

# Clinical Chemistry



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## FOREWORD

The ACS SYMPOSIUM SERIES was founded in 1974 to provide a medium for publishing symposia quickly in book form. The format of the SERIES parallels that of the continuing ADVANCES IN CHEMISTRY SERIES except that in order to save time the papers are not typeset but are reproduced as they are submitted by the authors in camera-ready form. As a further means of saving time, the papers are not edited or reviewed except by the symposium chairman, who becomes editor of the book. Papers published in the ACS SYMPOSIUM SERIES are original contributions not published elsewhere in whole or major part and include reports of research as well as reviews since symposia may embrace both types of presentation.

## PREFACE

This book is the result of the eighth annual series of lectures for continuing education sponsored by the Chicago Section of the American Chemical Society. It is also the first volume in the ACS Symposium Series to be sponsored by a local section of the ACS. The purpose of the lecture series and this volume is to present topics that have a clinical chemical basis but are also of interest to specialists in other clinical areas. Indeed, several contributors to this volume represent the disciplines of pediatrics, endocrinology, and hematology. The material included is appropriate to both the clinical aspects of the subject and to analytical and technological advances. Chapters in this book are concerned with etiology of disease and diagnosis as well as technical advances which can be applied more practically in the very near future. We believe the chapters are excellent examples of the integral part that the clinical chemist plays in modern medicine. The chapters on competitive protein binding assays and drug interference in laboratory testing were not originally presented as lectures but were added because of the current interest in these subjects.

The editors hope that this book will be as enlightening and useful to the reader as the lecture series was to the participating audience. It is a great pleasure to thank our contributors and publisher for their excellent cooperation, without which this volume would not have been possible. A special note of grateful acknowledgment goes to Florence Forman and Debra H. Forman for their meticulous editorial assistance.

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# Separation and Characterization of Hemoglobins

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Human red cells are ideally suited for studies of genetic disorders mainly because they can easily and at any degree of frequency be obtained in relatively large quantities. It is, therefore, not surprising that in the course of time many genetic disorders involving this cell have been detected. It is interesting that some of these result in a severe hemolytic anemia, while other genetically imposed changes might not affect the life nor the function of the cell. An excellent example is the hemoglobin molecule; in some variants the chemical change does not influence the properties of this protein, whereas in others the change occurs at a critical site affecting its stability and/or functional properties. Identification and characterization of these variants have become routine procedures in some laboratories. The results of these studies have led to extensive investigations of patients with unexplained hereditary hemolytic anemias, of structure-function relationships of normal hemoglobins and of variants found in patients with an unexplained alteration in their oxygen transport system, of the different types of genetic polymorphism, and of the incidence of selected variants in several populations. It therefore appears appropriate to summarize general aspects of the normal hemoglobins and of selected hemoglobin variants, to review methods used to identify and separate these proteins, and to discuss some procedures useful in the characterization of possible chemical changes in this molecule.

## Normal and Abnormal Hemoglobins

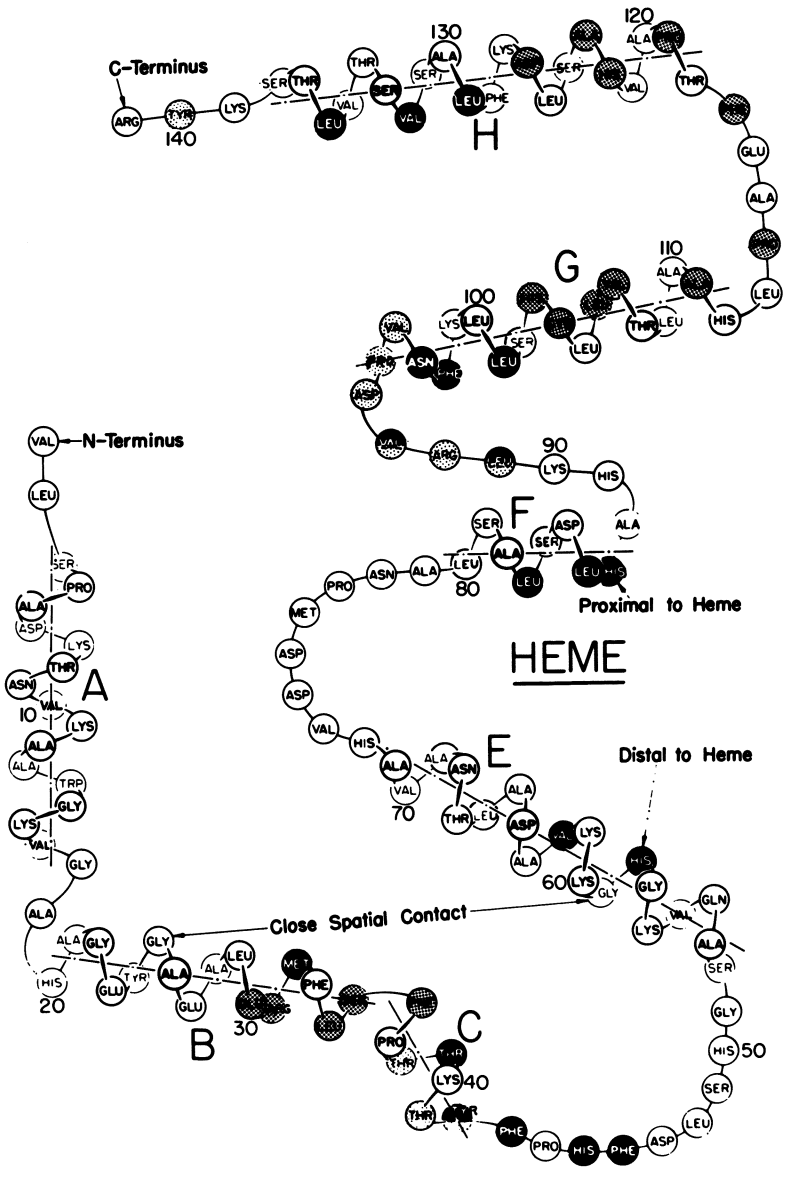
Hemoglobin is a large complex protein molecule; the atomic model of oxyhemoglobin at 2.8 Å resolution has been reported in 1968 by Perutz and coworkers, and a similar model of deoxy-hemoglobin in 1970 (1,2). The molecule is a spheroid, approx-



imately  $65 \text{ \AA} \times 55 \text{ \AA} \times 50 \text{ \AA}$ . It is composed of four polypeptide chains each resembling quite closely the myoglobin chain. The three dimensional structure of the subunits is held together by weak noncovalent bonds. The polar amino acid side chains are in contact with the solvent, and the nonpolar residues are located in the interior of the molecule or in regions which form the contacts between chains. The heme group is located in a pocket in each chain; residues in contact with heme are invariable (*i.e.* are the same in different mammalian hemoglobins) and the bonds between heme and chain are hydrophobic interactions. Contacts between like chains ( $\alpha$ - $\alpha$ ;  $\beta$ - $\beta$ ) are limited to salt bridges involving the terminal residues but contacts between unlike chains ( $\alpha$ - $\beta$ ) are many. The largest number of contacts is between the  $\alpha_1$  and  $\beta_1$  (34 residues with 110 atoms being within  $4 \text{ \AA}$ ). These are primarily contributed by amino acid residues of the B, G, and H helices. The  $\alpha_1$ - $\beta_2$  contact is formed by 19 residues (in helices C, F, and G) and is of importance for the functional interactions between subunits. The effect of deoxygenation causes an alteration in distance between iron atoms. The most pronounced change is in the  $\beta_1$ - $\beta_2$  distance, which increases 6 to  $7 \text{ \AA}$ . The  $\alpha_1$ - $\beta_1$  contact undergoes only slight change whereas drastic changes seem to occur in the  $\alpha_1$ - $\beta_2$  contact. Figures 1 and 2 (from reference 3) give further details on the structures of the  $\alpha$ - and  $\beta$ -chains of Hb-A and list the residues that participate in the contacts with heme, and between chains.

The major type of human adult hemoglobin (Hb-A<sub>0</sub>) is composed of two pairs of identical chains, and its formula can be written as  $\alpha_2\beta_2$ . The primary structure of the two chains has been known for several years. The second adult hemoglobin is Hb-A<sub>2</sub> and it constitutes 2.5 to 3.0 percent of the total hemoglobin. Hb-A<sub>2</sub> also consists of four polypeptide chains, two of these are  $\alpha$ -chains and the other two (the  $\delta$ -chains) differ from the  $\beta$ -chains of Hb-A<sub>0</sub> by amino acid substitutions in 10 positions. The formula of Hb-A<sub>2</sub> can be written as  $\alpha_2\delta_2$ . A small amount (less than one percent) of fetal hemoglobin (Hb-F) is also present in red cells of the human adult. The electrophoretically fast moving fraction Hb-A<sub>3</sub> (which separates as Hb-A<sub>1</sub> by chromatography) is a mixture of at least four distinct minor fractions. One of these fractions (Hb-A<sub>1c</sub>) is present in adult red cell hemolysates for 3.5 to 4.5 percent and is composed of two normal  $\alpha$ -chains and two  $\beta$ -chains each with the NH<sub>2</sub> terminal group being blocked by a carbohydrate. A second fraction (Hb-A<sub>1e</sub>) has been identified as a mixed disulfide of one molecule of Hb-A<sub>0</sub> and two glutathione residues. Increased quantities of Hb-A<sub>1e</sub> are found in aged red cell hemolysates.

Fetal hemoglobin is the major hemoglobin of the red cells of the fetus and the newborn. It has the characteristic property of being resistant to denaturation by alkali. Hb-F is composed of two  $\alpha$ -chains (the same as in Hbs A and A<sub>2</sub>) and two



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**Figure 1.** Two-dimensional presentation of the  $\alpha$ -chain of human hemoglobin. ●, residues in contact with the heme group; ⊗, residues that participate in the  $\alpha_1$ - $\beta_1$  contact; ⊕, residues that participate in the  $\alpha_1$ - $\beta_2$  contact (3).

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$\gamma$ -chains which differ from the  $\beta$ -chains in 39 positions. The formula of Hb-F can be written as  $\alpha_2\gamma_2$ . A minor hemoglobin fraction, Hb-F<sub>1</sub>, which is present for about 7 to 10 percent in a cord blood sample, has the structure of  $\alpha_2\gamma_2$  acetyl indicating that the NH<sub>2</sub> terminal glycol residue of the  $\gamma$ -chain is acetylated. Recently the existence of two structurally different  $\gamma$ -chains has been demonstrated; these two chains (the  $G_\gamma$ - and  $A_\gamma$ -chains) differ at a minimum in one position, namely position 136, which is occupied by a glycol residue in the  $G_\gamma$ -chain and by an alanyl residue in the  $A_\gamma$ -chain. The two chains are present at birth in a ratio ( $G_\gamma$  to  $A_\gamma$ ) of 7 to 3 whereas this ratio changes gradually after birth to the adult ratio of 2 to 3 (for references, see (3)).

Two additional hemoglobin chains exist which have been termed the  $\epsilon$ - and the  $\zeta$ -chain. The  $\epsilon$ -chain is found in two hemoglobin components in young human (and animal) embryos. One of these hemoglobins (Hb-Gower-2) has the probable structure of  $\alpha_2\epsilon_2$ , whereas Hb-Gower-1 ( $\epsilon_h$ ) lacks  $\alpha$ -chains. These hemoglobins are not present in a fetus 12 weeks and older, and are apparently specifically synthesized during embryonic development. A third embryonic hemoglobin (termed Hb-Portland-1) is composed of a pair of  $\zeta$ -chains and a pair of  $\gamma$ -chains (thus  $\zeta_2\gamma_2$ ) and has been found in very small amounts in normal newborn infants, and in increased amounts in newborns with D<sub>1</sub> trisomy. The  $\zeta$ -chain is likely an embryonic  $\alpha$ -chain (4,5).

The occurrence of several hemoglobin polypeptide chains ( $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$ ,  $\epsilon$ ,  $\zeta$ ) makes it necessary to assume the presence of at least six structural loci involved in the synthesis of the different hemoglobins. This number is larger because the two structurally different  $\gamma$ -chains ( $G_\gamma$  and  $A_\gamma$ ) are the product of non-allelic  $\gamma$ -chain structural loci. There is also increasing evidence that duplication of the  $\alpha$ -chain structural locus may have resulted in an  $\alpha$ -chain gene multiplicity in some human populations which is comparable to that found in several mammalian species. It is obvious that the marked developmental changes require additional mechanisms which regulate the rate of synthesis of certain polypeptide chains during various stages of development.

The demonstration of an abnormal form of hemoglobin (Hb-S) in sickle cell anemia by Pauling *et al.* in 1949 (6) was followed by the discovery of a large number of additional variants. Many are distinguished from Hb-A by electrophoretic or chromatographic methods. The number and types of abnormal hemoglobins that have been discovered thus far are indeed overwhelming. At the latest count (December, 1974) at least 135  $\beta$ -chain variants, 72  $\alpha$ -chain variants, 8  $\delta$ -chain variants, and 11  $\gamma$ -chain variants have been found. These include variants with single amino acid substitutions (the majority), variants with two substitutions (the  $\beta$ -chain variants, Hb-C-Harlem and Hb-Arlington Park), variants with deletion of one or more residues

(nine variants of this type have been discovered), and even a variant with an insertion (the  $\alpha$ -chain variant Hb-Grady). Moreover, there are 7 fusion hemoglobins, *i.e.* hemoglobins with either  $\delta\beta$ -,  $\beta\delta$ - or  $\gamma\beta$ -chains, and 5 hemoglobins with chains that are extended at the COOH- terminal end. Several of these variants have altered physical and/or functional properties interfering with the life-span of the red cell or with the normal oxygen delivery system. (For recent reviews see references 3, 7, 8; a list of variants is available upon request from the Director of the International Hemoglobin Information Center, Medical College of Georgia, Augusta, GA 30902, USA.)

The synthesis of an abnormal  $\alpha$ -chain will result in the presence of more than one abnormal hemoglobin since all normal hemoglobin types are composed of the same pair of identical  $\alpha$ -chains and of different pairs of non- $\alpha$ -chains. An adult heterozygous for an  $\alpha$ -chain abnormality produces several hemoglobin types, namely  $\alpha_2\beta_2$ ,  $\alpha_2\delta_2$ ,  $\alpha_2^G\gamma_2$ ,  $\alpha_2^A\gamma_2$ , and the abnormal variants  $\alpha_2^X\beta_2$ ,  $\alpha_2^X\delta_2$ ,  $\alpha_2^XG\gamma_2$ ,  $\alpha_2^XA\gamma_2$ . Four of these are readily observed in the blood of the adult heterozygote, while the  $\gamma$ -chain-containing components are usually identified through analyses of blood of a heterozygous newborn. An adult individual heterozygous for an  $\alpha$ -chain variant and for a  $\beta$ -chain variant will produce two additional hemoglobins since the components  $\alpha_2\beta_2^Y$  and  $\alpha_2^X\beta_2^Y$  are the result of the presence of a  $\beta$ -chain structural gene abnormality. The observation of two different  $\alpha$ -chain variants together with Hb-A in some members of a family of Hungarian origin (9, 10) has been explained by the presence of two non-allelic  $\alpha$ -chain structural loci. The absence of Hb-A in subjects with an apparent homozygosity for the  $\alpha$ -chain variant Hb-J-Tongariki (11,12) indicates that some human populations have multiple  $\alpha$ -chain structural genes while others do not.

Abnormalities of structure may lead to changes in function (for extensive reviews see references 3, 7, 8, 13, 14). A hemoglobin will function normally if, (a) the hemes are present and fixed in the appropriate position in each chain; (b) the iron remains in ferrous form in deoxyhemoglobin; (c) the molecule is able to change its configuration in the process of oxygenation-deoxygenation through changes at the  $\alpha_1\text{-}\alpha_2$ ,  $\beta_1\text{-}\beta_2$ ,  $\alpha_1\text{-}\beta_1$ , and  $\alpha_1\text{-}\beta_2$  contacts. Hemoglobin abnormalities can be classified by grouping the chemical replacements according to their positions in the chains. Such an analysis leads to four major subgroups. The *first* concerns variants with replacements of residues which are in contact with heme. This group includes several unstable hemoglobins and also the four M hemoglobins in which either the proximal or the distal histidyl residue is replaced by a tyrosyl residue. The *second* group consists of variants in which residues participating in the  $\alpha_1\text{-}\beta_1$  contact are replaced, and the *third* group that in which replacements of residues of the  $\alpha_1\text{-}\beta_2$  contact have occurred. Many of the structural changes occurring

in positions participating in the  $\alpha_1\text{-}\beta_2$  contact are associated with alterations in function. The *fourth* group concerns variants with replacements in external regions of the molecule which are usually without any direct effect on structure and function. There are notable exceptions such as some unstable hemoglobins and the notorious Hb-S which shows a decreased solubility in its deoxygenated form and will therefore be accompanied by pathological symptoms in homozygotes.

Some of the abnormal fetal hemoglobin variants are the product of an allele of the  $A_\gamma$  structural locus and others of an allele of the  $G_\gamma$  structural locus. The structural abnormality in all eight variants of Hb-A<sub>2</sub> has been determined.

Nine variants resulting from deletions have been described; all are  $\beta$ -chain variants. Deletions leading to shortening of the chain (from one to five residues) usually have severe functional consequences. It is likely that the genetic event causing the deletion of one or more of the corresponding nucleotide base triplets is the result of an unequal crossing over between homologous genes. Hb-Grady is a variant in which the  $\alpha$ -chains are extended by three residues because of a tandem repetition of a short sequence (15). The underlying genetic alteration responsible for the  $\alpha$ -Grady chain appears to be a tandem duplication of nine base pairs which might have arisen by a process of mismatched intragenic crossing over.

Another group of hemoglobins are the fusion hemoglobins of which seven have been reported. The three Lepore hemoglobins are composed of two normal  $\alpha$ -chains and two chains that are part  $\delta$ -chain (NH<sub>2</sub>-terminal region) and part  $\beta$ -chain (COOH-terminal region). The  $\delta\beta$ -chain of Hb-Lepore is, like the  $\beta$ -chain and the  $\delta$ -chain, 146 amino acid residues long. The genetic event leading to the formation of these hybrid chains involves a nonhomologous crossing-over between corresponding points of the  $\delta$  and  $\beta$  structural genes. Three "anti-Lepore" hemoglobins, *it est* variants with a chain that is part  $\beta$ -chain (NH<sub>2</sub>-terminal region) and part  $\delta$ -chain (COOH-terminal region), have recently been described (Hbs-Miyada; P-Congo; P-Nilotic). This  $\beta\delta$  hybrid chain apparently is the product of a gene which is the counterpart of that synthesizing the  $\delta\beta$  hybrid chain of the Lepore hemoglobins. The discovery of the  $\gamma\beta$ -chain of Hb-Kenya (16, 17) is important because it has indicated for the first time that the  $\gamma$ ,  $\delta$ , and  $\beta$  structural genes are closely linked. The chain is a  $\gamma$ - $\beta$ -chain in which the crossing-over has occurred between residues 80 and 87. The structure of this chain suggests that the nonhomologous crossing-over has removed parts of a  $\gamma$  and the  $\beta$  structural genes and the entire  $\delta$  structural gene.

Hb-Constant Spring (18, 19) was the first variant discovered that has 31 additional residues at the COOH-terminus of the  $\alpha$ -chain. Comparable hemoglobins are Hb-Icaria and Hb-Koya-Dora, and Hb-Tak which has 10 additional residues at the COOH-terminus

of the  $\beta$ -chain (20). It seems possible that these variant chains have either arisen from a crossing-over with a gene for a protein unrelated to hemoglobin or through a mutation of a terminal codon thus permitting elongation of the protein. Hb-Wayne, the fourth variant with an elongated  $\alpha$ -chain, results from the deletion of a single base in the DNA of the  $\alpha$ -chain causing a "frameshift" which results in a sequence of different amino acids which are coded by the new succession of codons.

Different hemoglobins lacking  $\alpha$ -chains have been described. They consist solely of  $\beta$ -chains ( $\beta_4$  or Hb-H),  $\gamma$ -chains ( $\gamma_4$  or Hb-Bart's),  $\delta$ -chains ( $\delta_4$ ), or  $\epsilon$ -chains ( $\epsilon_4$  or Gower-1). In most instances these variants are present in patients with  $\alpha$ -thalassemia in which a decreased synthesis of  $\alpha$ -chains will lead to the formation of an excess of non- $\alpha$ -chains. The existence of different forms of  $\alpha$ -thalassemia (heterozygous and homozygous forms of  $\alpha_1$ -thalassemia and of  $\alpha_2$ -thalassemia, and Hb-H disease) has been noted; these forms are best characterized by the amount of  $\gamma_4$  present at time of birth (for references, see (3)). Homozygosity for  $\alpha_1$ -thalassemia is incompatible with life because the major hemoglobin in this condition at time of birth,  $\gamma_4$ , has a greatly increased affinity for molecular oxygen and is unable to release it to the tissues. The heterogeneity of  $\alpha$ -thalassemia is difficult to explain and the genetics of the abnormality is complex; it is considered possible that more than one  $\alpha$  structural loci are involved.

In a second type of thalassemia the synthesis of specific non- $\alpha$ -chains is impaired. The heterozygous form of  $\beta$ -thalassemia is characterized by a mild hypochromic microcytic anemia, by an increased level of Hb-A<sub>2</sub> (4 to 7 percent), and, in the majority of the cases, by an elevated Hb-F (1 to 15 percent). In homozygotes, the synthesis of  $\beta$ -chains is greatly decreased and as a result a severe anemia may develop after birth when the  $\gamma$ -chain production ceases.  $\beta$ -Thalassemia, like  $\alpha$ -thalassemia, is a heterogeneous condition which can be concluded from the differences in suppression of  $\beta$ -chain synthesis. In some cases  $\beta$ -chain production is completely absent (the  $\beta^0$  type) whereas, in others, a considerable  $\beta$ -chain synthesis can be observed (the  $\beta^+$  type). The severe anemia will lead, by a mechanism which is still not completely understood, to an increased production of  $\gamma$ -chains. Both types ( $G\gamma$ - and  $A\gamma$ -chains) are present and are produced in heterozygotes in either one of two distinct ratios, namely 7:3 as in the newborn or 2:3 as in the normal adult (for a summary see 21).

Other types of thalassemia concern the suppression of both  $\delta$ -chain and  $\beta$ -chain production ( $\delta\beta$ -thalassemia), and the existence of a  $\delta$ -thalassemia and a  $\gamma$ -thalassemia.

Changes in the quantities of the various normal hemoglobin components during developmental stages can be explained in terms of ill-defined regulatory mechanisms which control the rate of synthesis of the polypeptide chains. Such mechanisms have to

be responsible for the unequal suppression of the two types of  $\gamma$ -chains ( $G\gamma$  and  $A\gamma$ ) after birth and the simultaneous stimulation of the  $\beta$ - and  $\delta$ -chain production. It is of great interest that a silent structural  $\beta$ -chain locus is present in Caprini species (goats, sheep) which is activated during (experimental) anemia and which is stimulated by erythropoietic factors of yet undefined nature. Apparently humoral factors may have important functions in the regulation of gene activities.

The condition known as the hereditary persistence of fetal hemoglobin (HPFH) is characterized by the presence of 5 to 35 percent Hb-F in the heterozygote and in 100 percent Hb-F in the homozygote. It has been postulated that this anomaly, which is clinically benign, is caused by deletion of specific structural loci. The condition is rather heterogeneous. For instance, only  $A\gamma$ -chains are found in the Hb-F from carriers of the Greek type of HPFH and only  $G\gamma$ -chains in the Hb-F from carriers of a certain type observed in India and among blacks, whereas both  $A\gamma$ - and  $G\gamma$ -chains are present in varying ratios in the Hb-F of the HPFH condition that is most frequently observed in blacks. In most forms of HPFH the production of  $\beta$ - and  $\delta$ -chains is completely absent *in cis* of the HPFH determinant.

Only a few of the many hemoglobin variants occur in high frequencies in certain parts of the world. The Hb-S and Hb-C abnormalities are observed mainly in blacks, and occur in tropical Africa and in emigré populations. Hb-D-Los-Angeles is common in certain areas of India and Hb-E is rather exclusively found in the population of Southeast Asia. The various forms of  $\beta$ -thalassemia are known to occur mainly in the population of Mediterranean countries and in Asia, with a specific form being present in the Negro. The  $\alpha$ -thalassemias are primarily found in the Far East and the Mediterranean area with again a specific form in the Negro race.

The complexity of the problem of hemoglobin heterogeneity has made it necessary to use rather advanced biochemical investigative procedures. Many analyses require advanced protein chemical techniques. The second part of this review describes some of these methods.

### Identification And Characterization Of Hemoglobins

Hematological Methods. Hematological analyses can include the determination of the total hemoglobin concentration (in g%), the packed cell volume (PCV in %), the red blood cell count (in  $10^6/\text{mm}^3$ ) and reticulocytes count (in %), calculation of the red cell indices, examination of a blood film, tests to demonstrate the presence of inclusion bodies and of sickle cells, tests to evaluate the distribution of fetal hemoglobin (Hb-F) inside the red cells, the red cell osmotic fragility, the concentration of serum iron (SI), total iron binding capacity (TIBC), and the survival time of the red cells. Details of all



methods will not be presented; they are available in appropriate laboratory manuals (22-26).

A search for Heinz bodies is helpful in the detection of an unstable hemoglobin, of  $\alpha$ -thalassemia, homozygous  $\beta$ -thalassemia and related abnormalities, because hemoglobin often precipitates in the red cells of patients with one of these disorders. Inclusion bodies may consist of precipitated unstable hemoglobin, of  $\beta$ -chains (in  $\alpha$ -thalassemia), or of  $\alpha$  chains (in  $\beta$ -thalassemia).

Anemia is a constant feature of the thalassemias, the Lepore hemoglobinopathies and of many (unstable) hemoglobinopathies. Morphological changes of the red blood cells are not always characteristic. Determination of red cell indices is of help in the diagnosis of  $\beta$ -thalassemia, and the MCH and MCV values are useful in distinguishing milder forms of  $\beta$ -thalassemia from other forms of hypochromic anemia. It is not always possible to differentiate between iron deficiency anemia and heterozygous  $\beta$ -thalassemia, and the determination of the serum iron content, the total iron binding capacity, and the level of Hb-A<sub>2</sub> is of decisive importance. The presence of inclusion bodies supports the diagnosis of thalassemia. In Hb-H disease the number of inclusion bodies depends on the amount of Hb-H that is present, and in heterozygous  $\alpha$ -thalassemia only a few cells will contain inclusion bodies. In homozygous  $\beta$ -thalassemia and related disorders the number of inclusion bodies is increased considerably after splenectomy.

Handling Of Blood Samples. A relatively small (5 to 10 ml) sample is sufficient for nearly all hematological and biochemical studies; the material is collected by venipuncture with heparin, oxalate, or EDTA as anticoagulant. When micro-techniques are used a small sample can be collected in heparinized hematocrit tubes from a fingerstick or on filter paper from which the hemoglobin can be eluted with a few drops of a hemolyzing solution. Blood samples should be stored in the refrigerator for a period not exceeding 4 weeks. Shipment requires that the sample of blood or the saline washed red cells be kept cold but not frozen. Addition of a small amount of penicillin and/or streptomycin is useful in preserving the samples that are shipped around the world. Hemoglobin solutions are prepared by centrifugation of packed red cells which are washed at least three times with 0.9 g% NaCl and are hemolyzed by addition of one volume of distilled water and about 0.3 volume of carbon tetrachloride, chloroform, or toluene. Hemoglobin solutions should be stored in the cold for not longer than four weeks, but can be kept at -50°C unchanged for a longer period of time.

Electrophoretic Methods. Abnormal hemoglobins are usually detected by electrophoresis either at pH 8.5 - 9.0 or at

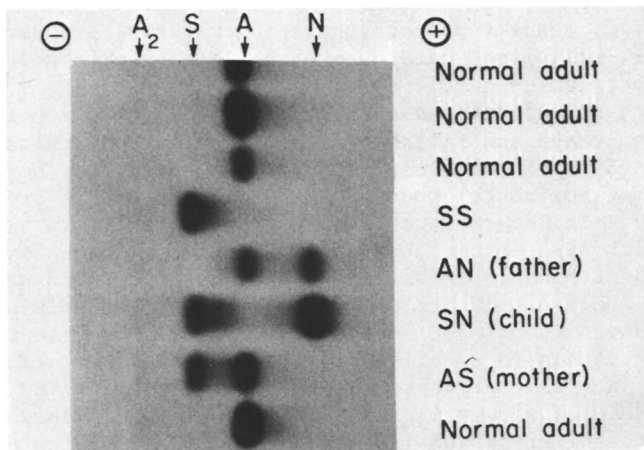
pH 6.0 - 7.0. Many types of supporting material are used, such as: paper, cellulose-acetate, starch-block, starch gel, agar gel, and acrylamide gel.

*Starch gel electrophoresis* at pH 8.8 - 9.0 is considered the most accurate and reliable method even in routine testing programs. Several commercial systems are available, but simple, inexpensive, horizontal models can be constructed in every laboratory. The starch that is used is partially hydrolyzed starch which has certain physical properties allowing superior separation of hemoglobin variants. The buffers which have been used most successfully for the preparation of the gels are tris-EDTA-borate buffers, pH 8.6 - 9.0. The buffer in the electrode vessels is usually 3 to 4 times more concentrated resulting in a discontinuous system that gives improved resolution mainly of slowly migrating minor hemoglobin components. A phosphate buffer system (pH 6.5 - 7.0) is useful for detecting small quantities of the hemoglobins H and Bart's; these variants will both move toward the anode while Hb-A remains at the origin. Figure 3 gives some examples of separations that can be obtained.

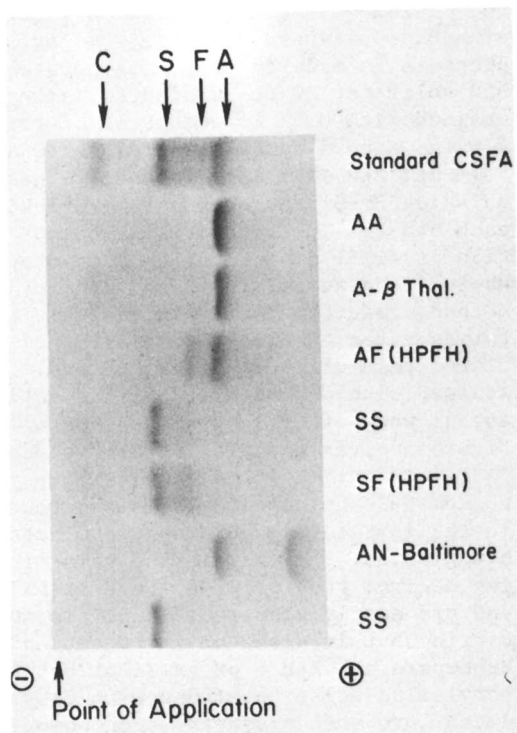
*Cellulose acetate electrophoresis* is a simple, fast, and sensitive method for preliminary testing of abnormal hemoglobins. Cellulose acetate media may differ in pore size, distribution, volume, length of cellulose chain, and degree of acetylation of the cellulose. Plates on which eight samples can be applied simultaneously are satisfactory for screening purposes. Electrophoresis is made in tris-EDTA-borate buffer, pH 9.0, at about 350 volts for 20 to 30 minutes, after which the plate is stained with 0.5% Ponceau S in 5% trichloroacetic acid, Figure 4. The method can be adapted for quantitative purposes.

*Citrate agar electrophoresis* has the advantage of distinguishing Hb-S from Hb-D and hemoglobins C, E, and O-Arab from each other. The method is also most useful for the demonstration of small amounts of Hb-A in samples from persons with Hb-S- $\beta^+$  thalassemia. There are many modifications of this method; however, the commercial kit marketed by Helena Laboratories gives excellent results. All methods use an 0.05 M citric acid-citrate buffer, pH 6.0. The agar slides can be stained with O-dimethylbenzidine until a blue color begins to appear whereafter they are washed with distilled water. Figure 5 gives examples of some separations that can be obtained.

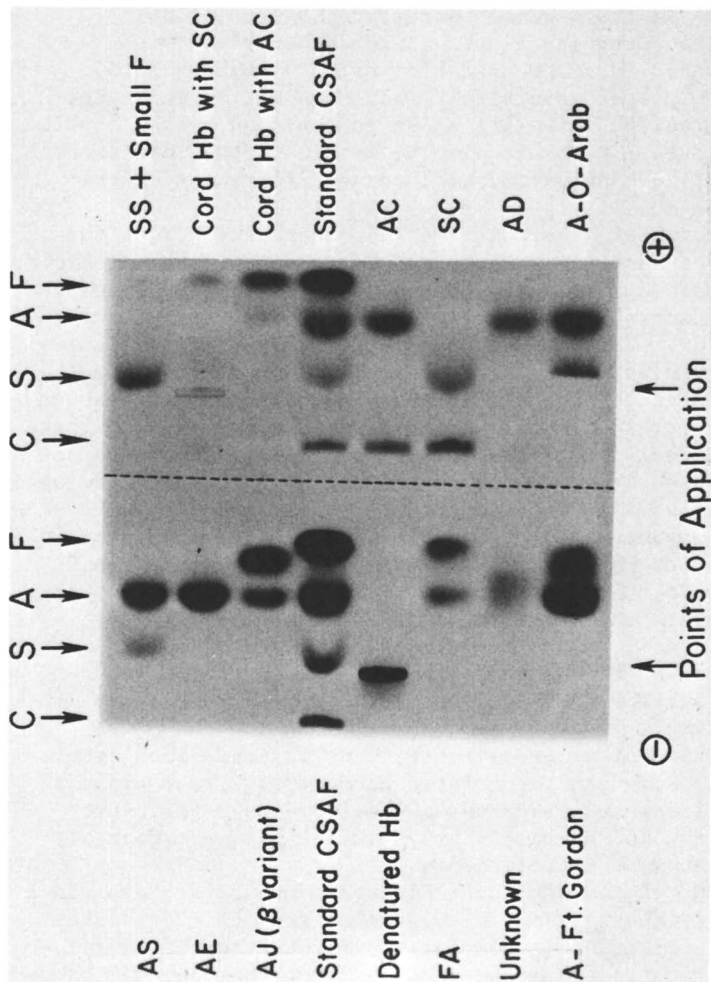
*Isoelectric focusing* is a more recently developed method. Successful application of this method depends upon the difference in the isoelectric points of at least 0.02 pH units between the hemoglobins. Polyacrylamide gels are most popular as a supporting medium; they provide great stability, excellent resolution, and are easily prepared. Analysis in polyacrylamide gel can be carried out in slabs or in tubes, and the hemoglobins can best be separated with a pH gradient between pH 6 and 8 in 4% polyacrylamide gels, developed with carrier ampholyte. When introduced into such a system, each hemoglobin will migrate to the



**Figure 3. Starch gel electrophoresis of hemoglobins. Tris-EDTA-boric acid buffer, pH 9.0. O-Dianisidine stain.**



**Figure 4. Cellulose acetate electrophoresis of hemoglobins. Variations in the quantities of Hb-A<sub>2</sub> are hardly detectable (compare the samples 2 and 3 from top). Hb-N-Baltimore is a β-chain variant in which lysyl residue in position 95 is replaced by a glutamyl residue.**



**Figure 5.** Citrate agar electrophoresis of hemoglobins. The plate is stained with O-dimethylbenzidine.

Two sets of samples have been analyzed on one plate with a standard mixture of the hemoglobins C, S, A, and F occupying the fourth position from the top. The data show that the hemoglobins A, F, S, and C are readily separated; that the hemoglobins E and D8 do not separate from Hb-A; and that some β-chain variants can readily be separated from Hb-F, Hb-A, Hb-S, or Hb-C (examples are the unidentified Hb-J, Hb-Fort Gordon (see also Figure 14), and Hb-O-Arab).

pH that is its isoelectric point, and will focus into a sharp band. The separated hemoglobins can be quantitated either with the use of a densitometer without staining or by elution of each band.

The techniques mentioned here provide a basic approach to the study of hemoglobinopathies since the identification of many variants is dependent upon their net charge. However, certain changes in the tertiary structure of a hemoglobin variant may also alter its electrophoretic behavior. This is well documented for many unstable variants such as for Hb-Köln which migrates more slowly than Hb-A but in which the neutral valyl residue in position  $\beta 98$  is replaced by the neutral methionyl residue. It should also be mentioned that differences in the level of heme oxidation will cause differences in the electrophoretic mobility.

The selection of a certain electrophoretic technique for the detection of abnormal hemoglobins in a large number of blood samples is based on simplicity, convenience and economy; cellulose acetate electrophoresis is, therefore, most often the method of choice. This technique allows the separation of many commonly observed variants but differentiation of rarer hemoglobin variants and of the different forms of thalassemia-abnormal hemoglobin combinations is often difficult to make. Citrate agar electrophoresis has a restricted application for large scale screening purposes because many of the common hemoglobin variants have a mobility similar to that of Hb-A. This technique, however, is most important for the differentiation of Hb-S from Hb-D, of Hb-A from Hb-F, of Hb-O-Arab and Hb-E from Hb-C, and of the AS, AC, SS, CC, and SC conditions in newborns. Isoelectric focusing separates the hemoglobins F and A, and also the hemoglobins D and S. Minor hemoglobin components are more readily detected by this method than by any other electrophoretic procedure. The technique has not yet been adapted to routine screening.

The detection of abnormal hemoglobins in cord blood samples is usually made with electrophoretic procedures. Four types of hemoglobin variants can be present, namely  $\gamma$ -chain variants,  $\beta$ -chain variants, and Hb-Bart's or  $\gamma_4$  indicating some form of  $\alpha$ -chain deficiency or  $\alpha$ -thalassemia.

All eleven  $\gamma$ -chain variants, discovered thus far, exhibit a change in electrophoretic mobility, and starch gel electrophoresis is the recommended method for their detection. Quantitation of the variant can best be done by chromatography on columns of either DEAE-Sephadex or CM-Cellulose. The quantities of some variants in heterozygotes differ greatly. For instance, the relative amount (expressed in  $\%F_x/F_{Total}$ ) varies from 20-25% (F-Malta-I) to 10-15% (most  $\gamma$ -chain variants) to 5-6% (F-Malta-II). Hybridization studies or chain composition analyses are often needed to identify the abnormality as a  $\gamma$ -chain abnormal hemoglobin.

The  $\alpha$ -chain variants are characterized by the presence of two abnormal components, an abnormal Hb-F ( $\alpha_2^X\gamma_2$ ) and an abnormal Hb-A ( $\alpha_2^X\beta_2$ ). Of these two, the  $\alpha_2^X\gamma_2$  component dominates and the  $\alpha_2^X\beta_2$  component is often difficult to detect. The methods of choice are starch gel electrophoresis and anion-exchange chromatography using DEAE-Sephadex or DE-52 Cellulose. Chain analyses of these isolated hemoglobin components will lead to a definitive identification.

The demonstration of  $\beta$ -chain variants in heterozygotes is complicated by the presence of the large amount of Hb-F. Another obstacle is the nearly identical electrophoretic mobilities of Hb-A and the minor Hb-F<sub>1</sub> component. Despite these difficulties, abnormalities such as AS, SS, AC, CC, SC, and others can readily be detected using cellulose acetate electrophoresis, starch gel electrophoresis, acid agar electrophoresis, and by CM-Cellulose microchromatography to be described in a separate section.

Hb-Bart's or  $\gamma_4$  can best be demonstrated by either cellulose acetate or starch gel electrophoresis. The amount of Hb-Bart's can differ from 1% to over 80% dependent on the abnormality involved. Quantitative determination is most accurately made by CM-Cellulose or CM-Sephadex chromatography.

**Macrochromatographic Methods.** These procedures use cation exchangers, such as Amberlite-IRC-50, carboxymethyl-Cellulose (CMC) and carboxymethyl-Sephadex (CMS), and the anion exchangers diethylaminoethyl-(DEAE)-Cellulose and DEAE-Sephadex.

**Cation-exchange chromatography** - The CMC-chromatographic method was developed in 1958; presently the best material available is the microgranular, pre-swollen CM-52 with a capacity of 1.0 mEq/g dry weight (Whatman). The cation-exchanger is stirred with distilled water for at least 12 hr and equilibrated with 0.01 M sodium phosphate buffer, pH 6.7, containing 100 mg KCN/1000 ml. Hemoglobin samples are dialyzed overnight against a similar buffer at 4°C, and 50 to 60 mg oxyhemoglobin in 1.0 to 2.0 ml volume is applied to a 1.8 X 35 cm column. The chromatogram is developed at room temperature with a pH gradient obtained by mixing 0.01 M sodium phosphate buffers of selected pH values (between 7.2 and 8.2) with the 0.01 M sodium phosphate buffer, pH 6.9, contained in a constant volume mixing flask. Absorbance readings of the effluent are made at 415 nm in 1 cm cuvettes using any suitable (semi-automated) spectrophotometer. Quantities of the various zones are calculated as percent of the total amount of hemoglobin recovered from the column. The procedure can easily be automated and several chromatograms can be developed simultaneously when a constant volume delivering pump is used.

The procedure is the method of choice for the separation of Hb-A<sub>2</sub> from slow-moving hemoglobins such as Hb-C, Hb-C-Harlem, Hb-O-Arab, Hb-I-Indonesia, and Hb-Agenogi. Many of these variants can also be distinguished from each other by this procedure. Exceptions are Hb-S and Hb-E, which are eluted together with

Hb-A<sub>2</sub>, and chromatography on DEAE-Sephadex or DEAE-Cellulose is required for the quantitation of Hb-A<sub>2</sub> in blood from Hb-S heterozygotes and homozygotes. Since some Hb-D variants elute at pH values slightly different from that of Hb-S, it is possible to obtain an (incomplete) separation of Hb-D and Hb-S and of Hb-D and Hb-A<sub>2</sub>. Minor hemoglobins, such as F<sub>1</sub>, A<sub>1</sub>, and C<sub>1</sub>, emerge first from the column and are followed by their respective major hemoglobin component. Figure 6 gives some examples of resolutions that can be obtained.

In certain instances the replacement of CMC by CM-Sephadex (C-50) may have advantages. Excellent separation of various major and minor hemoglobin components can be observed when 80 to 120 mg of hemoglobin are chromatographed on a 50 X 0.9 cm column of CM-Sephadex with 0.05 M tris-maleic acid buffers (containing 100 mg KCN/1000 ml) of pH values between 6.5 and 7.5, as developers. However, such a system is time-consuming and highly sensitive to small variations in the pH values of the eluting buffer system.

*Amberlite IRC-50 chromatography* is well suited for the isolation and purification of major and minor hemoglobin fractions. The development of a chromatogram is greatly dependent on the problem involved and an initial adaptation of the elution system is required prior to routine application. The resin is Amberlite IRC-50 of the appropriate mesh size (either 200-250 or 250-325 mesh/sq. inch) and the developers are sodium phosphate buffers of different molarities and with a pH varying between 6.7 and 7.2. The chromatogram is developed at a slow flow rate (5-6 ml/hr) at 6°C; separation of the various components requires that this temperature be kept constant within 0.2°C. At the end of the chromatogram the columns are warmed to 28°C while the flow-rate is increased to approximately 10 ml/hr. The absorbance of each fraction is measured in a 1 cm cuvette at 415 nm and preferably also at 280 nm.

*Anion-exchange chromatography* - The use of DiEthylAminoEthyl-anion exchangers for the separation of hemoglobin components was introduced in 1962; analyses with DEAE-Sephadex are carried out exclusively with DEAE-Sephadex A-50, capacity  $3.5 \pm 0.5$  mEq/g dry weight; particle size 40-120  $\mu$  (Pharmacia Fine Chemicals, Inc.). The dry material is suspended and equilibrated in a large volume of 0.05 M tris-HCl developer, pH 8.5, under occasional stirring. The columns used for analytical purposes are about 1.0 X 65 cm and the resin height is about 50 cm. Variable amounts (30-80 mg) of oxyhemoglobin are chromatographed; the hemolysate is dialyzed overnight against a selected tris-HCl developer. The chromatograms are developed at room temperature by applying a pH gradient to the column in the same manner as described for the CMC-chromatographic procedure; the flow-rates are kept at 14 to 16 ml per hr and the effluent is collected in 4 ml fractions. The gradient usually requires mixing of developers with pH values 7.9, 7.5, 7.1, 6.8, 6.5, with a developer

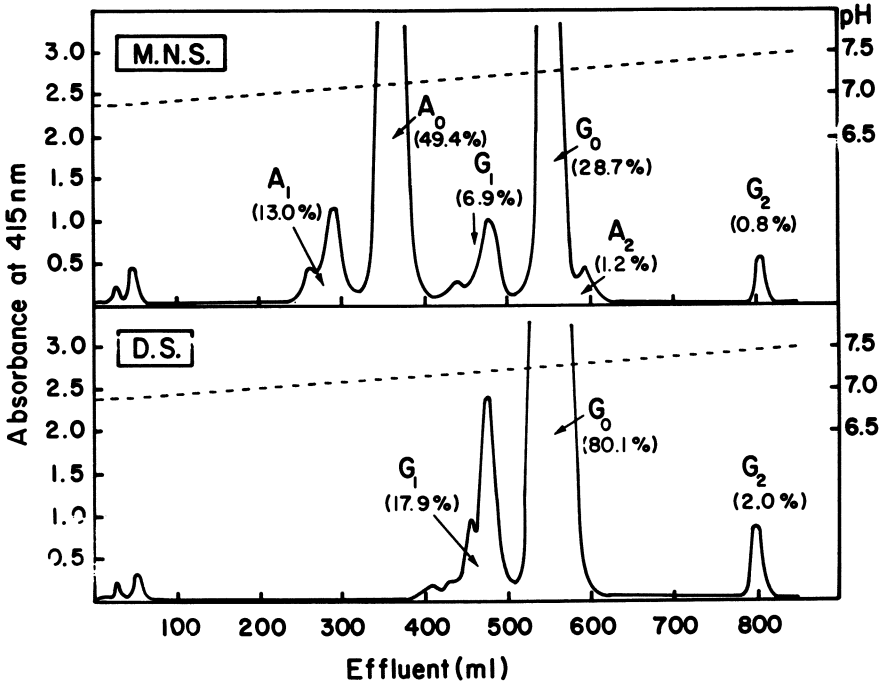


Figure 6. CM-cellulose chromatography of hemoglobin. Top panel: heterozygous Hb-G<sub>α</sub>. Note the incomplete separation of Hb-G<sub>α</sub> and Hb-A<sub>2</sub>. Bottom panel: homozygous Hb-G<sub>α</sub>. Note the complete absence of Hb-A. Hb-G<sub>α</sub> was identified as Hb-G-Philadelphia or α<sub>2</sub><sup>68</sup>Lysβ<sub>2</sub>. The broken line represents pH values of the effluent.



pH 8.3, contained in a constant mixing flask. The effluent fractions are analyzed for hemoglobin concentration by measuring the absorbance at 415 nm. The use of an open two-vessel gradient system might facilitate the operation without considerable loss in resolution of the hemoglobin components. The order in which the more common hemoglobins are eluted from the column is as follows: Hb-A<sub>2</sub>' or B<sub>2</sub>; Hb-A<sub>2</sub> and Hb-C which elute together; Hb-S; Hb-A<sub>0</sub>; Hb-A<sub>1</sub> + Hb-F<sub>0</sub>. With exceptions such as Hb-A<sub>2</sub> and Hb-C, Hb-A<sub>0</sub> and the minor variant Hb-S<sub>1</sub>, Hb-F<sub>0</sub> and the minor components Hb-A<sub>1</sub>, a complete separation of various hemoglobin types can be obtained.

Chromatography of hemoglobins on columns of DEAE-Cellulose (DE-52 microgranular, preswollen Whatman) often results in excellent separations of many variants, because the hemoglobins are eluted as sharp, narrow zones generally widely separated from each other. The hemoglobin zones are eluted from the DE-52 columns at a distinctly higher pH value than from a similar DEAE-Sephadex column.

Microchromatographic Methods. During the past two years rapid, inexpensive, miniaturized column chromatographic methods for the separation of hemoglobins have been developed. These methods are designed for the qualitative detection and quantitative determination of hemoglobins in normal and abnormal conditions and cover the quantitation of Hb-A<sub>2</sub>; the detection of Hb-S, Hb-C; other abnormal Hbs; differentiation of various conditions in adults; and the detection of hemoglobinopathies especially sickle cell anemia at birth (27, 28, 29, 30).

Conventional chromatographic procedures for hemoglobins have used columns about 1 to 10 cm in diameter and 20 to 100 cm in length for analytical and preparative purposes. However, microchromatographic procedures use small Pasteur pipets, 0.5 cm in diameter and only 8 cm long. The general procedure is as follows: after a plug of cotton has been lightly tapped into the restricted part of a Pasteur pipet, a 0.5 X 6 cm column is poured with the equilibrated ion exchanger. In special cases, the column may be 0.5 X 15 or 20 cm. The sample which may be whole blood or conventional hemolysate is diluted with water or appropriate buffer, placed on the column, allowed to run in, and then covered with the appropriate developer. The column is next attached by rubber tubing to a supply of developer in a funnel or manifold. Flow rates of developer are 20-40 ml per hr and procedures are usually completed in 30-120 min.

