose electrophoresis (pH 8.6) and citrate agar electrophoresis (pH 6.4) provides quick identification of hemoglobins S, C, D, A, and F. An experienced person combining these data and information from globin chain electrophoresis with urea—2-mercaptoethanol, TEB buffer; pH 8.9, and TEB citrate buffer (pH 6.0) can accurately identify most of the commonly known variants. Isoelectric focusing shows a good potential for replacing electrophoresis as a routine screening procedure for hemoglobin abnormality in the adult and newborn and during the antenatal period. Although the ultimate identification of an abnormal hemoglobin is confirmed only by structural analysis, the solubility testing or measurement of HbF is a help in many hemoglobinopathies. The presence of unstable hemoglobin is detected by heat or isopropanol stability tests, and a variant with abnormal oxygen affinity is evaluated by P_{50} measurements.

The quantitation of HbA_2 and *in vitro* globin chain synthesis analysis are required for thalassemia evaluation. Thalassemia is more easily diagnosed in the newborn than the adult because of the presence of Hb Bart's. The gene mapping with the use of restriction enzymes and a cloned DNA probe, though still in its infancy, promises to be useful in the accurate prenatal diagnosis of hereditary disorders of hemoglobins. This procedure, like structural analysis, is still beyond the capabilities of clinical laboratories. Immunoidentification, with its high specificity and sensitivity combined with IEF, appears to be ideal for future routine identification and quantitation of hemoglobins of clinical significance.

REFERENCES

1. Abraham, E. C., Huisman, T. H. J., Schroeder, W. A., Pace, L. A., and Grussing, L. *J. Chromatogr.* **143,** 57 (1977).
2. Allen, R. C., Harley, R. A., and Talamo, R. C. *Am. J. Clin. Pathol.* **62,** 732 (1974).
3. Alter, B. P., Modell, C. B., Fairweather, D., Hobbins, J. C., Mahoney, M. J., Frigoletto, F. D., Sherman, B. S., and Nathan, D. G. *N. Engl. J. Med.* **295,** 1437 (1976).

4a. Bank, A., and Mark, P. A. *Nature* (*London*) **212,** 1198 (1966).
4b. Bank, A., Braverman, A. S., O'Donnell, J. V., and Marks, P. A. *Blood* **31,** 226 (1968).
5a. Bassett, P., Beuzard, Y., Garel, M. C., and Rosa, J. *Blood* **51,** 971 (1978).
5b. Bassett, P. *J. Chromatogr.* **227,** 267–304 (1982).

6. Benz, E. J., Jr., and Forget, B. G. *Semin. Hematol.* **11,** 525 (1974).
7. Betke, K., Marti, H. R., Kleihauer, E., and Butikofer, E. *Klin. Wochenschr.* **38,** 529–532 (1960).