*Quantitative determination of Hemoglobin A<sub>2</sub> by microchromatography* - By means of this simple method (29, 30), Hb-A<sub>2</sub> may be rapidly and accurately determined in blood samples from subjects with and without Hb-S or Hb-D. The procedure makes use of DEAE-Cellulose, inexpensive laboratory glassware, and allows one technician to determine the level of Hb-A<sub>2</sub> in 50 samples a day. Whole blood samples, red cell hemolysates, and blood

collected on filter paper can be analyzed with comparable accuracy. The anion exchanger, diethylaminoethylcellulose, is equilibrated by mixing with an equal volume of 0.05 M tris-HCl buffer, pH 8.5, which contains 100 mg KCN/1000 ml. After nearly all material has settled, the supernatant with the fines are removed, and the equilibration procedure is repeated three times. The DE-52 is stored as a slurry; the supernatant volume of buffer is about 0.7 of that of the settled anion exchanger. A 6 to 7 cm column is prepared by pipetting the DE-52 cellulose slurry with a Pasteur pipet. One drop of a 10 g% hemoglobin solution is diluted with 5 to 6 drops of distilled water and applied on the top of the column. Whole blood or blood spots collected on filter paper can also be used. The column is developed with 0.05 M tris-HCl developers, pH 8.3, containing 100 mg KCN/l. The Hb-A<sub>2</sub> fraction is eluted as a sharp band in 6 to 8 ml and is collected in a tube graduated at 10 ml. The remaining hemoglobin is eluted with a second developer (0.05 M tris-HCl, pH 7.0, containing 100 mg KCN/l) and collected in a tube graduated at 25 ml. The volumes of the two fractions are adjusted to 10 and 25 ml, respectively, and the optical densities determined at 415 nm. The procedure is also suitable for the quantitation of Hb-A<sub>2</sub> in hemolysates containing Hb-S using a 0.5 X 15 cm column. This procedure requires that the Hb-A<sub>2</sub> zone is eluted with pH 8.35 developer, Hb-S with pH 8.20 developer, and the remaining hemoglobin with pH 7.0 developer.

A modification of the microchromatographic procedure for Hb-A<sub>2</sub> which utilizes DEAE-Cellulose allows the quantitation of Hb-A<sub>2</sub> without interference from any Hb-S in the sample. Elution of Hb-A<sub>2</sub> with a 0.2 M glycine-0.01% KCN developer is much less sensitive to minor changes in pH of the developer, but is greatly dependent on the pH of the ion exchanger. About 100 gm of DEAE-Cellulose is mixed with sufficient developer to give a total volume of 400 ml. This procedure is repeated twice whereafter the ion exchanger is resuspended in fresh developer. After stirring for 10 min, the pH of the stirred *suspension* is adjusted to 7.5 with about 12-15 mEq of HCl. After settling, some supernatant fluid is removed so that the volumes of settled ion exchanger and supernatant fluid are about equal. Next, a test chromatogram is made with a fresh sample from a Hb-S heterozygote. If Hb-A<sub>2</sub> does not emerge in the first 3-4 ml and Hb-S in the next 15-20 ml of the developer, the pH of the suspension is readjusted under stirring and another test is made. If necessary, further reduction of pH and testing is made until the "correct" pH is reached. Subsequent preparations of DE-52 should be adjusted to this pH. If the pH has decreased or has been adjusted to too low value it cannot satisfactorily be raised to the proper value.

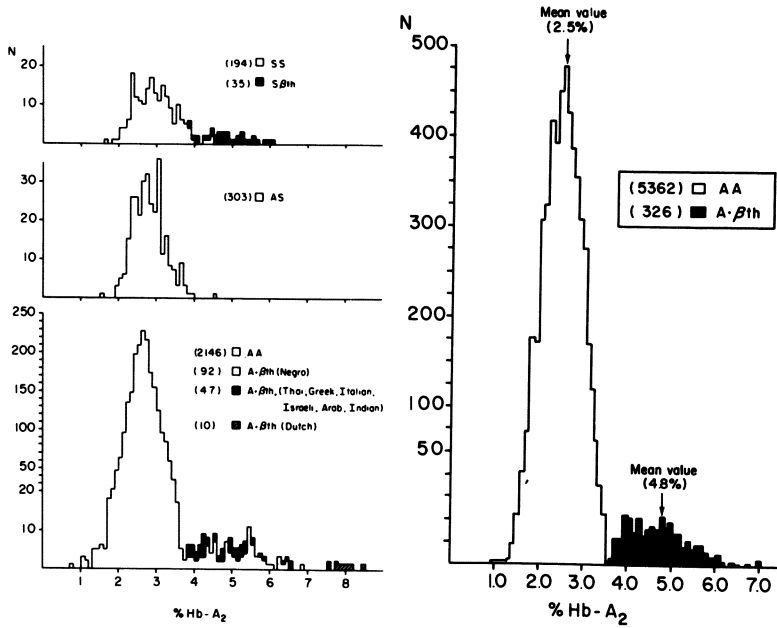
More than 15,000 samples have now been studied by these methods, and application of these methods has been made in the Augusta area, in California, in Yugoslavia, and in Ghana. The total number of individuals participating in the study in

Georgia was more than 5,000. The majority participated in a testing program in a school district. Others were from subjects participating in testing programs in other areas in the USA or abroad; included are 10 subjects from Holland with a heterozygosity for the Dutch type of  $\beta$ -thalassemia. Figure 7A presents some of the Hb-A<sub>2</sub> data. Differentiation of A- $\beta$ -thalassemia from AA, and of S- $\beta$ -thalassemia from SS can readily be made and in only a few known SS patients did the Hb-A<sub>2</sub> levels fall within the S- $\beta$ -thalassemia range. The Hb-A<sub>2</sub> level in the Dutch  $\beta$ -thalassemia heterozygote is considerably higher than that of persons with the same condition but from another ethnic origin. It is worth noting that the Hb-A<sub>2</sub> levels in persons with AS or SS are only slightly higher than those in normal persons.

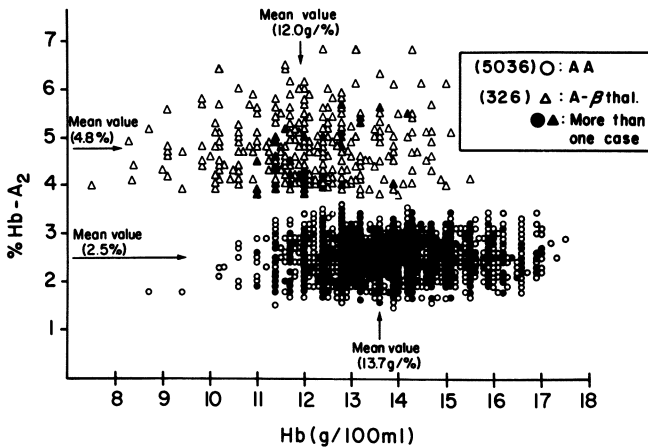
The studies in Sr. Macedonia, Yugoslavia, were only concerned with the evaluation of the usefulness of the microchromatographic procedure for the determination of Hb-A<sub>2</sub> and thus for the detection of the  $\beta$ -thalassemia heterozygote. Nearly 6,000 persons participated in the study. All participants were school children from five Macedonian cities who voluntarily participated in these testing programs; parents and relatives of known patients with a  $\beta$ -thalassemia homozygosity are also included in this review. Figure 7B gives the Hb-A<sub>2</sub> levels in all subjects; no overlap between the values of the normal persons and those of the  $\beta$ -thalassemia heterozygotes was apparent. The values are comparable to those found in the survey in Georgia (Figure 7A). Figure 8 presents the relationship between the hemoglobin level and the Hb-A<sub>2</sub> level in more than 5,000 normal adults and in 326  $\beta$ -thalassemia heterozygotes. Again no overlap is evident. The  $\beta$ -thalassemia heterozygote could readily be identified by the elevated level of the Hb-A<sub>2</sub> alone, although a slightly decreased Hb level and a considerable decrease in the MCH level were helpful observations.

Hb-A<sub>2</sub> can also be quantitated by electrophoresis. The most accurate procedures are starch block electrophoresis, cellulose acetate electrophoresis, and isoelectric focusing.

*Microchromatographic Testing Methods for Sickle Cell Anemia and Related Disorders in the Adult.* The procedures described in the preceding Section are, in fact, applicable to the detection of hemoglobinopathies in the adult, and any quantitative determination of Hb-A<sub>2</sub> immediately becomes a qualitative test for abnormal Hb's. If, instead of the very small zone of Hb-A<sub>2</sub>, a prominent zone moves in the same way, Hb-C (or Hb-E) is indicated. Hb-S (or D), if present, is readily apparent as is Hb-A although Hb-F in large percentages might be confused with Hb-A. The quantitative procedure for Hb-A<sub>2</sub> has been used as a qualitative test for Hb-S and Hb-C under suboptimal conditions in Ghana. Of the 2,350 samples that were studied there, 2,005 could be reevaluated by starch gel electrophoresis. The same identification by both methods was made in 98.9% of the cases. Misidentifications were due to such technical problems as 1) changes in pH of buffer because of the growth of mold; 2) high temperature (33°C)



**Figure 7.** Microchromatography on DEAE-cellulose. *A*, The levels of Hb-A<sub>2</sub> in normal adults, in β thalassemia heterozygotes, in Hb-S traits, and in patients with sickle cell anemia and Hb-S-β-thalassemia (Georgia survey). *B*, The levels of Hb-A<sub>2</sub> in normal adults and in β-thalassemia heterozygotes (Macedonia survey).



**Figure 8.** Microchromatography on DEAE-cellulose. The relationship between the total hemoglobin levels and the levels of Hb-A<sub>2</sub> (Macedonia survey).

and high relative humidity (80-90%) which sometimes caused erratic behavior of the hemoglobins; and 3) lack of a pH meter which required that the proper chromatographic conditions be attained by repeated chromatograms of a control sample until correct behavior was obtained. Despite these suboptimal conditions, only 1% of the identifications were in error. Table I summarizes some of the data. At least 25% of the participants had a hemoglobinopathy. The testing programs in the village population and the school district did not uncover additional sickle cell anemia patients or patients with SC disease; these patients were already seen by the physician of the hospital. The method has also been used in the Augusta area to quantitate Hb-S in nearly 300 AS cases and Hb-C + A<sub>2</sub> in 150 AC cases. Percentages were comparable to those by more conventional chromatographic methods.

These microchromatographic procedures allow:

- a. A rapid and accurate identification of the most common hemoglobin variants AS, AC, SS, SC, and CC.
- b. A rapid and accurate quantitation of Hb-A<sub>2</sub> in samples from normal persons or from  $\beta$ -thalassemia heterozygotes.
- c. A slight modification will allow the quantitation of Hb-A<sub>2</sub> in Hb-S (or Hb-D) containing samples, and will aid in the differentiation between SS and S- $\beta$ -thalassemia.
- d. Samples to be studied can be analyzed as whole blood, red cell hemolysate, or as blood collected on filter paper. These samples should preferably be kept in the cold and in the dark, and should be studied within 20 to 30 days after collection.
- e. The method can readily be adapted to testing under less optimal conditions when identification of variants by electrophoresis is not possible.
- f. The microchromatographic procedure does not permit the identification of "fast-moving" variants nor does it separate the hemoglobins A and F. However, a combination of two methods, starch gel electrophoresis and microchromatography on columns of DEAE-Cellulose, will allow the identification of many different hemoglobin variants in various combinations.

*Cord Blood Testing for Sickle Cell Anemia and Related Disorders by Microchromatographic Methods.* The CM-Sephadex procedure easily detects Hb-S and Hb-C at birth but the diffuse zone of Hb-A has on occasion been difficult to see. Substitution of CM-Cellulose for CM-Sephadex has yielded a superior microchromatographic method, and the compact, well-defined zones of the CM-Cellulose column facilitate the interpretation of the results even though the amount of sample is only 20% as great. The CM-Cellulose method is as simple and rapid as the original CM-Sephadex procedure (27, 28).

Developer A is 0.03 M tris-HCl; 0.025 M NaCl; 0.01% KCN at pH 6.1 and Developer B is 0.03 M tris-HCl; 0.04 M NaCl; 0.01% KCN at pH 6.3. CM-Cellulose (CM-52, microgranular and preswollen,

**TABLE I**  
**Data in Number of Cases Obtained by Microchromatography**  
**During a Survey in Ghana**

|                                       | AA<br>(%)          | AS<br>(%)         | AC<br>(%)         | SS        | CC       | SC        | Oth-<br>ers |
|---------------------------------------|--------------------|-------------------|-------------------|-----------|----------|-----------|-------------|
| Village<br>(4)                        | 378 (77.9)         | 71 (14.6)         | 34 ( 7.0)         | 0         | 2        | 0         | 0           |
| School<br>(1)                         | 519 (72.4)         | 117 (16.3)        | 79 (11.0)         | 0         | 1        | 1         | 0           |
| Hospital<br>Clinics                   | 828 (72.1)         | 173 (15.1)        | 107 ( 9.3)        | 15        | 4        | 21        | 0           |
| <b>Total<br/>(n=2350)</b>             | <b>1725 (73.4)</b> | <b>361 (15.4)</b> | <b>220 ( 9.4)</b> | <b>15</b> | <b>7</b> | <b>22</b> | <b>0</b>    |
| <b>By Starch Gel Electrophoresis:</b> |                    |                   |                   |           |          |           |             |
| <b>Total<br/>*(n=2005)</b>            | <b>1433 (71.5)</b> | <b>317 (15.8)</b> | <b>175 ( 8.7)</b> | <b>8</b>  | <b>7</b> | <b>23</b> | <b>42**</b> |

\* Microchromatographic analyses were made in Ghana, and starch gel electrophoresis in the USA. 112 samples lost in transport; 145 samples not mailed; 88 samples not identifiable.

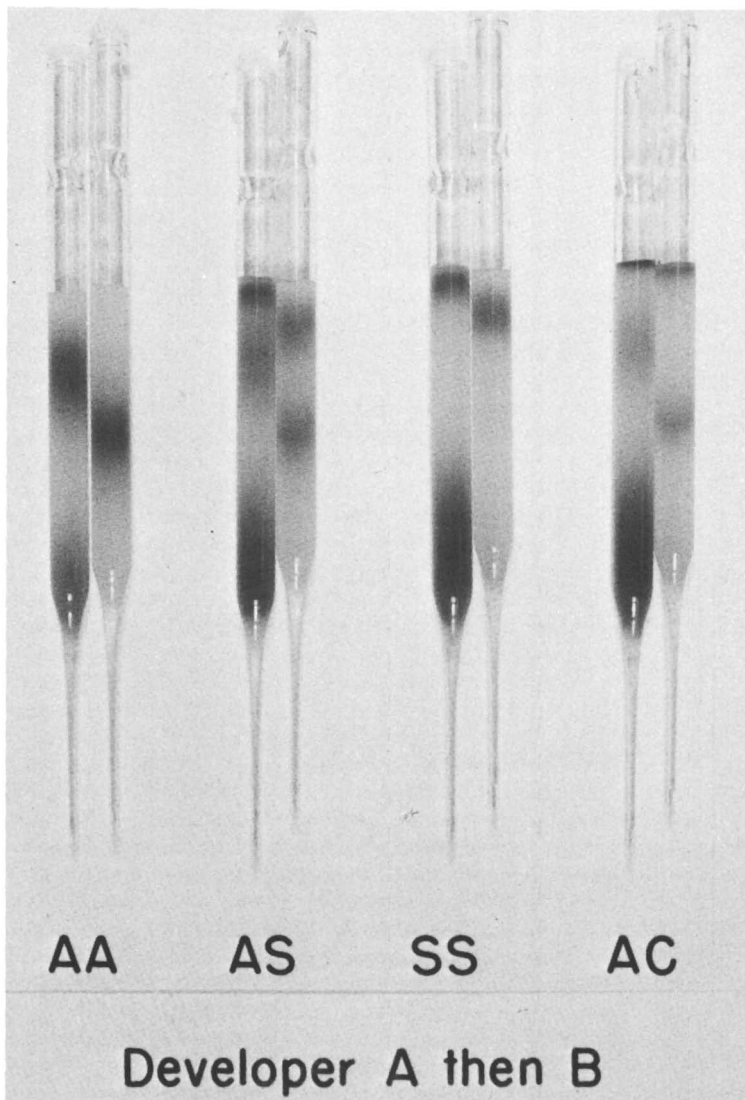
\*\* 32 cases with A-HPFH; 8 cases with Hb-A<sub>2</sub>' heterozygosity; 1 case with AN and 1 case with SN.

Reeve Angel and Co., Clifton, N.J. 07104) is equilibrated with Developer A, and the pH of the stirred suspension is adjusted to 6.1 with 6 N hydrochloric acid. Several settlings in Developer A should be made in order to remove fine particles. Supernatant buffer and settled resin should be equal in volume for the slurry that is used to pour the column. One column, 6 cm in length, requires about one gram of CM-52. A 0.02 ml portion of blood, 0.3 ml of Developer A, and 0.2 ml of 0.004 M maleic acid are mixed, and hemolysis is carried out for five minutes at room temperature. This sample is carefully applied without disturbing the top of the column and allowed to flow in. The tube above the column is filled with Developer A, and the column is attached with rubber tubing to a manifold. The chromatogram is developed with 40-50 ml of Developer A with a liquid head of about 50-60 cm to give a flow rate of approximately 20-25 ml per hour. If a strongly fixed hemoglobin is present after this development, the solvent above the column is replaced by Developer B and 6-8 ml are passed through the column.

The appearance of chromatograms after the development with Developer A is shown by the left chromatogram of each pair in Figure 9 for cord bloods from AA, AS, SS, and AC children. The Hb-F in the sample remains partly on the columns, but some is in the effluent. Hb-A forms a zone with limits between about 10 and 25 mm from the top whereas Hb-S is in a 2-3 mm zone that has barely moved from the top and Hb-C remains in the upper 0.5-1 mm. The hemoglobin zones are compact and well visible against the white of the CM-Cellulose column. Hb-S and Hb-C may be distinguished by the fact that Hb-C is very strongly fixed whereas Hb-S moves slightly down the column. The conclusion is substantiated by change to Developer B with the result seen in the right chromatogram of each pair in Figure 9. Because tris has only meager buffering action at pH 6.1, fluctuations in the pH of ion exchanger and solutions may occur and may result in abnormal chromatographic behavior. Buffered conditions may be obtained by substituting bis-tris which has a  $pK_a$  of about 6.5, and virtually identical behavior results if Developer A is constituted with 0.03 M bis-tris-HCl; 0.03 M NaCl; 0.01% KCN at pH 6.2.

The validity of diagnosis by this technique has been examined by comparing more than 2,000 samples by the CM-Cellulose procedure, the original CM-Sephadex procedure, and by starch gel electrophoresis. It is interesting that occasionally the AS condition at birth is not diagnosed by the electrophoretic technique.

The Determination of Fetal Hemoglobin (Hb-F). Specific properties of Hb-F have given rise to various methods for its detection and quantitation which are based on electrophoretic and chromatographic mobilities of Hb-F, on its slow rate of



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*Figure 9. Chromatograms of cord blood with the indicated conditions on  $0.5 \times 6$  cm columns of CM-cellulose. The left-hand member of each pair shows the result after completion of chromatography with Developer A. The right-hand member shows the added movement that occurs with subsequent use of Developer B and thereby distinguishes Hb-S and Hb-C (28).*



denaturation at pH 10.5-11, its specific ultraviolet spectral absorption, and on its immunological specificity. The method used for the demonstration of Hb-F in the red cells is based on the difference in rate of dissociation into subunits that exists between the hemoglobins A and F at pH values below 4.

The *acid elution technique* is a useful tool for the demonstration of increased amounts of Hb-F as well as for the evaluation of the distribution of Hb-F in red cells. The method is described in detail in references 31, 32, and 33. Cells containing Hb-F are densely stained with erythrosin and cells with Hb-A appear as ghost cells; intermediate cells are stained more or less pink. Reticulocytes with Hb-A sometimes resemble intermediate cells and may also show some intracellular granulation. Inclusion bodies are visible in eluted cells as compact particles of differing sizes. Figure 10 gives some examples. The method is ideally suited to demonstrate the presence of newborn red cells in the maternal circulation. The method is also widely used for the evaluation of the distribution of Hb-F within red cells, mainly to differentiate between the HPFH condition and the  $\beta$ - or  $\beta\delta$ -thalassemias. Evaluation of F cell smears in such cases is difficult; the term "equal distribution" usually indicates the presence of Hb-F in each red cell but not necessarily in the same amount.

The laboratory quantitation of Hb-F by *alkali denaturation* ( $\%F_{AD}$ ) is one of the more difficult routine procedures. Accurate and reproducible results are often achieved only in laboratories where the procedures are in daily use. Three methods are available which are discussed in detail in references 24 and 25. Comparative studies of these three procedures have demonstrated that the method of Betke *et al* (34) is the most reliable for the quantitation of low percentages of Hb-F (below 10 to 15%).

Hb-F can also be separated from Hb-A and many of its variants by both *anion- and cation-chromatography*. However, in most instances Hb-F elutes together with the minor adult hemoglobin Hb-A<sub>1</sub> thus preventing its accurate quantitation. Recently, a method has been devised (35) which is based on the fact that the  $\alpha\gamma$  dimer contains four isoleucyl residues, 35 leucyl and 15 phenylalanyl residues, whereas the  $\alpha\beta$  dimer contains no isoleucine, 36 leucyl and 15 phenylalanyl residues. Thus, the determination of the content of isoleucine in relation to leucine and phenylalanine can be used as a measure of the relative amount of Hb-F in mixtures. The procedure involves the chromatographic isolation of the Hb-F containing zone and the determination of the isoleucyl, and phenylalanyl content in a 72 hour acid hydrolysate of the hemoglobins in this zone. This procedure is presently the most accurate method for the quantitation of Hb-F. However, it has the great disadvantage of being laborious and not easily applicable to clinical problems. The development of new microchromatographic procedures,

using either DE-52 columns or CM-Sephadex columns, might be important particularly when these techniques can be simplified and used in routine analyses.

Detection of Sickling Hemoglobins. *Microscopic methods* will aid in detecting Hb-S and related variants with the same structural abnormality such as Hb-C-Harlem because these hemoglobins have greatly decreased solubility in the deoxygenated state which causes a distortion of the cell membrane. The cells generally take on an oak leaf or maple leaf shape and under prolonged deoxygenation the sickle shape appears. The procedure has been done in many ways usually using fingertip blood. The blood is covered with a cover glass, sealed with petroleum jelly, and examined for sickling properties after 2 and 24 hr, or a reducing agent is added (usually a freshly prepared 2% solution of sodium metabisulfite is mixed in excess with blood and allowed to stand for 5 min). The methods are simple and popular but are subject to a number of errors. False results can be obtained when blood is drawn within 4 months after transfusion, when it is old, exposed to heat, contaminated with bacteria, or allowed to dry during testing. Abnormally shaped erythrocytes can easily be confused for sickling types. Hb-F when present in sufficient quantity and in every cell will inhibit sickling. Plasma is essential to demonstrate sickling; prolonged washing of cells with 0.9 g% NaCl will inhibit sickling.

The *solubility test*, which is specific for the demonstration of Hb-S or Hb-C-Harlem, was originally developed by Itano in 1953 (36), and many modifications, both bad and good, have been published (for references see (25)). All are based on the original principle that deoxy-Hb-S is less soluble in concentrated phosphate buffers than are all other hemoglobins. Many of the new procedures have added saponin to the buffers so that whole blood can be used. The most simple method uses these buffers: a potassium phosphate buffer, pH 6.5 and 2.50 M, and a phosphate buffer, 1.00 M, which is prepared by the proper dilution of the 2.50 M phosphate buffer. About 1 ml each of the 2 buffers are placed in separate test tubes. One or two drops of hemolysate are pipetted in each tube, and, after mixing, 3-5 mg  $\text{Na}_2\text{S}_2\text{O}_4$  are added to deoxygenate the hemoglobin. When Hb-S is present the content of the tube with the 2.50 M phosphate buffer will become turbid while that of the tube with the 1.00 M phosphate buffer remains clear. This method has not given false-positive or false-negative results in over 50,000 samples tested and checked by electrophoretic procedures. The use of whole blood shortens the procedure considerably but it also introduces additional errors due mainly to the presence of cellular debris, low hemoglobin concentrations in blood samples from patients with a severe anemia, and high levels of plasma proteins in conditions such as multiple myeloma and others. The use of hemolysate also eliminates the false-positive readings seen in samples from

patients with an unstable hemoglobin variant when Heinz bodies are present in the erythrocytes.

The *mechanical instability of oxy-hemoglobin S* has been used as a new screening method for Hb-S. Such a test does not exclude unstable hemoglobins and other variants with even a slightly decreased stability and will likely not distinguish between heterozygotes and homozygotes.

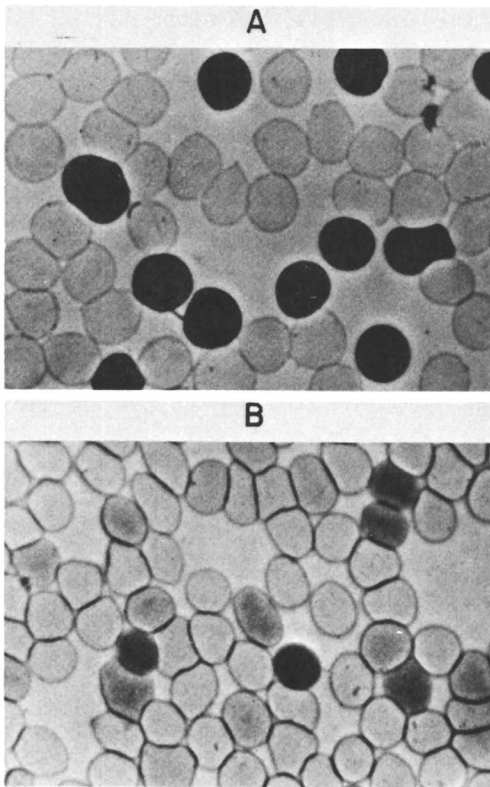
Detection of Unstable Hemoglobins. The stability of the hemoglobin molecule depends to a great extent on bonds between the chains, between chains and the heme groups, and on bonds within the chains. Thus, when the replacement of an amino acid residue affects one of these many bonds, the stability of the molecule can be impaired. The hemoglobin readily precipitates in the red cell and *intraerythrocytic inclusion bodies* can appear leading to increased cell destruction with subsequent anemia. These bodies are more easily observed in a blood sample from patients with such an anomaly after the spleen has been removed; Figure 11 demonstrates Heinz bodies in the blood of such a patient.

Other tests useful for the detection of unstable hemoglobins are the heat stability test, the isopropanol precipitation test, and the PCMB or PMB precipitation test.

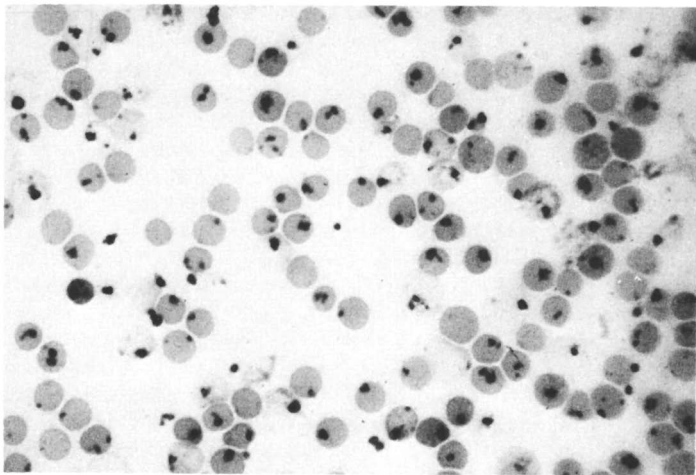
The *heat stability test* was first applied to blood from patients with Hb-H disease and later to patients with Heinz body anemias. In this test the heat stability of the hemoglobin variant in hemolysate is compared with that of a normal control. The hemolysates are incubated in a phosphate buffer at 60°C to 70°C, and hemoglobin precipitation evaluated either visually or spectrophotometrically. Precipitation of normal hemoglobin is minimal when incubated at 60°C for 30 minutes, but many unstable hemoglobins are almost completely denaturated under these conditions. Figure 12 gives some examples.

The *Isopropanol precipitation test* is a simple screening test, recently described by Carrell and Kay (37). Hemolysate is mixed with an isopropanol-tris-buffer, pH 7.4, incubated at 37°C for various times and the hemoglobin precipitation evaluated either visually or spectrophotometrically. Technical details are also given in reference 25.

The *PCMB precipitation test* is a most useful test for the detection of an unstable hemoglobin and is based on reaction of hemoglobin with p-chloromercuribenzoate (PCMB). PCMB was first used by Bucci and Fronticelli (38) for the isolation of native hemoglobin chains. Modification of their method has broadened its applicability to hemoglobin variants that dissociate more readily. When normal hemoglobin reacts with PCMB the protein dissociates into subunits but no precipitation occurs. However, many unstable hemoglobins dissociate more readily when exposed to PCMB and often the abnormal chain precipitates. Electrophoretic examination of the PCMB treated hemolysate will,



*Figure 10. Red cell smears after the application of the acid elution technique. A, artificial mixture of cells from newborn and adult. B, blood sample from a patient with Fanconi's anemia (Hb-F<sub>AD</sub>: 14.6%).*



*Figure 11. The formation of Heinz bodies in a subject with an unstable hemoglobin (courtesy of Dr. E. Kleihauer, Ulm, Germany)*

therefore, allow evaluation of the extent of the dissociation and of the precipitation of the unstable subunit. The same method can be used on a preparative scale for the isolation of a large quantity of the abnormal chain.

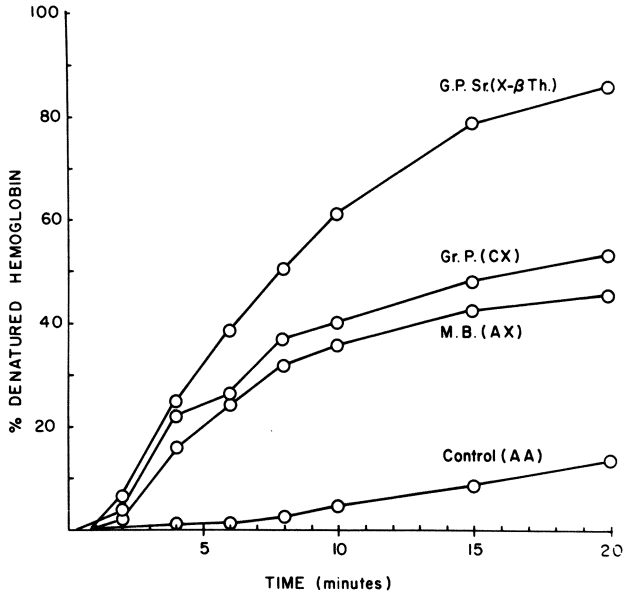
Unstable hemoglobin variants are often found in persons with unexplained hemolytic anemias. Below are some guidelines for their detection.

1. Red cells of a freshly collected blood sample will form Heinz bodies in the presence or absence of phenylhydrazine in a greater number than red cells of a similar blood sample from a normal control.
2. The stability of hemoglobin in a freshly prepared red cell hemolysate at temperatures varying between 50° and 70° is distinctly less than that of a normal control. The unstable variant will also show decreased stability at 37° in the presence of the isopropanol-tris buffer, pH 7.4.
3. An increased dissociation of the hemoglobin molecule to monomers can be demonstrated after treatment with p-chloromercuribenzoate; often, the unstable subunit will precipitate irreversibly.
4. Free  $\alpha$ - and  $\beta$ -chains can be demonstrated in a freshly prepared hemolysate using starch gel electrophoresis at alkaline pH.
5. Often unstable hemoglobins have a decreased number of heme groups. This number can be calculated from the optical densities of a solution of the cyanferri derivative of the isolated variant at 540 and at 280 nm using normal Hb-A as control.

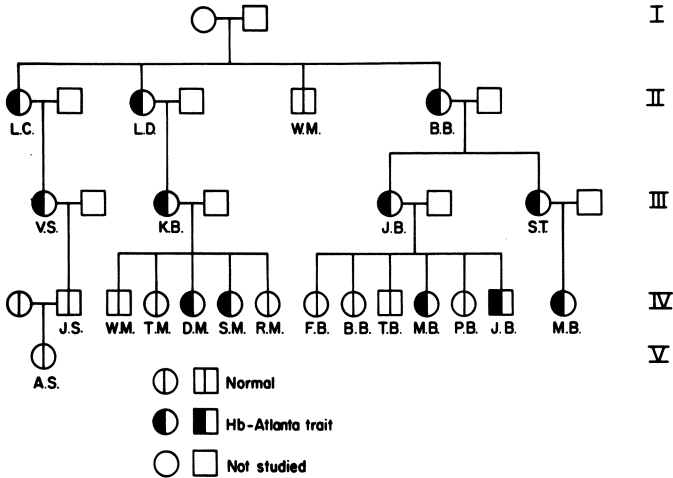
Unstable variants are often found in a single member of a family, and are the result of a new mutation. However, occasionally many members in a large family are heterozygous for such a variant; these persons are not easily identified unless some specific techniques are used. A typical example is the family with Hb-Atlanta, a recently discovered variant in which leucyl residue in position 75 (E19) is replaced by a prolyl residue (39). Figure 13 presents the pedigree of the family in which twelve members in three generations carry the variant. Hematological data are given in Table II; only a mild hemolytic anemia is present in the heterozygotes. The presence of the variant was confirmed by four instability tests.

#### Detection of Variants With Altered Functional Properties.

When substitutions in either  $\alpha$ - or  $\beta$ -chains involve amino acid residues that participate in the contact with heme or the contact between chains, changes in functional properties can occur and the determination of the oxygen affinity of the blood sample or of an isolated hemoglobin variant is desirable. Oxygen affinity is affected by temperature, pH, salt concentration, the level of 2,3-diphosphoglycerate (2,3-DPG), and to a lesser extent by the concentration of the hemoglobin. The concentration of 2,3-DPG in blood changes rather rapidly after collection and a



**Figure 12.** Heat denaturation curves of hemoglobin from three members of a family with Hb-Leslie, a newly discovered unstable variant with a deletion of residue 131 of the β-chain. G.P.Sr. has Hb-Leslie-β-thalassemia; the %Hb-Leslie is 85% (DEAE-Sephadex chromatography); Gr.P. has Hb-Leslie-Hb-C; %Hb-Leslie is 28%; M.B. has Hb-Leslie trait; %Hb-Leslie is 28%.



**Figure 13.** The pedigree of a Caucasian family with Hb-Atlanta or  $\alpha_2\beta_2^{75}Leu \rightarrow Pro(E19)$

**TABLE II**  
**Hematological and Hemoglobin Stability Data on 22 Members of the Family with Hb-Atlanta**

| Case <sup>a</sup> | Condi-<br>tion <sup>b</sup> | WBC<br>10 <sup>3</sup> /mm <sup>3</sup> | Hb<br>g% | PCV<br>% | RBC<br>10 <sup>6</sup> /mm <sup>3</sup> | MCV<br>μ <sup>3</sup> | MCH<br>μg | MCHC<br>% | Heat <sup>c</sup><br>Stabil-<br>ity | Iso-C<br>pro-<br>panol | Free <sup>c</sup><br>α<br>Chains | Heinz <sup>c</sup><br>Bodies |
|-------------------|-----------------------------|---|----------|----------|---|-----------------------|-----------|-----------|-------------------------------------|------------------------|----------------------------------|------------------------------|
| II. L.C.          | AX                          | -                                       | 9.6      | 31       | 3.35                                    | 91                    | 29        | 31        | +                                   | +                      | +                                | +                            |
| L.D.              | AX                          | 11.6                                    | 11.6     | 36.5     | 4.05                                    | 88                    | 28.5      | 32        | +                                   | +                      | +                                | +                            |
| W.M.              | AA                          | 6.0                                     | 14.3     | 44       | 5.48                                    | 81                    | 25.5      | 31.5      | -                                   | -                      | -                                | -                            |
| B.B.              | AX                          | 7.6                                     | 11.4     | 36       | 3.75                                    | 98                    | 32        | 32.5      | +                                   | n.d.                   | +                                | +                            |
| III. V.S.         | AX                          | 6.1                                     | 10.6     | 33.5     | 3.65                                    | 89                    | 29        | 32.5      | +                                   | +                      | +                                | +                            |
| K.B.              | AX                          | 7.2                                     | 12.4     | 37       | 4.06                                    | 92                    | 31        | 33.5      | +                                   | +                      | +                                | +                            |
| J.B.              | AX                          | 6.8                                     | 11.9     | 38       | 4.35                                    | 88                    | 29        | 32.5      | +                                   | n.d.                   | +                                | +                            |
| S.T.              | AX                          | 11.1                                    | 11.3     | 37.5     | 4.40                                    | 86                    | 27        | 31        | +                                   | n.d.                   | +                                | +                            |
| IV. J.S.          | AA                          | 7.9                                     | 16.0     | 47.5     | 5.38                                    | 87                    | 29.5      | 33.5      | -                                   | n.d.                   | -                                | -                            |
| W.M.              | AA                          | 7.1                                     | 14.1     | 43.5     | 5.19                                    | 83                    | 27.5      | 32.5      | -                                   | n.d.                   | -                                | n.d.                         |
| T.M.              | AA                          | 7.3                                     | 12.9     | 38       | 4.75                                    | 81                    | 27.5      | 34        | -                                   | n.d.                   | -                                | n.d.                         |
| D.M.              | AX                          | 11.0                                    | 11.8     | 37       | 4.24                                    | 85                    | 28        | 32        | +                                   | +                      | +                                | +                            |
| S.M.              | AX                          | 7.7                                     | 13.3     | 40       | 4.01                                    | 98                    | 33.5      | 33.5      | +                                   | +                      | +                                | +                            |
| R.M.              | AA                          | 7.9                                     | 13.0     | 38.5     | 4.68                                    | 81                    | 28        | 34        | -                                   | -                      | -                                | -                            |

TABLE II - Continued  
 Hematological and Hemoglobin Stability Data on 22 Members of the Family with Hb-Atlanta

| Case <sup>a</sup> | Condi-<br>tion <sup>b</sup> | WBC<br>10 <sup>3</sup> /mm <sup>3</sup> | Hb<br>g% | PCV<br>% | RBC<br>10 <sup>6</sup> /mm <sup>3</sup> | MCV<br>μ <sup>3</sup> | MCH<br>μg | MCHC<br>% | Heat <sup>c</sup><br>Stabil-<br>ity | Iso- <sup>c</sup><br>pro-<br>panol | Free <sup>c</sup><br>α<br>Chains | Heinz <sup>c</sup><br>Bodies |
|-------------------|-----------------------------|---|----------|----------|---|-----------------------|-----------|-----------|-------------------------------------|------------------------------------|----------------------------------|------------------------------|
| IV. F.B.          | AA                          | 7.1                                     | 11.8     | 36       | 4.56                                    | 80                    | 27.5      | 34        | -                                   | n.d.                               | -                                | -                            |
| B.B.              | AA                          | 6.8                                     | 14.1     | 42       | 4.95                                    | 85                    | 27.5      | 32.5      | -                                   | -                                  | -                                | -                            |
| T.B.              | AA                          | 5.9                                     | 14.6     | 43.5     | 5.00                                    | 87                    | 28        | 32.5      | -                                   | -                                  | -                                | -                            |
| M.B.              | AX                          | 8.2                                     | 10.9     | 34       | 3.86                                    | 87                    | 27        | 31        | +                                   | +                                  | +                                | +                            |
| P.B.              | AA                          | 7.0                                     | 13.0     | 39.5     | 4.50                                    | 87                    | 28        | 31        | -                                   | -                                  | -                                | -                            |
| J.B.              | AX                          | 7.3                                     | 11.3     | 35       | 3.99                                    | 87                    | 27.5      | 32        | +                                   | +                                  | +                                | +                            |
| M.B.              | AX                          | 10.2                                    | 9.2      | 29.5     | 3.98                                    | 73                    | 22.5      | 30.5      | n.d.                                | n.d.                               | +                                | +                            |
| V. A.S.           | AA                          | 6.3                                     | 11.9     | 35.5     | 4.33                                    | 81                    | 27.5      | 33.5      | -                                   | n.d.                               | -                                | -                            |

a. See Figure 13; ages of the children in generation IV vary between 1 and 26 years.

b. X refers to Hb-Atlanta

c. + refers to a positive test and - to a negative test; n.d.: not determined.



rapid method is desirable for the determination of its concentration. Red cell hemolysates can be stripped of 2,3-DPG by dialysis against 0.2 M NaCl for 24 hours followed by dialysis against a desired buffer, or by passage of the red cell hemolysate through a Sephadex G-25 column previously equilibrated with buffer used in the oxygen equilibrium analyses.

The oxygen content of blood and other test samples can be measured with the well-known Van Slyke apparatus or spectrophotometrically. Automated instruments for measuring the oxyhemoglobin content in blood are available; for instance the system IL517 of the Instrumentation Laboratory Inc., (Lexington, Mass.). This system consists of a gas mixing module, oxygen monitor, tonometer, digital pH/blood gas analyzer, and CO-oximeter, and allows the construction of an oxygen dissociation curve (eight equilibrations requiring 15 ml blood) in approximately 2 hrs. Figure 14 gives examples of two dissociation curves, both being determined in freshly collected blood samples at pH 7.38 and at 37°C. The curve of subject A is normal with a P<sub>50</sub> value of 26 mm Hg. That of subject B is most unusual, and its shape indicates the presence of two only partially interacting hemoglobin types one of which completely lacks subunit interaction. Subject B is a 20 year old black marine with a severe erythrocytosis; he has a normal 2,3-DPG level/g Hb, but is heterozygous for Hb-Fort Gordon, a  $\beta$  chain variant in which tyrosyl residue in position 145 is replaced by an aspartyl residue (unpublished data).

Detection of Met(Ferri-)Hemoglobins (Hb-M). Detection of these variants can be made by starch gel electrophoresis of the ferri-derivatives of hemoglobins in red cell hemolysate using a phosphate buffer, pH 7.0 (25). However, some methemoglobin variants can be separated from normal Hb-A at pH 9.0 (40). Nearly all methemoglobin variants exhibit absorption spectra that are different from that of normal ferrihemoglobin. The absorption spectra are determined after the hemolysate is dialyzed overnight at 4°C against an 0.1 M phosphate buffer, pH 6.4 or 7.1, using an automatic recording spectrophotometer. A total hemoglobin concentration of 0.6 to 0.8 mg/ml is required. Table III presents characteristic spectral maxima observed for the known methemoglobin variants. Some methemoglobin variants vary in the rate of reaction with KCN.

Structural Analyses of Hemoglobin Variants. It has become impossible to characterize nearly any abnormal hemoglobin by its electrophoretic and/or chromatographic mobility only. This is most strikingly demonstrated by the fact that over fifty different variants behave similar to Hb-S in electrophoresis. Characterization, therefore, often requires detailed structural analyses or the demonstration of a property unique to a specific variant. Some of the techniques used in these studies will be

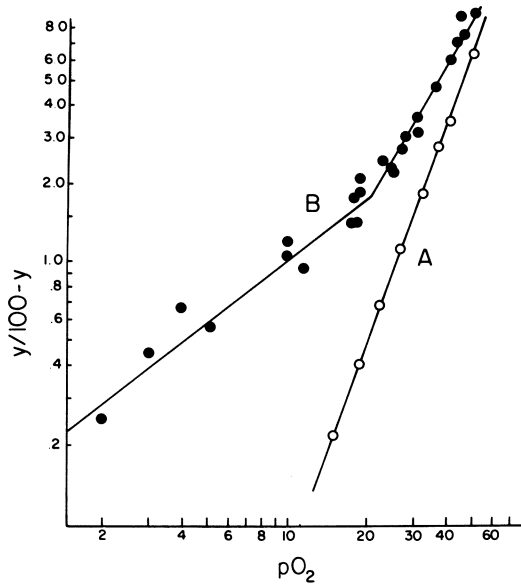


Figure 14. Oxygen equilibrium curves of freshly collected blood samples from the author (A) and from a patient with Hb-Fort Gordon with an increased oxygen affinity (B). Hb-Fort Gordon has been identified as  $\alpha_2\beta_2$  (145 Tyr  $\rightarrow$  Asp).

TABLE III

Spectral Characteristics of Methemoglobin Variants

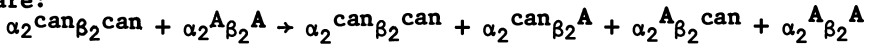
| Variants    | Amino Acid Substitution               | Spectroscopic Maxima at pH 7.1 | Velocity of -CN Reactivity and CN-Ferri-Hb Spectrum |
|-------------|---------------------------------------|--------------------------------|---|
| M-Boston    | $\alpha 58$ (E7)His $\rightarrow$ Tyr | 495,600 nm                     | Slow & incomplete                                   |
| M-Saskatoon | $\beta 63$ (E7)His $\rightarrow$ Tyr  | 490,602 nm                     | Fast, normal  |
| M-Iwate     | $\alpha 87$ (F8)His $\rightarrow$ Tyr | 490,610 nm                     | Fast, normal  |
| M-Hyde Park | $\beta 92$ (F8)His $\rightarrow$ Tyr  | 490,610 nm                     | Normal  |
| M-Milwaukee | $\beta 67$ (E11)Val $\rightarrow$ Glu | 500,622 nm                     | Fast, normal  |

mentioned in this section and examples of structural analyses of an  $\alpha$ -chain variant and a  $\beta$ -chain variant will be discussed.

*Structural information gained from electrophoretic procedures* will aid in detecting an  $\alpha$ -chain abnormality because such a variant will be present as two abnormal bands, one major abnormality ( $\alpha_2^X\beta_2$ ) and one minor abnormality ( $\alpha_2^X\delta_2$ ). The absence of the minor abnormality does not exclude an  $\alpha$ -chain abnormality since its electrophoretic mobility may be similar to that of a known normal hemoglobin type. The electrophoretic pattern in a subject heterozygous for the  $\beta$ -chain abnormality Hb-S or Hb-D and for the  $\delta$ -chain variant Hb-A<sub>2</sub> resembles closely that of a person who is heterozygous for a Hb-D that is abnormal in the  $\alpha$ -chain.

A detailed study of the structure of a hemoglobin variant still requires at least 100 to 200 mg of the pure protein. The protein can best be isolated by chromatographic techniques.

Identification of the abnormality in one of the two types of chains can be made by *hybridization* or by *electrophoretic identification* of the chains. The principle of the hybridization procedure is the formation of hybrid hemoglobins from a mixture of two hemoglobins by dissociation at low pH and subsequent random recombination of the subunits at neutral or slightly alkaline pH. Canine hemoglobin is well suited for hybridization experiments with human hemoglobin types. The final products from dissociation and recombination experiments of this hemoglobin ( $\alpha_2^{\text{can}}\beta_2^{\text{can}}$ ) with normal human Hb-A ( $\alpha_2^{\text{A}}\beta_2^{\text{A}}$ ) are:



The two hybrid hemoglobins are easily demonstrated by electrophoresis.

The presence of individual chains in a hemoglobin variant can also be demonstrated by electrophoresis at alkaline pH after the protein has been dissociated into its subunits through exposure to 6 M urea in the presence of  $\beta$ -mercaptoethanol. The buffer is either a barbital buffer or a tris-EDTA-boric acid buffer, pH 8.0 - 8.6, and contains 6 M urea and  $\beta$ -mercaptoethanol. Dissociation of the hemoglobin into subunits is best accomplished in a mixture of 1 ml 10 g% Hb (or whole hemolysate), 4 ml 6 M urea barbital or tris-EDTA-boric acid buffer, and 1 to 1.5 ml  $\beta$ -mercaptoethanol. After 30 minutes to 1 hour the sample is subjected to cellulose acetate or starch gel electrophoresis. Each chain has a specific mobility and an alteration in electrophoretic mobility easily identifies the abnormal chain. Schneider recently published a detailed description of the technique (41).

After it has been determined which chain in the variant is aberrant, specific structural studies are required. Several procedures are available which differ from one laboratory to the other and include chain separation by column chromatography, modification of the sulfhydryl groups through reaction with

ethylenimine, digestion of the chain with proteolytic enzymes, separation of the resulting peptides by column chromatography or by fingerprinting, and lastly, amino acid analyses and sequential analyses. No detailed description of these procedures will be given; instead, the structural analyses of two variants will be discussed in some detail as examples of approaches that can be followed.

Structural Analyses of Hb-St. Claude or  $\alpha_2^{127}(\text{H10})\text{Lys}\rightarrow\text{Thr}\beta_2$   
(reference 42)

This  $\alpha$  chain variant was found in a 47 year old healthy male under investigation for a chronic low-back complaint. Routine hematological investigation showed a hemoglobin concentration of 15.6 g/100 ml, a packed red cell volume of 45%, normal erythrocyte morphology, and no indication of excessive hemolysis. The same abnormal hemoglobin was found in one son. The propositus is one of ten children living in various parts of Canada. While conducting the family study, it was discovered that another part of this family (including the father, a brother, and a niece of the propositus) was found by other investigators to have a Hb-J $_{\alpha}$  with an amino acid substitution thought to reside in the core.

This electrophoretically fast-moving variant was readily isolated by DEAE-Sephadex chromatography. Hybridization analyses with canine Hb confirmed the suggestion that the abnormality was located in the  $\alpha$ -chain. The  $\alpha$ -chain was separated from the  $\beta$ -chain on 1.7 X 15 cm columns of CM-Cellulose using the method of Clegg *et al* (43). The CM-Cellulose used was CM-52 (Reeve Angel, Clifton, N.J.) and the developers were 8 M urea- $\beta$ -mercaptoethanol phosphate buffers, pH 6.7 - 7.1. Figure 15 shows the separation obtained. The zone containing the  $\alpha$ -St. Claude chain was passed through 2.5 x 50 cm columns of Sephadex G-25 in 0.5% formic acid to remove the urea, and the protein zone isolated and recovered by freeze-drying. The  $\alpha$  chain was digested with trypsin (Worthington Biochemical Corp., TPCK trypsin) for 6 hours at room temperature in a pH stat at pH 8.9. Trypsin equal to 0.5% of the substrate by weight was added at 0 and at 1 hr. The pH of the digest was lowered to 6.5, and the insoluble core separated by centrifugation and stored.

The soluble tryptic peptides of 130 mg  $\alpha$  chain of Hb-St. Claude were separated on 0.9 x 60 cm columns of Chromobead resin type P (Technicon Instruments, Dowex 50-X4) at 37°C using the procedure described earlier (16). The method uses a gradient of volatile pyridine-acetic acid buffers of differing molarities and pH as follows: first gradient, 666 ml 0.1 M, pH 3.1, and 333 ml 1.0 M, pH 5.0; and second gradient, 166 ml 1.0 M, pH 5.0, and 332 ml 2.0 M, pH 5.0. The amino acid composition of isolated fragments was determined with a Spinco model 121 automated amino acid analyzer (Beckman Instruments)

equipped with high-sensitivity cuvettes and an Infotronic model CRS-110A integrator. Samples were hydrolyzed for 24 hr and in some instances for 72 hr at 110°C under reduced pressure with 6 N HCl.

Figure 16 gives the separation of the tryptic peptides. Many fragments are eluted as single peaks. However, in some zones at least two peptides are present, and rechromatography on columns of Dowex 1-X2 is necessary for further purification. Peptides T-1 through T-11 originate from the 99 residues in NH<sub>2</sub>-terminal position and T-14 is of the Tyr-Arg dipeptide in COOH-terminal position (see Figure 1 for the sequence of the normal  $\alpha$ -chain). Amino acid analyses of these peptides and their location in the chromatogram usually reveal if the fragment contains a variation or not. Peptides T-12 (residues 100 through 127) and T-13 (residues 128-139) are not observed because they form the insoluble core; however, considerable quantities of the T-13 peptide have been observed in several chromatograms where it is eluted in the T-1 and T-11 region.

The insoluble core of the normal  $\alpha$  chain consists of this fragment:

|  |     |     |
|--|-----|-----|
| 100  | 105 | 110 |
| Leu-Leu-Ser-His-Cys-Leu-Leu-Val-Thr-Leu-Ala-Ala-His-Leu-Pro- |     |     |
| 115  | 120 | 125 |
| Ala-Glu-Phe-Thr-Pro-Ala-Val-His-Ala-Ser-Leu-Asp-Lys-Phe-Leu- |     |     |
| 130  | 135 | 139 |
| Ala-Ser-Val-Ser-Thr-Val-Leu-Thr-Ser-Lys                      |     |     |

The cysteinyl residue in the core was oxidized with performic acid according to the method of Hirs (44), and the oxidized core was digested with chymotrypsin for 8 hours at room temperature in a pH stat at pH 8.9. Enzyme equal to 0.5% of the substrate by weight was added at 0 and at 2 hr. The chymotryptic peptides were also separated by Dowex 50-X4 chromatography. Figure 17 gives examples of separation that have been obtained. From the peptide fragments of the normal  $\alpha$  chain listed in the figure it becomes evident that splits have occurred between residues 101-102, 109-110, 117-118, 122-123, 127-128, 128-129, and 136-137, and that all fragments were recovered. The same is true for the chymotryptic peptides of the core of the  $\alpha$  chain of Hb-St. Claude. However, one extra zone (see arrow) is observed and two zones (see arrows) are missing. The latter two correspond to residues in positions 123-127 and 123-128, respectively. The amino acid composition of the extra zone was the same as that of the first missing zone except for a replacement of the normally occurring lysyl residue for a threonyl residue; thus, the  $\alpha$  chain of Hb-St. Claude had a Lys $\rightarrow$ Thr substitution in position 127 which corresponds to the 10th position of the H-helix. The substitution does not affect the stability nor the function of the protein and the presence of the variant in the heterozygote does not have clinical consequences. The substitution explains the electrophoretic and chromatographic

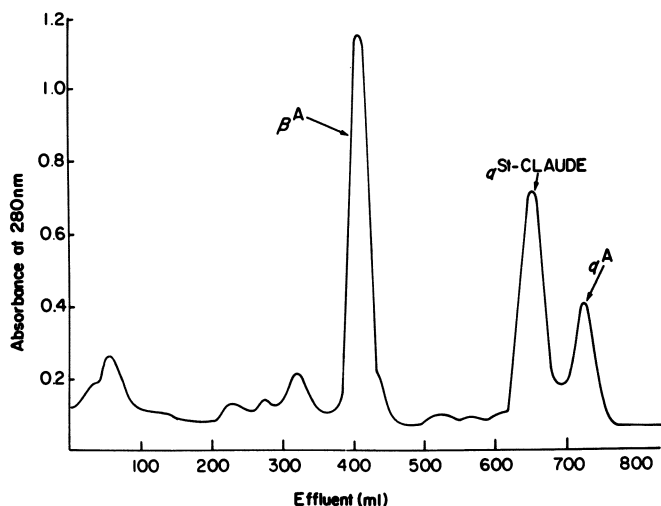


Figure 15. The separation of the chains from globin of 280 mg of partially purified Hb-St. Claude on a  $1.7 \times 15$  cm column of carboxymethylcellulose in 8M urea

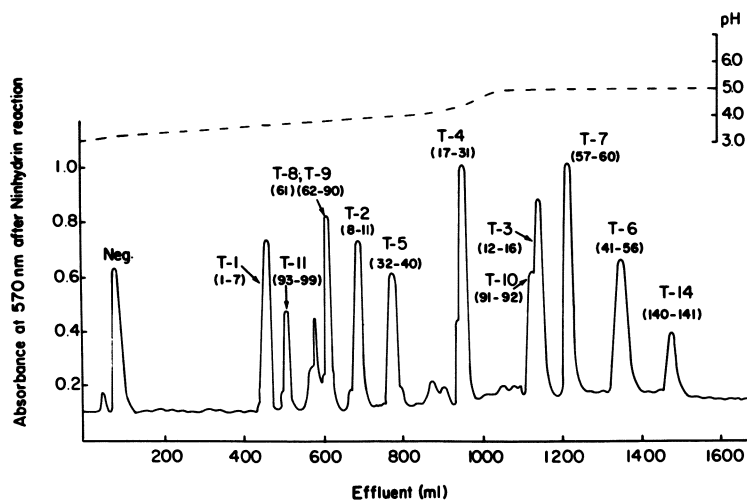
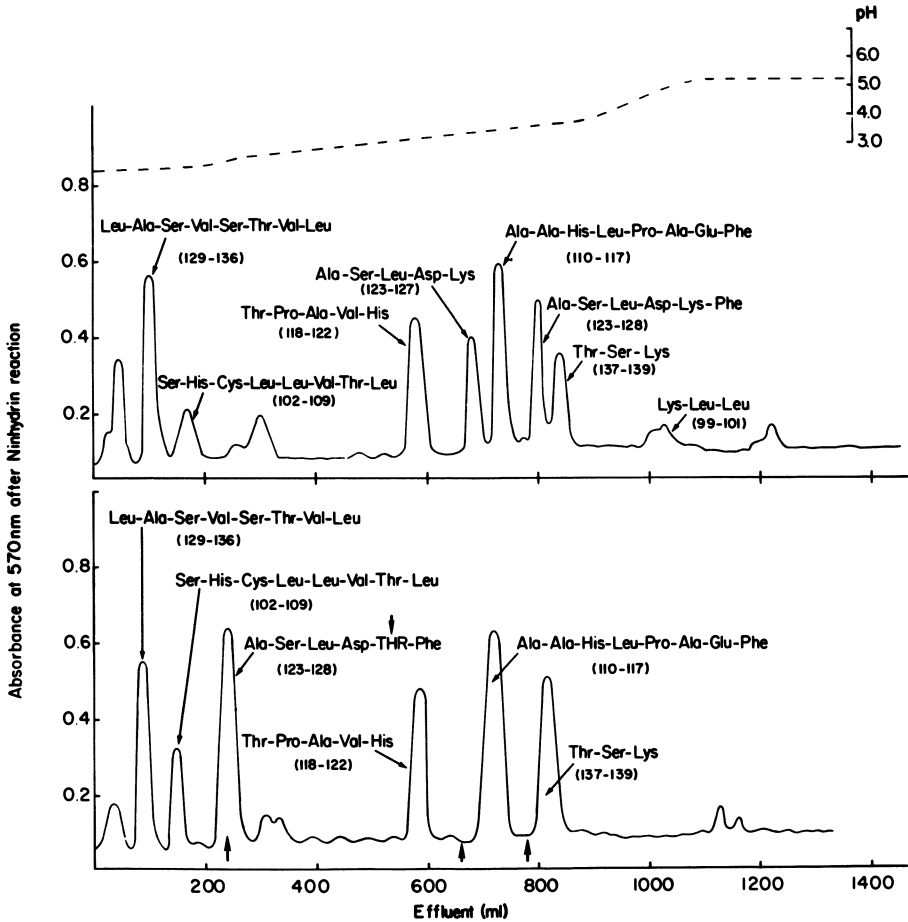


Figure 16. Chromatographic separation of peptides of a tryptic hydrolysate of the  $\alpha$  chain of Hb-St. Claude on a  $0.9 \times 60$  cm column of Chromobead resin type P at  $37^\circ\text{C}$ . The pH gradient is indicated by the broken line. T-1, T-2, etc. refer to the tryptic peptides and the numbers underneath to the positions in the chain. Several peptides are pure and give satisfactory analyses without further purification.



**Figure 17.** Chromatographic separation of peptides of chymotryptic hydrolysates of the oxidized cores of the  $\alpha$ -chain of Hb-A (top panel) and of the  $\alpha$ -chain of Hb-St. Claude (bottom panel) on  $0.9 \times 60$  cm columns of Chromobead resin type P at  $37^\circ\text{C}$ . The pH gradient is indicated by the broken line. Peptides are identified by their sequences and by the positions they occupy in the  $\alpha$ -chain.

properties of the variant.

Structural Analyses of Hb-Shepherds Bush or  $\alpha_2\beta_2^{74}(E18)Gly\rightarrow Asp$

The variant was found in a 22 year old woman with chronic hemolytic anemia characterized by a persistent reticulocytosis, development of gallstones requiring cholecystectomy, frequent episodes of jaundice, dark urine, and falling PCV value. Her mother, maternal grandfather, and sister have a similar clinical picture. In all patients red cell enzymes are elevated with a reticulocytosis of about 10%, and 2,3-DPG levels are normal. Heinz body preparations are positive as is the heat stability test. Electrophoretic examination of hemolysate showed the presence of a fast-moving variant which was readily separated from Hb-A by chromatography on DEAE-Sephadex. The abnormal  $\beta$ -chain was isolated by CM-Cellulose chromatography as described before, and converted into the S-2-aminoethyl (AE) derivative (45). Treatment of the  $\beta$ -chain with ethylenimine converts the -SH groups into the -S-CH<sub>2</sub>-CH<sub>3</sub> groups. The structure of AE-cysteine is similar enough to that of lysine that peptide bonds involving this derivative will also serve as a substrate for trypsin. An added advantage is that the peptides containing the AE-cysteinyl residue have a greatly increased solubility.

Tryptic peptides from a digest of 120 mg AE- $\beta$ -chain were separated by Dowex 50-X4 chromatography; the chromatogram is depicted in Figure 18. Many peptides were eluted in single zones, but several occurred together. However, all peptides, T-1 through T-15, were recovered (residues 1-146; see Figure 2). Peptide T-12 occurred in two fragments; T-12a is the NH<sub>2</sub>-terminal fragment with a AE-cysteinyl residue in COOH-terminal position. Some larger fragments (T-10, T-11, T-14, 15; T-7, 8) were also observed in small quantities indicating an incomplete tryptic digestion. Several zones required rechromatography using 0.6 X 60 cm columns of Dowex 1-X2 according to the method of Schroeder (46). Figure 19 gives an example of the separation of two peptides that occurred together in the original Dowex 50 chromatogram.

Peptide T-9 was eluted from the Dowex 50 column in front of the T-14 peptide instead of between the T-14 and T-1 peptides. Amino acid analysis indicated that one of the two normally occurring glycyl residues (in positions 69 and 74, respectively) was replaced by an aspartyl residue. To decide which glycyl residue was replaced, two approaches were followed. First, degradation of the isolated peptide by the phenylthiohydantoin-dansylation procedure (47, 48) determined its NH<sub>2</sub>-terminal sequence (a Val-Leu-Gly-Ala-NH<sub>2</sub>-terminus was established, thus suggesting that glycyl residue in position 74 was involved). Second, the peptide was digested with thermolysin for 3 hr at 37°C in a volatile buffer at pH 7.4; this enzyme will hydrolyze peptide bonds with alanyl, aspartyl, leucyl, and asparaginyl residues in COOH-terminal position. The thermolytic fragments



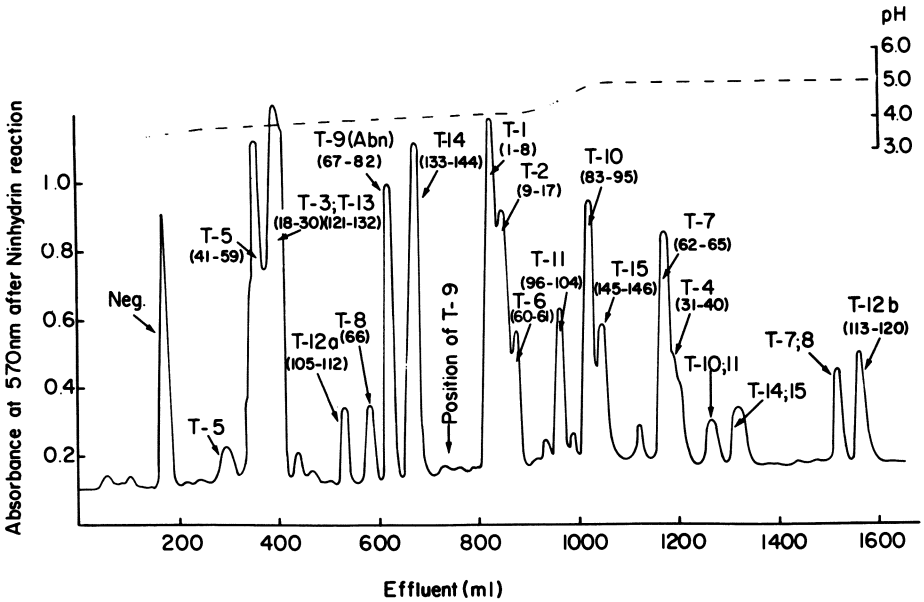


Figure 18. Chromatographic separation of peptides of a tryptic digest of the AE- $\beta$ -chain of Hb-Shepherd's Bush on a  $0.9 \times 60$  cm column of Chromobead resin type P at  $37^\circ\text{C}$ . The pH gradient is indicated by the broken line. Peptides are identified by the positions occupied in  $\beta$ -chain.

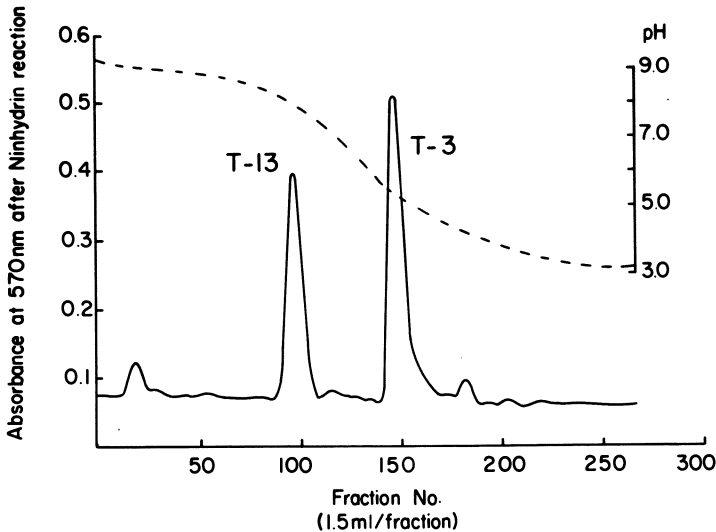


Figure 19. Chromatographic separation of peptides  $\beta$ T-3 and  $\beta$ T-13 on a  $0.6 \times 60$  cm column of Dowex 1-X2. The broken line indicates the pH gradient.



were separated on a column of Dowex 1-X2, and five fragments were recovered as indicated in Figure 20. Examination of the amino composition of these fragments left no doubt as concerns the introduction of a charged amino acid residue in the heme pocket causing considerable disruption to this internal structure and an instability of the protein. It is likely that the side chain of the aspartyl residue at E18 of the  $\beta$ -chain interferes with threonyl residue  $\beta$ 84 (EF8) and phenylalanyl residue  $\beta$ 85 (F1) which are located opposite to the aspartyl residue in position 74, probably weakening the hydrophobic forces which hold the heme pocket together (49).

### Concluding Remarks

There are many important reasons for an intensive study of hemoglobin variants. First, the number of variants that have been discovered is steadily increasing, and it may well be that some 100 million persons have a variant of Hb-A or a type of thalassemia. Second, homozygosity and even heterozygosity for some variants and the thalassemias, may cause considerable health problems because these conditions (Hb-S, Hb-E,  $\beta$ -thalassemia,  $\alpha$ -thalassemia in particular) affect several million persons in various racial and/or ethnic groups. Third, identification of hemoglobin variants in persons suffering from unexplained hemolytic anemias or from an unexplained erythrocytosis has aided in explaining these conditions. Fourth, analyses of structural variants have offered data of great importance for an insight in the structure-function relationships of the hemoglobin molecule. Fifth, the discovery of several unusual variants has given insight into the genetic mechanisms that control protein synthesis in man.

It seems likely that these investigations when continued will uncover additional variants of importance for the understanding of basic biological phenomena and disease states.

### Literature Cited

1. Perutz, M. F.; Muirhead, H.; Cox, J. M.; and Goaman, L. C. G.; "Three-dimensional Fourier Synthesis of Horse Oxyhemoglobin at 2.8 Å Resolution: (II) The Atomic Model". *Nature* (1968), 219, 131.
2. Bolton, W.; and Perutz, M. F.; "Three Dimensional Fourier Synthesis of Horse Deoxyhaemoglobin at 2.8 Å Resolution". *Nature* (1970), 228, 551.
3. Huisman, T. H. J.; "Normal and Abnormal Human Hemoglobins". *Advan. Clin. Chem.* (1972), 15, 149.
4. Capp, G. L.; Rigas, D. A.; and Jones, R. T.; "Evidence for a New Haemoglobin Chain ( $\zeta$ -Chain)". *Nature* (1970), 228, 278.
5. Jones, R. T.; Personal communication (1974).

6. Pauling, L.; Itano, H. A.; Singer, S. J.; and Wells, I. C.; "Sickle Cell Anemia, a Molecular Disease". *Science* (1949), 110, 543.
7. Perutz, M. F.; and Lehmann, H.; "Molecular Pathology of Human Haemoglobin". *Nature* (1968), 219, 902.
8. Schroeder, W. A.; "Hemoglobin Structure and Function. The Proteins", H. Neurath and R. Hill, Eds., Academic Press, New York, in press.
9. Hollán, S. R.; Szelényi, J. G.; Brimhall, B.; Duerst, M.; Jones, R. T.; Koler, R. D.; and Stocklen, Z.; "Multiple Alpha Chain Loci for Human Haemoglobins: Hb J-Buda and Hb G-Pest". *Nature* (1972), 235, 47.
10. Brimhall, B.; Duerst, M.; Hollán, S. R.; Stenzel, P.; Szelényi, J.; and Jones, R. T.; "Structural Characterizations of Hemoglobins J-Buda [ $\alpha$  61 (E 10) Lys $\rightarrow$ Asn] and G-Pest [ $\alpha$  74 (EF 3) Asp $\rightarrow$ Asn]". *Biochim. Biophys. Acta.*, (1974), 336, 344.
11. Gajdusek, D. C.; Guiart, J.; Kirk, R. L.; Carrell, R. W.; Irvine, D.; Kynoch, P. A. M.; and Lehmann, H.; "Haemoglobin J Tongariki ( $\alpha$  115 Alanine $\rightarrow$ Aspartic Acid): The First New Haemoglobin Variant Found in a Pacific (Melanesian) Population". *J. Med. Genet.* (1967), 4, 1.
12. Abramson, R. K.; Rucknagel, D. L.; and Shreffler, D. C.; "Homozygous Hb J Tongariki: Evidence for Only One Alpha Chain Structural Locus in Melanesians". *Science* (1970), 169, 194.
13. Weatherall, D. J., guest editor; "The Haemoglobinopathies". *Clinics in Haematology* (1974), 3, No. 2.
14. Williams, W. J.; Beutler, E.; Allan, J. E.; and Rundles, R. W.; "Hematology", Chapter 11, "Erythrocyte Disorders - Anemias Related to Abnormal Globin", McGraw-Hill Book Company, New York, 1972.
15. Huisman, T. H. J.; Wilson, J. B.; Gravely, M.; and Hubbard, M.; "Hemoglobin Grady: the First Example of a Variant with Elongated Chains Due to an Insertion of Residues". *Proc. Natl. Acad. Sci.*, (1974), 71, 3270.
16. Huisman, T. H. J.; Wrightstone, R. N.; Wilson, J. B.; Schroeder, W. A.; and Kendall, A. G.; "Hemoglobin Kenya, the Product of Fusion of  $\gamma$  and  $\beta$  Polypeptide Chains". *Arch. Biochem. Biophys.* (1972), 153, 850.
17. Kendall, A. G.; Ojwang, P. J.; Schroeder, W. A.; and Huisman, T. H. J.; "Hemoglobin Kenya, the Product of a Gamma-beta Fusion Gene: Studies of the Family". *Am. J. Hum. Genet.*, (1973), 25, 548.
18. Clegg, J. B.; Weatherall, D. J., and Milner, P. F.; "Haemoglobin Constant Spring - A Chain Termination Mutant?" *Nature* (1971), 234, 337.
19. Efremov, G. D.; Wrightstone, R. N., Huisman, T. H. J.; Schroeder, W. A.; Hyman, C.; Ortega, J.; and Williams, K.; "An Unusual Hemoglobin Anomaly and its Relation to

- $\alpha$ -Thalassemia and Hemoglobin - H Disease". *J. Clin. Invest.*, (1971), 50, 1628.
20. Flatz, G.; Kinderlerer, J. L.; Kilmartin, J. V.; and Lehmann, H.; "Haemoglobin Tak: A Variant with Additional Residues at the End of the  $\beta$ -Chains". *Lancet*, (April 10, 1971), 732.
  21. Huisman, T. H. J.; Schroeder, W. A.; Efremov, G. D.; Duma, H.; Mladenovski, B.; Hyman, C. B.; Rachmilewitz, E. A.; Bouver, N.; Miller, A.; Brodie, A. R.; Shelton, J. R.; Shelton, J. B.; and Apell, G.; "The Present Status of the Heterogeneity of Fetal Hemoglobin in  $\beta$ -Thalassemia; An Attempt to Unify Some Observations in Thalassemia and in Related Conditions". *Proc. Third Cooley's Anemia Conf.* April, 1973. *Ann. N. Y. Acad. Sci.*, (1974), 232 107.
  22. Huisman, T. H. J.; "Normal and Abnormal Hemoglobins". *Advan. Clin. Chem.* (1963), 6, 231.
  23. Huisman, T. H. J.; "Human Hemoglobins in Biochemical Methods in Red Cell Genetics", Ed. Y. Yunis, p. 391, Academic Press, New York, 1969.
  24. Jonxis, J. H. P.; and Huisman, T. H. J.; "A Laboratory Manual on Abnormal Haemoglobins", Second Edition, Blackwell, Oxford, 1968.
  25. Efremov, G. D.; and Huisman, T. H. J.; "The Laboratory Diagnosis of Hemoglobinopathies". *Clinics in Haematology*, (1974), 3, No. 2, 527.
  26. Schmidt, R. M.; and Brosious, E. M.; "Laboratory Methods of Hemoglobinopathy Detection", U. S. Department of Health, Education, and Welfare, Public Health Service, 4th Edition, April 1973.
  27. Schroeder, W. A.; Jakway, J.; and Powars, D.; "Detection of Hemoglobins S and C at Birth: A Rapid Screening Procedure by Column Chromatography". *J. Lab. Clin. Med.*, (1973), 82, 303.
  28. Schroeder, W. A.; Huisman, T. H. J.; Powars, D.; Evans, L.; Abraham, E. C.; and Lam, H.; "Microchromatography of Hemoglobins". IV. "An Improved Procedure for the Detection of Hemoglobins S and C at Birth". *J. Lab. Clin. Med.*, in press.
  29. Efremov, G. D.; Huisman, T. H. J.; Bowman, K.; Wrightstone, R. N.; and Schroeder, W. A.; "Microchromatography of Hemoglobins". II. "A Rapid Method for the Determination of Hemoglobin A<sub>2</sub>". *J. Lab. Clin. Med.*, (1974), 83, 657.
  30. Huisman, T. H. J.; Schroeder, W. A.; Brodie, A. R.; Mayson, S. M.; and Jakway, J.; "Microchromatography of Hemoglobins". III. "A Simplified Procedure for the Determination of Hemoglobin A<sub>2</sub>". *J. Lab. Clin. Med.*, in press.
  31. Betke, K.; and Kleihauer, E. F.; "Fetaler und bleibender Blutfarbstoff in Erythrozyten und Erythroblasten von menschlichen Feten und Neugeborenen". *Blut*, (1958), 4, 241.

32. Kleihauer, E.; "Fetales Hämoglobin und fetale Erythrocyten". *Archiv. Kinderheilk. Beih.*, 53, F. Enke-Verlag, Stuttgart, Germany, 1966.
33. Kleihauer, E.; "Determination of Fetal Hemoglobin: Elution Technique", in "The Detection of Hemoglobinopathies", p. 20, R. M. Schmidt, T. H. J. Huisman, and H. Lehmann, editors. CRC Press, publishers, 1974.
34. Betke, K.; Marti, H. R.; and Schlicht, I.; "Estimation of Small Percentages of Foetal Haemoglobin". *Nature*, (1959), 184, 1877.
35. Schroeder, W. A.; Huisman, T. H. J.; Shelton, J. R.; and Wilson, J. B.; "An Improved Method for Quantitative Determination of Human Fetal Hemoglobin". *Anal. Biochem.*, (1970), 35, 235.
36. Itano, H. A.; "Solubilities of Naturally Occurring Mixtures of Human Hemoglobin". *Arch. Biochem. and Biophys.*, (1953), 47, 148.
37. Carrell, R. W.; and Kay, R.; "A Simple Method for the Detection of Unstable Hemoglobins". *Br. J. Haematol.*, (1972), 23, 615.
38. Bucci, E.; and Fronticelli, C.; "A New Method for the Preparation of  $\alpha$  and  $\beta$  Subunits of Human Hemoglobin". *J. Biol. Chem.*, (1965), 240, 551.
39. Hubbard, M.; Winton, E.; Lindeman, J. G.; Dessauer, P. L.; Wilson, J. B.; Wrightstone, R. N.; and Huisman, T. H. J.; "Hemoglobin Atlanta or  $\alpha_2\beta_2^{75Leu\rightarrow Pro}$  (E19) an Unstable Variant Found in Several Members of a Caucasian Family". *Biochim. Biophys. Acta*, (1975), 386, 538.
40. Efreinov, G. D.; Huisman, T. H. J.; Stanulovic, M.; Zurovec, M.; Duma, H.; Wilson, J. B.; and Jeremic, V.; "Hemoglobin M Saskatoon and Hemoglobin M Hyde Park in Two Yugoslavian Families". *Scand. J. Haematol.*, (1974), 13, 48.
41. Schneider, R. G.; "Differentiation of Electrophoretically Similar Hemoglobins - Such as S, D, G, and P; or A<sub>2</sub>, C, E, and O - by Electrophoresis of the Globin Chains". *Clin. Chem.*, (1974), 20, 1111.
42. Vella, F.; Galbraith, P.; Wilson, J. B.; Wong, S. C.; Folger, G. C.; and Huisman, T. H. J.; "Hemoglobin St. Claude or  $\alpha_2^{127(H10)}\beta_2^{Lys\rightarrow Thr}$ ". *Biochim. Biophys. Acta.*, (1974), 365, 318.
43. Clegg, J. B.; Naughton, M. A.; and Weatherall, D. J.; "Abnormal Human Haemoglobins. Separation and Characterization of the  $\alpha$  and  $\beta$  Chains by Chromatography, and the Determination of Two New Variants, Hb-Chesapeake and Hb-J-Bangkok". *J. Mol. Biol.*, (1966), 19, 91.
44. Hirs, C. H. W.; "Performic Acid Oxidation", in "Methods in Enzymology", p. 197, Vol. XI, "Enzyme Structure", C. H. W. Hirs, Editor, Academic Press, New York, 1967.
45. Jones, R. T.; "Structural Studies of Aminoethylated

- Hemoglobins by Automatic Peptide Chromatography". Cold Spring Harbor Symp. Quant. Biol., (1964), 29, 297.
46. Schroeder, W. A.; "Separation of Peptides by Chromatography on Columns of Dowex 1 with Volatile Developers", in "Methods in Enzymology", p. 214, Vol. XXV, "Enzyme Structure, Part B", C. H. W. Hirs and S. N. Timasheff, Editors, Academic Press, New York, 1972.
  47. Gray, R.; "Sequence Analysis with Dansyl Chloride", in "Methods in Enzymology", p. 333, Vol. XXV, "Enzyme Structure, Part B", C. H. W. Hirs and S. N. Timasheff, Editors, Academic Press, New York, 1972.
  48. Woods, R.; and Wang, Kung-Tsung; "Separation of Dansyl Amino Acids by Polyamide Layer Chromatography". Biochim. Biophys. Acta., (1967), 133, 369.
  49. White, J. M.; Brain, M. C.; Lorkin, P. A.; Lehmann, H.; and Smith, M.; "Mild 'Unstable Haemoglobin Haemolytic Anemia' caused by Haemoglobin Shepherds Bush (874 (E18) Gly→Asp)". Nature, (1970), 225, 939.

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## Measurement of Calcitropic Hormones in Clinical Medicine

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### Parathyroid Hormone (PTH)

**Methodology.** The radioimmunoassay of PTH is currently difficult to carry out. It requires meticulous attention to the vagaries of  $^{131}\text{I}$  or  $^{125}\text{I}$  labeling of the polypeptide, to its adsorption to glassware, to techniques of separation of bound and free [ $^{131}\text{I}$ ]- or [ $^{125}\text{I}$ ]-labeled PTH, and to nonspecific factors in serum which interfere with immune reactions. Extensive quality control measures are essential because of the clinical significance attached to increased values for PTH in serum by clinicians (i.e. recommending surgical exploration of the neck). Because of this latter important consideration, it is probably crucial for laboratories performing PTH radioimmunoassay for clinical purposes to maintain a consultation service to aid physicians in the interpretation of results.

The development of an individual assay for PTH requires great effort and dependence on chance. To provide a complete and competent service, it is important to have both an extremely sensitive assay, and also assays specific for amino terminal ( $\text{NH}_2$ -) and carboxyl terminal ( $\text{COOH}$ -) regions of the PTH molecule (see below). Sufficient human PTH is not available at the present time for even a single large immunization effort. Antisera must be produced with either bovine or porcine PTH with the hope of obtaining a high affinity cross reacting antiserum. Both bovine and porcine PTH (crude extracts) are extremely expensive unless extraction and purification is done in individual laboratories, and procedures available for the latter are time consuming and difficult. The commercial availability of "good" bovine PTH for labeling is limited and only a few research laboratories have preparations of standard human PTH.

It is hoped that current work being carried out on the amino acid sequence of human PTH (1-4) will be successful, so that synthetic preparations of important regions of the hormone can become commercially available. This would partially



solve some of the reagent problems. Although large programs for the collection of human parathyroid adenomas and hyperplastic glands are ongoing for the purpose of obtaining sufficient human PTH for complete amino acid sequence analysis, the availability of this tissue is clearly the central, rate-limiting problem in making progress in chemically characterizing human PTH; the expansion of gland collection efforts is important.

Circulating Immunoreactive PTH (iPTH) and Different Uses of NH<sub>2</sub>- and COOH-Specific Assays. The major secretory product of parathyroid tissue is the native 9,500 M.W. polypeptide (5), although it is possible that fragments of this molecule are secreted also (6,7). Once secreted, the 9,500 M.W. PTH is metabolized into amino terminal and carboxyl terminal fragments (8). In fact, the COOH fragments predominate over the 9,500 M.W. PTH in the circulation to the extent of 5:1 to 20:1 (7,9,10). This predominance is probably due to the long survival time of COOH-fragments in serum (hours) as compared with the 9,500 M.W. PTH (minutes) (11,12). The 9,500 M.W. PTH and its synthetic (bovine), amino terminal, (1-34) polypeptide are biologically active, but its carboxyl terminal region is biologically inert (13).

These relationships are confirmed by direct measurements on serum, using assays, which are specific for either "NH<sub>2</sub>-PTH" or "COOH-PTH". Concentrations of COOH-iPTH are 5-20 times higher than NH<sub>2</sub>-iPTH (9-10). However, the clinical utility of the two assays differs markedly (4,10,14). Assays specific for "COOH-PTH" are superior to "NH<sub>2</sub>" assays in assessing the chronic state of parathyroid function and conversely, "NH<sub>2</sub>" assays are superior to "COOH" assays in assessing the acute secretory status of the parathyroid glands as well as in the demonstration of step-up changes in iPTH levels in serum samples obtained during differential venous catheterization of the neck and mediastinum for localization of hyperfunctioning parathyroid tissue. Although the reasons for these differences are not entirely clear, they are probably related to the marked differences in the survival times of 9,500 M.W. PTH and -COOH fragments in the circulation and to the fact that the major secretory product of parathyroid tissue is the 9,500 M.W. species of PTH.

In a practical sense, routine fasting serum samples are examined in our laboratory using a very sensitive COOH-specific PTH assay (antiserum GP 1M, guinea pig anti-porcine PTH). It distinguishes between normal and hyperparathyroid individuals with 90% efficiency, and patients with primary hyperparathyroidism (HPT) and ectopic hyperparathyroidism due to non-parathyroid cancer (EHPT) with 80% efficiency; iPTH values are lower for a given degree of hypercalcemia in EHPT than in HPT because, in general, the quantity of COOH fragments in the serum of EHPT patients is lower (15,16,17). Additionally, the COOH-specific assay yields low or undetectable values in hypercalcemia of non-

parathyroid origin (i.e., sarcoid, vitamin D intoxication, and hyperthyroidism).

On the other hand, serum samples obtained during provocative and suppression tests of parathyroid function by means of differential venous catheterization of the neck and mediastinum are examined with a very sensitive  $\text{NH}_2$ -specific assay (antiserum CH 14M, chicken anti-bovine PTH). Unfortunately, we do not yet know how to interpret provocative and stimulation tests in patients with hyperparathyroidism with respect to determining the degree of autonomy of hyperfunctioning parathyroid tissue. Using  $\text{NH}_2$ -specific assays, serum iPTH increases with induced hypocalcemia and decreases variably with induced hypercalcemia in patients with parathyroid adenoma. We are currently carrying out systematic comparative studies of "COOH" and " $\text{NH}_2$ " assays of sera obtained during stimulation and suppression of serum iPTH levels in normal individuals and patients with either primary parathyroid adenoma or hyperplasia. It is hoped that significant differences will be observed which will allow the separation of normals from patients with mild hyperparathyroidism as well as the preoperative separation of patients with parathyroid adenoma and parathyroid hyperplasia.

### Calcitonin (CT)

Methodology. Several radioimmunoassays of human calcitonin (hCT) have been developed in the past 5 years (18-20). Their greatest utility has been in the definitive diagnosis of patients with medullary carcinoma of the thyroid gland (MTC) and, recently, in identifying family members of these patients who have occult MTC.

Synthetic hCT is not commercially available now. Most assays have been developed by using hormone supplied as gifts from the CIBA-Geigy Co., Basel, Switzerland for  $^{131}\text{I}$  or  $^{125}\text{I}$  labeling, immunization and standards although several useful antisera have been produced by immunization with crude extracts of medullary carcinoma tissue.

Utility. There is general agreement that the concentration of immunoreactive CT in normal serum is extremely low (< 200 pg/ml). However, there is evidence for immunoheterogeneity of calcitonin in the serum of MTC patients (21) and it is possible that studies in the future will demonstrate this phenomenon in normal sera as well. This might explain some reports of higher concentrations of immunoreactive CT in normal subjects (22).

Basal values for serum immunoreactive CT in patients with overt MTC are almost always increased above 1000 pg/ml, and relatively insensitive assays are capable of measuring these levels. However, many MTC patients' relatives with occult MTC have values below 100 pg/ml (20 pg/tube if serum concentrations

in incubation mixtures are 20%). This is particularly true when systematic examination of family members of a patient with proved MTC with provocative testing is anticipated since some of these family members may have normal basal concentrations (23).

Provocative testing has centered on the ability of calcium infusion and pentagastrin administration to increase serum immunoreactive hCT. With most assays, increases in immunoreactive CT greater than 500 pg/ml are not observed in normal subjects with either of these secretagogues; increases above this level are generally considered to be consistent with the presence of MTC and sufficient evidence (especially in a family member) to recommend thyroidectomy. Recently, the preliminary results of a systematic comparison between the calcium infusion and pentagastrin test in 4 patients with proved MTC were reported by Sizemore and Go (24). Although further studies will be required, the data suggested that the pentagastrin test produces more definitive increases in immunoreactive hCT and has the advantage of eliciting much earlier responses (1-5 minutes for pentagastrin vs. 1-5 hours for calcium infusion).

### Vitamin D and Metabolites

**Methodology.** Vitamin D, a sterol, occurs naturally in plants as vitamin D<sub>2</sub> (ergocalciferol) and is produced by ultraviolet irradiation of ergosterol. In man vitamin D<sub>3</sub> (cholecalciferol), the natural form of the vitamin, is produced by ultraviolet irradiation of 7-dehydrocholesterol in the skin. The fact that vitamin D<sub>2</sub> is a major food additive and the over-the-counter therapeutic form of vitamin D produces a serious problem in evaluating endogenous vitamin D<sub>3</sub> and its metabolites in human serum. Available ligand binding assay techniques generally fail to distinguish between D<sub>2</sub> and D<sub>3</sub>; special chromatographic techniques (long column, 60 Cm, Sephadex LH-20) are required to do this (25).

Vitamin D<sub>3</sub> enters the skin microcirculation after formation and is bound to a specific globulin in the serum. It and vitamin D<sub>2</sub> which is absorbed from the gut are subsequently metabolized to the 25-hydroxy derivative (25-OH-D) in the liver by an enzyme system which may or may not be regulated. The subsequent release of 25-OH-D from the liver is not well understood. There is evidence that it is secreted into the bile and subsequently reabsorbed by the intestine. The relative importance of this "enterohepatic" process and the release of this metabolite directly into the circulation from the liver is not known (26). It is clear however, that 25-OH-D<sub>3</sub> is the major circulating metabolite of vitamin D<sub>3</sub>. It circulates almost completely bound to a specific serum globulin.

The methods now used to measure 25-OH-D are competitive protein-ligand binding assays that use either serum globulin (diluted rat serum) (27,28) or a vitamin D-deficient rat kidney

cytosol preparation (29,30) as binding proteins, crystalline 25-OH-D<sub>3</sub> as standard and <sup>3</sup>H-25-OH-D<sub>3</sub> as radioactive ligand. Both methods require that the serum or plasma be extracted (after addition of <sup>3</sup>H-25-OH-D<sub>3</sub> for recovery and marker purposes) with either chloroform-methanol or ether and that the extracts be chromatographed on silicic acid or Sephadex LH-20 columns. The column fractions containing previously added <sup>3</sup>H-25-OH-D are dried over N<sub>2</sub>, taken up in ethanol, and assayed. Recovery of labeled or stable 25-OH-D is 65-85%.

The sensitivity of these competitive binding assays for 25-OH-D<sub>3</sub> (0.1 - 0.5 ng) is sufficient for the measurement of the large quantities of this metabolite circulating in serum in normal subjects (range 11-55 ng/ml, mean 27 ng/ml).

The 25-OH-D is further metabolized in the kidney to 1,25 dihydroxycholecalciferol (1,25(OH)<sub>2</sub>D) which is considered to be the major physiologically important, tissue-active metabolite of vitamin D. It circulates in extremely low concentrations (< 100 pg/ml of serum). Assay of 1,25(OH)<sub>2</sub>D is extremely tedious. It is done by competitive binding technique using a combined intestinal cell cytosol and chromatin binding system, biosynthetic <sup>3</sup>H-1,25(OH)<sub>2</sub>D<sub>3</sub> as labeled ligand and synthetic 1,25(OH)<sub>2</sub>D<sub>3</sub> as standard (31).

**Utility.** Insufficient data is available on the measurement of 1,25(OH)<sub>2</sub>D<sub>3</sub> for evaluation of its utility in clinical medicine. A major breakthrough in methodology will be needed before routine application will be possible. This could come with the development of a battery of radioimmunoassays for the measurement of all of the vitamin D metabolites. So far, however, the development of antibodies to vitamin D and its metabolites has been limited by apparently irreversible changes in the important B ring of the sterol which occur during its conjugation to immunogenic proteins.

The basic clinical tool used at the present time is the competitive ligand binding assay for 25-OH-D. Although concentrations are low in the serum of patients with osteomalacia and vitamin D deficiency rickets, we have recently noted the interesting paradox that levels can be only 1/2 normal in the face of overt bone disease (32). This had led us to propose that substrate levels of 25-OH-D<sub>3</sub> available to the hydroxylase in kidney which is responsible for the conversion of 25-OH-D<sub>3</sub> to the tissue active metabolite, 1,25(OH)<sub>2</sub>D<sub>3</sub>, may be rate limiting for this enzyme.

One of the major problems in the treatment of patients with vitamin D compounds (i.e., patients with hypoparathyroidism) has been the unpredictable development of hypercalcemia and the syndrome of vitamin D intoxication. Although this problem may become less serious when some of the faster-acting metabolites and analogues of metabolites of vitamin D become available for clinical use, it is likely that monitoring of serum levels of

25-OH-D<sub>3</sub> may prove to be helpful in anticipating overdosage. However, systematic studies which correlate vitamin D dosage, 25-OH-D<sub>3</sub> serum concentrations, and serum calcium in patients such as these, have not yet been reported.

#### Literature Cited

1. Brewer, H. B.; Fairwell, T.; Ronan, R.; Sizemore, G. W.; Arnaud, C. D.; "Human Parathyroid Hormone: Amino-Acid Sequence of the Amino-Terminal Residues 1-34". *Proc. Natl. Acad. Sci. USA*, (1972), 69, 3585-3588.
2. Niall, H. D.; Sauer, R. T.; Jacobs, J. W.; Keutmann, H. T.; Segre, G. V.; O'Riordan, J. L. H.; Aurbach, G. D.; Potts, J. T. Jr.; "The Amino-Acid Sequence of the Amino-Terminal 37 Residues of Human Parathyroid Hormone". *Proc. Natl. Acad. Sci. USA*, (1974), 71, 384-388.
3. Brewer, H. B.; Fairwell, T.; Rittel, W.; Littledike, T.; Arnaud, C. D.; "Recent Studies on the Chemistry of Human, Bovine and Porcine Parathyroid Hormones". *Am. J. Med.* (1974), 56, 759-766.
4. Arnaud, C. D.; Brewer, H. B. Jr.; "Parathyroid Hormone: Structure and Immunoheterogeneity" in "Methods in Radioimmunoassay, Toxicology and Related Areas". *Progress in Analytical Chemistry*, pp. 45-75, Vol. 8, Simmons, I. L. and Ewing, G. W., Editors, Plenum Press, New York and London, 1974.
5. Habener, J. F.; Powell, D.; Murray, T. M.; Mayer, G. P.; Potts, J. T. Jr.; "Parathyroid Hormone: Secretion and Metabolism in Vivo". *Proc. Natl. Acad. Sci. USA*, (1971), 68, 2986-2991.
6. Arnaud, C. D.; Sizemore, G. W.; Oldham, S. B.; Fischer, J. A.; Tsao, H. S.; Littledike, T.; "Human Parathyroid Hormone: Glandular and Secreted Molecular Species". *Am. J. Med.* (1971), 50, 630-638.
7. Silverman, R.; Yalow, R. S.; "Heterogeneity of Parathyroid Hormone: Clinical and Physiologic Implications". *J. Clin. Invest.* (1973), 52, 1958-1971.
8. Segre, G. V.; Niall, H. D.; Habener, J. F.; Potts, J. T. Jr.; "Metabolism of Parathyroid Hormone: Physiologic and Clinical Significance". *Am. J. Med.* (1974), 56, 774.
9. Segre, G. V.; Habener, J. F.; Powell, D.; Tregear, G. W.; Potts, J. T. Jr.; "Parathyroid Hormone in Human Plasma: Immunochemical Characterization and Biological Implications". *J. Clin. Invest.* (1972), 51, 3163-3172.
10. Arnaud, C. D.; Goldsmith, R. S.; Bordier, P. J.; Sizemore, G. W.; Larsen, J. A.; Gilkinson, J.; "Influence of Immunoheterogeneity of Circulating Parathyroid Hormone on the Results of Radioimmunoassays of Serum in Man". *Am. J. Med.* (1974), 56, 785-793.
11. Canterbury, J. M.; Riess, R.; "Multiple Immunoreactive

- Molecular Forms of Parathyroid Hormone in Human Serum". *Proc. Soc. Exp. Biol. Med.* (1972), 140, 1393-1398.
12. Goldsmith, R. S.; Furszyfer, J.; Johnson, W. J.; Fournier, A. E.; Sizemore, G. W.; Arnaud, C. D.; "Etiology of Hyperparathyroidism and Bone Disease During Chronic Hemodialysis. III. Evaluation of Parathyroid Suppressability". *J. Clin. Invest.* (1973), 52, 173-180.
  13. Potts, J. T. Jr.; Keutman, H. T.; Niall, H. D.; Tregear, G. W.; Habener, J. F.; O'Riordan, J. L. H.; Murray, T. M.; Powell, D.; Aurbach, G. D.; "Parathyroid Hormone: Chemical and Immunochemical Studies of the Active Molecular Species" in "Endocrinology 1971" (Proceedings of the Third International Symposium), pp. 333-349, Taylor, S., Editor, William Heinemann, London, 1972.
  14. Arnaud, C. D.; "Parathyroid Hormone: Coming of Age in Clinical Medicine". *Am. J. Med.* (1973), 55, 577-581.
  15. Riggs, B. L.; Arnaud, C. D.; Reynolds, J. C.; Smith, L. H.; "Immunologic Differentiation of Primary Hyperparathyroidism From Hyperparathyroidism Due to Nonparathyroid Cancer". *J. Clin. Invest.* (1971), 50, 2079-2083.
  16. Benson, R. C.; Riggs, B. L.; Pickard, B. M.; Arnaud, C. D.; "Radioimmunoassay of Parathyroid Hormone in Hypercalcemic Patients With Malignant Disease". *Am. J. Med.* (1974), 56, 821.
  17. Benson, R. C.; Riggs, B. L.; Pickard, B. M.; Arnaud, C. D.; "Immunoreactive Forms of Circulating Parathyroid Hormone in Primary and Ectopic Hyperparathyroidism". *J. Clin. Invest.* (1974), 54, 175-181.
  18. Tashjian, A. H. Jr.; Howland, B. G.; Melvin, K. E. W.; Hill, C. S. Jr.; "Immunoassay of Human Calcitonin: Clinical Measurement, Relation to Serum Calcium and Studies in Patients With Medullary Carcinoma". *N. Engl. J. Med.* (1970), 283, 890-895.
  19. Deftos, L. J.; "Immunoassay of Human Calcitonin, I Method". *Metabolism* (1971), 20, 1122.
  20. Sizemore, G. W.; Go, V. L. W.; Kaplan, E. L.; Sanzenbacher, L. J.; Holtermuller, K. H.; Arnaud, C. D.; "Relations of Calcitonin and Gastrin in the Zollinger-Ellison Syndrome and Medullary Carcinoma of the Thyroid". *N. Engl. J. Med.* (1973), 288, 641-644.
  21. Sizemore, G. W.; Heath, H. III; "Immunochemical Heterogeneity of Calcitonin in Plasma of Patients With Medullary Carcinoma of the Thyroid". *J. Clin. Invest.* (1975), In Press.
  22. Sanman, N. A.; Hill, C. S. Jr.; Beceiro, J. R.; Schultz, P. N.; "Immunoreactive Calcitonin in the Thyroid and in Maternal and Cord Serum". *J. Lab. Clin. Med.* (1973), 81, 671.
  23. Melvin, K. E. W.; Miller, H. H.; Tashjian, A. H. Jr.; "Early Diagnosis of Medullary Carcinoma of the Thyroid Gland

- By Means of Calcitonin Assay". *N. Engl. J. Med.* (1971), 285, 1115-1120.
24. Sizemore, G. W.; Go, V. L. W.; "Stimulation Tests For Diagnosis of Medullary Carcinoma". *Mayo Clin. Proc.* (1975), 50, 53-56.
  25. Haddad, J. G.; Hahn, T. J.; "Natural and Synthetic Sources of Circulating 25-Hydroxyvitamin D in Man". *Nature* (1973), 244, 515-517.
  26. Arnaud, S. B.; Goldsmith, R. S.; Lambert, P.; Go, V. L. W.; "25-Hydroxyvitamin D<sub>3</sub>: Evidence of an Enterohepatic Circulation in Man". *Proc. Soc. Exp. Biol. and Med.*, In Press.
  27. Belsey, R.; De Luca, H. F.; Potts, J. T. Jr.; "Competitive Binding Assay for Vitamin D and 25-OH-Vitamin D". *J. Clin. Endocrinol. Metab.* (1971), 33, 554-558.
  28. Belsey, R.; Clark, M. B.; Bernat, M.; Nold, J.; Holick, M. F.; De Luca, H. F.; Potts, J. T. Jr.; "The Physiologic Significance of Plasma Transport of Vitamin D and Metabolites". *Am. J. Med.* (1974), 57, 50-56.
  29. Haddad, J. G.; Chuy, K. J.; "Competitive Protein-Binding Radioassay for 25-Hydroxycholecalciferol". *J. Clin. Endocrinol. Metab.* (1971), 33, 992-995.
  30. Haddad, J. G.; Stamp, T. C. B.; "Circulating 25-Hydroxyvitamin D in Man". *Am. J. Med.* (1974), 57, 57-62.
  31. Brumbaugh, P. F.; Haussler, D. H.; Bressler, R. Houssler, M. R.; "Radioreceptor Assay for 1 $\alpha$ 25-Dihydroxyvitamin D<sub>3</sub>". *Science* (1973), 183, 1089-1091.
  32. Arnaud, S. B.; Arnaud, C. D.; Bordier, P. J.; Goldsmith, R. S.; Flueck, J. A.; "The Interrelationships Between Vitamin D and Parathyroid Hormone in Disorders of Mineral Metabolism in Man" (Proceedings of 2nd Vitamin D Symposium), Weisbaden, West Germany, Oct., 1974, In Press.