8. Bhagavan, N. V. editor *In* "Biochemistry" 74, 371–797, 2nd ed. Lippincott, Philadelaphia, Pennsylvania, 1972.
9. Briere, R. O., Golias, T., and Batsakis, J. G. *Am. J. Clin. Pathol.* **44,** 695 (1965).
10. Boggs, D. R. *Blood* **44,** 699 (1974).
11. Bunn, H. F. *Hemoglobin* **2,** 1 (1978).
12. Capp, G. L., Rigas, D. A., and Jones, R. T. *Nature (London)* **228,** 278–280 (1970).
13. Carrell, R. W., and Kay, R. A. *Br. J. Haematol.* **23,** 615–619 (1972).
14. Charache, S., and Conley, C. L. *Ann. N.Y. Acad. Sci.* **165,** 37 (1969).
15a. Chernoff, A. L. *N. Engl. J. Med.* **253,** 322 (1955).
15b. Chernoff, A. L. *Blood* **13,** 116 (1958).
16. Clegg, J. B., Weatherall, D. J., and Milner, P. F. *Nature (London)* **234,** 337 (1971).
17. Conn, H. O. *N. Engl. J. Med.* **251,** 417 (1954).
18. Cooley, T. B., and Lee, P. *Trans. Am. Pediatr. Soc.* **37,** 29 (1925).
19. Schneider, R. G. *CRC Crit. Rev. Clin. Lab. Sci.* **9,** 243–271 (1978).
20. Dacie, J. V., Grimes, A. J., Meisler, A., Steingold, L., Hemstead, E. H., Beaven, G. H., and White, J. C. *Br. J. Haematol.* **10,** 388 (1964).
21. Danon, D. *J. Clin. Pathol.* **16,** 377–382 (1963).
22a. Diesseroth, A., Velez, R., and Nienhuis, A. W. *Science (Washington, D.C.)* **191,** 1262 (1976).
22b. Diesseroth, A., Nienhuis, A., Anderson, W. F., Turner, P., Velez, R., Ruddle, F., Lawrence, J., Creagen, R., and Kucherkpati, R. *Cell* **12,** 205–218 (1977).
23. Dix, D., Cohen, P., Kingsley, S., Senkbell, J., and Sexton, K. *Clin. Chem.* **25,** 877 (1979).
24. Dozy, A. M., and Huisman, T. H. J. *J. Chromatogr.* **40,** 62 (1969).
25. Drysdale, J. W., Righetti, P. G., and Bunn, H. F. *Biochim. Biophys. Acta* **229,** 42 (1971).
26. Efremov, G. D., Huisman, T. H. J., Bowman, K., Wrightstone, R. N., and Schroeder, W. A. *J. Lab. Clin. Med.* **83,** 657 (1974).
27. Emerson, C. P. *Arch. Intern. Med.* **97,** 1 (1956).
28. Emmel, V. E. *Arch. Intern. Med.* **20,** 586 (1917).
29a. Gerald, P. S., and Diamond, L. K. *Blood* **13,** 835–844 (1958).
29b. Gerald, P. S., and Ingram, V. M. *J. Biol. Chem.* **236,** 2155 (1961).
30. Gianazza, E., Righetti, P. G., Bjellquist, B., Ek, K., Gorg, A., and Westermeier, R. *Protides Biol. Fluid* **30,** 603–606 (1982).
31a. Graver, F. A., Baker, M. B., Jones, C. S., Gravely, M., Altay, G., and Huisman, T. H. J. *Science (Washington, D.C.)* **196,** 1334–1336 (1977).
31b. Graver, F. A. *Lab. Manag.* **16,** 25–34 (1978).
32a. Grimes, A. J., and Meisler, A. *Nature (London)* **194,** 190–191 (1962).
32b. Grimes, A. J., Meisler, A., and Dacie, T. V. *Br. J. Haematol.* **10,** 281–290 (1964).
33. Henson, J. B., Carver, J. R., and Wilson, J. B. *J. Chromatogr.* **98,** 443–448 (1980).
34. Herrick, J. B. *Arch. Intern. Med.* **6,** 517 (1910).
35a. Huehns, E. R., Dance, N., Beaven, G. H., Hecht, F., Keil, J. V., and Motulsky, A. G. *Nature (London)* **201,** 1095–1097 (1964).
35b. Huehns, E. R., and Shooter, E. M. *Haemoglobin. Sci. Prog.* **52,** 353 (1964).
35c. Huehns, E. R., and Shooter, E. M. *J. Med. Genet.* **2,** 48 (1965).
36a. Huisman, T. H. J., and Dozy, A. M. *J. Chromatogr.* **7,** 180 (1962).
36b. Huisman, T. H. J., and Dozy, A. M. *J. Chromatogr.* **19,** 160 (1965).
36c. Huisman, T. H. J., and Dozy, A. M. *J. Chromatogr.* **32,** 723 (1968).
36d. Huisman, T. H. J., and Jonxis, J. H. P. "The Hemoglobinopathies," Vol. 6. Dekker, New York, 1977.