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## Practical Concepts of Competitive Protein Binding Assays

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The terminology used in the field of competitive protein binding procedures has become very complex and may be confusing for a newcomer in this area. It will therefore be necessary to define a few of the commonly used terms.

Antigens may be defined as substances which, when injected into an animal, will elicit an immunologic response resulting in the production of antibodies. Molecules with a molecular weight less than 10,000 usually will not elicit such a response. An example of this type of compound would be cortisol. To make cortisol antigenic it is coupled to albumin thereby resulting in a complex with a molecular weight greater than 10,000. When this complex is injected into an animal, antibodies that will bind to cortisol will be produced. In this case, cortisol is termed a haptén, that is, a molecule which on its own cannot elicit the production of antibodies but which can react with certain preformed antibodies. The term ligand may be used as a general term to include both antigens and haptens.

Antibodies are a group of serum proteins called gamma globulins or immunoglobulins, that are produced by an animal in response to exposure to foreign substances (antigens). The antibody molecules produced will react in vitro with a great degree of specificity towards the antigens. However, some substances structurally similar to the antigen that originally produced the antibodies may also react with them. This is termed a cross-reaction. For example, the antibodies produced against insulin will cross-react with proinsulin.

Radioimmunoassay is a competitive protein binding assay which utilizes an antibody as the binding protein. This assay also employs a highly purified antigen which has been radio-labeled (tagged).

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Radioassay is a competitive protein binding assay which employs a natural binding protein instead of an antibody. For example, transcortin is the binding protein for cortisol in nature and also in the radioassay procedure for cortisol.

Titre is a term used to express the strength of an antiserum. Thus, an antiserum with a high titre would indicate that it would be able to be diluted and used to perform a large number of assays.

Sensitivity may be defined as the lowest concentration of the material being assayed that can be distinguished from zero concentration.

Specificity may be defined as the freedom from interferences by substances other than those intended to be measured.

### Principle (1,2)

The basic theory of competitive protein binding assays employing a radioactive label is as follows:

P = Material to be measured

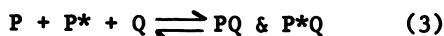
P\* = Radioactive label of this material

Q = Binding protein (natural binding protein or antibody, depending on the assay).

Combining components P & Q and P\* and Q in equal quantities yields:

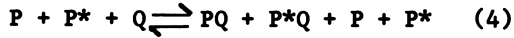


Thus, it can be seen that by adding P or P\* to Q individually, a complex PQ or P\*Q is obtained. By combining both P and P\* with Q simultaneously the following situation obtains:



In this one reaction both PQ and P\*Q are generated simultaneously. These complexes can be referred to as either the bound fraction of P or P\*. However in radioassay and radio-immunoassay techniques, the concern is with the bound fraction of P\* (i.e., P\*Q), since it is this complex that is measured. If a fixed amount of Q and P\* is used and the amount of Q is adjusted so that it can only bind 50% of the P\* added, the fol-

lowing equilibrium is obtained:



This equation illustrates the components of a competitive protein binding assay system. That is, the reaction system contains both radioactive and non-radioactive free ligand ( $P^*$  and  $P$ ) and both radioactive and non-radioactive protein bound ligand ( $P^*Q$  and  $PQ$ ). This type of assay assumes that binding protein will have the same affinity for the labeled or non-labeled material that is being measured. Although this assumption is not always completely valid, it usually causes no problems of consequence with most radioassays or radioimmunoassays.

In order to obtain reproducible results, near equilibrium must be established in the reagent system. This is usually accomplished by combining the material to be measured with the binding protein under fixed conditions. The time required to achieve this equilibrium may vary from several hours to days depending on the system used (3,4).

After the addition of the non-radioactive material, the labeled material is added to the mixture and the mixture allowed to proceed toward equilibrium. In equilibrium analysis the order of addition of reagents is not critical as long as sufficient time is allowed for establishing equilibrium of the complete mixture (3,4).

Furthermore, from equation (4) it can be seen that when the amount of  $P^*$  and  $Q$  are fixed, the more  $P$  added the smaller will be the quantity  $P^*Q$ . Therefore, if various known amounts of  $P$  are added and  $P^*Q$  is measured in a suitable counter, the relationship between radioactivity and concentration of  $P$  can be established. From this a standard curve can be drawn and used to determine the amount of  $P$  in an unknown solution.

In order to quantitate  $P^*Q$  by counting, it must be separated from the reagent mixture so that it can be counted in the absence of free  $P^*$ .

### Separation Techniques

Four types of techniques for separating the bound fraction  $P^*Q$  from the reagent mixture are in common usage, loosely termed: double antibody, solid phase, charcoal adsorption and solution precipitation. The first type is used with radioimmunoassay methods specifically, while the other three types can be used with both radioassay and radioimmunoassay methods.

In the double antibody method of separation, a second antibody, produced by injecting the first antibody into another animal, is utilized. This antibody is used to combine with and form an insoluble complex with the first antibody. After

allowing sufficient time for the precipitation reaction, separation of the precipitate from the reagent mixture is accomplished by centrifugation and decantation or aspiration of the supernatant. Although this method of separation is the most specific, a problem arises here in that the precipitate is of low density, thus requiring a minimum centrifugal force of about 2500 to 3000 x g to achieve separation. In addition, the centrifugation should be performed in the cold since heat tends to break up antibody complexes.

In the solid phase type of separation the antibody or binding protein is bound to an inert material such as Sephadex or glass beads yet is still free to react with the ligand. Once equilibrium in the reagent mixture is attained, centrifugation in a common laboratory centrifuge, followed by decantation of the supernatant is all that is required for separation of bound and free label.

The precipitation method of separation involves the addition of salts such as ammonium sulfate or solvents such as polyethylene glycol to the reagent mixture to cause precipitation of the large molecular weight bound species. These methods of precipitation lack specificity and work well only when there is a large difference between the molecular weight of the material being measured and that of the bound complex of it.

A fourth separation technique employs charcoal. Charcoal has adsorption characteristics such that most organic molecules will adhere to its surface. However, charcoal is also very porous, thereby allowing smaller molecules to enter into its pores and become trapped in them. The charcoal is first coated with dextran or hemoglobin which covers its outer surface. Since both of these compounds are very large they cannot enter the pores, thus the pores are still free to hold small molecules. When the coated charcoal is added to the assay reagent mixture, the free (unbound) material is adsorbed while the larger bound fraction is not. Final separation is accomplished by centrifugation and decantation of the supernatant.

In the first three methods of separation discussed, the bound label has been contained in the precipitate and it is this radioactivity which is measured in the assay. In the charcoal method, the bound label fraction is in the supernatant and thus it is the supernatant which is counted. From a theoretical point of view the best method of separation is the double antibody method due to its specificity. However, because of the need for a high capacity refrigerated centrifuge, this method is not used as commonly as would be expected. The most convenient type of separation from an operational point of view is the solid phase technique. This type of separation also has a good degree of specificity because of the fact that the binding protein is the only material attached to the solid material which may then be separated by centrifugation. This method is limited, however, by the technology needed to attach

the binding proteins to the solid material in a workable form. The other two types of separation procedures can be used adequately after some experience; however, they are prone to other problems and are not as specific as the double antibody or solid phase separation methods. There are, however, a great many commercially available kits utilizing charcoal separation and one should therefore become familiar with this technique and its problems. The coated charcoal can lose its adsorption properties after a period of time in solution, and one has to be extremely careful in making sure that the charcoal suspension is kept homogeneous so that a uniform amount is dispensed to the assay mixture.

### Standard Curves

After separating the bound label from the free label and counting the former in a gamma or beta counter depending upon the isotope used, a standard curve is plotted. The standard curve may be presented in a number of ways. The most common forms of presentation are shown in Figures 1 and 2. These are, in Figure 1, a plot of the percent bound vs. log of the concentration (dose) of a series of standards. Percent bound may be represented as  $B/B_0$ ,  $B$  being defined as the ligand bound at any concentration of standard and  $B_0$  defined as the zero dose tube, that is the tube that contains no non-radioactive ligand. This form of data expression is referred to as a plot of  $B/B_0$  vs log dose, and yields a sigmoid shaped curve. In doing an assay the values for  $B/B_0$  are determined for each standard and unknown and then the standard curve is plotted. The concentration of the unknown is then read off the standard curve opposite its  $B/B_0$  value. This sigmoid shaped standard curve, because of its linear portion, simplifies data handling. A mathematical transform of the  $B/B_0$  vs log dose is shown in Figure 2. This logit of  $B/B_0$  vs log dose is a widely used method of standard curve presentation (5,6,7). Logit  $B/B_0$  is defined as follows:

$$\text{logit } (B/B_0) = \ln (B/B_0)/(1 - B/B_0)$$

This latter transform is used to linearize the usual sigmoid curve produced in plotting  $B/B_0$  vs log dose. This transform may be accomplished by using a computer, logit paper or a table of logit values.

### Counting Equipment

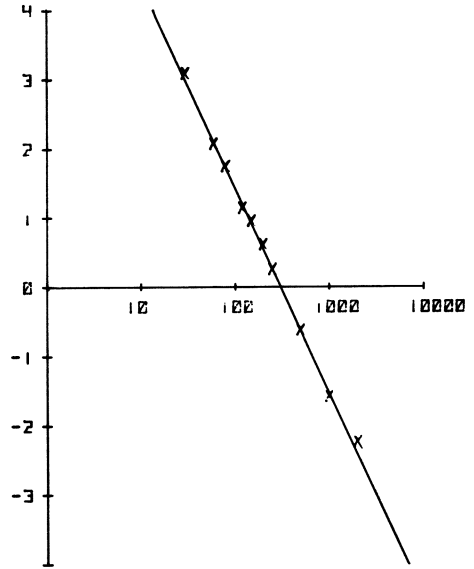


Figure 1. Plot of the percent bound vs. log of the concentration of a series of standards (dose)

LOGIT VS LOG CONCENTRATION

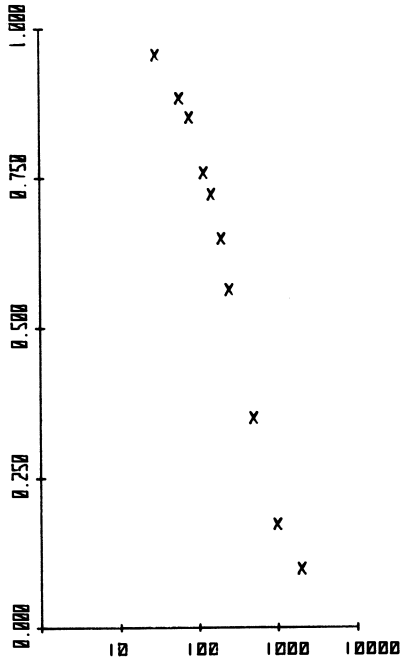


Figure 2. Mathematical transformation of the B/B<sub>0</sub> vs. log dose

B/B0 VS LOG CONCENTRATION

The counting equipment used to establish quantitation in radioassay and radioimmunoassays may be either a gamma counter or a liquid scintillation counter depending upon the isotope being used. Most assays use  $^{125}\text{I}$  as a label and require a gamma counter; however, a few of the steroid hormones are tritium labeled and require a liquid scintillation counter. If only one counter can be purchased, then a gamma counter is the instrument of choice since most assays are now performed with gamma emitting isotopes. Table I lists the isotopes in common usage for competitive protein binding assays.

There are several manufacturers of counting equipment and most manufacturers will be able to supply manual, semi-automatic or completely automatic counting equipment.

With a manual counter each tube has to be positioned by hand. This system would be used for low volume or small batch sized assays. The decision most people have to make is whether to get a counter capable of counting only 100 tubes or counters capable of handling 300 to 500 samples. With the 100 tube counters usually a lister printer is included which places the printout on adding machine tape with the tube number next to it. This type of instrument is suitable for a laboratory that is involved in a limited number of assays, say 3 or 4 at the most. The reasons are that counting time can be slow since the changing cycle, that is the time to go from one sample to the next, is not always fast on these instruments. Another factor is that the efficiency of these small counters may not be as reliable as the more expensive counters, therefore, requiring more counting time per sample to acquire good counting statistics. Finally, a lister printer is used which necessitates manual data handling. This usually means that even blank tube counts must be subtracted by hand from each sample tube count. In the larger counter, printout is via a teletype with a paper tape punch. These counters are usually very efficient and have fast sample changing times. They usually can automatically subtract out background and most can even give you the dose response factor,  $B/B_0$ , as the print out, thereby eliminating the need to calculate this.

### Data Handling

After counting the samples and obtaining a printout from the counter the data must be arranged into a standard curve of some type from which patient results can be determined. In order to do this, a simple calculator may be used to aid in the mathematical manipulations. A desktop computer into which the data is entered may be used to generate a standard curve automatically along with the unknown values which are automatically read from the curve. If a paper tape print out

TABLE I  
COMMON ISOTOPES USED IN  
COMPETITIVE PROTEIN BINDING ASSAYS

| Nuclide               | Half Life | Type of Emission |
|-----------------------|-----------|------------------|
| $^{125}_{53}\text{I}$ | 60 d      | $\gamma$         |
| $^3_1\text{H}$        | 12.3 y    | $\beta$          |
| $^{57}_{27}\text{Co}$ | 270 d     | $\gamma$         |
| $^{75}_{34}\text{Se}$ | 120 d     | $\gamma$         |

is available from the teletype output of the counter, data handling is made even easier in that the tape can be read by a tape reader and fed automatically into the computer. This eliminates the time it takes to enter numbers manually. The only other consideration is whether to keep the computer on line with the counter and eliminate the paper tape. This system is predicated only if a counter is running continually day and night since the cost of the computer itself is approximately the same, whether on line or off line. By being on line the computer can be used for nothing else in the laboratory.

### Miscellaneous Equipment

Besides counting equipment, automatic pipettes and dilutors are also needed in doing competitive protein binding assays. These can range from a manual pipetter ( $\approx$  \$65), semi-automatic dilutor-pipetter ( $\approx$  \$2,000) or fully automatic diluting-pipetting station ( $\approx$  \$18,000). When only a few assays, consisting of a small number of samples, are run in a laboratory, the manual type pipette can be used. When several different assays of between 10 and 25 patients per assay are being done, the semi-automated pipettes are time saving and enhance precision. The fully automated pipette stations are recommended if the number of samples run is very large. If it is not, the time and inconvenience of cleaning these systems between runs and of using a large amount of reagent to prime them tends to preclude their use.

### Assay Set Up

Assays are usually set up according to the instructions provided with the reagents. These directions are usually very detailed and should be referred to in establishing an assay. However, the following illustrates the most common type of assay and may be helpful in explaining some of the various nomenclature used in the field of competitive protein binding assays.

1. Total activity tubes: These tubes contain only the isotope used in the assay in the same volume as that in each of the remaining tubes of the assay. These tubes are run to assess total activity (T) used in the assay and to determine  $B_0/T \times 100$  which usually should be between 35% and 50% in order to assure that the assay has a useable sensitivity.



2. NSB (non-specific binding) tubes: These tubes contain all reagents except the binding protein. They will therefore show those counts that cause an error in that they are due to free label being counted as bound.
3. Zero dose tubes ( $B_0$ ): These tubes contain all components except unlabeled material being assayed.
4. Finally, tubes for the standard curve are followed by the quality control tubes and the unknown tubes.

The background for each particular system should be determined periodically. This can be accomplished by counting an empty tube overnight in the counter and then by determining the counts per minute. This background is then subtracted from all tubes before any calculations are performed.

In evaluating different assay systems it is convenient to use a table of  $B/B_0$  vs dose. By comparing these values for different assays a comparison of the sensitivity of the assay can be made. Thus an insulin assay with 50% binding at a concentration of 40 microunits would show much more sensitivity than an assay with a 50% binding occurring at 100 microunits, since the more the dose is lowered, the greater the percent binding becomes.

### Isotope Safety (8)

Since the amount of radiation present in most radioassay methods is quite small, relatively few special precautions are needed. The basic factors to be kept in mind in dealing with isotopes are time, distance and shielding. The isotope storage should be located as far from personnel as possible. Radiation can be reduced by proper shielding. Normally gamma sources exceeding 10  $\mu\text{Ci}$  are packaged in small lead containers and should be kept in these containers except when material is being withdrawn. Each laboratory should have an isotope safety officer who has the responsibility of knowing and enforcing the safety rules which will vary with the type and amount of isotope used. This person would be responsible for monitoring the radiation areas with a portable radiation detector (Geiger Mueller Counter). Radiation workers who are working with gamma emitters or with beta emitters having a maximum energy greater than 0.2 Mev should wear film badges (these badges are provided by various companies). This badge is a sealed photographic film to measure exposure to ionizing radiation. The badges are then sent to the company and the exposure in rems is calculated and reported each month.

### Disposal of Radioactive Waste

The level of radioactivity encountered in the usual radioimmunoassay procedures is low enough so that liquid wastes may be disposed of in the sink with running water. In calculating the amount of radioactive material that may be disposed of via the sewage system from one building, one must know the water usage. This may be obtained from the water bill. The allowable quantity of  $^{125}\text{I}$  in sewage is  $4.0 \times 10^{-5} \mu\text{Ci/ml}$  of water usage. This is equal to  $1.13 \mu\text{Ci}$  per cubic foot of water usage.

### Radioisotope Licensing:

This rule varies from state to state. Some materials are exempt from Nuclear Regulatory Commission or State licensing requirements. Most institutions already have an institutional license which would specify the safety officer. It would be well for the clinical chemistry laboratory to check with this individual before beginning to use radioactive materials. If there is no license, many manufacturers of isotope materials will assist the laboratory in obtaining the proper license.

### Conclusion

While much care has to be used in performing competitive protein binding assays, most well-equipped and staffed clinical laboratories should have no serious problem in undertaking such assays. The biggest problem that may be encountered is the selection of a dependable and reliable manufacturer for reagents. Problems that may arise are non-purity of standards and label non-specificity of antibodies or the inability to maintain any of these characteristics from lot to lot. It therefore is a good practice to evaluate a few manufacturers before selecting one for routine use.

### Literature Cited

1. Besch, P.K.; *Clinical Radioassay Procedures: A Compendium*. AACC (1975).
2. Odiell, W. D.; and Daughaday, W.; *Principles of Competitive Protein-Binding Assays*. J. B. Lippincott (1971).
3. Zettner, A.; *Principles of Competitive Binding Assays (Saturation Analyses) I Equilibrium Techniques*. Clin. Chem. (1973), 19, 699.
4. Zettner, A; and Duly, P.; *Principles of Competitive Binding*

- Assays (Saturation Analyses) II Sequential Saturation.  
Clin. Chem. (1974), 20, 5.
5. Rodbard, D. et al.; Statistical Quality Control of Radioimmunoassays. J. Clin. Endocrinol. and Metab. (1968), 28, 1412.
  6. Rodbard, D.; Statistical Quality Control and Routine Data Processing for Radioimmunoassays and Immunoradiometric Assays. Clin. Chem. (1974), 20, 1255.
  7. Rodgers, R. C.; Radioimmunoassay Theory for Health Care Professionals. Hewlett Packard (1974).
  8. Bermes, E.W., Jr.; and Forman, D.T.; "Basic Laboratory Principles and Procedure" Fundamentals of Clinical Chemistry, 2nd ed., pp. 1-59, N.W. Tietz (ed.), W. B. Saunders, Philadelphia (1976).

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## Prenatal Detection of Genetic Diseases

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Perhaps the most important development in the general area of genetic counseling in the past several years has been the development of techniques for the prenatal diagnosis of genetic diseases. These techniques have made it possible to convert genetic counseling from an essentially passive endeavor to one in which the counselors and parents, working together, can take steps to alter the genetic risks to which the latter are exposed (1). This discussion, which will deal with the broad aspects of prenatal diagnosis, will be divided into two parts. The first will constitute a general background and theoretical introduction to the subject, while the second will describe the experience which we and others have had in the application of the various techniques of prenatal diagnosis. For further background and detail, the reader is referred to several other recent reviews of the subject (2-6).

As the name implies, the objective of making a prenatal diagnosis of a genetic disease is to determine whether a fetus believed to be at risk for having some genetic disease actually does have that disorder. For the most part the information which is obtained is used by the parents to decide whether they wish to continue the pregnancy or have it terminated. In the beginning stages of the introduction of prenatal diagnosis into medical practice, it was a general policy not to undertake prenatal diagnosis unless the family seemed committed to terminating the pregnancy if the fetus were abnormal. The principal reason for this policy was to protect the interests of the fetus in view of the uncertainty about the risks associated with the procedure. However, as time has passed and the safety of the procedure has been better established, more prenatal diagnoses are being performed to reassure the parents that the fetus is normal without necessarily providing information leading to termination of the pregnancy (7). In numerical terms, this still represents a very small proportion of the procedures

which are actually done.

### Frequency and Types of Genetic Disease

When the newborn population is examined it is found that between 2 and 4% of individuals, depending on how the figures are calculated, have some type of birth defect which is considered to be genetic in origin (8,9). The term "birth defect" is used in a general sense and encompasses all types of structural, metabolic, and other abnormalities which derive from genetic or other prenatal causes. The birth defects detected postnatally represent only a small fraction of the totality of abnormalities which result from genetic aberrations, but most of the latter result in early spontaneous abortions (10) and do not constitute indications for prenatal diagnosis.

The genetic disorders of the newborn can be divided into three general categories, and these are shown in Table I. The first category is that of the chromosomal disorders compatible with viability, and they affect about 0.6% of the newborn population. The second is the group of mendelian disorders which result from abnormalities of single genes. It is estimated that approximately 0.4% of the newborn population are affected with such conditions. This figure does not, however, include the large number of individuals who would be affected by erythroblastosis fetalis due to Rh incompatibility if anti-Rh-immune globulin were not administered. Likewise, it does not include those genetic conditions which have particularly high frequencies in restricted ethnic or racial groups. The best examples of the latter are glucose-6-phosphate dehydrogenase (G6PD) deficiency and sickle cell anemia (0.25% incidence) in the Black population, thalassemia in certain Mediterranean and Oriental populations, and Tay-Sachs disease in Ashkenazi Jews (0.04% incidence). While many of the single gene disorders are biochemical in nature, at least in so far as is possible to define specific biochemical abnormalities, not all can be so characterized. Several of them represent disorders of morphogenesis, with malformation of one or several organs, or disorders in function for which biochemical explanations do not presently exist. In absolute numbers, therefore, the frequency of frankly biochemical genetic disorders, often designated as the inborn errors of metabolism, is quite low.

The third category of genetic disorders includes these conditions in which the genetic components are less well defined and are influenced by non-genetic or "environmental" factors which are poorly understood. These conditions are referred to as the polygenic or multifactorial disorders and can be further subdivided into two groups. The first is the group of the common congenital malformations, including cleft palate and lip, spina bifida and anencephaly, club feet,

congenital heart defects, pyloric stenosis, congenitally dislocated hips, and renal anomalies. In the aggregate, these disorders affect over 1% of the newborn population. The second group of multifactorial disorders, and the one for which it is impossible to obtain accurate incidence figures, is the group of relatively common diseases of adulthood which have genetic components to them. Included in this group are diabetes mellitus, schizophrenia, allergies, and certain forms of malignancy.

In terms of their suitability for prenatal diagnosis, the first two categories of genetic diseases, the chromosome abnormalities and single gene defects, especially those that are biochemical in nature, constitute the conditions most amenable to the techniques presently available. However, at least one of the multifactorially determined congenital malformations (the neural tube defects) can also be diagnosed prenatally.

The major impetus to the development of methods for the prenatal detection of genetic disorders derives, in historical terms, from the roughly simultaneous development of three major techniques (11-14). One was the technique, and the willingness to use it, for obtaining samples of amniotic fluid early in gestation. The second was the development of techniques for the culture of human cells *in vitro*, and the third was the development of better techniques for cytogenetic analysis. As will be described below, with the availability of these three techniques it became possible first to work out methods for the examination of fetal chromosomes, and then, by extension, to devise ways of determining other characteristics of the fetus.

If the object of prenatal diagnosis is to determine the state of the fetus who is suspected of having some genetic abnormality, how can its condition actually be assessed? The range of possibilities for making such assessments will be outlined in the next section, starting with the most direct and going to the least direct. Although discussions of prenatal diagnosis are not usually presented in this way, this approach should help the reader conceptualize the problems associated with attempting to learn about an organism which cannot readily be approached.

### Fetal Visualization

Direct Visualization. The most direct way of examining a fetus is to look directly at it, and an instrument has been developed for this purpose -- the fetoscope (15). This instrument is a modified endoscope which can be inserted into the amniotic cavity through a 14 gauge needle. Since this needle is fairly large in diameter, there is a significant possibility of uterine or placental bleeding associated with its introduction. In addition, lack of flexibility interferes with

Table I

Frequency of genetic disorders in the English newborn population

Chromosomal DisordersAutosomal

|                  |             |
|------------------|-------------|
| Trisomy 21**     | 0.13%       |
| Trisomy 18**     | 0.03        |
| Trisomy 13**     | 0.02        |
| Translocations** | 0.01        |
| Deletions**      | <u>0.01</u> |
| Total autosomal  | 0.20%       |

Sex chromosome

|                          |              |
|--------------------------|--------------|
| XO and X deletions**     | 0.02         |
| Other "severe" defects** | 0.01         |
| XXY**                    | 0.1          |
| XXX**                    | 0.1          |
| XYY**                    | 0.1          |
| Others**                 | <u>0.015</u> |
| Total sex chromosome     | 0.35%        |

|                             |       |
|-----------------------------|-------|
| Total chromosomal disorders | 0.55% |
|-----------------------------|-------|

Monogenic (mendelian) DisordersAutosomal recessive

|                                    |             |
|------------------------------------|-------------|
| Mental retardation, severe         | 0.08%       |
| Cystic fibrosis                    | 0.05        |
| Deafness, severe (several forms)   | 0.05        |
| Blindness, severe (several forms)  | 0.02        |
| Adrenogenital syndrome             | 0.01        |
| Albinism                           | 0.01        |
| Phenylketonuria                    | 0.01        |
| Other amino acidurias*             | 0.01        |
| Mucopolysaccharidoses, all forms** | 0.005       |
| Tay-Sachs disease**                | 0.001       |
| Galactosemia**                     | 0.0005      |
| Others*                            | <u>0.05</u> |
| Total autosomal recessive          | 0.30%       |

Table I (Continued)

|   |                      |
|---|----------------------|
| <b>X-linked</b>                             |                      |
| Duchenne muscular dystrophy***              | 0.02%                |
| Hemophilias A and B***                      | 0.01                 |
| Others*, ***                                | 0.02                 |
| <b>Total X-linked</b>                       | <u>0.05%</u>         |
| <b>Autosomal dominant</b>                   |                      |
| Blindness (several forms)                   | 0.01%                |
| Deafness (several forms)                    | 0.01                 |
| Marfan syndrome                             | 0.005                |
| Achondroplasia                              | 0.005                |
| Neurofibromatosis                           | 0.005                |
| Myotonic dystrophy                          | 0.005                |
| Tuberous sclerosis                          | 0.005                |
| All others                                  | <u>0.015</u>         |
| <b>Total autosomal dominant</b>             | <u>0.06%</u>         |
| <b>Total monogenic disorders</b>            | <b>0.41%</b>         |
| <b><u>Multifactorial Disorders</u></b>      |                      |
| <b>Congenital malformations</b>             |                      |
| Spina bifida and anencephaly**              | 0.45%                |
| Congenital heart defects                    | 0.4                  |
| Pyloric stenosis                            | 0.3                  |
| Talipes equinovarus (club feet)             | 0.3                  |
| Cleft lip and palate                        | 0.1                  |
| Dislocated hips                             | 0.1                  |
| Others                                      | <u>0.2</u>           |
| <b>Total congenital malformations</b>       | <u>1.85%</u>         |
| "Common diseases"                           | ? (>>1%)             |
| <b>Total multifactorial disorders</b>       | <u>&gt;&gt;1.85%</u> |
| <b>Total frequency of genetic disorders</b> | <b>&gt;&gt;2.81%</b> |

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\*Diagnosable in utero (some)

\*\*Diagnosable in utero

\*\*\*Fetal sex can be determined



manipulation of the instrument once it is within the uterus, and the narrow field of vision makes it difficult to adequately examine all parts of the fetus. Nevertheless, it has been possible with such an instrument to visualize fetal fingers and other aspects of the external surface of the fetus, and in theory such examination should be useful in diagnosing major structural abnormalities. However, because of the difficulties already mentioned, the fetoscope does not presently enjoy much popularity as a practical tool for prenatal diagnosis, although it is being used to assist in the sampling of fetal blood from placental vessels (16,17). Before the fetoscope can be more widely used, it will be necessary to develop a very fine flexible instrument with a wide field of vision.

#### Visualization by Radiological and Ultrasound Techniques.

Other less direct visualization systems have also been and are being used, with varying degrees of success. At the present time, radiological (x-ray) examinations of the fetus are mainly limited to a determination of the structure of fetal bones. This is of potential usefulness in those disorders which are characterized by the absence or gross structural abnormality of a specific bone or bones (18), but such examinations have not always proved to be reliable. Again, it will be necessary to have improvements in radiologic technique, such as are exemplified by xerography and image intensification, before radiologic examination of the fetus will attain the degree of usefulness that it might have for prenatal diagnosis.

The method of fetal visualization that has thus far been the most helpful makes use of ultrasonic scanning. In regular obstetrical practice this technique is used to diagnose twin pregnancies, to localize the placenta, and to determine fetal age by measuring the width of the fetal head. The ability to examine the fetal head by the currently available techniques makes ultrasonic scanning useful for the prenatal diagnosis of anencephaly, one of the multifactorially determined congenital malformations (19). In this condition, the cranium and brain are not properly formed, and it is the absence of the cranium which is detected by ultrasonic scanning. While this disorder is relatively infrequent in the general population, its frequency is greater than 1% in the offspring of couples who have already had a child affected with the same or a related (spina bifida) neural tube disorder. Coupled with a determination of amniotic fluid  $\alpha$ -fetoprotein concentrations (see below), ultrasonic scanning leads to a secure diagnosis of anencephaly. However, like the other methods, the present techniques of ultrasonic scanning are still capable of considerable improvement, and one such improved method, gray scale scanning, is now being introduced (20). In theory, this technique has the capability of providing much sharper resolution of fetal structures, both external and internal.

### Sampling of Fetal Blood and Tissues

Even if visualization techniques were developed to the point of allowing a complete and accurate examination of the external aspects of the fetus, many genetically determined disorders would still not be diagnosable, either because they did not produce any external physical abnormalities or because the abnormalities would not as yet have become apparent. Therefore, the next most direct method of examining the fetus is to obtain a sample of fetal tissue. As might be imagined, the direct sampling of tissues of an infant or child is not always an easy thing to do, and the direct sampling of fetal tissues is even less so. The most readily accessible and easily sampled tissue after birth is blood, and it is not surprising that the most serious attempts to sample fetal tissues have also been concerned with the obtaining and examination of fetal blood. The prenatal situation is particularly favorable for this since it is not really necessary to obtain blood from the fetus itself. With suitable techniques, fetal blood can be obtained from the placenta, either by puncture of a placental vessel under direct visualization or by aspiration from the placenta with ultrasonic guidance (15-17,21,22). The principal abnormalities which have so far come under investigation and seem to be amenable to this approach are those of hemoglobin structure and synthesis. While the major hemoglobin synthesized by the fetus after the first few months in utero is fetal hemoglobin (hemoglobin F), which is composed of  $\alpha$ - and  $\gamma$ -chains, the fetus also makes  $\beta$ -chains from very early on. Therefore, in addition to being able to detect all of the  $\alpha$ -chain abnormalities, it is also possible to make the diagnosis of  $\beta$ -chain abnormalities as well. Of the latter, the most common are sickle cell anemia, a structural abnormality of the  $\beta$ -chain, and  $\beta$ -thalassemia, a condition characterized by decreased  $\beta$ -chain synthesis. To diagnose hemoglobin abnormalities, small quantities of fetal blood are obtained from the placenta and incubated with radioactive amino acids in vitro. Since there is usually an admixture of maternal blood, it is necessary to determine the relative proportions of maternal and fetal red cells and to make the appropriate corrections to the data. After incubation, the hemoglobin is isolated, fractionated by chromatographic techniques, and the radioactivity in the individual chains determined (22,23). With these methods it is possible to assess whether the various chains are made in normal amounts and whether abnormal chains are being synthesized. Because of changes in the relative rates of chain synthesis -- for example, the ratio of  $\beta$ - to  $\gamma$ -chains in normal fetuses increases from 7.5 to 10% between the 6th and 16th weeks of development -- it is necessary to have well defined control information if a decision is to be made about whether a significant decrease in the rate of synthesis of a particular chain has occurred (22).

At the present time the sampling of fetal blood is still problematic at best. Fetal blood in sufficient amounts for analysis cannot be obtained with absolute certainty and with the degree of safety associated with the sampling of amniotic fluid (17). Therefore, we are still not yet at the stage of being able to analyze fetal blood with the same ease that we can analyze other biochemical properties of the fetus from cultured cells and amniotic fluid. And, to date, the obtaining of fetal serum, as opposed to red cells, has been even less satisfactory. Since many of the constituents that could be assayed for in the prenatal detection of genetic disorders are present in maternal serum in relatively large concentrations, even a small contamination of the fetal serum would make reliable assessment of the latter quite difficult. Therefore, while it would be desirable to be able to determine the amounts of antihemophilic globulin (factor VIII) and other coagulation factors to diagnose the clotting disorders and of  $\text{Cl}^-$ -esterase inhibitor, to diagnose hereditary angioneurotic edema, such determinations are not presently feasible. Similarly, other serum proteins of future genetic interest would be  $\alpha_1$ -antitrypsin, ceruloplasmin, complement, and the immunoglobulins.

The direct sampling of other fetal tissues such as skin (24) and possibly even the internal organs has been discussed but has not been used in practice. Likewise, biopsy and analyzing the placenta has been advocated but not used for prenatal diagnosis (25). Therefore, for most types of information, indirect sampling of the fetus through analysis of the amniotic fluid and its constituents is commonly used. It must be emphasized that amniotic fluid and its constituents are a few biochemical and physiological steps removed from the fetus itself, and results obtained from their examination must be interpreted with caution.

### Amniocentesis

The amount of amniotic fluid which surrounds the fetus increases with fetal size and age. The ability to obtain amniotic fluid for analysis is basically a mechanical problem of having an amniotic cavity large enough to enable the obstetrician to safely insert a needle and withdraw an amount of fluid sufficient for study. With current techniques it is considered reasonably safe to do so at 13 or more weeks of fetal development, at which time there is 150 ml or more of amniotic fluid (26). The amount of fluid withdrawn is generally of the order of 10 to 15 ml, 10% or less of the total amniotic fluid volume, and this volume is rapidly replaced by the fetus. The amniotic fluid is obtained by inserting a needle into the uterus through the midline of the lower abdomen. This procedure, referred to as amniocentesis, is relatively non-traumatic physically, and if handled properly, psychologically. The

risks to the mother and to the fetus seem to be minimal, but absolute safety cannot be claimed and the possibility of inducing a miscarriage cannot be dismissed (see below).

#### Direct Analysis of Amniotic Fluid Constituents

In most instances, the cellular component of the amniotic fluid, which consists of diverse cells of fetal origin, are placed in culture and used for further analysis. However, there are instances in which uncultured cells have been used for direct cytologic or biochemical analysis and in which the soluble components of the fluid have been directly analyzed (27-29). The biochemical analysis of uncultured cells has not proven to be highly reliable (30,31). The cell population itself is quite heterogeneous in composition and variable with regard to the proportion of viable and nonviable cells. The use of uncultured cells for ultrastructural examinations has also been advocated, especially for the diagnosis of the lysosomal storage diseases, and there is evidence that amniotic cells of fetal origin will show the presence of various bodies characteristic of lysosomal storage (27,32). However, this requires the availability of an appropriate electron microscope facility, and most groups do not have such facilities at their disposal.

Uncultured cells have also been used to a limited extent for fetal sex determination. This is possible because the cells can be appropriately stained and examined for the presence of sex chromatin, characteristic of female cells, and for the fluorescent Y-body, characteristic of male cells (33,34). However, these cytologic techniques are not completely reliable, and it is still desirable to determine fetal sex by direct chromosome analysis (35).

The soluble components of the amniotic fluid have also been subjected to various types of analyses. Thus far, examination of the concentrations of various metabolites or degradation products in the fluid have not proven to be useful for the diagnosis of inborn errors of amino acid or mucopolysaccharide metabolism (36,37). Likewise, it has not been possible to make specific diagnoses of disorders associated with deficiencies of particular proteins by examination of amniotic fluid. While certain proteins, deficiencies of which are associated with inherited disease, are present in amniotic fluid (38,39) -- for example,  $\alpha_1$ -antitrypsin, Cl<sup>-</sup>-esterase inhibitor, ceruloplasmin,  $\beta$ -lipoprotein, and immunoglobulin G (IgG) -- it still has to be determined how much of these materials are fetal in origin as opposed to being maternal, and how well the concentration in the amniotic fluid reflects the actual state of the fetus (40). The one constituent of the cell-free amniotic fluid which has proven to be very useful for prenatal diagnosis is  $\alpha$ -fetoprotein, and this will be discussed below. A few prenatal diagnoses have

been made by analyses of enzymes in cell-free amniotic fluid. However, such methods are not considered highly reliable (41) and are little used.

### Cultured Amniotic Fluid Cells

As a result of these difficulties, most of the work with amniotic fluid is concerned with the analysis of cultured cells. At the present time it is believed that three types of cells can be grown from the amniotic fluid: an epithelioid type of cell, a fibroblast-like cell, and a so-called "amniotic fluid cell" (AF cell) intermediate in characteristics between the other two (42). While the AF and fibroblast-like cells grow reasonably well in culture, the epithelioid cells grow very slowly and are difficult to work with. Of all of the cells originally applied to a culture plate when a culture is initiated, relatively few (on the order of several hundred) actually attach and grow out. Therefore, cultures derived from amniotic fluid represent a set of cell clones rather than a mass population (42). The media and general techniques for culturing amniotic fluid are not greatly different from those used for the culturing of other human cells, and success seems to be more a function of the skill of the person handling the cells than of any particular culture condition.

Two types of information can be obtained from cultured amniotic fluid cells. The first, which constitutes the major proportion of the work done in the area of prenatal diagnosis of genetic disorders, is the chromosome constitution of the fetus. The second is the biochemical status of the fetus, at least in so far as certain defined enzyme pathways are concerned.

Cytogenetic Analysis and Sex Determination. Since amniotic fluid cells are fetal in origin, they have the chromosome complement of the fetus itself. Therefore, the chromosomes of the fetus can be examined by karyotyping the cultured amniotic fluid cells. There are two principal reasons for examining fetal chromosomes. One, the numerically less frequent reason, is to determine the sex of the fetus; the other is to determine whether the fetus has a chromosome abnormality. Fetal sex determination is not, at present, performed so that couples can choose the sex of their baby. While there may not be any significant ethical or sociological (43) reasons to oppose sex determination for this reason, the lack of sufficient facilities has made this an indication of extremely low priority. The reason for ascertaining the sex of a fetus, in so far as the prenatal diagnosis of genetic disease is concerned, is to determine whether the fetus is a male or female in situations in which the parents are at risk of having a child with an X-linked disorder which affects only males. If the fetus is a male, it will have a 50% risk of being affected. This risk, in such

conditions as Duchenne muscular dystrophy, hemophilia A, and some of the other serious X-linked disorders, is considered to be quite high (see 44 for complete list). If the fetus is female, the risk of being affected is extremely low (except in the rare situation in which the father is affected), and families which undertake to have the sex of the fetus determined because of X-linked problems do so because they are anxious to prevent the birth of a male who might be affected.

The more general reason for examining the chromosomes of the fetus and the single most important indication for prenatal diagnosis is to determine whether some type of chromosome abnormality is present. Several of the more common chromosome abnormalities are listed in Table I. The chromosome abnormalities can be divided into two broad categories, those of the autosomes (non-sex chromosomes) and those of the sex chromosomes. Of the autosomal abnormalities, by far the most common is trisomy 21, the presence of an extra chromosome 21. This abnormality is associated with Down's syndrome (mongolism), a condition characterized by mental retardation and major and minor congenital malformations. The other autosomal trisomies, trisomies 18 and 13, are less frequent but considerably more severe than trisomy 21 and usually result in early death. Abnormalities involving other autosomes are also infrequent, but can produce a wide variety of abnormalities and are generally always associated with significant mental retardation.

The sex chromosome abnormalities are generally less severe in terms of their effects on mental development, but two of them, the XO and XXY abnormalities, are associated with sterility and other abnormalities in individuals who develop as females or males, respectively. The two other sex chromosome abnormalities, XXX and XYY, are more problematic. The XXX constitution does not produce significant abnormalities in the females who have it but can, in some individuals, be associated with a modest degree of mental slowness (45). The XYY constitution, in the males who have it, appears to be associated with increased stature and, at least in some individuals, with a tendency toward antisocial behavior (46, 47). While the absolute incidence of such behavior of XYY individuals is probably quite low, it is still thought to be higher than in a chromosomally normal population. These two sex-chromosome abnormalities have been specifically mentioned because they have engendered considerable concern about what types of counseling should be given in situations in which they are detected prenatally.

While all chromosomal abnormalities can in principle be detected by prenatal diagnosis, fetal karyotyping is not at present being done as a general screening procedure. Rather, it is done under circumstances in which there appears to be a significantly increased risk of having a child with a chromosome abnormality. For the most part, this increased risk is associated with trisomy 21, although it can, in certain rare instances,

be associated with other chromosomal abnormalities as well. While the overall population incidence of trisomy 21 is between 1 in 700 and 1 in 600 live births (48, 49), the frequency of its appearance in any given family is determined by two factors. One is the age of the mother at the time of the infant's birth. The mean figure of about 1 in 600 live births is representative of the risk to a mother who is 35 years of age at the time the child is born. For women in the third decade, the risk is considerably less, 1 in 1000 or lower. However, by the time a woman reaches 40 years of age, the risk of having a child with Down's syndrome is between 1 and 2% (based on the newborn population) and this risk may reach as high as 4% by the time she is 45. Although it is impossible to define what constitutes a high risk for having a child with Down's syndrome, it is commonly accepted that a woman of age 40 or greater is in a high risk category. However, for many families and physicians this period of high risk begins even as early as age 35.

The second major factor which influences the frequency of Down's syndrome is the prior birth of a child with the same disorder (50). In such instances, the chance of having another affected child is considerably greater than the age-specific incidence of the disorder, and is generally in the range of 1 to 2%. Again, for the individuals involved, this is considered to be a high risk, particularly in view of the fact that they have already had an abnormal child. Down's syndrome can occur with even higher frequencies, sometimes reaching as high as 20%, in the relatively rare families in which one of the parents, usually the mother, is the carrier of a balanced translocation involving chromosome 21.

As has already been mentioned, all chromosome abnormalities can be detected in the fetus. For that reason, some individuals have suggested that wide-scale routine prenatal screening be carried out (51). However, the greater the population screened, the lower will be the proportion of fetuses found to be affected. In economic terms, this would elevate the cost of case detection to high, perhaps prohibitive, levels. Putting economics aside, the most cogent reason for not undertaking wide-scale screening, at least at this time, may be the still undefined upper limit of complications of the procedure, whether these be induction of miscarriage, misdiagnosis, or the like. It has generally been felt, at least to this time, that prenatal diagnosis should not be undertaken in circumstances in which the risk of having an affected child is significantly less than the risk of the procedure itself. As confidence in the safety of the procedure has increased there has been a relaxation in the age restrictions for prenatal diagnosis. But, it is not at all clear that the overall complication rate will even reach a point of being significantly below 0.1 to 0.2%, the risk to a 30 to 35 year old mother of having a child with a chromosome abnormality.

**Biochemical Analysis.** The second principal use of cultured amniotic fluid cells is to determine the biochemical status of the fetus. Again, it should be pointed out that the individual biochemical disorders are quite low in incidence, but many of them are very serious in their manifestations and can occur with relatively high frequencies in specific families. If two parents are carriers of a gene for an autosomal recessive condition, then the risk to each child of having the disorder is 25%.

The biochemical disorders diagnosable in utero are those which have enzymatic representations in the types of cells which can be cultured from the amniotic fluid. At the present time approximately 25 to 30 disorders have actually been diagnosed (table II) and another 35 are capable of diagnosis (52,53). Many of the disorders which have already been diagnosed are those in the lysosomal storage disease group, conditions which frequently cause early neurologic degeneration, sometimes in association with skeletal abnormalities and visceral enlargement. In nearly all instances, the diagnosis is made by direct determination of the activity of the enzyme involved in the disorder, although occasionally a somewhat more indirect approach can be used. For example, cystinosis is detected in cultured cells by assessment of the ability of the cells to handle cystine, even though the precise enzymatic defect is not known (54).

There are important methodologic considerations which apply to the use of cultured amniotic fluid cells for the detection of biochemical disorders. The first is that the enzymes which can be sampled are those which are usually present in fibroblasts or fibroblast-like cells. Therefore, conditions such as phenylketonuria and glycogen storage disease type I, which are associated with deficiencies of enzymes present only in liver and kidney, are not amenable to this approach. The same also pertains to enzyme deficiencies affecting other specific tissues. The one present exception to this is histidase, an enzyme characteristic of epithelial cells. In this instance, it has been possible to select clones of epithelioid cells from the mixed cell population of the amniotic fluid cell cultures and to analyse them for this enzyme (55).

The second methodologic consideration relates to the type of cells which are used as controls for the biochemical determinations. While less a problem now, there was a tendency in some early investigations to use cultured skin fibroblasts or similar cells as controls for the cultured amniotic fluid cells. It has been demonstrated that such controls are not valid and that there may be significant differences in enzyme concentrations between two cell types (56). For this reason, normal amniotic fluid cells themselves must be used as controls for amniotic fluid cell cultures being subjected to enzymologic investigation.