36e. Huisman, T. H. J., and Prins, H. K. *J. Lab. Clin. Clim. Acta* **2,** 307 (1951).
36f. Huisman, T. H. J., Wrightstone, R. N., Wilson, J. B. *Arch. Biochem. Biophys.* **153,** 850–853 (1972).
36g. Huisman, T. H. J., Schroeder, W. A., Brodie, A. N., Mayson, S. M., and Jakway, J. *J. Lab. Clin. Med.* **86,** 700 (1975).
37. Ingram, V. M. *Biochim. Biophys. Acta* **36,** 402 (1959).
38. International Committee for Standardization in Hematology *Blood* **52,** (1977).
39. International Hemoglobin Information Center *Hemoglobin* **3,** 104–481 (1979).
40. Itano, H. A. *Arch. Biochem. Biophys.* **47,** 148–159 (1953).
41. Jeppsson, J. O., and Berglund, S. *Clin. Chim. Acta* **40,** 153 (1972).
42. Kamuzora, H., and Lehmann, H. *Nature (London)* **256,** 511 (1975).
43. Kan, Y. W., and Dozy, A. *Lancet* **2,** 910 (1978).
44. Kleihauer, E., Braun, H., and Betke, K. *Klin. Wochenschr.* **35,** 637 (1957).
45. Lehman, H., and Huntsman, R. G. "Man Haemoglobin" Lippincott, Philadelphia, Pennsylvania, 1974.
46. Lehninger, A. L. (Ed.) "Biochemistry" 2nd ed., p. 167. Worth, New York, 1975.
47. Lie-Injo, L. E., Pillay, R. D., and Thuraisingham, V. *Blood* **28,** 830–839 (1966).
48. Makler, M. T., and Pesce, A. J. *J. Clin. Pathol.* **74,** 673–676 (1980).
49. Marengo-Rowe, A. J. *J. Clin. Pathol.* **18,** 790 (1965).
50. McDonald, M. J., Shapiro, R., Bleichman, M., Solway, J., and Bunn, H. F., *J. Biol. Chem.* **253,** 2327–2332 (1978).
51. Miller, W. L. *J. Pediatr.* **99,** 1 (1981).
52. Milner, P. F., and Gooden, H. *J. Clin. Pathol.* **64,** 58 (1975).
53. Monte, M., Beuzard, Y., and Rosa, J. *Am. J. Clin. Pathol.* **66,** 753 (1976).
54. Motulsky, A. G. *N. Engl. J. Med.* **288,** 31 (1973).
55. Nastev, D., Efremov, G. D., and Pethov, G. *Hemoglobin* **1,** 445 (1977).
56. Nathan, D. G. *N. Engl. J. Med.* **286,** 586 (1972).
57. National Institute of Child Health and Human Development (National Registry for Amniocentesis Study Group) *JAMA J. Am. Med. Assoc.* **236,** 1471 (1976).
58a. Nienhuis, A. W., and Benze, J., Jr. *N. Engl. J. Med.* **297,** 1430–1436 (1977).
58b. Nienhuis, K., and Betke, K. *Klin. Wochenschr.* **46,** 47 (1968).
59. Orkin, S. H., Aller, B. P., Altay, C., Mahoney, M. J., Lazarus, H., Hobbins, J. C., and Nathan, D. G. *N. Engl. J. Med.* **299,** 166 (1978).
60. Ottolenghi, S., Comi, P., Giglioni, B., Tolstoshev, P., Lanyon, W. G., Mitchell, G. J., Williamson, R., Russo, G., Musumeci, S., Schiliro, G., Tsistraltis, G. A. *Cell* **9,** 71–80 (1976).
61a. Pauling, L., Itano, H. A., Singer, S. J., and Wells, I. C. *Science (Washington, D.C.)* **110,** 543 (1949).
61b. Pauling, L., Corey, R. B., and Branson, H. R. *Proc. Natl. Acad. Sci. USA* **37,** 205 (1951).
62. Pearson, H. A., Obrien, R. T., McIntosh, S., Aspnes, G. T., and Yang, M. M. *JAMA J. Am. Med. Assoc.* **227,** 420 (1974).
63a. Perutz, M. F. *Sci. Am.* **211,** 64–76 (1964).
63b. Perutz, M. F. *Proc. R. Soc. Med.* **173,** 113–140 (1969).
63c. Perutz, M. F. *Nature (London)* **228,** 551 (1970).
63d. Perutz, M. F. *Sci. Am.* **239,** 92 (1978).
63e. Perutz, M. F., Kendrew, J. C., and Watson, H. C. *J. Mol. Biol.* **13,** 669–678 (1965).
63f. Perutz, M. F., and Lehmann, H. *Nature (London)* **219,** 29–131 (1968).
64. Portier, A., Detraverse, P., Duzer, A., Destaing, F., and Porot, J. F. *Presse Med.* **68,** 1760–1763 (1960).