The third and potentially most severe methodologic problem



**Table II**  
**Biochemical Disorders Diagnosed Prenatally (Second Trimester)**

| <u>Disease</u>   | <u>Metabolic Defect</u>                                   | <u>Reference</u> |
|--|---|------------------|
| Acid phosphatase deficiency                                      | Lysosomal acid phosphatase deficiency                     | <u>73</u>        |
| Adenosine deaminase deficiency (combined immunodeficiency)       | Adenosine deaminase deficiency                            | <u>74</u>        |
| Argininosuccinic aciduria  | Argininosuccinase deficiency                              | <u>75</u>        |
| Cystinosis   | Cystine accumulation                                      | <u>54</u>        |
| Fabry disease  | Ceramidetrihexoside $\alpha$ -galactosidase deficiency    | <u>76</u>        |
| Galactokinase deficiency   | Galactokinase deficiency                                  | <u>77</u>        |
| Galactosemia   | Galactose-1-phosphaturidyltransferase deficiency          | <u>78</u>        |
| G <sub>M</sub> <sub>1</sub> gangliosidosis, type I (generalized) | $\beta$ -Galactosidase deficiency                         | <u>79</u>        |
| G <sub>M</sub> <sub>1</sub> gangliosidosis, type II (juvenile)   | $\beta$ -Galactosidase deficiency                         | <u>80</u>        |
| Gaucher disease  | $\beta$ -Glucosidase deficiency                           | <u>70</u>        |
| Glycogen storage disease, type II (Pompe)                        | $\alpha$ -1,4 Glucosidase deficiency                      | <u>81</u>        |
| Hunter syndrome  | $\alpha$ -L-Iduronic acid-2 sulfatase deficiency          | <u>82, 83</u>    |
| Hurler syndrome  | $\alpha$ -L-Iduronidase deficiency                        | <u>82, 83</u>    |
| Hypophosphatasia   | Alkaline phosphatase deficiency                           | <u>84</u>        |
| Krabbe disease   | Galactocerebroside- $\beta$ -galactosidase deficiency     | <u>69, 72</u>    |
| Lesch-Nyhan syndrome   | Hypoxanthine guanine phosphoribosyltransferase deficiency | <u>85</u>        |
| Maple syrup urine disease  | Branched-chain keto-acid decarboxylase deficiency         | <u>86</u>        |
| Metachromatic leuko-   | Arylsulfatase A deficiency                                | <u>87</u>        |

Table II (Continued)

| <u>Disease</u>   | <u>Metabolic Defect</u>                         | <u>Reference</u> |
|--|---|------------------|
| dystrophy  |   |                  |
| Methylmalonic aciduria<br>(B <sub>12</sub> -resistant) | Methylmalonyl-CoA mutase<br>deficiency          | <u>88</u>        |
| Mucopolipidosis II (I-<br>cell disease)                | Multiple lysosomal enzyme<br>deficiencies       | <u>89</u>        |
| Niemann-Pick disease                                   | Sphingomyelinase deficiency                     | <u>68, 71</u>    |
| Sandhoff disease                                       | β-N-Acetyl-hexosaminidase<br>A and B deficiency | <u>27</u>        |
| Sanfilippo disease,<br>type A                          | Heparin sulphamidase defi-<br>ciency            | <u>90</u>        |
| Tay-Sachs disease                                      | β-N-Acetylhexosamidase A<br>deficiency          | <u>28</u>        |
| β-thalassemia  | Decreased hemoglobin<br>β-chain synthesis       | <u>22</u>        |
| Xeroderma pigmentosum                                  | Endonuclease deficiency                         | <u>91</u>        |

is that which relates to the amount of material required for analysis. For most of the conditions that have been studied, conventional methods of enzyme analysis have been employed. This has necessitated the growing of fairly large quantities of cells, a procedure frequently requiring between 4 and 8 weeks of culture (57). There is a limited time available between the earliest period at which an amniocentesis can be performed and the latest at which a pregnancy can be terminated. As a result, such long culture periods can lead to great difficulties, psychologic and otherwise, for both the patients and the physician. For this reason, micromethods have been developed for the assay of enzymes in limited numbers of cells. Such methods have been applied to cultured amniotic cells and make use of microspectrophotometry and/or microfluorometry. The amniotic fluid cells are generally grown directly on thin plastic foils, and after a relatively short time in culture (5-10 days), are freeze dried and their number or dry mass determined. They are then incubated with substrates in very small quantities of medium and examined with the appropriate instrumentation. By this means, it has been possible to make the diagnosis of several conditions using fewer than a thousand cells, sometimes even fewer than 100, rather than the several million cells which are generally needed at present (58,59). Since the instrumentation for such micro assays is expensive and the techniques highly specialized, it would seem reasonable to have centralized laboratories which can make this methodology available to those who need it. While such laboratories exist in Europe, these methods are not readily available at the present time in the United States.

### $\alpha$ -Fetoprotein Determinations

Perhaps the most indirect way of sampling the fetus is to assess something which the fetus causes to happen even though it is not itself an intrinsic part of the birth defect. Such sampling has become the basis of the diagnosis of the neural tube abnormalities, spina bifida with anencephaly and meningo-myelocoele, and mention has already been made of determination of  $\alpha$ -fetoprotein in the amniotic fluid. This protein is made by the fetus and is present in only low concentrations in amniotic fluid and maternal serum during pregnancy. However, in the presence of open neural tube defects, as well as of a limited number of other defects (such as omphalocele or the fetal nephrotic syndrome), large quantities of  $\alpha$ -fetoprotein appear in the amniotic fluid (60-62). Using sensitive immunochemical methods for assaying  $\alpha$ -fetoprotein concentrations, it has been possible to reliably make the diagnosis of the open neural tube defects during the second trimester of pregnancy. This diagnostic procedure is gradually being introduced as a screening procedure in all cases in which amniocentesis is performed, since ample amniotic fluid is available after the cells are removed for

culture.

While the concentration of  $\alpha$ -fetoprotein in the maternal serum is also frequently elevated when the mother is carrying a fetus with an open neural tube defect, such elevations occur only about 65% of the time (63). Therefore, the determination of  $\alpha$ -fetoprotein in maternal serum cannot be used as a specific diagnostic procedure. However, it has been suggested that it might be a useful screening procedure for all pregnant women, particularly those who are not undergoing amniocentesis for some other indication. While not all affected fetuses would be detected, a sizable proportion would, and this could lead to a significant reduction in the incidence of a condition associated with significant disability.

#### Experience with Prenatal Diagnosis

The San Francisco Series. With the above as background, the experience in San Francisco and the accumulated experience of several groups throughout the world will be discussed. At the time of this writing, over 700 patients have undergone amniocentesis in San Francisco for the prenatal diagnosis of a wide variety of genetic disorders. The figures to be discussed here are based on the fully analyzed first 500 cases (64) (earlier reports of the first 50 and 100 cases have already appeared) (65, 66). Before giving actual outcomes, there are two technical points worthy of mention. First, an attempt was made to determine whether the time required to obtain a cytogenetic diagnosis was a function of the stage of pregnancy at which the amniotic fluid was obtained. Over the time span of 14 weeks to 21 weeks after the last menstrual period, there did not appear to be any significant relationship between stage of gestation and time to diagnosis, and the mean time to diagnosis was approximately 3 1/2 weeks (65). Likewise, this time was not affected by the experience of the technician who was doing the work and seemed to be more related to inherent differences in the proliferative capacity of the amniotic cells derived from different individuals (65). For biochemical investigations, as already mentioned, considerably longer periods of time were required.

A total of 448 of the 500 pregnancies were monitored for cytogenetic indications exclusive of fetal sex. Of these, 332 were for advanced maternal age, with 172 of that group being between 35 and 39 years of age and 160 being 40 years or over. Of the former group, four fetuses had Down's syndrome, while of the latter, two had Down's syndrome, two had other autosomal trisomies, and one had an unidentified centromeric fragment. Taken together, and keeping in mind the limited size of the sample, these figures indicate a 2.7% frequency of chromosomal abnormalities in the age group 35 years and older. Of this group, 1.8% had Down's syndrome, with the frequency of Down's syndrome being 2.3% in the 35 to 39 year old group and 1.3% in the 40 and over

group. If the other abnormalities are included, the frequency of chromosomal aberrations in the 40 and older group was 3.1%. However they are interpreted, these figures are higher than would be expected from the usual newborn population survey statistics, particularly in the age group between 35 and 39. The reasons for this are unclear. It does not seem to be a statistical artifact, since similar results have been obtained in the cumulative experience of many centers. It is possible that some sort of bias of which we are presently unaware is operating.

The second most frequent group being monitored for cytogenetic indications was that composed of women who had a previous child with Down's syndrome due to a simple trisomy 21. Of that group, one fetus had an XO karyotype, one had Down's syndrome and one had an abnormal karyotype with an extra band on the short arm of chromosome 7 (7p+). Again, keeping the size of the sample in mind, this gives a frequency of Down's syndrome of 1% and of all chromosome abnormalities of 3.2%. The former figure is within the expected range but the latter is higher than might have been expected. Twenty-one other women underwent amniocentesis for a variety of different cytogenetic indications, and no abnormal fetuses were detected. In one case, however, the fetal karyotype was quite abnormal, with a previously unrecognized translocation involving chromosomes 2 and 14. When this abnormality was first recognized in the cultured cells, it was impossible to determine whether the fetus would be normal or not. The parents elected to carry the pregnancy to term, and a normal fetus with the previously detected chromosome abnormality was delivered.

Nineteen women underwent amniocentesis for the determination of fetal sex. Several different X-linked abnormalities constituted the indications for this procedure, and these included hemophilia A, hemophilia B, Duchenne muscular dystrophy, optic albinism, X-linked mental retardation, the Lesch-Nyhan syndrome (due to deficiency of hypoxanthine-guanine phosphoribosyltransferase, and Fabry's disease (due to deficiency of an  $\alpha$ -galactosidase). Fourteen of the fetuses were male, including one which turned out to be a set of twins, and most of the male pregnancies were terminated. The sex determination being carried out for Fabry's disease is of particular interest, since in this case it was desired to find out whether the fetus was a female. The father of the child was affected, and the family was concerned about having a female who would by necessity be heterozygous for the condition and subject to manifesting the disorder. Of the X-linked disorders, muscular dystrophy was the most common indication. While it was not possible in our cases to determine whether the fetuses were or were not affected, it may, on the basis of a recent report, be possible to do so (67).

Although, as has been mentioned, inherited biochemical disorders are rare, 17 out of the 500 pregnancies have been monitored because of a possibility of having a child with a hereditary

biochemical defect. In two instances, a woman underwent amniocentesis in more than one successive pregnancy. In our series, prenatal diagnosis was undertaken for the following conditions: Niemann-Pick disease (sphingomyelinase deficiency), Gaucher disease ( $\beta$ -glucosidase deficiency), Krabbe disease (galactocerebrosidase- $\beta$ -galactosidase deficiency), Pompe disease or glycogen storage disease type II ( $\alpha$ -glucosidase deficiency), metachromatic leukodystrophy (arylsulfatase A deficiency), galactosemia (galactose-1-phosphate uridylyltransferase deficiency), maple syrup urine disease (branched chain ketoacid decarboxylase deficiency), cystinosis (abnormal storage of cystine), and  $\beta$ -thalassemia (decreased synthesis of hemoglobin  $\beta$  chains). Of this group, six affected individuals were detected, not statistically different from the five expected for a 25% recurrence risk (22, 68-70). Of considerable interest were the results of the pathologic examination of three fetuses affected with lysosomal storage diseases, Niemann-Pick disease, Gaucher disease, and Krabbe disease (70-72). Each of these fetuses presented unequivocal chemical and ultrastructural evidence for the disease process and indicated that even a fetus midway through gestation is demonstrably affected by the genetic abnormality.

The monitoring of pregnancies for neural tube defects is the most recent of the indications for prenatal diagnosis. In families in which one child has been affected, the risk of recurrence is approximately 4%. Of the 16 pregnancies in which amniocentesis was done to determine the level of  $\alpha$ -fetoprotein, no affected fetuses were found.

In these 500 cases, there were 26 culture failures, an incidence of about 5%. Nine women of this group underwent a second amniocentesis, and successful cultures were obtained. The other 17, or 3.4% of the total, did not obtain any results, either because of spontaneous abortions prior to the time a second amniocentesis could be performed (in four) or because the family elected not to proceed. There were no instances of severe or significant fetal injury, although small linear "scars" were noted on four fetuses. Three fetuses had abnormalities which were not related to the procedure itself, including spina bifida, osteogenesis imperfecta, and congenital heart disease. One set of undetected triplets also occurred, in addition to the twins already mentioned. Ten abortions occurred following amniocentesis, three occurring within one week of the procedure and associated with fever, and seven not associated with fever and occurring between 18 and 50 days after amniocentesis itself, the former group which comprises 0.4% of the total, must be considered a direct complication. However, even this conclusion must be tempered by the fact that in the week prior to a scheduled amniocentesis, 20 women had spontaneous abortions.

Since the introduction of the prenatal diagnosis in San Francisco in 1970, there has been virtually an exponential increase in the demand for this service. This demand has been

almost entirely for chromosomal indications and mainly by women in the older childbearing years. With the introduction of the determination of  $\alpha$ -fetoprotein concentrations for the detection of neural tube abnormalities, there has also been a similar increase, although numerically much less great, in the requests for prenatal diagnosis of neural tube defects. The number of cases monitored for fetal sex determination and for biochemical indications has remained fairly constant since 1971.

Finally, it is of some interest to examine the cumulative experience of many prenatal diagnostic centers. These figures have been gathered and analyzed by Dr. Mitchell S. Golbus. A total of 3,452 pregnancies have been studied and reported, 2,889 for cytogenetic indications, 181 for sex determination, 155 for biochemical disorders, and 227 for neural tube defects. For women 35 to 39 and 40 and over, the frequencies of abnormal fetuses were 2.3% and 3.8%, respectively. When previous trisomy 21 was the indication, 1.5% of the fetuses were affected, but 18.9% were affected when one of the parents carried a balanced translocation. Of the pregnancies monitored for fetal sex determination, 45.3% had male fetuses, and 20% of fetuses at risk for biochemical disorders were found to be affected. The overall incidence of fetal abnormality, including all male fetuses in the sex determination group, was 6.4%. If the latter group is removed and only specific diagnoses are considered, 4.3% of the tested fetuses were abnormal.

#### Literature Cited

1. Epstein, C. J.; "Genetic Counseling - Past, Present and Future" in "Birth Defects and Fetal Development, Endocrine and Metabolic Factors", ed. K. S. Moghissi, Charles C. Thomas Publisher, Springfield, Illinois, 1974.
2. Nadler, H. L.; "Prenatal Detection of Genetic Disorders". *Adv. Hum. Genet.*, (1972), 3, 1-37.
3. Dorfman, A., ed.; "Antenatal Diagnosis", University of Chicago Press, Chicago, 1972.
4. Milunsky, A.; "The Prenatal Diagnosis of Hereditary Disorders", Charles C. Thomas, Springfield, 1973.
5. Symposium on Intrauterine Diagnosis; "Birth Defects: Original Article Series", (1971), 7, 5.
6. Epstein, C. J.; "Prenatal Diagnosis". *Adv. Int. Med.*, (1975), 20, 325-344.
7. Garlinger, P.; Hecht, F.; Prescott, G. H.; Wyandt, H. E.; Tolby, B. E.; Milbeck, R. L.; and Overton, K.; "Restrictive Consent and Amniocentesis". *New Eng. J. Med.*, (1973), 288, 1028.
8. Edwards, J. H.; "The Mutation Rate in Man". *Prog. Med. Genet.*, (1974), 10, 1-16.
9. Carter, C. O.; "Genetics of Common Disorders". *Brit. Med. Bull.*, (1969), 25, 52-57.

10. Boué, J.; Boué, A.; and Lazar, P.; "The Epidemiology of Human Spontaneous Abortions with Chromosomal Abnormalities", *Aging Gametes, Int. Symp. Seattle*, (1975), 330-348.
11. Fuchs, F.; and Riis, P.; "Antenatal Sex Determination". *Nature (Lond.)*, (1956), 177, 330.
12. Steele, M. W.; and Breg, W. T.; "Chromosome Analysis of Human Amniotic Fluid Cells". *Lancet*, (1966), 1, 383-385.
13. Jacobson, C. B.; and Barter, R. H.; "Intrauterine Diagnosis and Management of Genetic Defects". *Am. J. Obstet. Gynecol.*, (1967), 99, 796-807.
14. Nadler, H. L.; "Antenatal Detection of Hereditary Disorders". *Pediatrics*, (1968), 42, 912.
15. Levine, M. D.; McNeil, D. E.; Kaback, M. M.; Frazer, R. E.; Okada, D. M.; and Hobel, C. J.; "Second-Trimester Fetoscopy and Fetal Blood Sampling: Current Limitations and Problems". *Amer. J. Obstet. Gynecol.*, (1974), 120, 937-943.
16. Valenti, C.; "Antenatal Detection of Hemoglobinopathies". *Am. J. Obstet. Gynecol.*, (1973), 115, 851-853.
17. Hobbins, J. C.; and Mahoney, M. J.; "Fetal Blood Drawing". *Lancet*, (1975), 2, 107-109.
18. Omenn, G. S.; Figley, M. M.; Graham, C. B.; and Heinrichs, W. L.; "Prospects for Radiographic Intrauterine Diagnosis - The Syndrome of Thrombocytopenia with Absent Radii". *N. Engl. J. Med.*, (1973), 288, 777-778.
19. Campbell, S.; Johnston, F. D.; Holt, E. M.; and May, P.; "Anencephaly: Early Ultrasonic Diagnosis and Active Management". *Lancet*, (1972), 2, 1226-1227.
20. Campbell, S.; "The Prenatal Detection of Fetal Abnormality by Ultrasonic Diagnosis" in *Birth Defects, Proc. 4th Int. Conf.*, ed. A. G. Motulsky and W. Lenz, Excerpta Medica, Amsterdam, (1974), 234-239.
21. Kan, Y. W.; Valenti, C.; Guidotti, R.; Carnazza, V.; and Rieder, R. F.; "Fetal Blood Sampling in Utero". *Lancet*, (1974), 1, 79-80.
22. Kan, Y. W.; Golbus, M. S.; Klein, P.; and Dozy, M.; "Successful Application of Prenatal Diagnosis in a Pregnancy at Risk for Homozygous  $\beta$ -thalassemia". *New Engl. J. Med.*, (1975), 292, 1096-1099.
23. Hollenberg, M. D.; Kaback, M. M.; and Kazazian, H. H. Jr.; "Adult Hemoglobin Synthesis by Reticulocytes from the Human Fetus at Mid-Trimester". *Science*, (1971), 174, 698-702.
24. Kadotani, T.; Sato, H.; Ohama, K.; and Takahara, H.; "A Technical Note on the Antenatal Chromosome Analysis by Trans-abdominal Fetal Skin Biopsy". *Jap. Jour. Human Genet.*, (1971), 16, 42-47.
25. Hahnemann, N.; "Early Prenatal Diagnosis; A Study of Biopsy Techniques and Cell Culturing from Extraembryonic Membranes". *Clin. Genet.*, (1974), 6, 294-306.
26. Lind, T.; and Hytten, F. E.; "Relation of Amniotic Fluid Volume to Fetal Weight in the First Half of Pregnancy".



- Lancet, (1970), 1, 1147-1149.
27. Desnick, R. J.; Krivit, W.; and Sharp, H. L.; "In Utero Diagnosis of Sandhoff's Disease". *Biochem. Biophys. Res. Commun.*, (1973), 51, 20-26.
  28. O'Brien, J. S.; Okada, S.; Fillerup, D. L.; Veath, M. L.; Adornato, B.; Brenner, P. H.; and Leroy, J. G.; "Tay-Sachs Disease: Prenatal Diagnosis". *Science*, (1971), 172, 61-64.
  29. Nadler, H. L.; and Gerbie, A. B.; "Enzymes in Noncultured Amniotic Fluid Cells". *Am. J. Obstet. Gynecol.*, (1969), 103, 710-712.
  30. Cox, R. P.; Douglas, G.; Hutzler, J.; Lynfield, J.; and Dancis, J.; "In-utero Detection of Pompe's Disease". *Lancet*, (1970), 1, 893.
  31. Sutcliffe, R. G.; and Brock, D. J. H.; "Enzymes in Uncultured Amniotic Fluid Cells". *Clin. Chim. Acta*, (1971), 31, 363-365.
  32. Hug, G.; Schubert, W. K.; Soukup, S.; "Prenatal Diagnosis of Type-II Glycogenosis". *Lancet*, (1970), 1, 1002.
  33. Mukherjee, A. B.; Blattner, P. Y.; and Nitowsky, H. M.; "Quinacrine Mustard Fluorescence of Sex Chromatin in Human Amniotic Fluid Cell Cultures". *Nature (New Biol.)*, (1972), 235, 226-229.
  34. Valenti, C.; Lin, C. C.; Baum, A.; Massobrio, M.; and Carbonara, A.; "Prenatal Sex Determination". *Am. J. Obstet. Gynecol.*, (1972), 112, 890-895.
  35. Tishler, P. V.; Lamborot-Manzur, M.; and Atkins, L.; "Polymorphism of the Human Y Chromosome: Fluorescence Microscopic Studies on the Sites of Morphological Variation". *Clin. Genet.*, (1972), 3, 116-122.
  36. Matalon, R.; Dorfman, A.; and Nadler, H. L.; "A Chemical Method for the Antenatal Diagnosis of Mucopolysaccharidoses". *Lancet*, (1972), 1, 798-799.
  37. Elsas, L. J.; Priest, J. H.; Wheeler, F. B.; Danner, D. J.; and Pask, B. A.; "Maple Syrup Urine Disease: Coenzyme Function and Prenatal Monitoring". *Metabolism*, (1974), 23, 569-579.
  38. Gitlin, D.; and Biasucci, A.; "Development of  $\gamma$ G,  $\gamma$ A,  $\gamma$ M,  $\beta_1$ C/ $\beta_1$ A, C'1 Esterase Inhibitor, Ceruloplasmin, Transferrin, Hemopexin, Haptoglobin, Fibrinogen, Plasminogen,  $\alpha_1$ -Antitrypsin, Orosomuroid,  $\beta$ -Lipoprotein,  $\alpha_2$ -Macroglobulin, and Prealbumin in the Human Conceptus". *J. Clin. Invest.*, (1969) 48, 1433-1446.
  39. Kohler, P. F.; "Maturation of the Human Complement System. I. Onset Time and Sites of Fetal C1q, C4, C3, and C5 Synthesis". *J. Clin. Invest.*, (1973), 52, 671-677.
  40. Cedergvist, L. L.; Queenan, J. T.; Gadow, E. C.; and Litwin, S. D.; "The Origin of Gamma G Globulins in Human Amniotic Fluid". *Am. J. Obstet. Gynecol.*, (1972), 113, 838-840.
  41. Nadler, H. L.; Bigley, R. H.; and Hug, G.; "Prenatal Detection of Pompe's Disease". *Lancet*, (1970), 2, 369-370.

42. Hoehn, H.; Bryant, E. M.; Karp, L. E.; and Martin, G. M.; "Cultivated Cells from Diagnostic Amniocentesis in Second Trimester Pregnancies. I. Clinical Morphology and Growth Potential". *Pediatr. Res.*, (1974), 8, 746-754.
43. Westhoff, C. F.; and Rindfuss, R. R.; "Sex Preselection in the United States: Some Implications". *Science*, (1974), 184, 633-636.
44. McKusick, V. A.; "Mendelian Inheritance in Man", 4th ed., Baltimore, John Hopkins Press, 1975.
45. Krone, L. R.; Prichard, L. L.; Bradshaw, C. L.; Jones, O. W.; Peterson, R. M.; and Dixson, B. K.; "Antenatal Diagnosis of an XXX Female. A Dilemma for Genetic Counseling". *West. J. Med.*, (1975), 123, 17-21.
46. Hook, E. B.; "Behavioral Implications of the Human XYY Genotype". *Science*, (1973), 179, 139-150.
47. Noël, B.; Duport, J. P.; Revil, D.; Dussuyer, I.; and Quak, B.; "The XYY Syndrome: Reality or Myth?" *Clin. Genet.*, (1974), 5, 387-394.
48. Penrose, L. S.; and Smith, A. F.; "Down's Anomaly". Churchill, London, 1966.
49. Zellweger, H.; and Simpson, J.; "Is Routine Prenatal Karyotyping Indicated in Pregnancies of Very Young Women?" *J. Pediatr.*, (1973), 82, 675-677.
50. Mikkelsen, M.; and Stene, J.; "Genetic Counselling in Down's Syndrome". *Hum. Hered.*, (1970), 20, 457-464.
51. Stein, Z.; Susser, M.; Guterman, A. V.; "Screening Programme for Prevention of Down's Syndrome". *Lancet*, (1973), 1, 305-310.
52. Golbus, M. S.; "The Antenatal Diagnosis of Genetic Disorders," in "Office Gynecology," ed. R. Glass, Williams & Wilkins, Baltimore, in press.
53. Littlefield, J. W.; Milunsky, A.; and Atkins, L.; "An Overview of Prenatal Genetic Diagnosis", in *Birth Defects, Proc. 4th Int. Conf.*, ed. A. G. Motulsky and W. Lenz, *Excerpta Medica*, Amsterdam, 1974, 234-239.
54. Schneider, J. A.; Verroust, F. M.; Kroll, W. A.; Garvin, A. J.; Horger, E. O., III; Wong, V. G.; Spear, G. S.; Jacobson, C.; Pellett, O. L.; and Becker, F. L. A.; "Prenatal Diagnosis of Cystinosis". *Abst., Pediatr. Res.*, (1973), 7, 291.
55. Gerbie, A. B.; Melancon, S. B.; Ryan, C.; and Nadler, H. L.; "Cultivated Epithelial-Like Cells and Fibroblasts from Amniotic Fluid: Their Relationship to Enzymatic and Cytologic Analysis". *Am. J. Obstet. Gynecol.*, (1972), 114, 314-320.
56. Kaback, M. M.; and Howell, R. R.; "Infantile Metachromatic Leukodystrophy. Heterozygote Detection in Skin Fibroblasts and Possible Application to Intrauterine Diagnosis". *New Engl. J. Med.*, (1970), 282, 1336-1340.
57. Galjaard, H.; Fernandes, J.; Jahodova, M.; Koster, J. F.;

- and Niermeijer, M. F.; "Prenatal Diagnosis of Genetic Disease". *Bull. Europ. Soc. Hum. Genet.*, (1972), November, 79-91.
58. Haljaard, H.; Hoogeveen, A.; Keijzer, W.; DeWit-Verbeek, E.; and Vlek-Noot, C.; "The Use of Quantitative Cytochemical Analysis in Rapid Prenatal Detection and Somatic Cell Genetic Studies of Metabolic Diseases". *Histochem. J.*, (1974), 6, 491-509.
  59. Hösli, P.; "Microtechniques for Rapid Prenatal Diagnosis in Early Pregnancy", in *Birth Defects, Proc. 4th Int. Conf.*, ed. A. G. Motulsky and W. Lenz, Excerpta Medica, Amsterdam, 1974, 234-239.
  60. Brock, D. J. H.; and Sutcliffe, R. G.; "Alpha-Fetoprotein in the Antenatal Diagnosis of Anencephaly and Spina Bifida". *Lancet*, (1972), 2, 197-199.
  61. Kjessler, B.; Sherman, M.; Johansson, S. G. O.; Gustavson, K. H.; and Hultquist, A.; "Alpha-Fetoprotein in Antenatal Diagnosis of Congenital Nephrosis". *Lancet*, (1975), 1, 432-433.
  62. Milunsky, A.; Macri, J. N.; Weiss, R. R.; Alpert, E.; McIssac, and Joshi, M. S.; "Prenatal Detection of Neural Tube Defects. Comparison Between Alpha Fetoprotein and Beta-Trace Protein Assays". *Amer. J. Obstet. Gynecol.*, (1975). 122, 313-315.
  63. Wald, N. J.; Brock, D. J. H.; and Bonnar, J.; "Prenatal Diagnosis of Spina Bifida and Anencephaly by Maternal Serum - Alpha-Fetoprotein Measurement. A Controlled Study". *Lancet*, (1974), 1, 765-767.
  64. Golbus, M. S.; Personal Communication.
  65. Epstein, C. J.; Schneider, E. L.; Conte, F. A.; and Friedman, S.; "Prenatal Detection of Genetic Disorders". *Am. J. Hum. Genet.*, (1972), 24, 214-226.
  66. Golbus, M. S.; Conte, F. A.; Schneider, E. L.; and Epstein, C. J.; "Intrauterine Diagnosis of Genetic Defects: Results, Problems, and Follow-up of 100 Cases in a Prenatal Genetic Detection Center". *Am. J. Obstet. Gynecol.*, (1974), 118, 897-905.
  67. Toop, J.; and Emery, A. E. H.; "Muscle Histology in Fetuses at Risk for Duchenne Muscular Dystrophy". *Clin. Genet.*, (1974), 5, 230-233.
  68. Epstein, C. J.; Brady, R. O.; Schneider, E. L.; Bradley, R. M.; and Shapiro, D.; "In Utero Diagnosis of Niemann-Pick Disease". *Amer. J. Hum. Genet.*, (1971), 23, 533-535.
  69. Suzuki, K.; Schneider, E. L.; and Epstein, C. J.; "In Utero Diagnosis of Globoid Cell Leukodystrophy (Krabbe's Disease)". *Biochem. Biophys. Res. Commun.*, (1971), 45, 1363-1366.
  70. Schneider, E. L.; Ellis, W. G.; Brady, R. O.; McCulloch, J. R.; and Epstein, C. J.; "Infantile (Type II) Gaucher's Disease: In Utero Diagnosis and Fetal Pathology". *J. Pediatr.*, (1972), 81, 1134-1139.

71. Schneider, E. L.; Ellis, W. G.; Brady, R. O.; McCulloch, J. R.; and Epstein, C. J.; "Prenatal Niemann-Pick Disease: Biochemical and Histologic Examination of a 19 Gestational Week Fetus". *Pediatr. Res.*, (1972), 6, 720-729.
72. Ellis, W. G.; Schneider E. L.; McCulloch, J. R.; Suzuki K.; and Epstein, C. J.; "Fetal Globoid Cell Leukodystrophy (Krabbe's Disease): Pathological and Biochemical Examination". *Arch. Neurol.*, (1973), 29, 253-257.
73. Nadler, H. L.; and Egan, T. J.; "Deficiency of Lysosomal Acid Phosphatase; a New Familial Metabolic Disorder". *N. Engl. J. Med.*, (1970), 282, 302-307.
74. Hirschhorn, R.; Beratis, N.; Rosen, F.; Parkman, R.; Stern, R.; and Palmer, S.; "Adenosine-Deaminase Deficiency in a Child Diagnosed Prenatally". *Lancet*, (1975), 1, 73.
75. Goodman, S. I.; Mace, J. W.; Turner, B.; and Garrett, W. J.; "Antenatal Diagnosis of Argininosuccinic Aciduria". *Clin. Genet.*, (1973), 4, 236-240.
76. Brady, R. O.; Uhlendorf, B. W.; and Jacobson, C. B.; "Farby's Disease: Antenatal Detection". *Science*, (1971), 172, 174-175.
77. Donnell, G.; Personal Communication.
78. Fensom, A. H.; Benson, P. F.; and Blunt, S.; "Prenatal Diagnosis of Galactosemia". *Brit. Med. J.*, (1974), 4, 386-387.
79. Lowden, J. A.; Cutz, E.; Conen, P. E.; Rudd, N.; and Doran, T. A.; "Prenatal Diagnosis of GM<sub>1</sub> - Gangliosidosis". *N. Engl. J. Med.*, (1973), 288, 225-228.
80. Booth, C. W.; Gerbie, A. B.; and Nadler, H. L.; "Intra-uterine Detection of GM<sub>1</sub> - Gangliosidosis, Type 2". *Pediatr.*, (1973), 52, 521-524.
81. Nadler, H. L.; and Messina, A. M.; "In-Utero Detection of Type-II Glycogenosis (Pompe's Disease). *Lancet*, (1969), 2, 1277-1278.
82. Fratantoni, J. C.; Neufeld, E. F.; Uhlendorf, B. W.; and Jacobson, C. B.; "Intrauterine Diagnosis of the Hurler and Hunter Syndromes". *N. Engl. J. Med.*, (1969), 280, 686-688.
83. Neufeld, E. F.; "Mucopolysaccharidoses" in "Antenatal Diagnosis", ed. A. Dorfman, University of Chicago Press, Chicago, 1972, 217-228.
84. Benzie, R.; Doran, T. A.; Escoffery, W.; Gardner, H. A.; Hoar, D. I.; Hunter, A.; Malone, R.; Miskin, M.; and Rudd, N. L.; "Prenatal Diagnosis of Hypophosphatasia". *Birth Defects Conference, 1975 (abstract)*.
85. Boyle, J. A.; Raivio, K. O.; Astrin, K. H.; Schulman, J. D.; Seegmiller, J. E.; and Jacobson, C. B.; "Lesch-Nyhan Syndrome: Preventive Control by Prenatal Diagnosis". *Science*, (1970), 169, 688-689.
86. Dancis, J.; "Maple Syrup Urine Disease", in "Antenatal Diagnosis", ed. A. Dorfman, University of Chicago Press, Chicago, 1972, 123-125.

87. Nadler, H. L.; and Gerbie, A. B.; "Role of Amniocentesis in the Intrauterine Detection of Genetic Disorders". *N. Engl. J. Med.*, (1970), 282, 596-599.
88. Mahoney, M. J.; Rosenberg, L. E.; Waldenstrom, J.; Lindbald, B.; and Zetterstrom, R.; "Prenatal Diagnosis of Methylmalonicaciduria". *Pediatrics Res.*, (1973), 7, 342.
89. Warren, R. J.; Condron, C. J.; Hollister, D.; Huijing, F.; Neufeld, E. F.; Hall, C. W.; McLeod, A. G. W.; and Lorincz, A. E.; "Antenatal Diagnosis of Mucopolipidosis II (I-cell disease)". *Pediatrics Res.*, (1973), 7, 343.
90. Harper, P. S.; Lawrence, K. M.; Parkes, A.; Wusteman, F. S.; Kresse, H.; Von Figura, K.; Ferguson-Smith, M. A.; Duncan, D. M.; Logan, R. W.; Hall, F.; and Whiteman, P.; "Sanfilippo A Disease in the Fetus". *J. Med. Genet.*, (1974), 11, 123-132.
91. Ramsay, C. A.; Coltart, T. M.; Blunt, S.; Pawsey, S. A.; Gianelli, F.; "Prenatal Diagnosis of Xeroderma Pigmentosum". *Lancet*, (1974), 2, 1109.

# The Analytical Laboratory of Neonatology

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## Functions of the Pediatric Laboratory

Objectives. The objectives of the chemical laboratory of the Neonatology Division of a hospital are to:

1. Supply data obtained by analysis of various biological materials from the newborn.
2. To develop new procedures for improving the service to the Pediatric Department.
3. To act as an advisory and consultant center for problems related to clinical chemistry for the Department of Neonatology; and
4. To carry on an active program in research and development in order to improve the procedures for the understanding and the diagnosis of the disease process in the newborn.

The Newborn Infant. Figure 1 is a photograph showing the appearance of a 2-1/2 kilo premature infant as compared to a nurse's hand. This infant is not the smallest infant one has to contend with since premature infants weighing 600 grams with no genetic abnormalities are occasionally seen.

Figure 2 shows a similar infant that has become dehydrated as a result of not receiving any fluids for approximately 36 hours after birth. In another infant who weighed 1 kilo at birth, no fluids were administered for 27 hours. During that period of time, 144 grams of body weight was lost which comprises approximately 19% of the original weight of the infant.

The Newborn and the Laboratory. The wellbeing of the premature infant can be ascertained by measuring blood pH, electrolytes and other blood components on a routine basis. The maintenance of these infants' electrolyte balance and normal pH is shown in Figure 3. An infant placed on a high protein diet milk formula developed an acidosis, and when brought to normal pH



*Figure 1. Premature infant, well hydrated at birth*



*Figure 2. Premature infant similar to Figure 1, but deprived of fluids for 36 hr showing symptoms of dehydration*

responded with a rapid recovery of his initial weight and weight gain(1).

From the above, derives the fundamental concept that the newborn infant must be maintained in an adequate degree of hydration and in electrolyte balance in order for the infant to thrive. In some cases, where for one reason or other, the infant is not able to take fluids by mouth in the normal manner, one may need to resort to supplementary fluid therapy by vein. For a rational approach to this problem one needs to have available from the clinical chemical laboratory, rapid response in order to continuously monitor changes in electrolyte levels so that fluids can be modified so as to correct these abnormalities.

Parenteral Fluids. During the past ten years interest has been renewed in the total alimentation of the infant by vein (2). The motivation for this is the fact that neonates may suffer from some congenital malformation of the intestinal tract which would require surgical resection. If this is done, then one needs some outside way for alimentation, bypassing the intestine, until the intestine is able to heal and recover its normal function. This may take many weeks. A second source of motivation is the small premature infant weighing less than a kilogram, whose immature central nervous system and gastrointestinal tract make it difficult to establish nutrition by oral intake soon after birth. These also require total intravenous nutrition for a substantial period of time.

A major breakthrough occurred in intravenous alimentation in 1966 when it was shown in puppies that not only could one maintain an animal in fluid and electrolyte balance by total intravenous alimentation, but also that one could achieve weight gain, approaching that of the normal while the animals received nothing other than fluids intravenously (3). This was accomplished by the use of hyperosmotic glucose and protein hydrolysate solutions, along with the required amount of salt and vitamins to maintain the infant. An important contribution to this development was the development of materials such as the silicones, which could lie in the vein or artery of the infant without producing a tissue reaction. Because of the use of hypertonic solutions in this procedure some refer to this method of fluid administration as hypertonic alimentation. Figure 4 illustrates the route used for administering fluids for total alimentation.

In addition to these two applications, intravenous nutrition and fluid therapy has been given for many years to newborns with chronic diarrhea, malabsorption for various reasons, and other problems associated with disease.

Laboratory Service. As a minimum, the physician requires that a laboratory be available on a 24 hour a day basis, which



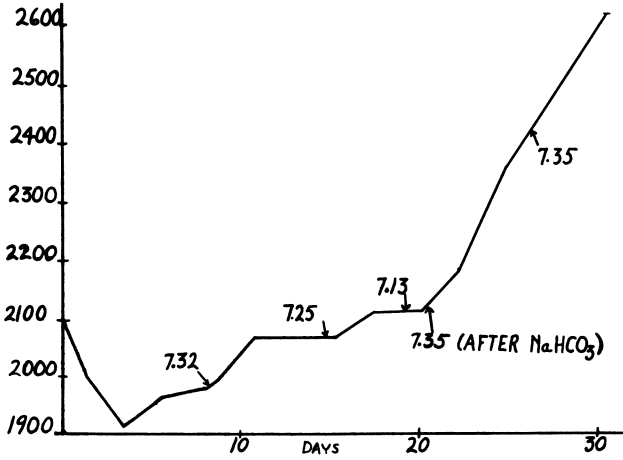


Figure 3. Effect of adjustment of pH to the normal range on weight gain in a premature infant

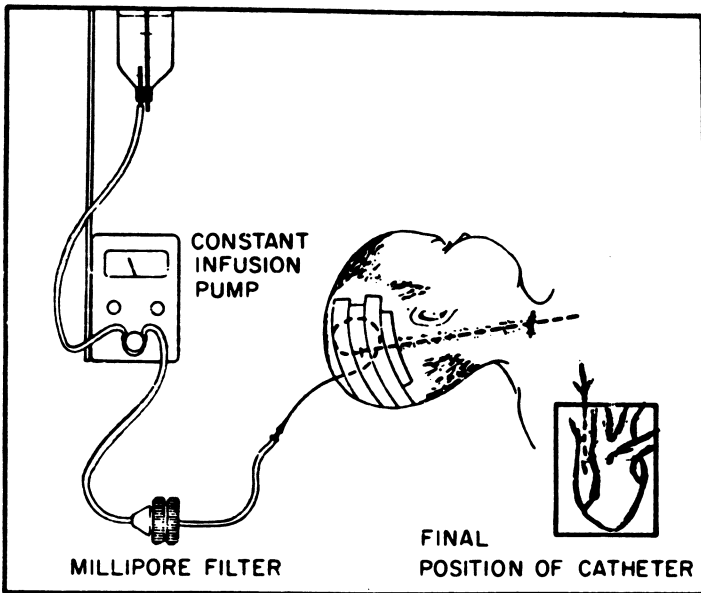


Figure 4. Total intravenous alimentation. Demonstration of method of I.V. administration (after Winters, et al.).

is capable of analyzing blood and urine samples for, at least, sugar, urea, protein, sodium, potassium, chloride, calcium, magnesium, and blood pH. With the small premature, and even with full term neonates, respiration may be a problem, it is important that the laboratory have available, on an emergency basis, methods for the analysis for pCO<sub>2</sub>, pO<sub>2</sub>, total CO<sub>2</sub>, oxygen content, hemoglobin and hematocrit values. It is advantageous to calculate P<sub>50</sub> values from the data so as to aid the physician in determining whether he is dealing with an abnormal oxygen transport system (4).

In addition, the physician with the aid of the clinical chemist, needs to calculate salt and water requirements for the newborn infant. Of prime importance is the determination of how much sodium bicarbonate is required to correct an acidosis, or in rare cases, how much ammonium chloride might be required to correct an alkalosis (5).

During the administration of fluids, the newborn infant requires that one periodically assay for the various components discussed above, so as to see whether the fluids being administered are serving their purpose or need to be changed. The newborn infant does not have the reserve capacity that the adult has in being able to buffer and adjust for various problems which may arise during fluid therapy. For this reason, the newborn infant during acute treatment may require repetitive analyses, at relatively short intervals. Even when the condition is stabilized it is customary for the pediatrician to require analyses for electrolytes at periodic intervals for the security of the patient.

In addition to the above, the laboratory of Neonatology needs to be equipped so as to screen newborn infants for the various genetic diseases. In the case of phenylketonuria, many states, including the State of Illinois, require that every infant be screened for this defect. Further, many hospitals are screening, in general, for aminoacidopathies in serum and urine of newborn infants. In some areas, screening is being done for the glycogenoses (6), lipidoses (7), and mucopolysaccharidoses (8). Screening for biochemical defects in the erythrocytes, such as the various enzymes of the Embden-Meyerhof sequence, and also the Shunt Mechanism (9), are required to be part of the armamentarium of the laboratory of Neonatology.

In certain areas, such as Chicago, where there is a high percentage of certain ethnic groups, certain defects, such as glucose-6-phosphate dehydrogenase are more common, and screening is useful in explaining some of the unexplained non-spherocytic hemolytic anemias and other abnormalities. In addition, the laboratory of Neonatology needs to be prepared to analyze for numerous metabolic intermediates which appear in increased amounts with certain genetic abnormalities. These include the porphyrins (10), porphobilinogen and ethanolamine phosphate (11), as typical examples.

Along with an effective electrolyte and screening program for genetic disease, the laboratory of Neonatology needs to have the capability of analyzing for other components in blood serum, which aid in the diagnosis of disease. These include such determinations as alkaline phosphatase, and various other enzymes, creatinine, uric acid and a host of other components which are normally assayed by the main clinical laboratory.

Toxicological problems among infants are relatively common. For example, it is now routine to follow the course of changes in Dilantin levels of patients who are being treated for various convulsive disorders (12). It is also important to be able to detect and assay for other drugs that are used in the newborn, such as various other anticonvulsants, salicylates, and a host of others.

Need for a Separate Pediatric Laboratory. From the above discussion, one can see that one may divide the analytical activities of the laboratory of Neonatology into four major classes.

1. Those associated with the acute and chronic treatment of newborns for respiratory, fluid and electrolyte problems.
2. Those associated with the screening of newborns for genetic diseases and for determining genetic diseases in newborns after some abnormalities are noted.
3. Those associated with drugs which may have been administered in excess to the infant or, more commonly, may have been administered correctly, where the physician requires that he be assured that the drug level is in the range that he intended.
4. Those associated with general clinical laboratory service assaying for numerous other components which are also done in the main laboratory of clinical chemistry.

It should be apparent from the above, that the problems of the laboratory of Neonatology are distinct from the main laboratory, and it would always be advisable that it be maintained as a separate unit, or at least in a separate section of the clinical laboratory of the institution in which clinical chemistry is being practiced.

### The Pediatric Laboratory As A Micro Analytical Unit

The Fundamental Analytical Problem. There is a problem,

which is unique to the laboratory of Neonatology and distinct from that of the main laboratory, that is the problem of the size of the sample to be analyzed. If one assumes that approximately 9% of the weight of the newborn is blood, and if the newborn weighs one kilogram, then one has a total volume of 90 ml of blood, of which about half is plasma. This should be compared to the 9 liters available from an adult who weighs 100 kilo. To withdraw 5 ml of blood from a 1 kilo premature, would then be almost equivalent to drawing 500 ml from an adult. One is therefore necessarily limited to the amount of blood to be drawn repetitively. Even if one is able to get into the vein of the newborn, one prefers to limit the total volume of blood that is drawn to less than 1 ml.

From the fingertip, earlobe or heel prick, one would like to stay within the limits of 100 - 200 $\mu$ l as a maximum. If one then draws 100 $\mu$ l of blood, then one cannot hope to have more than approximately 40 $\mu$ l of plasma, since the hematocrit of newborn infants is relatively high. This volume would be substantially lowered, in the dehydrated infant, with which one is working, normally.

Assuming that one had 50 $\mu$ l, and one were to use 10 $\mu$ l per analysis, then one could do 5 tests, which might be sodium, potassium, chloride, sugar and urea. However, experience has taught us that unless we do all of the tests required to evaluate the well-being of the infant, as far as his electrolyte balance is concerned, then it often turns out that the one test that we did not do is the most important. For example, in the experience of the author, in one case, a glucose level was not done on an infant who was admitted dehydrated. Subsequently, it turned out that the infant was suffering from hypoglycemia and was the offspring of a diabetic mother. In another case, the hematocrit value was not obtained for certain reasons. Subsequently, it was shown that even at birth, this newborn had a hematocrit value of only 28%, and had a severe hemolytic anemia. It is therefore the practice, in many laboratories of Neonatology, to set up a goal for determinations that need be done on the first sample of blood drawn from the infant. This would require as a minimum approximately 10 components. In order to meet this need one may resort to two different solutions:

1. A larger specimen, of the order of 0.5 ml may be routinely drawn. This in effect requires that this be taken by vein.
2. One may decrease the volume required per test so that fingertip blood may be utilized if one desires. This approach is the subject of subsequent discussion.

Measuring the sample. The question arises as to what the

lower limit of specimen volume can be sampled conveniently with a degree of accuracy required for the particular component being analyzed. In actual practice, there are at least two ways in which one may measure a small quantity of liquid. In the first method, a small quantity of material may be sampled directly for each test. Alternatively, this may then be mixed with a diluting fluid of larger volume, say of the order of 1 or 2 ml. In this case, if  $10\ \mu\text{l}$  were sampled, and diluted to 1 ml, then each  $100\ \mu\text{l}$  would actually contain  $1\ \mu\text{l}$  of specimen. This could then be measured with an accuracy of better than 1 or 2%.

In order to indicate the accuracy that can be obtained from a capillary pipette, one may refer to the so called Sahli pipette, which is approximately 80 mm from mark to tip. This pipette is used as a washout pipette and can measure samples which are readily reproducible to 1 part in 80, since it is a relatively simple thing for the eye to see 1 mm. On the other hand, if one places a bulb in the tube, and cuts the bore down at the mark by 50%, then this would decrease the error by a factor of 4. It is thus practicable to sample from a micropipette, which is used as a washout pipette, with greater accuracy than one can sample from a conventional macro pipette, which delivers 1 ml by blowout, or to deliver. Figures 5 and 6 illustrate various designs of micropipets (13,14).

It is advantageous to attach the micropipet to a sampler diluter. In this manner, the sample is aspirated by means of a plunger. The technician is not required to adjust the volume nor to make a judgement, because this is being done by a plunger, and accuracy is a function of the sampler diluter. If then one wipes off the tip and then ejects a diluting fluid, one could then proceed to measure out very small samples limited only on the nature of the tip. This can be seen in the sequence of events in Figure 7.

From a glass tip it is impracticable to measure specimens smaller than  $10\ \mu\text{l}$ . This is so because the holdback of the wetted glass, on the outside, is a significant portion of the volume sampled. If the tip is then dipped into the solution and then used as a washout pipet, a serious error would be introduced.

For these reasons, the plastic tip, or the siliconed glass tip have been used for micro measurements. With a plastic tip, there is no limit to the size of the specimen which may be sampled. For example, it is possible to sample amounts of the order of nanoliters, provided one resorted to a micro-manipulator. While the clinical chemist has not availed himself generally of this technique, the physiologist, has for many years, been measuring samples from within a single cell, or from perhaps, a renal tubule, so that the sample can then be subsequently analyzed. It awaits only an intelligent manufacturer to adapt the micromanipulator as a routine tool in the Pediatric

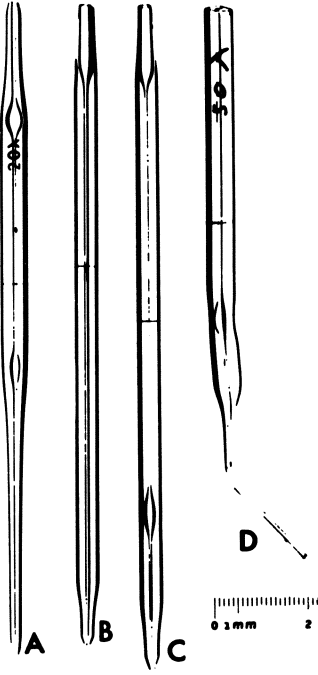


Figure 5. Examples of micro-pipets. A, pipet with slender tip; B, Sahli pipet; C, Accupipet with bulb; D, pipet with bulb and bent tip.

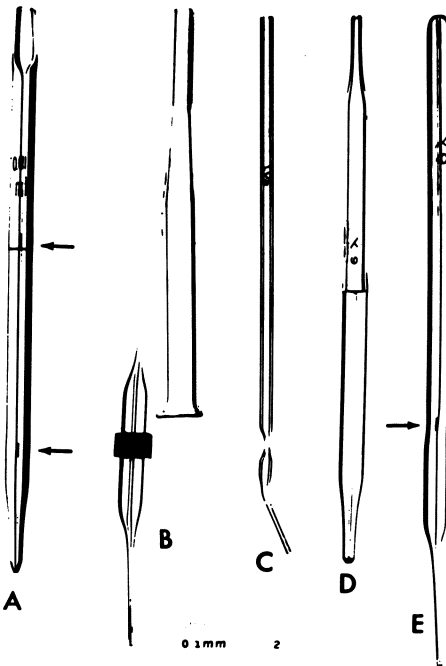
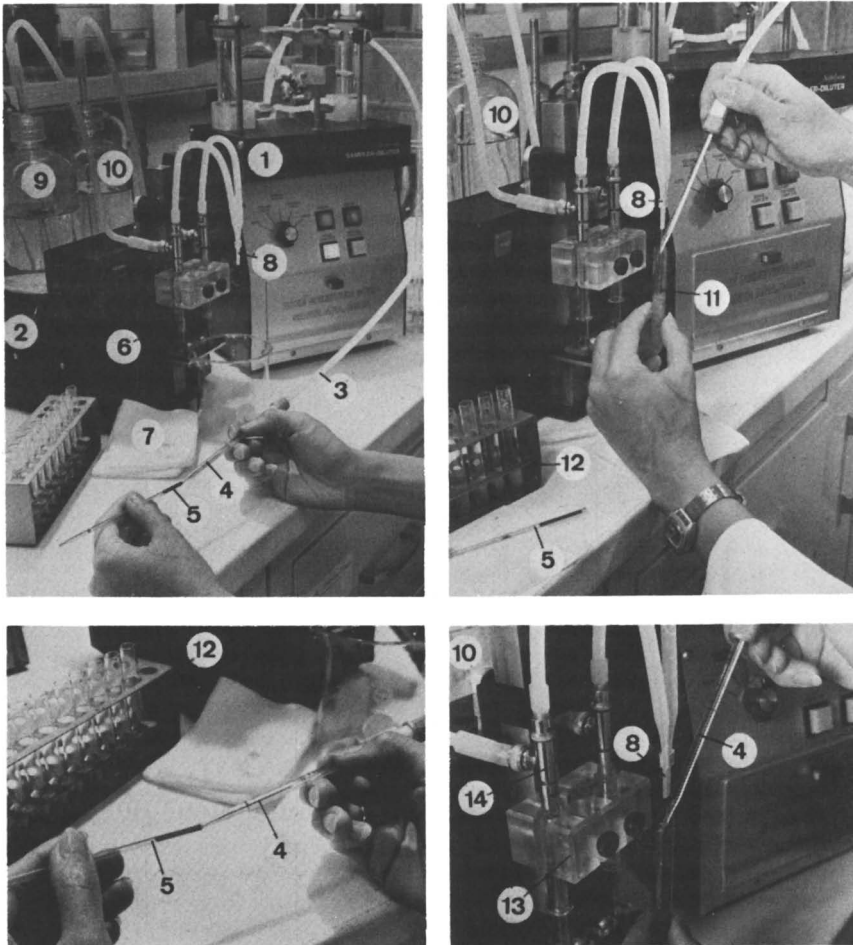


Figure 6. Self zeroing ultramicro pipets. A, constriction at two marks; B, overflow type with constriction at both ends; C, Lang-Levy pipet; D, another type of constriction pipet; E, Speedipet of Natelson.



**Figure 7.** Demonstration of the method of sampling from a capillary with a sampler-diluter and dispensing with a simultaneous dispenser for two reagents, so as to prepare a protein precipitate of the Somogyi type in two steps. 1, sampler-diluter; 2, dispenser (both made by Rohé Scientific Co., Santa Ana, Calif.); 3, connecting tube; 4, Sahli pipet; 5, blood collecting tube; 6, syringe for dispensing one of the reagents; 7, gauze for wiping off the tips of the pipet; 8, ganged tips for dispensing the two reagents; 9 and 10, zinc sulfate and barium hydroxide reagents, respectively; 11, test tube to receive the specimen and the reagent; 12, rack of test tubes already sampled; 13, block holding the two syringes; 14, valve.

Laboratory. This will eventually come about because this is the practical solution to the sampling of minute specimens. The micromanipulator, may be adapted to a sampler-diluter where amounts of the order of a few nanoliters are being sampled followed through the same tip by a diluting solution of several microliters.

At present, several of the instruments which are being utilized for enzyme analysis, such as the centrifugal analyzers (15), have been measuring samples of the order of 5  $\mu$ l. But this is deceptive, for the simple reason that in order to sample the 5 microliters, one requires 100 microliters or more to be present in the cup. Therefore, the sample size required is not the size that is being sampled, but the size that is needed in the sampling cup. This problem may be approached in several ways. The problem of sample control will now be discussed.

Sample Control. In figure 7, the technician is sampling with a sampler diluter from the capillary. Here we see what is called "sample control". None of the specimen is being wasted on the walls of a cup. All of it is being taken eventually into the capillary and then dispensed. With a setup as shown in Figure 7, synchronized with this diluter, is a multiple dispenser, which can dispense several reagents at the same time that the specimen is added to the tube.

In the particular example shown, zinc sulfate and barium hydroxide are being dispensed into the test tube so as to precipitate the proteins. The filtrate obtained is the filtrate from 10 microliters of serum. This can be used for several purposes and in the application being referred to, an amount equivalent to 3 microliters is being used for sugar determination, by the hexokinase procedure and an amount equivalent to 3 microliters is being used for urea estimation with diacetylmonoxime (15).

Figure 7, therefore, illustrates several practical methods of sampling. First, the sampling of a relatively large initial volume (10  $\mu$ l). This has the advantage that it can be measured with precision by the particular instrument employed; second, the removal of protein from the specimen, and, third, the taking of aliquots so that smaller volumes of sample can be removed with the same accuracy with which the initial sample was measured.

A similar procedure is used in the SMAC system of Technicon, where a modest sample of several hundred microliters is diluted and then aliquots are fed to various channels for analysis.

In summary, as far as measuring the initial specimen, it is recommended that the ordinary micro pipettes be aspirated by automatic means. It is also recommended that where large numbers of tests are to be performed on a single sample that



the initial specimen be diluted so as to have a reservoir from which to measure large numbers of aliquots. If this is followed, then with present technology it is not very difficult to set up procedures for determining sugar, urea, uric acid, creatinine enzymatically, and the various enzymes and electrolytes, on approximately 1 microliter aliquots. With this approach, a single drop of blood, which represents approximately 50 microliters would yield 20 microliters of plasma, which could then be utilized for 20 distinct tests.

It must be pointed out, that at present, this type of equipment cannot be readily purchased, but must be assembled from materials which are commercially available. The cost of a sampling station would be less than \$3,000. This would include 3 or 4 multiple dispensers, a single sample diluter and possibly a micromanipulator. If the micromanipulator is omitted, then the cost would be less than \$2,000.

Another approach to this problem is to use the type of syringe commonly referred to as the Eppendorf syringe. This is based upon the concept, originally introduced by Sanz, that plastic which does not wet, can be used to sample from different specimens without cross contamination (16). In the original Sanz system, he used a small bottle, which was pressed and then released to create a negative pressure, and aspirate the specimen, and then by positive pressure on the bottle, expel the specimen. In the Eppendorf system this is done with a plunger. The plunger retreats, aspirating the specimen, it then advances a greater distance than it retreated originally, expelling the specimen. The tip is disposable, so that a tip can be changed with each specimen, or can be changed after it becomes wetted by long use. This system is not very reproducible. It is difficult to measure an exact specimen volume and it is difficult to reproduce the volume but if one is satisfied with an accuracy of approximately  $\pm 5\%$ , this is useful. For this reason it has found wide use with the procedures using competitive binding systems of analysis. There the accuracy of the procedure is approximately  $\pm 12\%$  and the error introduced by the Eppendorf pipette is acceptable.

Obtaining The Specimen. Up to this time, the assumption has been made that a specimen in a capillary tube has been presented to the laboratory of Neonatology for analysis. I would like to address myself now to the problem of obtaining a specimen.

This problem is by far the most difficult of all problems presenting themselves to the laboratory of clinical chemistry. It is relatively simple to analyze a specimen for a host of components, as compared to the obtaining of the specimen in a dehydrated infant, particularly one who is in circulatory collapse.

The Pediatric Chemistry Committee of the AACC is now turning its attention to this particular problem as a separate study.

The procedures generally recommended are as follows. First, the blood collector must prepare himself for obtaining the blood. This includes the same procedure which one would undergo if one were initiating a surgical process. In other words, the operator needs to scrub his hands thoroughly to remove any contaminants which may be injurious to the infant. While a surgeon uses rubber gloves, it is not customary for the blood collector to do so. If the hands are properly washed, this is perfectly satisfactory, since one is not going to do extensive surgery.

The patient then needs to be prepared. In the case of the heel prick, a leg of the infant is massaged or warmed so as to increase circulation to the heel where the puncture is to be made. The site from which the collection of blood is made, is determined by the size of the infant. In prematures, where the fingers are extremely tiny, one has no choice but to obtain the specimen from the heel. In older children, the large toe may be used. In using the heel, one does not use the bottom of the heel because here the capillaries lie deep. One uses instead the back of the heel, where the capillaries come near the surface. Generally, sterile scapel blades or lances are used to puncture the skin and obtain the blood flow. Figure 8 illustrates the process of obtaining the specimen.

In Figure 8, it is noted that the blood is being taken from the bottom of the heel. This is not the correct technique. However, the principles discussed above are shown in the picture.

It is best to allow a drop to accumulate at the site where the puncture has taken place and then use the collecting tube which is held in a downward position to aspirate the specimen by a combination of capillarity and gravity. This can be seen in Figure 8. Note that the operator does not aspirate with suction. As each drop enters the capillary, the operator then moves the blood to the back of the capillary and then forward again, by rotating his wrists, while the second drop is collecting on the heel. In this way, the heparin that lines the wall of the capillary is equally distributed throughout the blood, and clotting is prevented. Prior to touching to the second drop, the blood is brought to the tip of the collecting tube so as to produce a continuous flow of blood.

If adequate flow does not occur after a reasonably deep puncture, sometimes massaging the leg downward will start the flow, sometimes merely waiting will start the flow. At the initial incision, there is a short period when the capillaries constrict. After a few minutes they relax and open up again. If one is patient, the blood flow will start. However, where no flow occurs, then one needs to resort to the bottom of the lobe of the ear, because while the blood flow may be restricted to the extremities in certain severe conditions, the flow to the head and the brain is not, and one can usually get an adequate amount of capillary blood from the ear. Once the blood

is collected, the bottom of the capillary is capped, with a plastic cap, which is commercially available, and the samples are brought to the laboratory where they are processed.

In the case of the capillary blood, it is extremely important that the specimen not be allowed to stand for extensive periods of time before centrifugation. If the blood is to be transferred to the pH meter, then the collecting tube is sealed at both ends during transportation. It is then aspirated into the pH instrument as soon as practicable since one needs a smooth even flow in order to aspirate a specimen into the conventional micro pH meter. After the whole blood has been sampled for various purposes, it is important that the remaining blood be centrifuged promptly. If not, it will clot. Subsequently, centrifuging with a clot will tend to hemolyze the blood. Erythrocytes will adhere to the wall and as they are pulled down by the clot, they will be ruptured. Those who do not observe these precautions will find that it is rather difficult to obtain unhemolyzed blood.

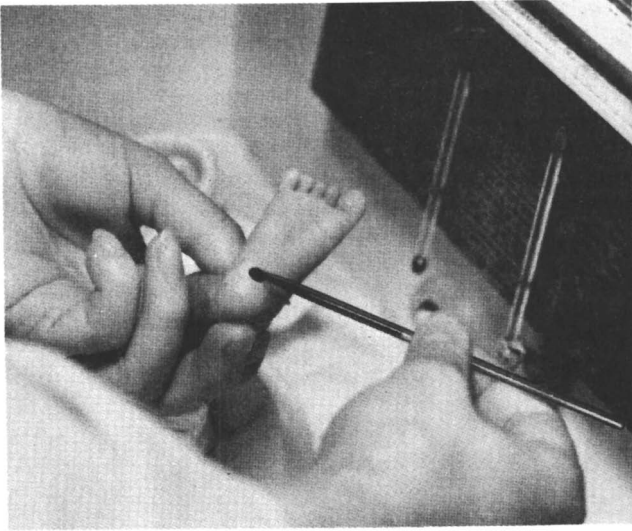
In the small premature, if the collection of capillary blood has been unsuccessful, then one needs to obtain blood from the scalp or the jugular vein. This is never attempted by the technician, but is done by the resident or member of the medical staff.

Generally, it is wise to rely on those individuals for obtaining capillary blood who are successful in doing so. Not everyone can obtain blood by fingertip from newborns readily, and this is particularly true for residents. If one finds a technician who is successful regularly in obtaining capillary blood from the infants, then that technician is the one who should be entrusted exclusively with that job. It is one area where skill is not always teachable. Certain individuals have a certain knack for obtaining blood where others do not.

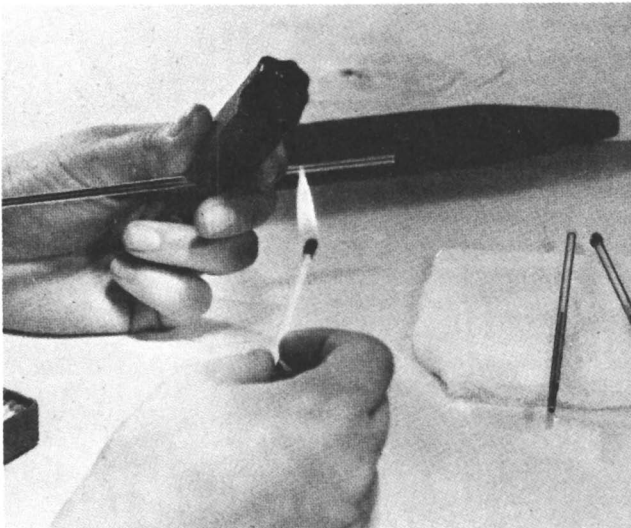
After the capillary has been filled, it is necessary to transport the capillary, usually, to the laboratory. For this purpose it is sealed at bedside. Most commonly it is capped with a plastic cap which is commercially available. However, it can be sealed with either sealing wax or with Kronig's cement, and then be brought to the laboratory. This sealing of the tube is shown in Figure 9.

After the tube has been sealed, it is brought to the laboratory and centrifuged. Usually, it is then cut by means of a file or an ampoule file at the interface, between the plasma and the cells. In Figure 10, the operator has just cut and broken the capillary above the plasma. Some prefer to do this first before cutting at the interface between the plasma and cells in order to remove any adhering blood particularly at the narrow tip of the capillary.

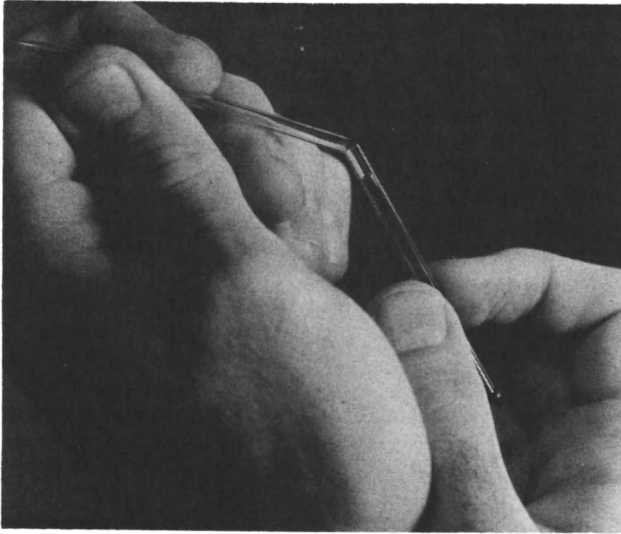
In Figure 11, the capillary with the plasma, is held in the left hand of the operator and the aspirating pipet is held in the right hand. Notice that the operator tilts the tube to



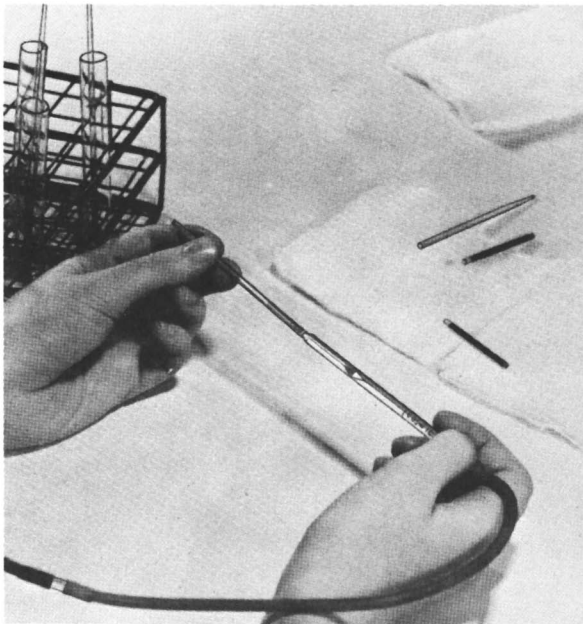
**Figure 8.** *Collecting the specimen from the infants heel*



**Figure 9.** *Sealing the specimen*



*Figure 10. Breaking the tube to separate the serum from the erythrocytes*



*Figure 11. Sampling from the cut tube*

bring the plasma to the end and then allows the plasma to run in by gravity and capillarity into the sampling pipette. As shown earlier, in Figure 7, this pipet could be used to sample with a sampler diluter.

The Specimen. One must keep in mind that the specimen to be analyzed, which is the blood of a premature or newborn infant, has certain characteristics of its own, which distinguishes it from blood which one normally obtains from the normal adult. First, bilirubin concentration is generally elevated. Values as high as 60 mg/l are not uncommon in normal infants, especially on the third day of life, when adjustment is taking place by extensive destruction of hemoglobin. Second, the erythrocytes of the neonate are rather fragile, and it is not uncommon to encounter blood which is partially hemolyzed. Third, with the destruction of the erythrocytes, and especially in view of the fact that the blood one obtains is not necessarily fasting blood, it is not uncommon for the blood of the premature to be turbid. All of these factors affect the choice of methodology which can be utilized in the laboratory of Neonatology.

The methodology selected for the laboratory of Neonatology needs to have as its prime objective, accuracy, that is specificity for what it is analyzing. This automatically eliminates many of the commercial automatic systems which are in common use, and which do not process the specimen adequately to remove interfering substances before analysis. The methodology discussed below is selected as being applicable to the type of specimen that is supplied to the analyst in the laboratory of Neonatology.

In addition to blood, certain types of specimens are submitted to the Pediatric laboratory which would not be commonly seen elsewhere. An example of this is sweat for analysis of chloride. The process of obtaining the sweat by iontophoresis usually falls to the personnel of the Laboratory of Neonatology (17). Stool for analysis of lipids and trypsin is more commonly submitted to the Laboratory of Neonatology than to the laboratory which services the adult population. The reason for this is that one is screening for certain intestinal diseases characteristic of infants and newborns which are rare in adults. Such conditions would be celiac disease, cystic fibrosis and others.

In addition to the specimens discussed above, it is common for the laboratory of Neonatology to process the erythrocytes and the leukocytes for enzymatic defects of genetic origin including aberrant hemoglobin identification. The laboratory which services the adult would be called upon to do these tests much less commonly.

In summary, the specimens which are submitted to the laboratory of Neonatology require that more extensive processing

be performed to remove interfering substances before the substance is assayed. However, as newer techniques develop, which are more specific, it is practicable to assay directly for the component in which one is interested. Examples of this would be the use of glucose analyzers which measure the oxygen generated with glucose oxidase and certain other specific ion electrodes which will react directly with the substances sought. Amperometric titration for chloride is an example where processing of the sample is not required.

The specimens submitted to the Laboratory of Neonatology, are also more varied and are usually analyzed on an individual basis. All of these factors need to be taken into account in setting up and operating a Pediatric Laboratory of clinical chemistry.

**Instrumentation and Methodology.** The instrumentation and methodology as applied to the Pediatric laboratory will now be discussed in relationship to particular components which are being assayed. The sequence chosen has no significance other than that these are the tests which are most frequently requested.

Before discussing these most commonly applied tests in detail, certain general principles should be presented. Converting a macro-method to a micro-method sometimes involves no more effort than decreasing all the volumes used in the procedure. This general procedure is well known to clinical chemists. A typical example would be the Folin method for doing sugar analysis, where a final volume of 25 ml was obtained. Since only 2 ml of solution are required for reading in a colorimeter, it is obvious that more than a 12 fold increase in sensitivity and decrease in sample size can be achieved merely by measuring out volumes which are smaller.

Dr. Sanz, from Switzerland, has, in the past, designed a system which was marketed commercially. The system was based on the philosophy that by keeping concentrations high a greater accuracy would be achieved. This was done by using very small volumes of sample of the order of 10 - 25  $\mu$ l, and then adding reagents, of approximately 100  $\mu$ l in volume. For this purpose, ultramicro cuvettes were designed which maintained the same light path. These are still in use today, the latest application being to the Perkin-Elmer Enzyme System. Figure 12 illustrates this principle.

In contradistinction to this philosophy, is the philosophy proposed by the author, for many years, even antedating Sanz's system, that the only micro specimen to be measured need be the original sample. Subsequent to that, methodology could be devised which would permit the sampling of larger volumes. These could then be handled more conveniently.

In order to bring this about, it was necessary to take advantage of certain fundamental principles of analytical chem-

istry. It was advocated that narrower band pass filters be used for increased sensitivity. For example, for the diacetyl method, for urea determination, the sensitivity with an interference filter is approximately 8 fold over that with a conventional colored piece of glass with a wide band pass. This can be seen in Figure 13.

Along the same lines it was advocated that colors be read at the absorption maximum rather than at some other convenient point. An example of this is the increase in absorbance one obtains when analyzing for bromide by means of gold chloride (18). As can be seen from Figure 14, the peak with gold bromide increases by a factor of more than 2 when the color is extracted into octanol and read at its absorption maximum (18).

Figure 14 also demonstrates the principle that sometimes extraction into an organic solvent increases the intensity of the color. This is true, for example, for ferric thiocyanate, where extraction into an organic solvent will increase the sensitivity of iron determination to that obtainable with the phenanthrolines. The advantage of the iron ferric thiocyanate method in organic solvents is the fact that it is insensitive to pH changes, which is not true for the phenanthroline procedures (19).

Another method that is commonly used for increasing sensitivity of a system is to increase the length of the light path. This is shown in Figure 15. In this case, the same volume is now read through a longer light path.

In Figure 15, comparison is made of the absorbance obtainable with a 1 centimeter light path as compared to an increased light path, with various instruments. Sensitivity is directly proportional to the length of the light path. This principle has become extremely important in the determination of trace gases. For example, if an individual has been exposed to a particular gas, such as fluorohydrocarbons, and has then left the area for substantial periods of time, up to as many as 2 or 3 days, it is possible to have him breathe into a cuvette which is several meters long, and is then placed in the path of an infrared beam. The gas can then readily be detected and identified by means of a spectrum drawn for the major lines.

In the authors experience, the amount of carbon dioxide in 10 microliters of blood can readily be determined by adding the blood to an acid, through which bubbles an inert gas. The CO<sub>2</sub> is then brought into the field of a long cuvette, of approximately 20" in length, and the carbon dioxide measured at the near infrared with a filter instrument. Instrumentation can be designed readily for measurement of the carbon dioxide content of as little as 1 $\mu$ l of plasma with this principle at the rate of approximately 40-60 per hour.

There are many techniques, which in their very nature permit the assay of very minute amounts of components in solution. The atomic absorption instrument is an example of such



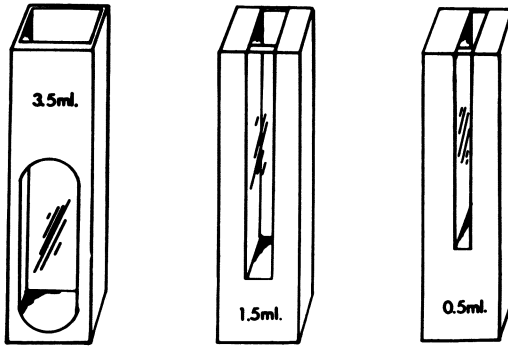


Figure 12. Maintenance of the same light path of 10 mm with smaller volumes

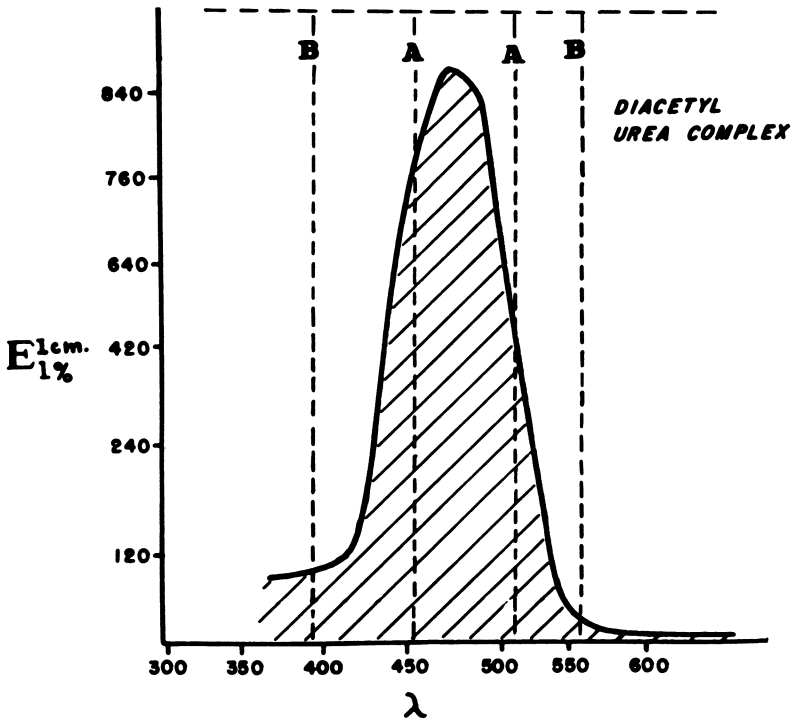
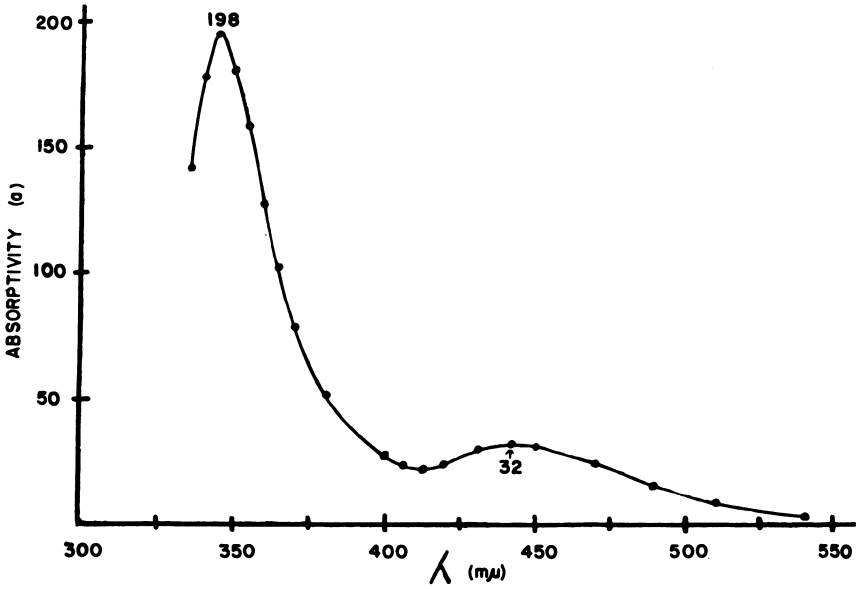
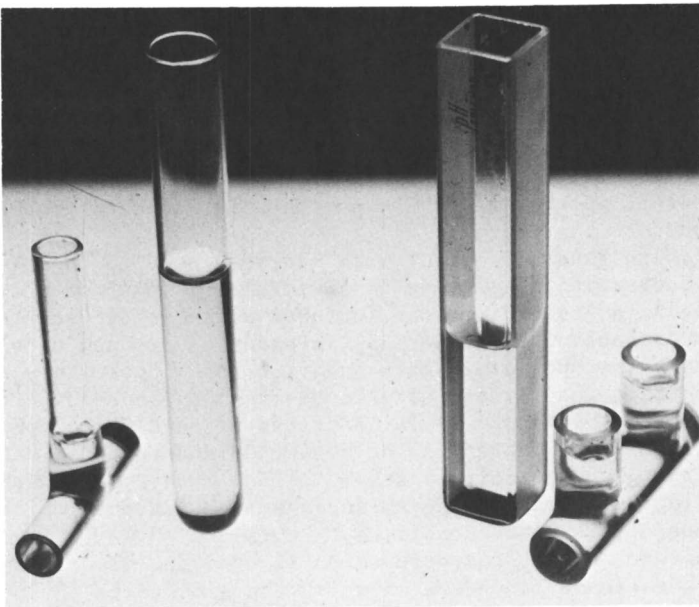


Figure 13. Demonstrating that a narrow band pass filter will increase sensitivity



*Figure 14. Increase in sensitivity by extraction into an organic solvent and choice of a high absorbance peak for bromide*



*Figure 15. Increasing sensitivity by lengthening the light path*

a device. In addition, fluorescence techniques readily measure concentrations of the order of 0.02 micrograms/ml for many substances. If fluorescence is measured in place of absorbance, increase in sensitivity of a factor of approximately 20 can be achieved when NADPH is being used.

Certain ion selective electrodes, such as the iodide and the chloride electrode, permit the assay of minute quantities if the electrode is properly designed for the purpose (20). Long range, many determinations which are presently being performed by chemical procedures, will be performed in the future with specific ion electrodes. Using the iodide electrode the high sensitivity of iodimetry is augmented manyfold.

How these and other principles are used in the Pediatric laboratory will now be discussed in connection with specific methodology which is recommended by the author for use in the laboratory of Neonatology.

### Assay for Specific Components

Glucose. There are four basic principles utilized for glucose analysis. The most widely used depends upon the reduction of some element such as copper or iron. This is the principle of the Folin-Wu method, the ferricyanide method, used on the Autoanalyzer, and the cupreine (21) method used on the SMA-12/60.

A second principle used widely for glucose analysis, is that of the oxidation of glucose enzymatically, mediated by the action of glucose oxidase with the formation of gluconic acid and hydrogen peroxide (22). In this procedure it is the hydrogen peroxide which is usually assayed for determination of glucose. This method suffers from the action of inhibitors which occur, particularly with patients in a diabetic coma and these need to be removed.

A third general method which has been used in the past, is the conversion of glucose to furfural, by means of strong sulfuric acid, condensing the furfural with certain substances such as orcinol or anthrone (23) in order to produce a color. While these methods are highly specific for glucose they suffer from the fact that traces of glucose from any source whatsoever will react. An example would be traces of dust which contain paper fragments. These will be converted and react in the same way as the glucose from the serum. Also in this procedure one is dealing with very strong sulfuric acid solutions, which are sometimes difficult to handle.

Another common procedure which is used for glucose assay is the hexokinase procedure in which the glucose is phosphorylated by means of ATP and then dehydrogenated with glucose-6-phosphate dehydrogenase measuring the absorption of NADPH

generated at the wavelength at 340nm, or by fluorescence. This method requires deproteinization by means of the Somogyi method in order to remove interference from hemoglobin (24). Figure 16 summarizes the reactions involved in the hexokinase procedure.

The hexokinase procedure can be carried out with the use of reagents which are supplied from various companies. From Table I it can be seen that these reagents vary in their specificity depending whether or not they have removed fructose hexokinase. However, as long as one is dealing with serum and not with a fructosuria, the procedure is specific.

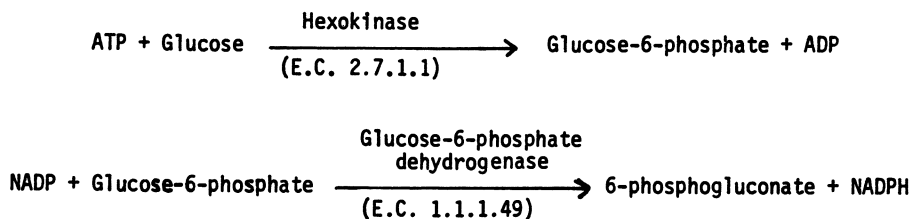
Many use the hexokinase procedure without protein precipitation. That this is a procedure which is not acceptable is illustrated in Table II where it is shown that unless hemoglobin is removed there will be interference in the results obtained. In severely hemolyzed blood, errors as high as 25% are not uncommon.

If the fluorometric procedure is used, with protein precipitation, then bilirubin will not interfere with the hexokinase procedure. Even if protein precipitation is not resorted to, the interference from bilirubin does not become significant until one is dealing with an infant in severe jaundice. This can be seen in Table III.

If the fluorometric method is used after protein precipitation then the glucose can be readily assayed on 1 $\mu$ l of plasma with the hexokinase procedure.

A widely used procedure is the method which uses o-toluidine. This method may be used directly if 25 $\mu$ l of serum is added to 2 ml of reagent(25). This suggests that if one wishes to go to smaller quantities then one needs to use an ultramicro cuvette. With the use of an ultramicro cuvette, one can readily perform this test on 5 $\mu$ l of serum provided that the total volume at the end of the test is less than 400 $\mu$ l in volume. This is a practical volume with some of the newer ultramicro cuvette systems used. Figure 17 illustrates the course of events during the heating of a solution with the o-toluidine reagent.

It will be noted that at 100°C that the color comes up rapidly, but then rapidly fades, and many have noticed that in the o-toluidine method unless one waits a substantial period of time, one is dealing with a fading color. However, it should be noted that when heated at 37°C that the color will not develop for at least 2 hours, but will yield a higher absorbance, because the color is more stable. A good compromise is to heat at temperatures of the order 55°C or 65°C in which case one can then conveniently read at the end of 10 or 15 minutes with adequate reading time for practical purposes. This is not done in most laboratories. Most laboratories continue to heat at 100°C, which is not the correct way to use this procedure.



*Figure 16. Glucose estimation by the hexokinase procedure*

**Table I**

**Comparison of the various hexokinase enzyme mixtures  
for specificity in glucose analysis**

| <u>Preparation</u> | <u>Sucrose,<br/>200 mg/100 ml</u> | <u>Mannose,<br/>100 mg/100 ml</u> | <u>Fructose,<br/>100 mg/100 ml</u> |
|--------------------|-----------------------------------|-----------------------------------|------------------------------------|
| Ref. #24           | 0                                 | 0.2                               | 18.9                               |
| Sigma              | 0                                 | 0.1                               | 4.7                                |
| Worthington        | 0                                 | 0                                 | 6.2                                |
| Calbiochem         | 0.5                               | 0.8                               | 15.5                               |
| Gen. Diagnostics   | 1.1                               | 2.4                               | 85.5                               |

\*Values are glucose equivalents in mg/100 ml.

Table II

Effect of hemolysis on the assay of glucose by the fluorescence-hexokinase method without protein precipitation.

| <u>Added Hemolysate<br/>(Hb, mg/100 ml)</u> | <u>Glucose Found<br/>(glucose, mg/100 ml)</u> |
|---|---|
| 0   | 69.0  |
| 100   | 71.9  |
| 200   | 75.3  |
| 300   | 79.3  |
| 1000  | 87.9  |
| 2000  | 95.7  |

Table III

Effect of bilirubin on the glucose fluorometric hexokinase procedure with and without protein precipitation.\*

| <u>Bilirubin<br/>(mg/100 ml)</u> | <u>Glucose (mg/100 ml)</u> |                 |
|----------------------------------|----------------------------|-----------------|
|                                  | <u>Pptd.</u>               | <u>Nonpptd.</u> |
| 2.7                              | 77                         | 77              |
| 5.4                              | 63                         | 59              |
| 7.8                              | 73                         | 71              |
| 11.2                             | 118                        | 113             |
| 18.1                             | 85                         | 85              |
| 21.1                             | 90                         | 85              |
| 40.0                             | 382                        | 377             |

\*Results are means of duplicate determinations. Serums are from the routine laboratory. Bilirubin levels are assayed values.

Exactly what is happening during the heating can be seen in Figure 18. In Figure 18 it will be noted that the color at the 635 nm maximum, fades with heat with the appearance of another color with a maximum at approximately 410 nm. As a result, on continued heating one observes a murky green solution as distinct from the clear green-blue solution observed when heated at lower temperatures.

The use of the lower temperature permits the performance of this test in the presence of substantial amounts of hemoglobin. Since hemoglobin has very little absorbance at 635 nm, then as much as 2% hemoglobin in the solution will not significantly affect the results obtained for glucose. This is shown in Figure 19. The reason for this is that hemoglobin is stable at 55°C or 65°C while it decomposes at 100°C. If a direct method of doing glucose is to be sought, then the toluidine method is probably the method of choice. Bilirubin and lipemia also do not interfere because if the method is done properly, the lipids will dissolve in the glacial acetic acid in which the test is being performed.

The use of aniline derivatives for the identification of sugars was originally used in connection with identification of sugars in general by thin layer chromatography. Table IV shows the response of various sugars to the toluidine reagent when the colors are being developed on the thin layer chromatogram. It will be noted that the use of this reagent at low temperatures can distinguish sugars from others which may have the same  $R_f$  values, but do not develop the color at the low temperature. Heating the chromatogram at low temperature and then at high temperature can distinguish these sugars. However, Table IV also points out the significant fact that the o-toluidine method is not specific for glucose determination.

In figure 7 a procedure was described for aspirating a sample from a capillary tube and simultaneously adding zinc sulfate and barium hydroxide solutions in order to produce a Somogyi filtrate. Aliquots of the supernatant are suitable for assay for glucose and urea by various procedures. The reason for this is the fact that zinc hydroxide precipitates uric acid, creatinine and other substances, such as low molecular polypeptides, along with the proteins, so that there results a solution which is clear with relatively few components.

Another procedure which has adequate sensitivity for determining the glucose in 1 microliter of serum or filtrate, is the method which uses copper reduction, and subsequently determination of the cuprous ion with a suitable reagent (15). However, one must be careful that one has obtained complete precipitation, for, if uric acid or any other impurities remain, false high values will be obtained. This would result in disaster for the hypoglycemic infant. To uncover this condition is often one of the major reasons for doing this test.

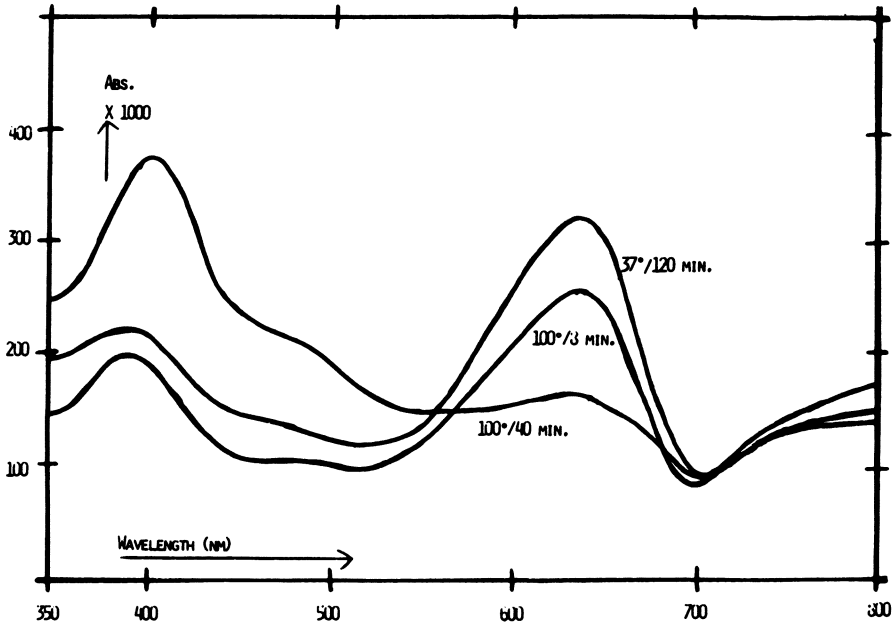


Figure 17. Change in absorbance pattern of reaction product of glucose and toluidine with heating

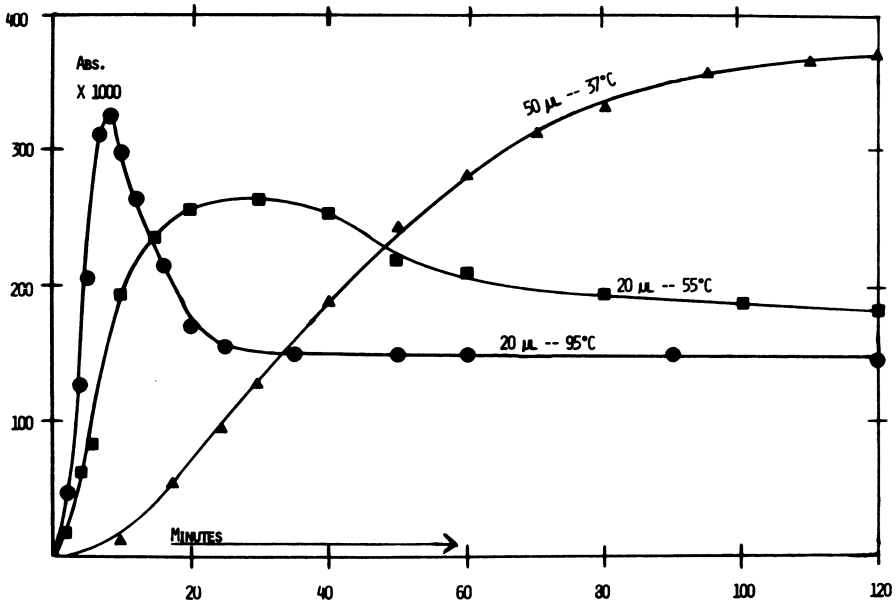


Figure 18. Absorbance change with time of the toluidine glucose reaction product at various temperatures



This problem removes the cupreine method as an acceptable procedure for the Pediatric Laboratory for glucose analysis under any circumstances.

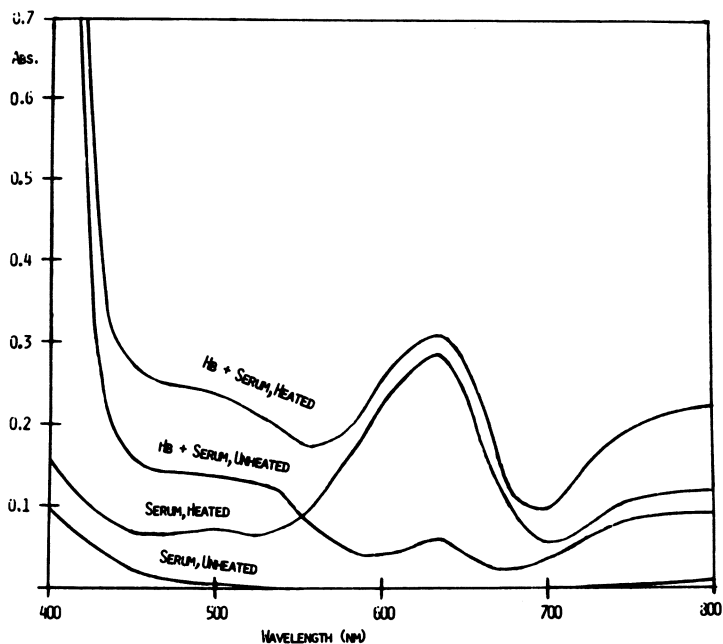
In addition to the method described above, there are at present, on the market, several glucose analyzers offered by several commercial companies, wherein the individual injects approximately 20 microliters of serum. This is then incubated with glucose oxidase and the peroxide or oxygen generated is measured with an oxygen sensing electrode. These instruments cannot perform the test on 1  $\mu$ l nor are they designed for that purpose at the present time.

In view of the above discussion, it should be clear why the author recommends that the laboratory of Neonatology precipitate proteins with a Somogyi filtrate, and then perform the hexokinase procedure on the filtrate. Neither the glucose oxidase procedure nor the toluidine method have adequate sensitivity without the use of special micro-equipment and special techniques.

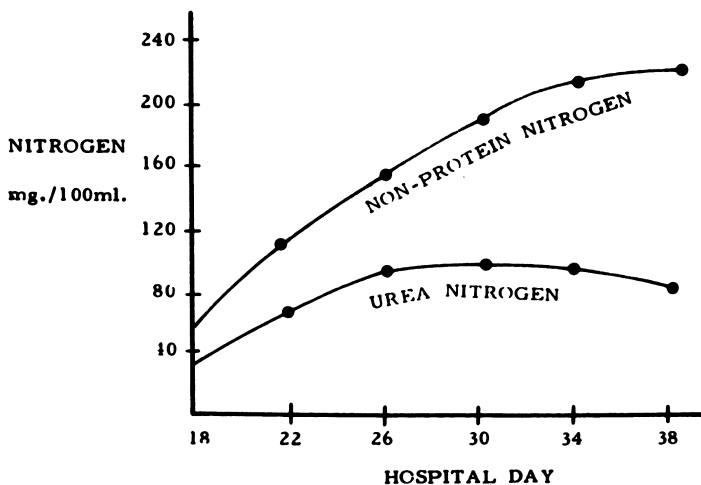
Urea. The diacetyl method continues as the most specific method for urea at present (26). Addition of thiosemicarbazide, antipyrine (15) or other additives remove the specificity of this procedure for urea. Other substances including ammonium and amines of various types begin to react, so that in uremia a significantly elevated value is obtained which begins to approach the nonprotein nitrogen level. This is extremely important because as the condition becomes worse, a greater and greater disparity occurs between urea and nonprotein nitrogen. If the urea is to be meaningful in this regard, it needs to be a specific procedure and not react with other substances. An example of this phenomenon in a patient is seen in Figure 20.

Recently, the old alkaline phenol method has been revived, and is being widely used in clinical laboratories, without protein precipitation(27). In this procedure, the serum is added to an alkaline phenol reagent, and the ammonia generated from urea is determined either after the action of urease or after strong alkaline treatment of the serum. The objection to this procedure is first, that all urease is rich in ammonia, and second, the color produced with alkaline phenol is not specific for ammonia. It will react with other compounds, especially for those that liberate ammonia. By this procedure one obtains a useful number from the point of view of determining whether the patient has nitrogen retention, but a value which is somewhere between a urea and an N.P.N. determination.

In the Beckman instrument, change in conductivity is used (28). The instrument uses 14  $\mu$ l for the test but samples from a cup which needs to contain at least 100  $\mu$ l of serum. The Beckman instrument suffers from certain faults. First, the standard for urea must contain the proper amount of saline or false values are obtained. Second, urine cannot be analyzed



*Figure 19. The effect of 2% hemoglobin solution on the color produced with toluidine on glucose*



*Figure 20. Demonstration of disparity between nonprotein nitrogen and urea levels in a patient with terminal kidney disease complicated by liver involvement*

for urea, except under very special conditions. Third, fluoride interferes, so that if blood is presented to the laboratory with fluoride, the test cannot be done. This requires that the laboratory convert to iodoacetate as a preservative. This, of course, is very difficult in the main laboratory, but in the Pediatric Laboratory, one can line the capillary in which the blood is collected with iodoacetate with very little difficulty, in order to preserve the sugar level, if the analysis is not to be done promptly. On the other hand, since very little time elapses in the Pediatric Laboratory between taking the specimen, and analysis, no preservative at all need be used, particularly for the urea determination, which is relatively stable. However, the fact that this instrument cannot even perform the test on aqueous solutions of urea, and urine, should eliminate it as an instrument to be recommended for any laboratory. Any instrument used for urea analysis should be able to perform the test under all conditions under which the laboratory operates. This includes the analysis of blood which has been taken in various ways, and the application to other specimens, such as urine, gastric aspiration or amniotic fluid.