65. Rilbe, H. *In* "Isoelectric Focusing" (N. Catsimpoolas, ed.), pp. 13–52. Academic Press, New York, 1976.
66. Rosenbaum, D. L. *Am. J. Med. Sci.* **252,** 726 (1966).
67. Roth, E. F., Jr., Rachmilewitz, E. H., Schifler, A., and Nagel, R. L. *Acta Haematol.* **59,** 237 (1978).
68. Sander-Haigh, L., Anderson, W. F., and Franche, V. *Nature (London)* **283,** 683–686 (1980).
69a. Schleider, C. T. H., Mayson, S. M., and Huisman, T. H. J. *Hemoglobin* **1,** 503 (1977).
69b. Schneider, R. G., Hosty, T. S., Tomlin, G., and Atkins, R. *Clin. Chem.* **20,** 74–77 (1974).
69c. Schneider, R. G. *Clin. Chem.* **20,** 1111–1115 (1974).
69d. Schneider, R. G., Hightower, B., Hosty, T. S., Ryder, H., Tomlin, G., Atkins, R., Brimhall, B., and Jones, R. T. *Blood* **48,** 629–637 (1976).
70a. Schroeder, W. A., Huisman, T. H., Shelton, J. R., Shelton, J. B., Kleinhauer, E. F., Dozy, A. M., and Robberson, B. *Proc. Natl. Acad. Sci. USA* **60,** 537–544 (1968).
70b. Schroeder, W. A., Jakway, J., and Power, D. *J. Lab. Clin. Med.* **82,** 303 (1973).
70c. Schroeder, W. A., Huisman, T. H. J., Powars, D., Evans, L., Abraham, E. C., and Lam, J. *J. Lab. Clin. Med.* **52,** 312 (1975).
70d. Schroeder, W. A., and Huisman, T. H. J. (Editors) "Chromatography of Hemoglobin," Vol. 9. Dekker, New York, 1980.
71. Schwartz, E. *Semin. Hematol.* **11,** 549 (1974).
72. Shepard, M. K., Weatherall, D. J., and Conley, C. L. *Bull. Johns Hopkins Hosp.* **110,** 293 (1962).
73. Singer, K., Chernoff, A. I., and Singer, L. *Blood* **6,** 413–429 (1951).
74. Smith, E. W., and Krevans, J. R. *Johns Hopkins Med. J.* **104,** 17 (1959).
75. Soderholm, J., Allestam, P., and Wadstrom, T. *FEBS Lett.* **24,** 89 (1972).
76. Souther, E. M. *J. Mol. Biol.* **98,** 503–517 (1975).
77. Spicer, K. M., Allen, R. C., Hallett, D., and Buse, M. G. *J. Clin. Invest.* **64,** 40–48 (1979).
78a. Stamatoyannopoulous, G., Sofroniadov, C., and Akrivekis, A. *Blood* **30,** 772–776 (1967).
78b. Stamatoyannopoulous, G., Fessas, P., and Papayannopoulous, T. *Am. J. Med.* **47,** 194–208 (1969).
78c. Stamatoyannopoulous, G., Bellingham, A. J., Lenfant, C., and Finch, C. A. *Annu. Rev. Med.* **22,** 221 (1971).
79. Stephens, A. D. *Br. J. Haematol.* **36,** 153 (1977).
80. Stryer, L. "Biochemistry," 2nd ed. Freeman, San Francisco, California, 1975.
81. Sunderman, F. W. *Am. J. Clin. Pathol.* **40,** 227 (1963).
82. Valla, F. *Am. J. Clin. Pathol.* **49,** 440 (1968).
83. Vesterberg, O. *Biochim. Biophys. Acta* **257,** 11 (1972).
84. Vinograd, J. R., Hutchinson, V. W. D., and Schroeder, W. A. *J. Am. Chem. Soc.* **81,** 3168 (1959).
85. Wagner, P. O. *Semin. Hematol.* **11,** 405 (1974).
86. Wasi, P., Na-Nakom, S., Poutrakul, S., Sookanek, M., Disthasongehan, P., Pornpatkul, M., and Panich, V. *Ann. N.Y. Acad. Sci.* **165,** 60 (1969).
87a. Weatherall, D. J. *Ann. N.Y. Acad. Sci.* **119,** 450 (1964).
87b. Weatherall, D. J., and Clegg, J. B. (Eds.) "The Thalassemia Syndromes" Blackwell, Oxford, 1972.

88. Witrobe, M. M., Lee, G. R., Boggs, D. R., Bithell, T. C., Athens, J. W., and Foerster, J. (Eds.) "Clinical Hematology" 8th ed., pp. 175–176. Lea & Febiger, Philadelphia, Pennsylvania, 1981.
89. Zimmerman, J., Grantir, R., Mummert, K., and Cioffi, R. *Milit. Med.* **139,** 313–315 (1974).
90. Zuelzer, W. W., Robinson, A. R., and Booker, C. R. *Blood* **17,** 393–408 (1961).
91. Weatherall, D. J., Clegg, J. B., Naughton, M. A. *J. Molecular Biol.* **19,** 91–108 (1966).

Index

G

H

N

O

P