The above discussion does not mean that the use of urease and subsequently the use of an ammonia electrode is not practicable for a urea determination. Unfortunately, the commercial company that produced the urea analyzer, chose a conductivity procedure, which happens to be unsuitable for the laboratory of Neonatology. Had they chosen the ammonia electrode, which happens to be a relatively good electrode, and is especially specific, since only ammonia and not potassium can pass an air space, then the instrument could have been made highly specific for urea. In this case an ammonia determination would be done initially and then subtracted by the computer, from the amount which has been generated subsequently. In any case, with present technology, sensitivity is not adequate to use less than approximately 15  $\mu$ l of serum.

For the present, for reasons discussed above, the diacetyl procedure is the method of choice for the Laboratory of Neonatology with equipment available at present. The importance of the assay for urea in the blood of infants is emphasized by the readiness with which the urea level responds to change in diet in the infant (1). This is seen in Table V.

Sodium and Potassium. For the electrolytes, sodium and potassium the flame photometer is the instrument of choice (29). This instrument permits readily the dilution of the serum 200 fold, for analysis, using an internal lithium standard. Most instruments require 1 ml for analysis. It is therefore practicable to measure out 5  $\mu$ l and dilute it to 1 ml. This is best done with a sampler-diluter of high precision. The tip of the diluter needs to be a drawn out polyethylene tip, or the 5  $\mu$ l will not be measured with any degree of accuracy.

TABLE IV  
Color developed at 37°C and 100°C for  
several sugars with toluidine.

| <u>Sugars tested</u> | <u>Color</u> | <u>Relative reading</u> |              |
|----------------------|--------------|-------------------------|--------------|
|                      |              | <u>37°C</u>             | <u>100°C</u> |
| Glucose              | Green        | 91                      | 175          |
| Galactose            | Green        | 117                     | 275          |
| Mannose              | Green        | 75                      | 187          |
| Fructose             | Green        | 0                       | 45           |
| Sorbose              | Green        | 0                       | 64           |
| Maltose              | Green        | 0                       | 15           |
| Lactose              | Green        | 0                       | 70           |
| Sucrose              | Green        | 0                       | 47           |
| Trehalose            | Green        | 0                       | 10           |
| Meliboise            | Green        | 7                       | 179          |
| Raffinose            | Green        | 0                       | 35           |
| Rhamnose             | Yellow       | 35                      | 29           |
| Xylose               | Red          | 205                     | 377          |
| Arabinose            | Red          | 129                     | 359          |
| Rucose               | Yellow       | 27                      | 12           |

Table V  
Urea nitrogen concentration in plasma of infants  
after five days on breast or cow's milk.

|           | Breast    | Cow       |
|-----------|-----------|-----------|
| Mean      | 12.5      | 17.4      |
| Range     | 8.7-15.4  | 9.4-23.9  |
| Std. Dev. | $\pm 1.9$ | $\pm 3.9$ |

Other methods for assaying for sodium and potassium such as with ion selective electrodes (30) or with the autoanalyzer, require much larger quantities of material. In addition, the ion selective electrodes, which have great potential, have not been developed at present to the point where they can be utilized in a practical way for micro sodium and potassium analysis. It is very important that one be able to distinguish between sodium concentration in specimens which differ by only 2 mEq/L. This requires that we have a reproducibility of 2 parts in 140, which is substantially less than 1%. This reproducibility can not be reached in an Autoanalyzer. It is practicable to reach this degree of precision however, in the routine laboratory of clinical chemistry with the commercially available flame photometers.

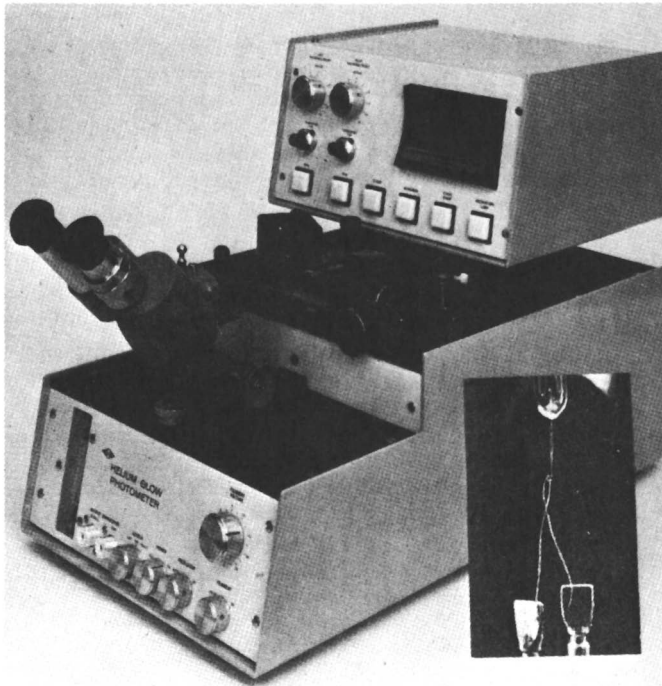
If one wishes to go to the nanoliter range there are instruments available such as one which operates with a glow discharge made by the American Instrument Company, which can determine sodium and potassium in a few nanoliters. In this procedure a glow discharge is set up between two electrodes with helium gas. On vaporization of the serum, the Na and K are excited by transfer of energy from excited He ions. At present, the inconvenience of this instrument makes it only of value in relationship to research in small animals. The design is seen in Figure 21.

Atomic absorption has not been effective for Na and K estimation for reasons discussed under Calcium Assay.

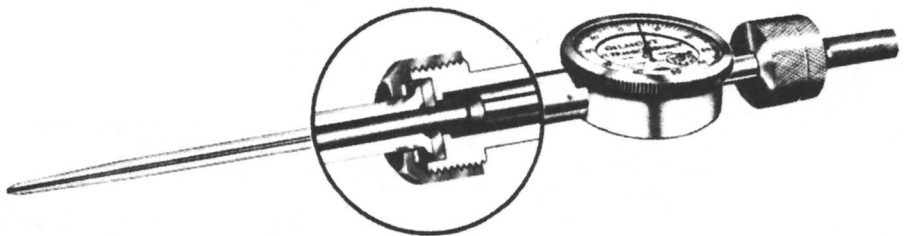
Chloride. Chloride analysis may be done in any one of 3 ways with adequate sensitivity for the Laboratory of Neonatology. It can be done amperometrically (31), by titration (32) or with the chloride electrode (33).

In the titrimetric procedure, the serum is diluted in 0.07 N nitric acid and titrated with a dilute mercuric nitrate solution in the presence of diphenylcarbazone (34). A purple color forms when excess mercury ions appear, over that needed to complex the chloride. Using an ultramicro buret this method has found wide use in the past. The microburets which are available such as the Rehburg buret are typified in Figure 22.

A second method which is now probably the most widely used method in the Pediatric Laboratory is to use amperometric titration. In this connection, a constant current flows through the solution. The silver dissolves and reacts stoichiometrically with chloride, precipitating silver chloride. When all of the chloride has reacted, there is a sharp increase in conductivity which is read as an end point. This instrument, therefore, measures the amount of time a current flows. Instruments are now available for which 5 microliters can be used routinely, rapidly, titration being of the order of about 20 seconds. More commonly 10 microliters is used with a longer time. This method is by far the most reproducible method available at



**Figure 21.** *Ultramicro instrument for Na and K estimation. Nano-liter specimen is placed on wire (inset) and is then vaporized in the glow discharge with helium (American Instrument Co.).*



**Figure 22.** *A type of ultramicro buret where the liquid is expelled by displacement of a plunger and readout on a dial indicator*

present.

A third method for chloride estimation which is commonly employed is the silver-silver chloride electrode. In this method the solution is usually diluted in a buffer, and the voltage generated measured. The problem with this procedure is that the electrode needs to be carefully maintained or problems occur. With larger volumes the chloride electrode has been very successful.

Colorimetric methods, such as those which depend upon the measurement of the color developed when excess mercury salts are added to a chloride solution, are not reproducible enough for practical purposes. They are widely used in automatic equipment, but for the purpose of maintenance and treatment of newborns, this data does not have the adequate accuracy and reproducibility.

As a microanalytical tool the method of amperometric titration is the most reproducible and is the method of choice for the Laboratory of Neonatology, for the analysis of chloride.

Calcium and Magnesium. When it comes to the elements, it is theoretically most advantageous to excite the element, and read the emission as the element returns to the ground state. The flame photometer is an application of this principle. In the flame photometer, the temperature of the hydrocarbon flame supplies the exciting energy. In order to excite elements, such as calcium and magnesium, the temperature of the hydrocarbon flame is not high enough. For this reason, some have used flames generated by the reaction of, for example, nitric oxide and acetylene. These are inconvenient for the chemistry laboratory. Others have used the flame, and selected minor lines for identification of calcium and magnesium (35). This was the principle of the calcium and magnesium system used by Coleman Instrument Company for many years. Actually, these systems failed, mainly because they were theoretically unsound to begin with. The carbon arc will yield temperatures high enough for obtaining the spectra for calcium and magnesium, but are inconvenient for the routine laboratory. In this class of excitation sources, is the plasma jet. Figure 23 shows the use of the plasma jet in welding. This is an everyday procedure, where the gas, such as helium or argon, or a mixture of gases, is excited by high frequency excitation, and escapes from an orifice containing the energy which is high enough so as to serve for welding stainless steel and other substances.

It is necessary to supply a spray into the plasma jet, in order to excite the elements. This has presented some difficulty and it is a mystery to the author as to why the plasma jet has not been more widely applied for the analysis of sodium potassium, calcium and magnesium. It has the advantage of safety, in that one inert gas (argon) would be used, and high sensitivity. In addition, four elements, calcium, magnesium,

sodium and potassium could be assayed just as easily as we do sodium, potassium today with a flame photometer.

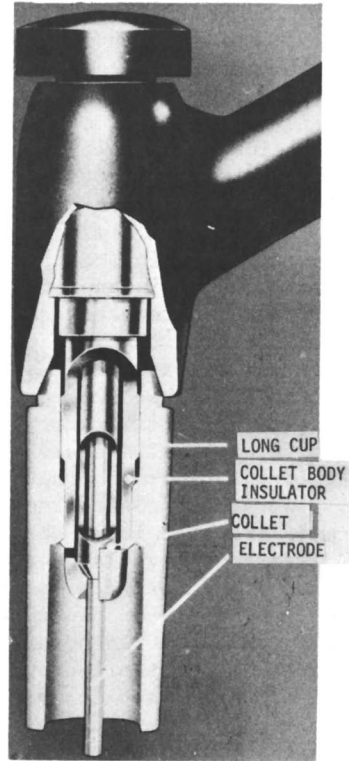
At present, calcium and magnesium are estimated almost exclusively by atomic absorption (36). Present instrumentation permits the dilution of the specimen to approximately 1 - 100 for calcium and even higher for magnesium. For many instruments, the two elements are not read out simultaneously such as is practicable for sodium and potassium with the flame photometer. The lower limit of serum volume at present, for the practical assay for calcium and magnesium in the laboratory of Neonatology, is approximately 10  $\mu$ l. The instruments are very readily automated, and it is not uncommon for results to be available at the rate of 240 per hour in the routine laboratory, where a typical atomic absorption instrument such as a Perkin-Elmer has been attached to an automatic feed system. In the author's laboratory, an Autoanalyzer turntable is used which rotates, at a speed, so that the 40 specimens can be assayed in a 10 - 15 minute period. Figure 24 shows the principle of atomic absorption. Note that an elongated absorption path is provided for higher sensitivity.

There are available also several kits for the assay of calcium, in 10 or 20 microliter samples by chelate formation colorimetrically or fluorimetrically. (Pierce Chem. Co., Rockford, Ill.). These are read either with the spectrophotometer or by spectrofluorometry. In our experience, while these systems can be used for approximate results, the plot of concentration versus reading curves are rather flat and only an approximation of the values can be obtained. This may be very important late at night or at times when the atomic absorption machine is down, but if the atomic absorption instrument is available it should be used in preference to these procedures.

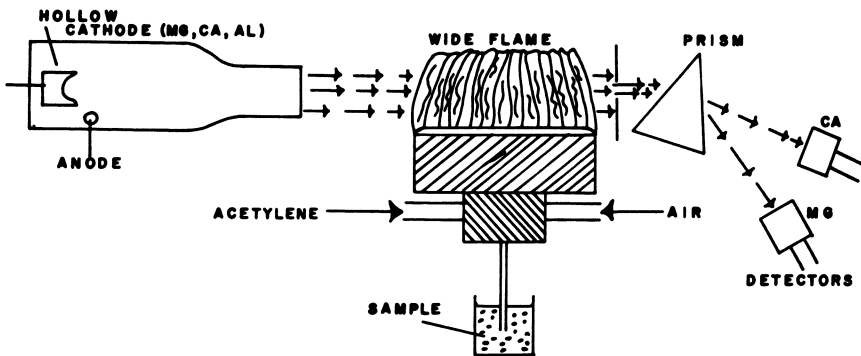
Assay of calcium and magnesium by specific ion electrodes (37) is not practicable on micro quantities today, although it is quite possible that these may become available in the near future, especially with the developments that are taking place in foreign countries in this area.

It must be pointed out that the atomic absorption system as used today, cannot accurately determine the calcium level of a solution. The reason for this is that results will vary depending upon the other elements present and the composition of the solution. Since it is impossible to duplicate every feature of the particular serum being analyzed, results have to be compared to standards which have been made up in serum dialysates. Such standards are available in the form of the Versatols where the calcium has been dialyzed out and then weighed back. This is distinct from substances such as Validate, which are used as controls and which values are results of analysis. The variability of serum composition has significantly widened what is now considered the "normal range" for serum Ca assay when done by atomic absorption (37a).





*Figure 23. Heliarc torch used in welding as an example of the plasma jet*



*Figure 24. The basic principle used in atomic absorption. The sample is sprayed into the flame, and the calcium and magnesium emission from the lamp is absorbed. The extent of absorption is measured on the detector and translated in terms of concentration.*

We have found that the use of serum standards for standardizing the instrument in the laboratory is useful. However, the serum standards cannot be used for urines. In urines, one runs into other problems and needs to use aqueous standards. Therefore, at present, while atomic absorption is the instrument of choice, there is much to be desired for the determination of calcium and magnesium in the routine laboratory of clinical chemistry.

**Bilirubin.** The most widely used method for bilirubin continues to be the method which couples bilirubin with the diazo reagent and reads the color formed (38). Unfortunately the sensitivity of this procedure is such that a lower limit of approximately 10 microliters of specimen is a limitation on the use of this procedure.

Recently, this problem has been approached by reverting to reading the color of the bilirubin directly. This used to be called an "Icterus Index", in which the color of the serum was compared to a dilute dichromate solution (39). However, the new approach has been to use two wavelengths. This can be used in either of two different procedures. In one procedure the reading is made at the peak for bilirubin which is at 453 nm and at an isosbestic point for hemoglobin (40). The effect on the reading due to hemoglobin is subtracted from the bilirubin value. Commercial instruments based on this principle have not been successful.

In another approach a reading is taken at 453 nm and also at 572 nm. The reason 572 nm is chosen is because, at this point, hemoglobin has the same molar absorbance as it has at 453 nm. Therefore, subtracting the reading at 572 nm is equivalent to subtracting the hemoglobin reading directly under the bilirubin peak. This system has another advantage in that the wavelengths at 453 and 572 are not far apart, and some correction is being made for lipemia and turbidity which might be present in the sample being tested (41).

Unfortunately, Perkin-Elmer who has produced this instrument has built the instrument for the purpose of measuring bilirubin in amniotic fluid. As a result, the instrument does not read above a level of approximately 2 mg/100 ml. Therefore, it is necessary to dilute the serum from newborns in order to be read in this instrument. However, if one standardizes by making a 1 to 5 dilution of all newborn serum, one can use this method for screening purposes. One looks forward to the further development of this instrument so that readings can be made over a wide range.

Still widely used is the method of scanning for bilirubin in amniotic fluid. A scan is run for some distance before and after 450 nm. From the baseline drawn between 2 points (e.g. 350 nm and 550 nm), one examines the height of the peak. Normally, no peak will be present. Where the bilirubin is

elevated, a peak will form with a maximum at approximately 450 nm. The rise above this baseline is called the O.D. 450 value which is the measure of the bilirubin concentration in amniotic fluid. Where one is dealing with amniotic fluid and therefore with a single specimen, this method is practicable, since it takes approximately 15 - 20 minutes per scan. This is too slow for the routine laboratory of Neonatology. In addition, the method is not sensitive enough to be utilized for small quantities such as 5 microliters unless special attachments are made to the spectrophotometer. Figure 25 shows a typical O.D. 450 scan for bilirubin (42,43).

Protein. In listing those procedures which have been used in the past for analysis of protein in serum using volumes of the order of 10  $\mu$ l of serum, one may include the Kjeldahl procedure, the color obtained with phosphotungstic acid on reduction by tyrosine in the protein (also commonly called the Folin-Ciocalteu reagent) and the biuret method. Of these, the Kjeldahl procedure has adequate sensitivity but is too time consuming (44). The Folin-Ciocalteu procedure, commonly referred to as the Lowry method in its micro version, is not specific for protein (45). Any phenol will react with this reagent, and high values will be obtained if the patient is being treated with salicylates. The biuret procedure is specific for protein, and can be made to react with small amounts of serum, suitable for use in a routine Laboratory of Neonatology (15,46). However, certain facts must be brought out when the biuret method is used.

The biuret method has been known since the turn of the century but was never used because of the interference of other substances in the serum, particularly turbidity, caused by lipids. This was corrected by Kingsley (46), when he introduced the idea of extracting the lipids with ether before applying the biuret reagent. The author has, for over 30 years, used a method which precipitates the proteins with Bloor's reagent (5). Bloor's reagent is made up of 1 part of ether and 3 parts of alcohol. The lipid extract and extract of bilirubin and heme are discarded by decantation after centrifugation and the biuret reagent is added to the residue. In this way, accurate values are obtained (15).

Recently, in a study of the various methods for doing proteins in amniotic fluid it was noted that values ranging up to 12% have been reported in literature. What was being measured in these cases was the turbidity and the presence of hemolysis in amniotic fluid. When the Bloor's reagent extract is applied, substantially lower and more consistent values were obtained (47).

The same is true for specimens of serum from the newborn. Those who attempt to apply the biuret method to newborn sera, end up with values in the normal infant, resembling those for

adult serum, namely of the order of 6.5 - 7.5%. Actually, serum protein levels in the newborn are at approximately 5.5% when the test is accurately performed (48). The effect of extracting of serum with Bloor's reagent before applying the biuret reaction is shown in Table VI. In Table VI, it will be noted that even with serum, when the lipids are not substantially elevated, there is an error of at least 10% in the direction of an overestimate. It is a fact that in many laboratories in this country, the protein determination has become of little value in the Pediatric Laboratory because of the fact that the determination is being performed by merely adding biuret reagent directly to serum and reading the hemolysis, bilirubin, lipemia and incidentally the protein and interpreting this as a protein determination.

It is recommended that the protein be precipitated and extracted by Bloor's reagent before adding the biuret reagent.

Blood Gases and Blood PH. For measurement of  $pCO_2$ ,  $pO_2$ , and pH there are 3 instruments on the market. One is marketed by Corning, another by Instrumentation Laboratories (IL) and the third by the Radiometer Corporation. The performance of each of these instruments is fairly good. However, only the Corning instrument is able to perform simultaneous  $pCO_2$ ,  $pO_2$ , and pH levels on samples of less than 150  $\mu$ l in size. The IL and the Radiometer instrument, require 0.5 ml of whole blood for the assays. In the case of the IL instrument, it is possible by maneuvering to utilize 250 microliters. In the case of the Corning instrument, the instrument is set to read the three parameters on 250  $\mu$ l and by special use of the instrument to do the determination on 125 microliters. Unfortunately, this creates a problem for the Pediatric Laboratory, since it requires that one obtain substantial amounts of capillary blood. This may be very difficult in the particular infant who is in most severe distress. At the present time there is a need for an instrument that can read all three parameters simultaneously on a maximum of 25 microliters of whole blood. This is not beyond the realm of modern technology.

The principle of the pH electrode has been discussed thoroughly in various treatises (49). The  $pCO_2$  electrode is the glass electrode separated from the blood by a thin Teflon membrane. The  $CO_2$  diffuses through the membrane changing the pH of the bicarbonate buffer, which is sensed with the pH electrode and then read out in terms of  $pCO_2$ . The  $pO_2$  is read by allowing the oxygen to diffuse through a polyethylene membrane (50,51). A constant current flows in an acid buffer, so as to deposit hydrogen gas and polarize the surface of a platinum electrode. As the oxygen diffuses to the platinum electrode the hydrogen reacts with oxygen to form water, depolarizing the electrode and increasing the current. The greater the rate at which the oxygen diffuses into the electrode, the higher

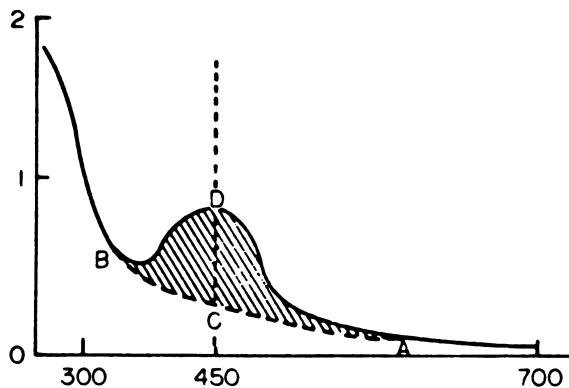


Figure 25. Typical scan, plotting absorbance vs. wavelength for amniotic fluid for bilirubin assay

Table VI

Effect of turbidity on the % protein found  
in serum as determined with and without  
eluting with Bloors Reagent. Results are in g/100 ml.

| Total Lipids<br>mg/100 ml | Replicates<br>Washed with<br>Bloors Rgt. |     |     | Replicates<br>(not washed) |      |      | Difference<br>of Means |
|---------------------------|--|-----|-----|----------------------------|------|------|------------------------|
| 592                       | 5.0                                      | 5.0 | 5.0 | 5.8                        | 5.7  | 5.8  | 0.8                    |
| 630                       | 6.2                                      | 6.3 | 6.2 | 7.1                        | 7.1  | 7.1  | 0.9                    |
| 855                       | 7.9                                      | 7.9 | 8.0 | 8.5                        | 8.6  | 8.5  | 0.6                    |
| 1080                      | 5.9                                      | 6.0 | 5.9 | 9.2                        | 9.2  | 9.4  | 3.3                    |
| 1350                      | 7.8                                      | 7.9 | 7.9 | 10.0                       | 9.9  | 10.0 | 2.1                    |
| 1755                      | 7.5                                      | 7.5 | 7.4 | 11.8                       | 11.8 | 11.7 | 4.3                    |

the current, and therefore, the higher the  $pO_2$ .

The difficulty with  $pO_2$  measurement is the fact that when measuring  $pO_2$  from blood, the blood is constantly being depleted of oxygen and low values are obtained. When standardizing the blood with gases, one is dealing with a constantly changing supply of gas through the electrode, and this source is not being depleted. As a result, the  $pO_2$  of blood oxygen tends to be underestimated in these systems. In some of the instruments, a correction factor is built in, to compensate for the effect, and in others, it is recommended that the blood be placed into the electrode, allowed to come to approximately equilibrium, and then a fresh specimen is put in to obtain the final result. The more often this is done, the closer one comes to the correct value. Unfortunately, this requires substantial amounts of blood. If one has approximately 1 ml blood, one can obtain a fairly reasonable reading.

Recently, interest has been aroused in obtaining  $P_{50}$  values on infants blood, especially in screening for genetic abnormalities (51). The  $P_{50}$  value is the  $pO_2$  value when the blood is half saturated. This is usually done by equilibrating the blood with oxygen at two known  $pO_2$  values. The blood is then taken and the oxygen content is measured at these two values. By interpolation on a chart, the  $pO_2$  value at half saturation is determined. In order to do this, a useful instrument is the Lex-O<sub>2</sub>-Con (Lexington Instrument Company, Waltham, Mass.). This is an instrument into which 20  $\mu$ l of whole blood is injected. The blood is then carried by means of a gas containing hydrogen and no oxygen to a cadmium electrode.

The oxidation of the electrode generates a current. The current is integrated and the total amount of current is then printed out in terms of oxygen content (52). In effect, this is a system of amperometric titration for oxygen. This instrument uses only 20  $\mu$ l of whole blood. It takes approximately nine minutes for each assay and this is a serious disadvantage.

The determination of total carbon dioxide is still being done where accuracy and precision are required by the use of a micro gasometer. This is shown in Figure 26. In this instrument the gas is liberated and the pressure produced by the carbon dioxide measured before and after it is reabsorbed into alkali. Attempts to measure carbon dioxide content by other means have not been particularly successful. In the autoanalyzer, the blood is acidified and the  $CO_2$  is then absorbed into a buffer, and the change in color of a phenolphthalein solution is moderated. This correlates rather poorly, with the actual  $CO_2$  content of the blood. Attempts to calculate total  $CO_2$  from  $pCO_2$  values and pH result in huge errors because of the fact that the  $pCO_2$  is related to the total  $CO_2$  by a logarithmic function, and a very small change in  $pCO_2$  results in a large change in total  $CO_2$ . Since the  $pCO_2$  is measured at present with much less accuracy than total  $CO_2$  can be measured in a gasometer (53),

54), this method is not to be accepted.

It is recommended for the present that in the Pediatric Laboratory the total CO<sub>2</sub> always be measured with a micro gasometer. (See Figure 26).

If CO<sub>2</sub> is removed from blood and passed through a one meter cuvette of an infrared spectrophotometer, and the absorption characteristic peak integrated, the CO<sub>2</sub> can be very accurately measured. Alternatively, CO<sub>2</sub> can be liberated from a solution and then collected in a weak buffer. The pH of this buffer can be brought back to its original value by electrometric titration, using a hydrogen electrode as a means of measurement. If this system is set up, carbon dioxide can be measured in extremely minute quantities. Unfortunately, in neither of the above cases have commercial companies taken the initiative and placed such instruments on the market. This is true in spite of the fact that the instrument for measuring carbon dioxide by amperometric titration is at present being used in the determination of carbon in organic specimens after combustion. The introduction of such instrumentation into the Pediatric Laboratory awaits development by a manufacturer.

Miscellaneous Serum Components. The discussion of the tests most commonly done in the Laboratory of Neonatology, above, illustrates the various types of instrumentation used. Here, various other procedures will be discussed at random in order to illustrate some basic principles, which have many applications.

Molecular diffusion (55) has been used in various ways in micro analysis. In Figure 27, is seen the Conway diffusion dish, in which the substance to be tested is placed on the outside, and the reagent is placed in the central cup. The cup is covered, and after a time, the substance being analyzed diffuses into the central cup where it produces an effect which can then be interpreted in various ways. In the Figure, carbon monoxide is being determined. This same method is very useful for alcohol determination, where dichromate oxidizes the alcohol after it diffuses into the dichromate from the blood.

A variation of this system is the rotator (Figure 28), which has come into importance in recent times, in the determination of ammonia in Reyes Syndrome (56). In this system, the blood is placed into a bottle, a stopper containing a glass rod, which has been dipped in dilute sulfuric acid is fit into the bottle. When the test is being performed, potassium carbonate is added quickly to the bottle, and the bottle is corked. On rotation, the ammonia diffuses from the blood to the sulfuric acid held on the glass tip. After a fixed period of time, the rubber stopper is removed, and the glass tip is dipped into an aqueous solution. Subsequently, reagents, such as Nessler's reagent, or the alkaline phenol reagent, are added to the solution, in order to generate a color in order to be read.

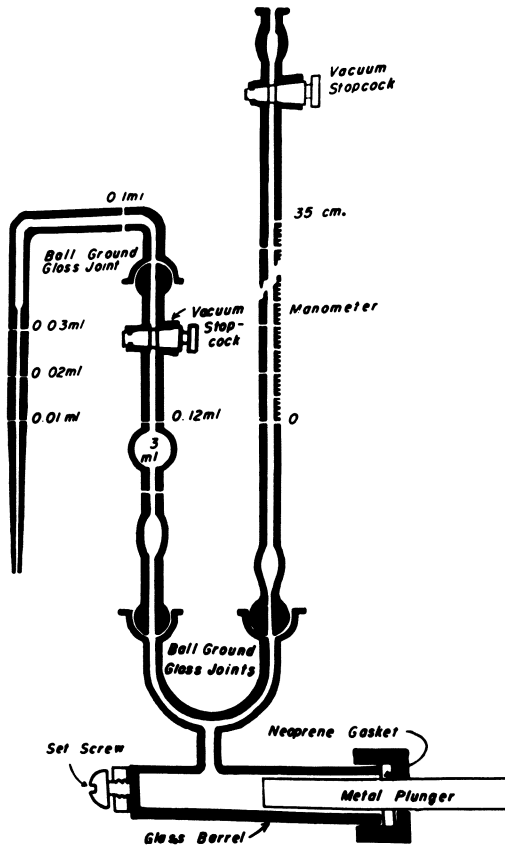


Figure 26. *The microgasometer for CO<sub>2</sub> assay*

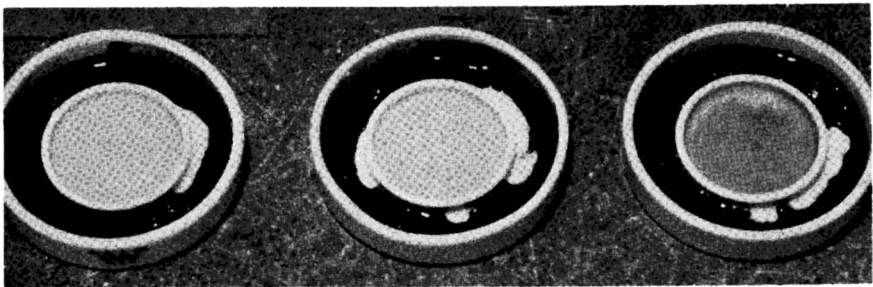


Figure 27. *Estimation of CO in blood with a Conway diffusion dish. Palladium chloride is reduced to black palladium powder in the center compartment by the diffused CO.*



**Amino Acids.** Thin-layer chromatography has found wide application in the clinical chemistry laboratory. An application that is practicable in the Laboratory of Neonatology is the screening of serum and urine for the amino acidopathies. In order to do this we use a micro ultrafiltering apparatus which has been designed in our laboratory (57). This is seen in Figure 29.

A 20 microliter sample is placed in a narrow tube which comprises the other part of an assembly. A Millipore filter is then placed in the lower part of the assembly. An instrument is then put together by screwing the upper part into the lower part and sealing the bottom of the lower part with an Allen screw. The assembly is now centrifuged as shown in Figure 30, where the operator is placing one of the micro ultrafiltering devices into the cup of the high-speed centrifuge.

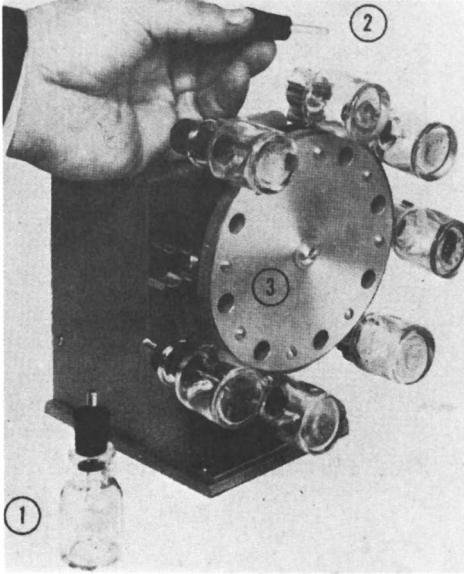
It is centrifuged at 12000 r.p.m. for between 10 and 20 minutes. The instrument is removed from the centrifuge and disassembled, removing the Allen screw. A capillary is inserted into the lower tube and the filtrate is then spotted on thin-layer chromatography. The results, comparing serum which has been filtered, and serum which has not been filtered, is shown in Figure 31. It can be readily seen that a smoother pattern with less trailing, and especially with very little material left at the origin is obtained after ultrafiltration.

Figure 32 shows typical abnormal patterns that are obtained in the Laboratory of Neonatology.

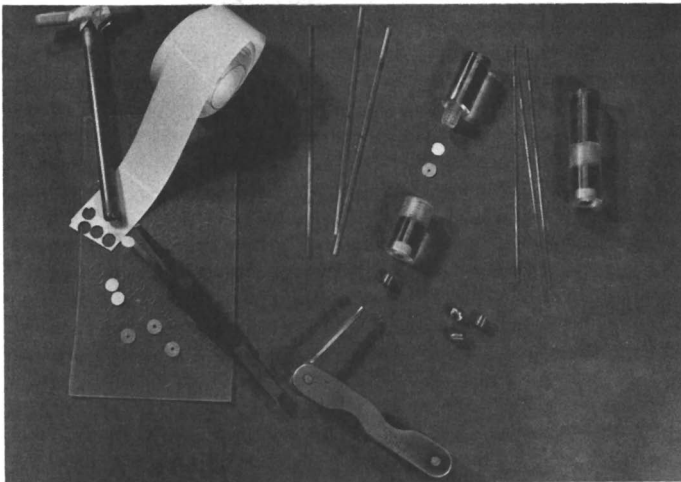
**Sampling With Capillaries.** Earlier it was pointed out that with the aid of a constriction pipette a sample can be measured accurately. A similar effect can be obtained by allowing the serum to enter a capillary open at both ends. The serum will reach the opposite end of the capillary if it is held at an angle and then stoppered, and if the capillary has been carefully calibrated an accurate volume can be measured out. Such capillaries are now available commercially, and have been in use in automated equipment in the authors laboratory for at least 10 years. It is now possible to take the capillary and empty its contents into a container for analysis, or into a stream for the purpose of determining any of the materials which can be determined with the autoanalyzer. Figure 33 shows an instrument which is used for this purpose (58).

With the instrument of Figure 33, the capillary is picked up, moved over, and emptied into a cup. The cup is shown in Figure 34. A diluting solution from the autoanalyzer pump drips into the cup continuously, creating the bubble pattern, since the solution is being removed more rapidly than it is being added. The capillary dispenser then presents the capillary to the flowing stream and blows out the specimen. In this way, the usual procedure for analysis can now proceed.

In our laboratory, we use this procedure routinely for



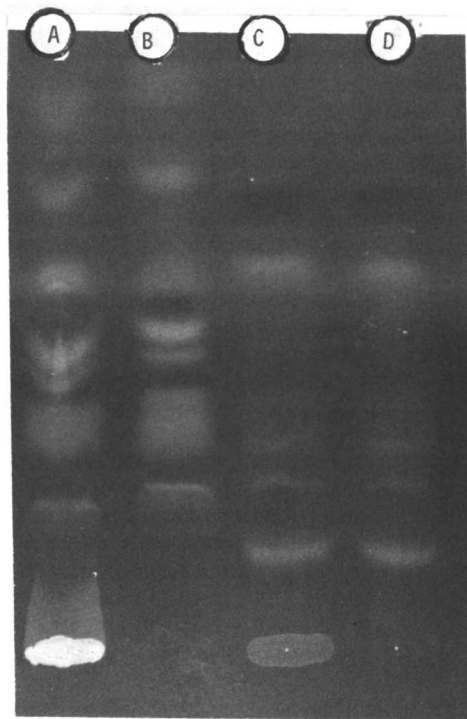
*Figure 28. Modification of the Conway technique using rotated bottles in place of the Conway diffusion dish. 1, bottle with a rubber stopper and glass plug containing an edged end which has been dipped in sulfuric acid; 2, roughed end of the glass plug; 3, rotator with clips holding the bottles in place.*



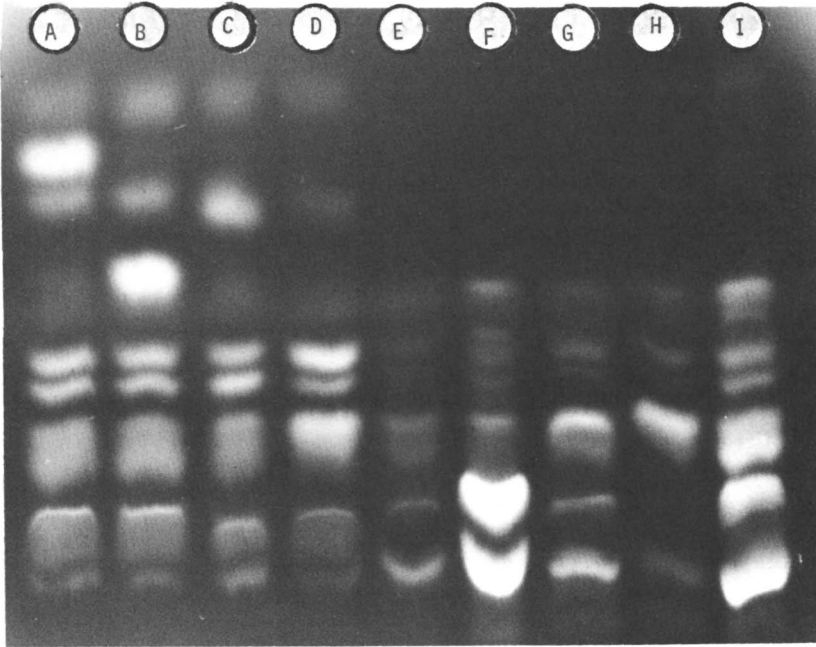
*Figure 29. Assembly for micro-ultrafiltration. The tweezers hold the Millipore filter, cut from the strip. The washers are shown below the tweezers. In the center, the top and bottom portion of the filter and washer are shown aligned with the Allen screw. An Allen wrench is used to tighten the screw in place. When assembled, it looks like the assembly on the right. The capillaries are used for measuring out the specimen and placing them in the upper chamber of the assembly.*



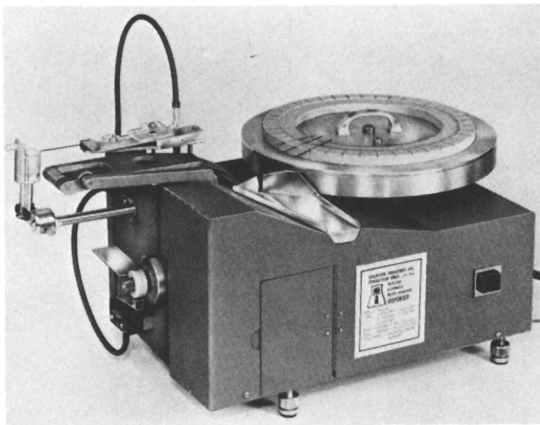
*Figure 30. Placing the assembly in the 12,000 r.p.m. micro centrifuge*



*Figure 31. Demonstrating the advantage of ultrafiltration before TLC. A, serum unfiltered; B, serum filtered; C, urine unfiltered; D, urine filtered.*



*Figure 32. Chromatograms of plasma and urine samples with various abnormalities. A, Phenylalaninemia; B, tyrosinemia; C, elevated plasma methionine seen in homocystinuria; D, glycinemia; E, normal urine; F, argininosuccinic aciduria; G, homocystinuria; H, hyperglycinuria; I, hyperlysinuria.*



*Figure 33. The micro sampler dispenser. The capillaries are shown on the turntable, and one capillary is in position, ready to be lifted to the vertical position to be emptied into the cup.*

phosphorus, uric acid and alkaline phosphatase. The method is particularly applicable to enzyme analysis, because as little as 25 microliters can be added. This can be followed by 5 or 6 ml of wash solution, so that there is no question about transfer of material from one specimen to another. This is particularly important with alkaline phosphatase, where values over 50 can be obtained in juxta position, to values as low as 2. This is a 25 to 1 differential. Figure 35 shows one of our technicians in our laboratory setting up and performing the test with a capillary dispenser.

Recently, we have developed a new version of this system, and this is shown in Figure 36. In this system, instead of the capillary being used as a blow-out pipette, the capillaries are used as wash-out pipettes. The capillaries are dispensed in a block, and the solution flows through the capillaries sequentially as they are presented to 2 press plates which apply pressure to the side of the block and maintain contact at all time. This done with O-rings and has an effect similar to a suction cup sliding along the block. In Figure 36, it shows how 3 capillaries can be simultaneously emptied into three different channels of an autoanalyzer so that three determinations can be done simultaneously.

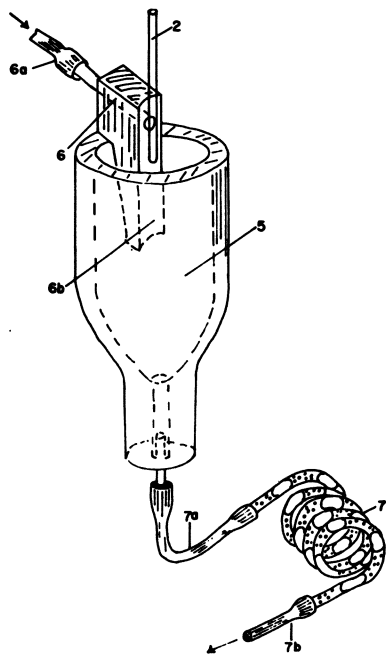
We do not use this procedure for glucose, but Figure 37 shows a series of glucose determinations being performed on 25 microliters of blood, illustrating the fact, that with this system, there may be no wash-out from specimen to specimen. In the procedure, determinations are done at 72 per hour.

Figure 37 illustrates the type of pattern one can obtain with the capillary system. Note complete return to baseline so that the area under the curve may be calculated by a computer for evaluation.

Lipid Screening. The problems of lipid analysis in the newborn is difficult because of the fact that most methods for analysis for lipids require substantial amounts of serum, yet a total lipid determination is very important in various types of disease. This problem can be solved by thin-layer chromatography (59). Figure 38 shows a typical pattern obtained when an extract from 10 microliters of serum is subjected to thin-layer chromatography. If these specimens are scanned, and an internal standard is run, one can obtain a rough approximation of the distribution of the various lipids in the serum. This is shown in Figure 39, in which a normal specimen is run in an adult.

In Figure 40, one can see the effect on the ratio of total to free cholesterol in a patient with alcoholic cirrhosis. Note that the cholesterol esters are markedly depressed in this patients serum and the free cholesterol band represents most of the cholesterol in the serum.

Another example is a case of acute pancreatitis. The low



*Figure 34. Cup for receiving the specimen from the micro sample dispenser. 2, capillary; 5, cup; 6 and 6A, assembly for leading the diluting fluid from the pump to be mixed with the contents of the capillary; 7, 7A, and 7B, assembly for mixing the sample with the diluting fluid before going into the autoanalyzer.*



*Figure 35. Technician adjusting the capillary dispenser for analysis*

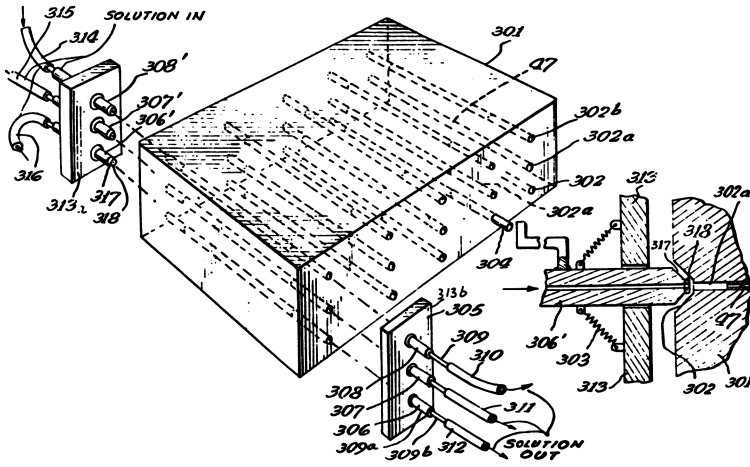


Figure 36. Instrument for emptying three capillaries simultaneously into three channels. 302 represents the various holes in the block; 304, capillary as it is being placed in the block; 306, 307, and 308, connections which are made each with a separate capillary so as to move the specimen from the capillary into the analyzing system. The inset on the right shows how the connector 306 moves into the depression in the block to make contact with the capillary holder. Number 47, capillary in this inset.

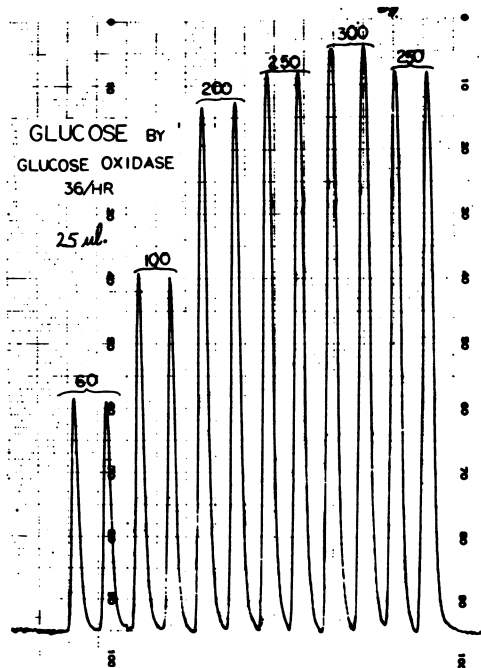
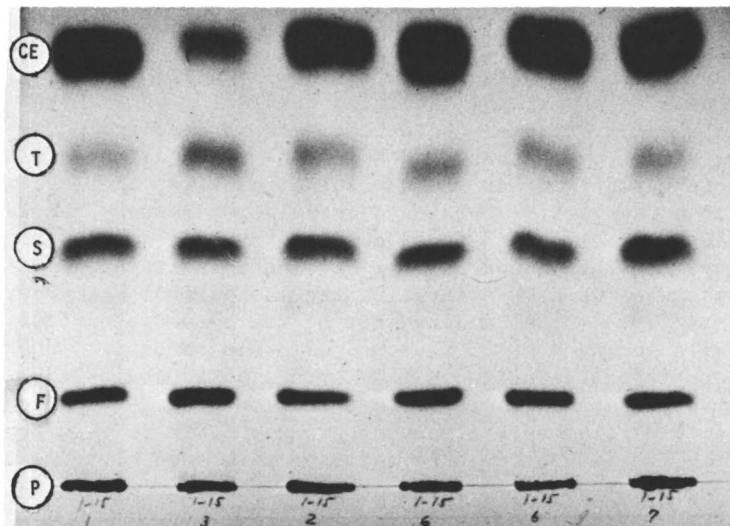
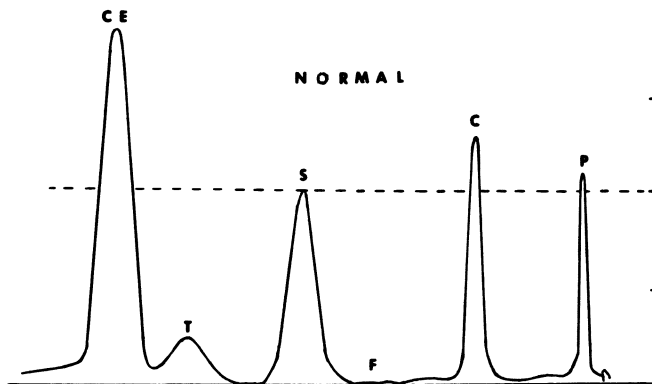


Figure 37. Pattern obtained for glucose by the glucose oxidase procedure using 25 ml in a capillary. Note the return to baseline between specimens.



*Figure 38. Patterns obtained from the extract of 10  $\mu$ l of serum for lipid fraction by thin-layer chromatography. In sequence, starting from the bottom, phospholipids, free cholesterol, cholesterol aniline as an internal standard, triglycerides, and cholesterol esters. The free fatty acids occur between cholesterol and the internal standard and are only barely visible in the print, on the extreme right. They are readily visible, normally, to the eye.*



*Figure 39. A lipid pattern from normal serum which has been scanned for density of the thin-layer chromatograph, showing the various peaks. P, phospholipids; C, cholesterol; F, free fatty acids; S, internal standard, T, triglycerides; CE, cholesterol esters.*



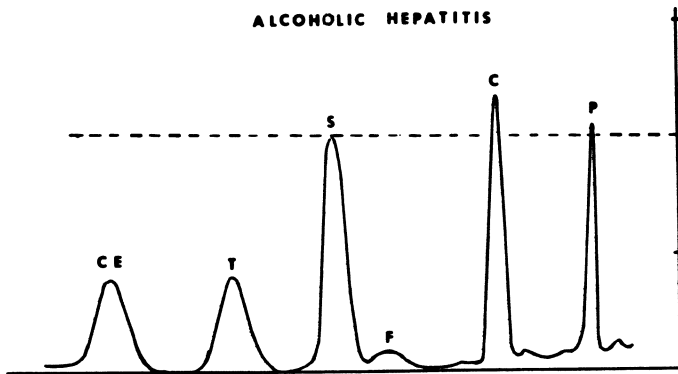
free cholesterol shows that the patient has liver involvement since it cannot be esterified.

The difficulty with this procedure is the method of staining. There is no problem obtaining resolution between the various lipid fractions. Recently, we have been exploring this system, and it seems that if one resorts to dyes in place of phosphotungstic acid, as we had done originally, there is a better opportunity for obtaining correlation between the values obtained chemically, and those obtained by thin-layer chromatography. We have found some improvements in staining characteristics when we have esterified the cholesterol by spraying with butyryl chloride, and iodinating the unsaturated fatty acids with iodine monochloride or iodine monobromide. Under these conditions, the lipids will take a basic stain, such as basic fuchsin, and hold it against elution procedures so as to produce a reasonably clear background. This is true, provided the dye is protected by spraying with some covering agent, such as mineral oil.

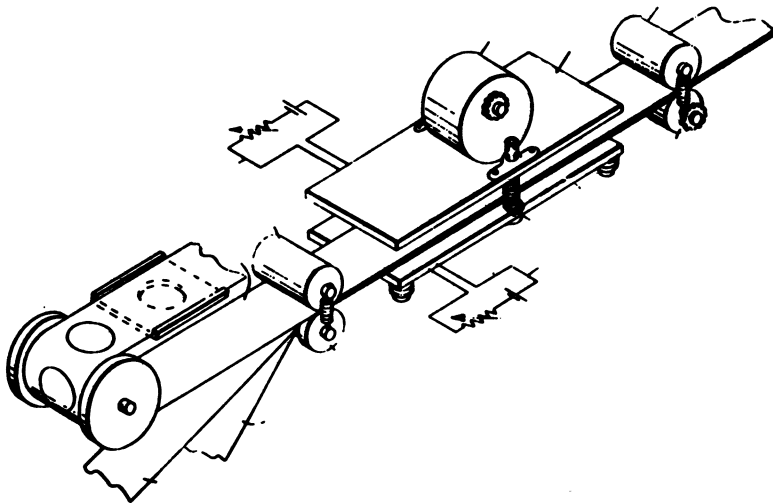
Tape System of Analysis. A tape system which is used widely for analysis in the Pediatric Laboratory is a system whose principle was developed by the author. A reagent is placed on a paper tape. The paper is covered with a membrane, such as cellophane, cellulose nitrate or cellulose acetate, porous to low molecular weight substances. Finally, the serum is placed above the porous membrane, so that diffusion of the components of serum take place and a stain is produced on the paper (60). This principle has been incorporated for example, with glucose oxidase, in the commercially available Dextro-Sticks. In addition, a similar principle is being applied by some for the analysis of components in urine (Ames Co., South Bend, Indiana).

The author has developed several variations of this system, but the general principle of this system is outlined in Figure 43. In this system a specimen is placed on a sample tape, which comprises Mylar carrying small discs of paper. The tape turns over and meets a porous tape, which in this case is cellophane, and the reagent tape which may be a toluidine succinate deposit on the lower tape, as an example. When the filtrate reaches the toluidine succinate pressure and heat is applied. A characteristic color is then formed, which is then ready by reflection on a densitometer (60). The principle of this system is shown in Figure 41.

This system potentially, has value for the Pediatric Laboratory. It needs further development, however. For example, a small drop of blood from a fingertip can be placed on an IBM card containing a small circle of filter paper. After air drying, this could then be brought down to the laboratory and placed on an assembly of cellophane covering a paper, moistened with a suitable reagent. When pressure is applied, the color



*Figure 40. Scan of the lipid pattern from a patient with alcoholic hepatitis. Note the decrease in the percentage of free cholesterol which has been esterified.*



*Figure 41. The principle of the tape system of analysis. The three tapes are the reagent tape on the bottom, cellophane in the center, and sample tape on top. The sample placed on the top tape folds over and is pressed by the press plate and warmed so that the material dialyses through the membrane. The stain is then formed and is read by reflecting densitometry on the reagent tape.*

is produced on the lower moist paper, which can then be removed and read in a densitometer. In this case, there is perfect identification of the specimen, since the IBM card on which the patients blood was placed originally, can have its information transferred to the reagent card.

### Future Developments

**Long Range Trends.** Long range, the trend in the laboratory of Neonatology is to move toward the use of smaller volumes of blood for greater amounts of information. A typical example would be the development of the specific ion electrodes, which have the potential for the estimation of the concentration of the components Na, K, Cl, Mg, Ca,  $pO_2$ ,  $pCO_2$ , and pH on minute quantities of whole blood directly on a single sample of the order of 10

Screening for the numerous disease of genetic origin on newborns will be expanded so that eventually a battery of tests will be utilized for the determination of the erythrocyte abnormalities and abnormalities of intermediate metabolism.

It is predicted that a compulsory program will be directed by governmental agencies in this direction.

The problems of the Laboratory of Neonatology are unique and distinct in many details from those of the general clinical chemistry laboratory. This requires a separate operation coordinated with the operation of the Pediatric Department of the hospital. It requires a highly sophisticated Chemist at the doctorate level as supervisor, trained in this area to insure that the results are meaningful and to supervise and accelerate the development of the techniques in this area. Developments in this direction are already taking place rapidly. The Committee on Pediatric Chemistry of the American Association of Clinical Chemists is now active in developing the list of normal values for the infant.

Probably, the most rapidly developing science in medicine today is the Science of Neonatology. It is important that the development of the laboratory keep pace.

### Literature Cited

1. Natelson, S.; Crawford, W. L.; and Munsey, F. A.; Correlation of Clinical and Chemical Observations in the Immature Infant. Ill. Dept. of Public Health Pub. Div. of Preventive Medicine (1952).
2. Heird, W. C.; Driscoll, J. M. Jr.; Schullinger, J. N.; Grebin, B; and Winters, R. W.; Intravenous alimentation in pediatric patients, J. Ped. (1972), 80, 351 - 372.
3. Dudrick, S. J.; Wilmore, D. W.; Vars, H. M.; and

- Rhoads, J.E.; Can intravenous feeding as the sole means of nutrition support growth in the child and restore weight loss in an adult. An affirmative answer. *Ann. Surg.* (1969), 169, 974 - 984.
4. Bellingham, A. J.; Detter, J. C.; and Lenfant, C.; Regulator mechanisms of hemoglobin oxygen affinity in acidosis and alkalosis. *J. Clin. Invest* (1971), 50, 700 - 706.
  5. Natelson, S.; *Techniques in Clinical Chemistry*, 3rd Edition. Chas. C. Thomas, Springfield, Ill., 1971.
  6. Huijing, F.; A rapid enzymic method for glycogen estimation in very small tissue samples. *Clin. Chim. Acta* (1970), 30, 567 - 572.
  7. Brady, R.O.; *Cerebral lipidoses*. *Ann. Rev. Med.* (1970), 21, 317 - 334.
  8. Constantopoulos, G.; and Dekaban, A. S.; Chemical definition of the mucopolysaccharidoses. *Clin. Chim. Acta* (1975), 59, 321 - 336.
  9. Beutler, E.; *Red Cell Metabolism: A Manual of Biochemical Methods*. Grune & Stratton, N.Y. (1971).
  10. Chu, T. C.; and Chu, E. J. H.; Porphyrin patterns in different types of porphyrins. *Clin. Chem* (1967), 13, 371 - 387.
  11. Vincke, H.; Hereditary hypophosphatasia. *Acta Paediat. Belg.* (1970), 24, 131 - 138.
  12. Wallace, J. E.; Microdetermination of diphenyl hydantoin in biological specimens by ultraviolet spectrometry. *Anal. Chem.* (1968), 40, 978 - 980.
  13. Natelson, S.; Routine use of ultramicro methods in the clinical laboratory. *Am. J. Clin. Path.* (1951), 21, 1153 - 1172.
  14. Bessey, O. A.; Lowry, O. H.; and Brock, M. J.; A method for the rapid determination of alkaline phosphatase with five cubic millimeters of serum. *J. Biol. Chem.* (1946), 164, 321 - 329.
  15. Natelson, S.; and Pochopiën, D. J.; Novel approach to the design of a Pediatric microanalytical laboratory. *Microchem.* (1973), 18, 475 - 485.
  16. Sanz, M. C.; Brechbühler, T.; and Green, I. J.; The ultramicro-determination of total and protein-bound iodine. *Clin. Chem.* (1956), 1, 570 - 576.
  17. Kaiser, E.; Kunstadter, R. H.; and Mendelsohn, R. J.; Electrolyte concentrations in sweat and saliva; comparison in patients with cysticfibrosis of pancreas and other conditions. *Amer. J. Dis. Child.* (1956), 92, 369 - 373.
  18. Natelson, S.; and Clark, M. K.; Estimation of bromide on fingertip blood in bromide intoxication. *Proc. Soc. Expt. Biol. Med.* (1955), 90, 723 - 725.
  19. Fischl, J.; Serum iron determination using direct color

- extraction. *Clin. Chim. Acta* (1960), 5, 164 - 170.
20. Moody, G. J.; and Thomas, J. D. R.; Selective Ion Sensitive Electrodes. Mellow Pub. Co., 276 Hempstead Rd., Watford Herts, England.
  21. Bittner, D. L.; U. S. Patent #3,282,649 (1966).
  22. Saifer, A.; and Gerstenfeld, S.; The photometric microdetermination of blood glucose with glucose oxidase. *J. Lab. Clin. Med.* (1958), 51, 448 - 460.
  23. Kapuscinski, V.; and Zak, B.; Use of perchloric acid filtrate and stabilized anthrone for determination of serum glucose. *Am. J. Clin. Path.* (1953), 23, 784 - 788.
  24. Tomisek, A. J.; and Natelson, S.; Fluorometric assay of ultramicro quantities of glucose with Somogyi filtrate and hexokinase. *Micro-chem. J.* (1974), 19, 54 - 62.
  25. Takahara, K.; Sasaki, M.; Tomisek, A. J.; and Natelson, S.; Sensitive o-Toluidine reagent for glucose assay at moderately elevated temperatures. *Microchem. J.* (1974), 19, 319 - 329.
  26. Natelson, S.; A rapid method for the estimation of urea in biological fluids as the urea diacetyl complex. *Am. J. Clin. Path* (1951), 21, 275 - 281.
  27. Chaney, A. L.; and Marbach, E. P.; Modified reagents for determination of urea and ammonia. *Clin. Chem.* (1962), 8, 130 - 132.
  28. Accord, W.; and Feldman, F. J.; Design consideration for a fully automated rate electrochemical glucose and urea nitrogen analyzer. *Clin. Chem.* (1974), 20, 903.
  29. Natelson, S.; The routine use of the Perkin-Elmer flame photometer in the clinical laboratory. *Am. J. Clin. Path.* (1950), 20, 463 - 472.
  30. Frant, M. S.; and Ross, J. W., Jr.; Potassium ion specific electrode with high selectivity for potassium over sodium. *Science* (1970), 167, 987 - 988.
  31. Cotlove, E.; and Nishi, H. H.; Automatic titration with direct read-out of chloride concentration. *Clin. Chem.* (1961), 7, 285 - 291.
  32. Gindler, E. M.; Mercurimetric determination of chloride in biologic fluids using 2-(8-Hydroxyquinoly-5-azo)-benzoic acid (QBA). *Clin. Chem.* (1968), 14, 1172 - 1175.
  33. Seligson, S.; McCormick, G. J.; and Sleeman, K.; Electrometric method for the determination of chloride in serum and other biological fluids. *Clin. Chem.* (1958), 4, 159 - 169.
  34. Driscoll, J. L.; and Martin, H. F.; Detection of brominism by an automated chloride method. *Clin. Chem.* (1966), 12, 314 - 318.
  35. Teloh, H. A.; Estimation of serum calcium by flame

- photometry. *Archives of Pathol.* (1958), 66, 474 - 481.
36. Pybus, J.; Feldman, F. J.; and Bowers, G. N., Jr.; Measurement of total calcium in serum by atomic absorption spectrophotometry with use of a strontium internal reference. *Clin. Chem.* (1970), 16, 998 - 1007.
37. Arnold, D. E.; Stansell, M. J.; and Malvin, H. H.; Measurement of serum ionic calcium using a specific ion electrode. *Am. J. Clin. Path.* (1968), 49, 627 - 634.
- 37a. Natelson, S.; Richelson, M. R.; Sheid, B.; and Bender, S. L.; X-ray spectroscopy in the clinical laboratory. *Clin. Chem.* (1959), 5, 519 - 531.
38. Dumas, B. T.; Perry, B. W.; Sasse, E. A.; and Straumfjord, J. V., Jr.; Standardization in bilirubin solutions. *Clin. Chem.* (1973), 19, 984 - 993.
39. Henry, R. J.; Golub, O. J.; Berkman, S.; and Segalove, M.; Critique on the icterus index determination. *Am. J. Clin. Path.* (1953), 23, 841 - 853.
40. Bilirubinometer, Model 10200 - American Optical Co., Buffalo, N. Y., 14215. Also the Sherwood Medical Industries, Bridgton, Missouri.
41. Dubin, A.; Application of multi-wavelength spectroscopy to analysis of amniotic fluid bilirubin, in *Amniotic Fluid*. Natelson, S.; Scommegna, A.; and Epstein, M. D., ed.; John Wiley, N. Y. (1974) pp. 191 - 197.
42. Alvey, J. P.; Obstetrical management of Rh incompatibility based on liquor amnii studies. *Amer. J. Obstet. Gynecol.* (1964), 90, 769 - 775.
43. Liley, A. W.; Liquor amnii analysis in the management of the pregnancy complicated by rhesus sensitization. *Am. J. Obstet. Gynecol.* (1961), 82, 1359 - 1370.
44. Dambacher, M.; Gubler, A.; and Haas, H. G.; A new time-saving method for determining nitrogen in biological material. *Clin. Chem.* (1969), 14, 615 - 622.
45. Daughaday, W. H.; Lowry, O. H.; Rosenbrough, N. J.; and Fields, W. S.; Determination of cerebrospinal fluid protein with the Folin phenol reagent. *J. Lab. Clin. Med.* (1953), 39, 663 - 665.
46. Kingsley, G. R.; Direct biuret method for determination of serum proteins as applied to photoelectric and visual colorimetry. *J. Lab. Clin. Med.* (1942), 27, 840 - 845.
47. Blumenfeld, T. A.; et al., eds.; Normal values for Pediatric Clinical Chemistry. *Am. Assoc. of Clin. Chem. Publication* (1974).
48. Natelson, S.; Penniell, R.; Crawford, W. L.; and Munsey, F. A.; Noncasein protein to casein ration of feeding formulas. Effect on blood component levels in normal infants. *Am. J. Dis. Child.* (1955), 89, 656 - 668.
49. Bates, R. G.; Determination of pH, Theory and Practice,

- 2nd Ed. Wiley, New York (1973).
50. Astrup, P.; and Rorth, M., eds.; Oxygen Affinity of Hemoglobin and Red Cell Acid Base Status. Academic Press, New York (1972).
  51. Natelson, S.; and Natelson, E. A.; Applied Clinical Chemistry, pp. 42 - 49. Plenum Press, New York (1974).
  52. Natelson, S.; Routine use of ultra-micro methods in the clinical laboratory. *Am. J. Clin. Path.* (1951), 21, 1153 - 1172.
  53. Menning, C. M.; and Natelson, S.; Improved methods of analysis for oxygen, carbon monoxide and iron on fingertip blood. *Clin. Chem.* (1955), 1, 165 - 179.
  54. Natelson, S.; and Barbour, J. H.; Equation and nomogram for approximation of alkali requirements in acidosis. *Am. J. Clin. Path.* (1952), 22, 426 - 439.
  55. Conway, E. J.; and O'Malley, E.; Microdiffusion methods. Ammonia and urea using buffered absorbance (revised methods for ranges greater than 10  $\mu$ /g N). *Biochem. J.* (1942), 36, 655 - 661.
  56. Seligson, D.; and Seligson, H.; A microdiffusion method for the determination of nitrogen liberated as ammonia. *J. Lab. Clin. Med.* (1961), 38, 324 - 330.
  57. Century, B.; Vorkink, W. P.; and Natelson, S.; Thin layer chromatographic screening of aminoacids in plasma and urine of newborns. *Clin. Chem.* (1974), 20, 1446 - 1450.
  58. Natelson, S.; Automatic analysis of microsamples contained in capillaries with a microsample dispenser. *Microchem. J.* (1968), 13, 433 - 458.
  59. Chedid, A.; Haux, P.; and Natelson, S.; Use of thin layer chromatography on silica gel for serum lipid fractionation and measurement in the routine clinical laboratory. *Clin. Chem.* (1972), 18, 384 - 390.
  60. Natelson, S.; Analysis of components in biological fluids in a gravity-free environment emphasizing procedures suitable for use in an orbiting laboratory. Progress report to National Aeronautics Space Agency, Contract #NAS 9-7934, NASA Headquarters, Washington, D.C., 20546 1966, 1967, 1968, 1969.

## Blood Gas Abnormalities

HARRY F. WEISBERG

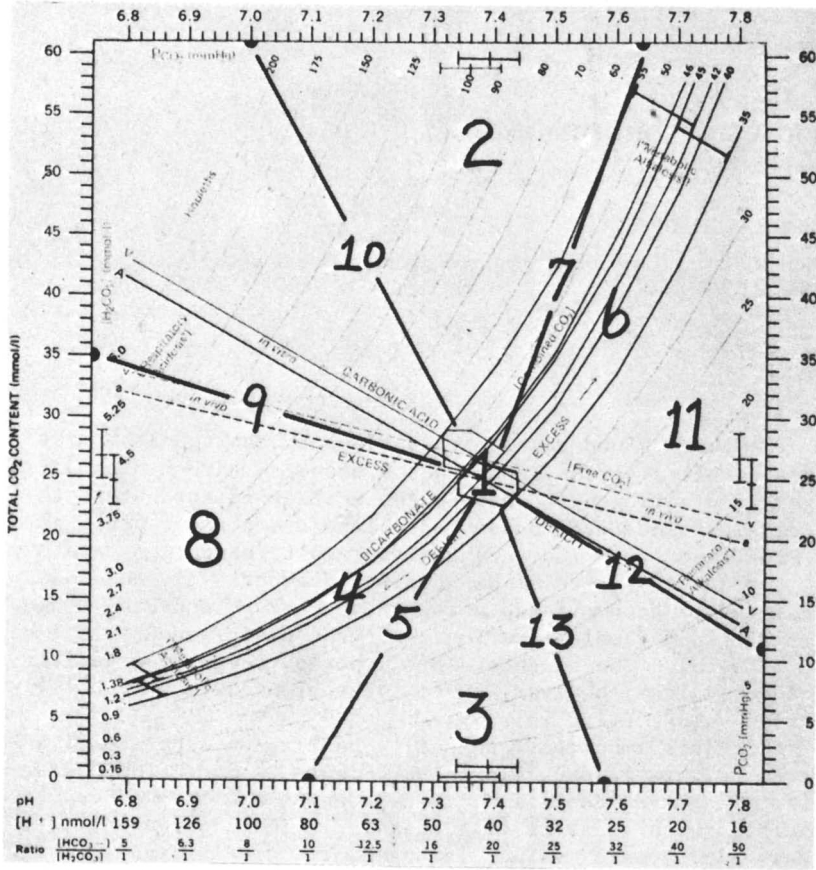
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The term "blood gases" has different meanings; the most accurate refers to the gases in the blood -- carbon dioxide, oxygen, and nitrogen -- whereas the most expedient refers to the data obtained from present-day laboratory instruments -- pH,  $P_{CO_2}$ , and  $P_{O_2}$ . Some confusion results since the data overlap two major areas of diagnosis. Diagnosis of "acid-base imbalance" is accomplished using values for pH and  $P_{CO_2}$  (though arterial blood samples are touted as the best, venous blood can also be used). The diagnosis of "hypoxia" requires arterial blood  $P_{O_2}$  values; however, three types of hypoxia have normal arterial values (vida infra).

Acid-base imbalance can easily be diagnosed by use of a diagram (Figure 1) or a flowchart (Figure 2) in which 13 diagnostic areas are identified. Knowledge of the pH and  $P_{CO_2}$  values can distinguish areas or diagnoses 1-7. The  $CO_2$  content or actual bicarbonate value is used to distinguish a "mixed" metabolic and respiratory abnormality from a "pure respiratory" abnormality -- area 8 from 9 or 10 and area 11 from 12 or 13. Base excess/deficit (previous base excess  $\pm$ ) or the  $\Delta CT$  (delta  $CO_2$  content) is necessary only to distinguish between an "acute" or "chronic" respiratory condition -- 9 vs 10 and 12 vs 13 (Figures 1 and 2). Figure 2 has many sets of data in parentheses; these are not necessary for the differential diagnosis of an acid-base imbalance. In similar fashion, the  $P_{O_2}$  values are not needed in the diagnosis of an abnormal condition of acid-base. Table 1 summarizes the data of Figures 1 and 2 and, in addition, gives the physio-pathological basis for the alterations of the (arterial)  $P_{O_2}$ .

A classification of the causes of hypoxia is presented in Table II. Only anoxic anoxia (due to decreased ambient oxygen or to respiratory disease) has arterial  $P_{O_2}$  values decreased (with accompanying decreased venous  $P_{O_2}$  values). However, hemic, ischemic, and histoxic hypoxia may be present (termed non-ventilatory hypoxia) with normal arterial  $P_{O_2}$  values. Thus the best evaluation of hypoxia is an analysis of both arterial and venous





Interpretation of Electrolyte, Acid-Base, and Oxygen Imbalance

Figure 1. Acid-base balance evolution diagram with 13 diagnostic areas (1)



TABLE I

Differential Diagnosis of Acid-Base Equilibrium.  
PO<sub>2</sub> values are arterial. From Weisberg (1).

| #          | pH       | Pco <sub>2</sub><br>or<br>[H <sub>2</sub> CO <sub>3</sub> ] | [CO <sub>2</sub> CT]<br>or<br>[HCO <sub>3</sub> <sup>-</sup> ] | ΔCT<br>or<br>BE/D | Po <sub>2</sub> ** | COMMENTS  |
|------------|----------|---|--|-------------------|--------------------|---|
| 1          | EupHemia |   |  |                   |                    |   |
| 1a         | N        | N   | N  | N                 | N                  | Normal blood "gases"<br>(Hemic hypoxia)<br>(Ischemic hypoxia)<br>(Histotoxic hypoxia)   |
| 1b         | N        | N   | N  | N                 | ↓                  | Q̇/Q̇ inequality<br>Shunts  |
| 2          | N        | ↑   | ↑  | ↑                 | ↓                  | Respiratory Acidosis, Compensated; see #8,<br>#9, and #10<br>Hypoventilation <sup>†</sup><br>(Shunts) <sup>‡</sup><br>(Q̇/Q̇ inequality) <sup>¶</sup><br>(Metabolic Alkalosis, Compensated) <sup>Δ</sup> ; see<br>#6, #7, and #11 |
| 3          | N        | ↓   | ↓  | ↓                 | ↑N                 | Respiratory Alkalosis, Compensated; see<br>#11, #12, and #13<br>Hyperventilation<br>(Metabolic Acidosis, Compensated); see<br>#4, #5, and #8  |
| HypopHemia |          |   |  |                   |                    |   |
| 4a         | ↓        | N   | ↓  | ↓                 | N                  | Metabolic Acidosis, Uncompensated<br>(Hemic hypoxia)<br>(Ischemic hypoxia)<br>(Histotoxic hypoxia)  |
| 4b         | ↓        | N   | ↓  | ↓                 | ↓                  | Q̇/Q̇ inequality<br>Shunts  |
| 5a         | ↓        | ↓   | ↓  | ↓                 | ↑N                 | Metabolic Acidosis, Partial compensation<br>(by Hyperventilation)   |
| 5b         | ↓        | ↓   | ↓  | ↓                 | N↓                 | Metabolic Acidosis & Shock; see #13b  |
| 8          | ↓        | ↑   | N  | ↓                 | N↓                 | Mixed Respiratory Acidosis & Metabolic<br>Acidosis  |
| 9          | ↓        | ↑   | ↑  | ↑N                | ↓                  | Respiratory Acidosis, Acute   |
| 10         | ↓        | ↑   | ↑  | ↑                 | ↓                  | Respiratory Acidosis, Chronic<br>(Partial compensation)   |

TABLE I (CONTINUED)

Differential Diagnosis of Acid-Base Equilibrium.  
PO<sub>2</sub> values are arterial. From Weisberg (1).

| Hyperpnea |   |   |   |   |    |  |
|-----------|---|---|---|---|----|--|
| 6a        | ↑ | N | ↑ | ↑ | N  | Metabolic Alkalosis, Uncompensated<br>(Hemic hypoxia)<br>(Ischemic hypoxia)<br>(Histotoxic hypoxia)  |
| 6b        | ↑ | N | ↑ | ↑ | ↓  | $\dot{V}/\dot{Q}$ inequality<br>Shunts   |
| 7         | ↑ | ↑ | ↑ | ↑ | ↓  | Metabolic Alkalosis, Partial compensation<br>(by Hypoventilation)  |
| 11        | ↑ | ↓ | N | ↑ | N† | Mixed Respiratory Alkalosis & Metabolic Alkalosis  |
| 12        | ↑ | ↓ | ↓ | N | †N | Respiratory Alkalosis, Acute°  |
| 13a       | ↑ | ↓ | ↓ | ↓ | ↑  | Respiratory Alkalosis, Chronic (Partial compensation)<br>Impaired diffusion  |
| 13b       | ↑ | ↓ | ↓ | ↓ | ↓  | Decreased ambient O <sub>2</sub><br>(Impaired diffusion)<br>(Shunt)<br>( $\dot{V}/\dot{Q}$ inequality)<br>"Inappropriate pulmonary ventilation in acutely ill patient"<br>eg, Myocardial infarct<br>Pulmonary embolus<br>Shock<br>Later pH‡; see #5b |

\* Arrows show direction and not extent of change; N = normal.  
 \*\* A "low" Po<sub>2</sub> may become "normal" or "increased" due to:  
     Hyperventilation  
     O<sub>2</sub> Therapy  
         Increased Concentration (100% should produce PaO<sub>2</sub> >600)  
         Increased pressure [hyperbaric chamber, respiratory ventilators (eg, PEEP or CPPB)]  
     Po<sub>2</sub> polarographic electrode "error" due to halogenated hydrocarbons (eg, Halothane)  
 † Po<sub>2</sub> usually <50  
 ‡ If present in addition to hypoventilation, Po<sub>2</sub> will be lowered further.  
 Δ Po<sub>2</sub> usually >50  
 ° Also transient hyperventilation in response to arterial puncture.

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TABLE II

Classification of Hypoxia. Modified from Weisberg (1).I. ANOXIC:A. AMBIENT ( $F_{I}O_2 \downarrow$ )

1. Altitude
2. O<sub>2</sub> Dilution ("Fire damp", "Black Damp")
3. Anesthetic or O<sub>2</sub> Therapy mishaps

B. RESPIRATORY:

1. ALVEOLAR HYPOVENTILATION  
("Ventilatory insufficiency")
2. DIFFUSION DEFECT  
("Alveolar-capillary block")
3. VENTILATION/CIRCULATION ( $\dot{V}/\dot{Q}$ ) INEQUALITY  
("Physiologic" shunt)
4. VENOUS-ARTERIAL SHUNT  
("Anatomic" shunt)

II. HEMIC:A. ANEMIC

1. Hemorrhage
2. Depressed marrow, etc.
  - a. X-ray
  - b. Phenylhydrazine

B. TOXIC

1. "Abnormal" Hemoglobin
  - a. CO-
  - b. Met-
  - c. Sulf-

TABLE II (CONTINUED)

Classification of Hypoxia. Modified from Weisberg (1).

- III. ISCHEMIC ("Stagnant", "Circulatory", "Hypokinetic"):
  - A. ISCHEMIA
    - 1. Hypovolemia
    - 2. Congenital defects
    - 3. Peripheral stasis
  - B. MINUTE-FLOW DISCREPANCY
    - 1. Decreased cardiac output
    - 2. Increased tissue demand for O<sub>2</sub>
- IV. HISTOTOXIC:
  - A. ENZYMATIC
    - 1. Cyanide
    - 2. Narcotics
    - 3. Alcohol
  - B. OXYGEN
  - C. "WATER & ELECTROLYTES"
    - e.g., Na↓

blood specimens (Table III).

A footnote in Table I (and Table VI) states that  $P_{O_2}$  values may be erroneously elevated due to the effect of halogenated hydrocarbons on the polarographic electrode. Severinghaus et al (2) reported that Halothane, a brominehydrocarbon anesthetic, can be polarographically reduced by the Clark-type oxygen electrode at polarizing voltages greater than 0.4 volt and also increases the sensitivity of the electrode to oxygen, resulting in as much as 10-fold spuriously elevated  $P_{O_2}$  values. This was confirmed for a different type of oxygen electrode which uses a polarizing voltage of 0.7 volt (3). However, the consensus of conversations with various anesthesiologists does not agree with these reports (4). Halothane does not affect the affinity of hemoglobin for oxygen (5).

A more common cause for "altered"  $P_{O_2}$  values is the age of the individual. Normal  $P_{O_2}$  values decrease with age; in addition, surgical procedures cause a temporary (post-operative period) decrease of the individual's  $P_{O_2}$  values. Various regression formulas have been presented describing the decrease of  $P_{O_2}$  with increasing age under normal conditions (6-12), after surgery (10, 13-17), and with oxygen therapy (10, 16).

I have used the following "rule-of-thumb" formulas to predict the expected  $P_{O_2}$  values under room air conditions in normal (equation 1) and post-operative (equation 2) patients; the "variation" of the prediction formulas is  $\pm 5$ .

$$P_{aO_2} = 105 - \frac{\text{age}}{3} \quad (1)$$

$$\bar{p}\text{-op } P_{aO_2} = 100 - \frac{\text{age}}{2} \quad (2)$$

Equations 3 and 4 are used when 100% oxygen is given to normal and post-operative patients, respectively; in this case the "variation" is  $\pm 25$ .

$$P_{aO_2} = 575 - \text{age} \quad (3)$$

$$\bar{p}\text{-op } P_{aO_2} = 500 - \frac{5}{4} \text{ age} \quad (4)$$

Dry air at sea level and 20° has partial pressures of 159, 0.3, and 601 mmHg, respectively, for the three gases, oxygen, carbon dioxide, and nitrogen. In ambient air (contains water vapor of 17 mmHg at 20°), the partial pressures of the gases

TABLE III  
Blood Gas Values Under Various Conditions

| CONDITION                       | PaO <sub>2</sub> | PaCO <sub>2</sub>          | PaO <sub>2</sub> > 600<br>on 100% O <sub>2</sub> | P <sub>v</sub> O <sub>2</sub> | P <sub>v</sub> CO <sub>2</sub> |
|---------------------------------|------------------|----------------------------|--|-------------------------------|--------------------------------|
| NORMAL VALUES                   | 95 (75-100)      | 40 (35-45)M<br>37 (32-42)F | 40 (30-50)                                       | 46 (42-55)M<br>43 (39-52)F    |                                |
| AMBIENT O <sub>2</sub> ↓        | ↓                | ↓                          | YES  | AS "ARTERIAL"                 |                                |
| <u>HYPOXIA:</u>                 |                  |                            |  |                               |                                |
| HYPOVENTILATION                 | ↓                | ↑(N)                       | YES  | AS "ARTERIAL"                 |                                |
| DIFFUSION DEFECT                | ↓                | ↓(N)                       | YES  | AS "ARTERIAL"                 |                                |
| Ṡ/Ḡ INEQUALITY                  | ↓                | ↑N(↓)                      | YES  | AS "ARTERIAL"                 |                                |
| SHUNTS                          | ↓                | N(↑↓)                      | NO   | AS "ARTERIAL"                 |                                |
| <u>NON-VENTILATORY HYPOXIA:</u> |                  |                            |  |                               |                                |
| HEMIC                           | N                | N                          |  | ↓                             | N                              |
| ISCHEMIC                        | N                | N                          |  | ↓                             | ↑                              |
| HISTOTOXIC                      | N                | ↓(N)                       |  | ↑                             | ↓(N)                           |
| HYPERVENTILATION                | ↑(N)             | ↓                          |  | AS "ARTERIAL"                 |                                |
| O <sub>2</sub> THERAPY          | ↑N               | (↑N↓)                      |  | AS "ARTERIAL"                 |                                |



are 156, 0.3, and 587, respectively; upon humidification at 37° (47 mmHg water vapor), the partial pressures are 150, 0.3, and 563. Alveolar air (end-pulmonary capillaries) has the same water vapor of 47 mmHg but the partial pressures of the gases are 104, 40, and 569 mmHg whereas the values for arterial blood (same 47 mmHg for water vapor) are 100, 40, and 573 mmHg. Note that because of differences in gas characteristics the carbon dioxide partial pressure is the "same" for arterial blood and alveolar air whereas alveolar  $P_{O_2}$  is higher but alveolar  $P_{N_2}$  is lower than in arterial blood. Expired air (at 37° with 47 mmHg water vapor) is a mixture of alveolar and humidified ambient air so that the partial pressures are 120, 27, and 566 mmHg for oxygen, carbon dioxide, and nitrogen. Venous blood values are 47, 40, 46, and 573 mmHg for the partial pressures of water, oxygen, carbon dioxide, and nitrogen.

In order to evaluate the ventilatory status of a patient the alveolar oxygen tension ( $P_{A_{O_2}}$ ) must be known. It can be determined from the "alveolar gas equation" (equation 5),

$$P_{A_{O_2}} = F_{I_{O_2}} (P_B - 47) - P_{A_{CO_2}} \left( F_{I_{O_2}} + \frac{1 - F_{I_{O_2}}}{R} \right) \quad (5)$$

in which  $P_A$  is the partial pressure of a gas in the alveoli,  $F_I$  is the fraction of the gas in the inspired air,  $P_B$  is the barometric pressure, 47 is the water vapor pressure at 37° at sea level, and  $R$  is the respiratory quotient (normal of 0.8, which may drop to below 0.5 in asphyxia). Substituting room air conditions at sea level results in

$$\begin{aligned} P_{A_{O_2}} &= 0.209 (760 - 47) - 40 \left( 0.209 + \frac{1 - 0.209}{0.8} \right) \\ &= 149 - 48 \\ &= 101 \end{aligned}$$

For most clinical purposes, using room air and considering arterial  $P_{CO_2}$  ( $P_{a_{CO_2}}$ ) the "same" as alveolar  $P_{CO_2}$  ( $P_{A_{CO_2}}$ ), equation 5 can be "simplified" to equation 6.

$$\begin{aligned} P_{A_{O_2}} &= 150 - \frac{5}{4} P_{a_{CO_2}} \quad (6) \\ &= 150 - 50 \\ &= 100 \end{aligned}$$

It may not be well known that the weather bureau reports of barometric pressure are corrected to sea level even though describing conditions in the mountains, etc. Table IV gives the barometric pressure at various altitudes; these should be used when applicable in equations 5 and 6. Representative cities and the variations in their respective altitudes are given in Table V.

The difference between alveolar and arterial  $P_{O_2}$  values is called the oxygen A-a gradient (also  $P_{(A-a)O_2}$  or  $D_{(A-a)O_2}$  or  $AaDo_2$ ) and is normally about 10 mmHg. It is used to evaluate ventilation/perfusion ( $\dot{V}/\dot{Q}$ ) inequalities. With increasing age, the gradient rises so that "normal" values for 70-80 year old individuals are 20-25 mmHg. Upon breathing 100% oxygen for 20 minutes the gradient is increased ("normal") up to 100 mmHg. Breathing room air, a simplification is given in equation 7; however, for patients on oxygen therapy, the  $P_{aO_2}$  should be subtracted from equation 5.

$$P_{(A-a)O_2} = 150 - \frac{4}{5} P_aCO_2 - P_aO_2 \quad (7)$$

In early pulmonary disease, a patient may be hyperventilating while breathing room air so that the  $P_aCO_2$  is decreased, yet the  $P_aO_2$  may be relatively normal; with such pulmonary pathology the  $P_{(A-a)O_2}$  will be increased. With frank pulmonary disease, oxygen therapy may mask the expected hypoxia so that the  $P_aO_2$  is normal or elevated; again the  $P_{(A-a)O_2}$  will be increased. On the other hand, in a patient with an elevated  $P_aCO_2$  (hypoventilation), if the  $P_{(A-a)O_2}$  is not elevated then the hypoventilation is probably due to extra-pulmonary factors such as narcosis, muscle weakness, paralysis, etc. For room air conditions, an increase of the  $\dot{V}/\dot{Q}$  ratio will show low  $P_{CO_2}$  and elevated  $P_{O_2}$  values with the "ultimate" being a point equal to the inspired room air,  $P_aO_2$  150 and  $P_aCO_2$  0 mmHg. A decrease of the  $\dot{V}/\dot{Q}$  ratio will show a fall of the  $P_{O_2}$  but a rather constant  $P_{CO_2}$ ; again the "ultimate" would be a point equal to mixed venous blood,  $P_aO_2$  40 and  $P_aCO_2$  46 mmHg. The oxygen gradient,  $AaDo_2$ , with the patient breathing 100% oxygen for 20 minutes, is used as a criterion for weaning a patient from a mechanical ventilator (19). An abnormally high  $AaDo_2$  gradient (above 350 mmHg) is an indication that the patient will need continued ventilatory support with high inspired fractions of oxygen.

The  $P_{(A-a)O_2}$  is also used in calculating the amount of shunt present (equation 8)

$$\% \text{ shunt} = \frac{0.03 \times P_{(A-a)O_2}}{0.03 \times P_{(A-a)O_2} + av}$$

**TABLE IV**  
**Conversions for Barometric Pressure and Altitude in Terms of mmHg per each 100 ft.**  
**Modified from Consolazio et al. (18).**

| ALTITUDE | 0   | 100 | 200 | 300 | 400 | 500 | 600 | 700 | 800 | 900 |
|----------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| 0        | 760 | 757 | 754 | 752 | 749 | 746 | 744 | 741 | 738 | 736 |
| 1000     | 733 | 730 | 728 | 725 | 722 | 720 | 717 | 714 | 712 | 709 |
| 2000     | 706 | 704 | 701 | 699 | 696 | 694 | 691 | 688 | 686 | 684 |
| 3000     | 681 | 678 | 676 | 674 | 671 | 668 | 666 | 664 | 661 | 659 |
| 4000     | 656 | 654 | 651 | 649 | 646 | 644 | 642 | 639 | 637 | 634 |
| 5000     | 632 | 630 | 628 | 626 | 623 | 621 | 618 | 616 | 613 | 611 |
| 6000     | 609 | 607 | 604 | 602 | 600 | 598 | 596 | 594 | 591 | 589 |
| 7000     | 586 | 584 | 582 | 580 | 578 | 576 | 573 | 571 | 569 | 567 |
| 8000     | 564 | 562 | 560 | 558 | 556 | 554 | 552 | 550 | 547 | 545 |
| 9000     | 543 | 541 | 539 | 537 | 535 | 533 | 530 | 528 | 526 | 524 |
| 10000    | 522 | 521 | 519 | 517 | 514 | 512 | 510 | 508 | 506 | 504 |
| 11000    | 502 | 501 | 499 | 497 | 495 | 493 | 491 | 489 | 487 | 485 |
| 12000    | 483 | 481 | 479 | 477 | 475 | 474 | 472 | 470 | 468 | 466 |

in which  $av$  is the difference in oxygen content of arterial and mixed venous blood or the oxygen uptake related to cardiac output. An approximation useful clinically is given in equation 9.

$$\% \text{ shunt} = \frac{P_{iO_2} - P_{aO_2}}{25} \quad (9)$$

Normally the shunt is 2-6%; for room air conditions equation 9 becomes

$$\frac{150 - 90}{25} = 2.4\%$$

To properly calculate the extent of a shunt, however, requires the patient to breathe 100% oxygen for 15-20 minutes. Table III illustrates that the low  $P_{aO_2}$  in hypoventilation, diffusion defect, and  $\dot{V}/\dot{Q}$  inequality is corrected by breathing 100% oxygen whereas in a true veno-arterial shunt it is not.

Table VI summarizes the material presented in the previous discussion. It correlates the changes in oxygen and carbon dioxide partial pressures, showing the pathological causes for the imbalances. In addition, it contains the various diagnoses of acid-base abnormality (using same numbers as in Figures 1 and 2 and Table I). Considering the format of Table VI as a tic-tac-toe set-up, we can label the nine portions by the letters A-I for identification in Table VII which gives examples of various conditions associated with such blood gas abnormalities (20-30).

|            |   |           |   |   |
|------------|---|-----------|---|---|
|            |   | $P_{O_2}$ |   |   |
|            |   | ↓         | N | ↑ |
| $P_{CO_2}$ | ↑ | A         | B | C |
|            | N | D         | E | F |
|            | ↓ | G         | H | I |

**TABLE V**  
**ALTITUDE VARIATIONS OF REPRESENTATIVE CITIES**

| <u>CITY</u>          | <u>ALTITUDE</u> | <u>COMMENTS</u>  |
|----------------------|-----------------|--|
| Buffalo              | 572→650         | Lake Erie  |
| Chicago              | 580→600         | Lake Michigan  |
| Cleveland            | 572→800         | Lake Erie  |
| Dead Sea             | -1300           | Lowest Spot; 23-25%<br>Salt (vs 4-6% for<br>Ocean)           |
| Death Valley, Calif. | -282            | T <sup>o</sup> >120 <sup>o</sup> F; Max 134.6 <sup>o</sup> F |
| Denver               | 5280            |  |
| Detroit              | 580→581         | Lake Huron   |
| Duluth               | 602→1200        | Lake Superior  |
| Lake Erie            | 572             |  |
| Huron                | 580             |  |
| Michigan             | 580             |  |
| Ontario              | 246             |  |
| Superior             | 602             |  |

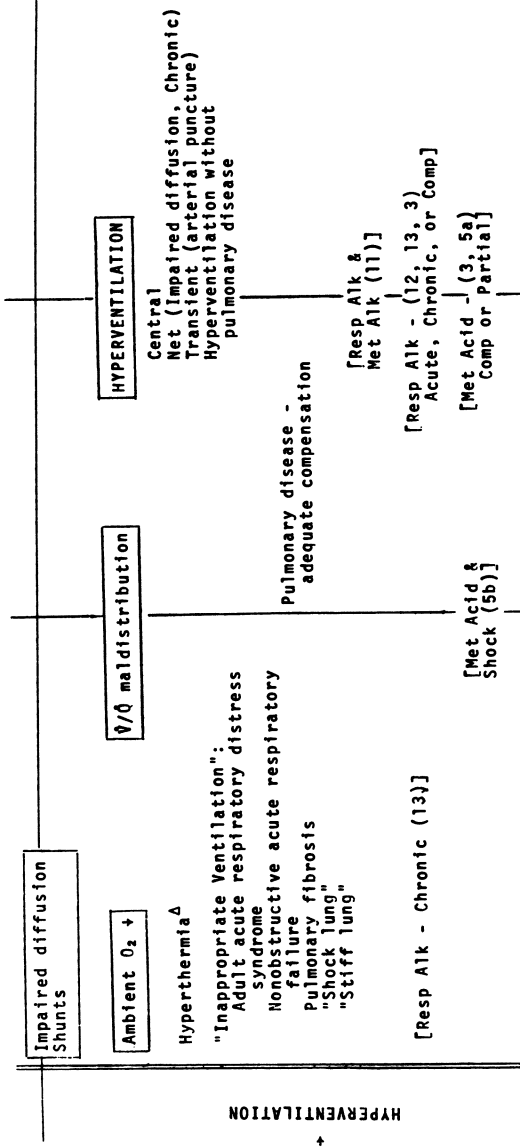
TABLE V (CONTINUED)

ALTITUDE VARIATIONS OF REPRESENTATIVE CITIES

| <u>CITY</u>     | <u>ALTITUDE</u> | <u>COMMENTS</u>   |
|-----------------|-----------------|---|
| Leadville, Col. | 10,200          |   |
| Mexico City     | 7347→8000       | Volcanoes →14,000   |
| Milwaukee       | 580→705         | Lake Michigan<br>Mitchell Field 693                       |
| Minneapolis     | 838→"1100"?     | Mid-Point Equator & N.<br>Pole at Falls of St.<br>Anthony |
| Philadelphia    | 150             |   |
| Phoenix         | 1100            |   |
| Pittsburgh      | 710→1365        |   |
| Rochester, NY   | 500→697         | "Along" Lake Ontario                                      |
| Salt Lake City  | 4255            | Salt Lake 27% Salt  |
| San Francisco   | 0→938           |   |
| Toronto         | 246→250         | Lake Ontario  |
| Tucson          | 2400            |   |

Table VI. Blood Gas Imbalance. Presented in part at ASCP-CAP Annual Meeting, San Francisco, Oct. 1972 (1); based on material from 20 and 21.

| $P_{aO_2}$<br>$P_{aCO_2}$ | $\uparrow$<br>HYPOXEMIA                             | N<br>$P_{aO_2}$   | $\uparrow$<br>HYPEROXEMIA                              |
|---------------------------|---|---|--|
|                           |   | $O_2$ Therapy <sup>o</sup>  | Hypothermia <sup>Δ</sup><br>$O_2$ Therapy <sup>o</sup> |
|                           |   | V/Q maldistribution - severe  |  |
| $\uparrow$                | HYPOVENTILATION                                     |   |  |
|                           | [Met alk - (2, 7)<br>Comp or Partial]               |   |  |
|                           | [Resp Acid - (9, 10, 2)<br>Acute, Chronic, or Comp] | [Resp Acid &<br>Met Acid (8)]   |  |
|                           | HYPOVENTILATION<br>Shunts                           | { General<br>Met  |  |
|                           |   | { "Local"<br>"Nonuniform"<br>"Regional"<br>"Uneven"   |  |
|                           | V/Q maldistribution                                 |   |  |
|                           |   | "NORMAL," BLOOD GASES   | $O_2$ Therapy <sup>o</sup>                             |
|                           |   | { (1b) Normal "Acid-Base" (1a) ]<br>{ (4b) Met Acid - Acute (4a) ]<br>{ (6b) Met Alk - Acute (6a) ] |  |
| $P_{aCO_2}$<br>N          | Pulmonary disease - poor compensation               | NON-VENTILATORY HYPOXIA   |  |
|                           |   | (1a, 4a, 6a)  | $P_{yO_2}$ $P_{yCO_2}$                                 |
|                           |   | Hemic   | $\uparrow$ N   |
|                           |   | Ischemic  | $\uparrow$   |
|                           |   | Histotoxic  | $\uparrow$ $\uparrow$ N $\uparrow$                     |
|                           |   | $\uparrow$ Also $P_{aCO_2}$ ; pH $\uparrow$   |  |



<sup>A</sup>Based on material from R. C. Kory (1970) and on Audio-Digest Fndn "Blood Gas Analysis" (Int Med Series 19: No. 17, Sept. 1, 1972). (Numbers and letters refer to acid-base diagnosis diagram [I-187A], flow-chart [I-187C], and table [I-188].)

<sup>a</sup>A "low" P<sub>O<sub>2</sub></sub> may become "normal" or "increased" due to:  
Hyperventilation  
O<sub>2</sub> Therapy:  
Increased Concentration

Increased Pressure (hyperbaric chamber, respiratory ventilators (eg, PEEP or CPPB), etc)  
P<sub>O<sub>2</sub></sub> polarographic electrode "error" due to halogenated hydrocarbons (eg, Halothane)

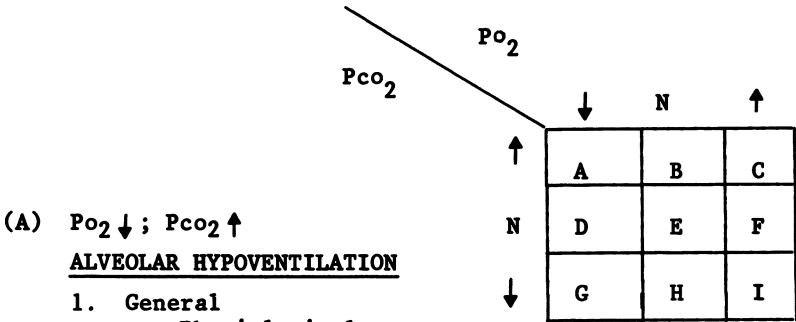
<sup>Δ</sup>If lab data uncorrected to temperature of patient!



TABLE VII

Conditions Associated with Blood Gas Imbalance

Modified from Weisberg (1)



ALVEOLAR HYPOVENTILATION

1. General

a. Physiological

- (1) Sleep
- (2)  $CO_2$  Retention
- (3) Metabolic Alkalosis

b. Impaired Movement of Respiratory Cage & Diaphragm

- (1) Abdominal Distension
- (2) Ankylosing Spondylitis
- (3) Ascites
- (4) Chest Injury
- (5) Kyphoscoliosis
- (6) Myxedema
- (7) Obesity
- (8) Pleural Disease

c. Neurological Impairment of Respiratory Drive & Cage

- (1) CNS Lesions of Respiratory Center
- (2) Muscular Dystrophy
- (3) Myasthenia Gravis
- (4) Narcotics & Drugs
- (5) Pickwickian Syndrome
- (6) Poliomyelitis

2. Net (Secondary to Pulmonary Disease)

a. Obstruction to Airways

- (1) Asthma, Severe
- (2) Bronchitis, Chronic
- (3) Cystic Fibrosis
- (4) Emphysema
- (5) Laryngeal Spasm

TABLE VII (Continued)

Conditions Associated with Blood Gas ImbalanceModified from Weisberg (1)

## b. Distortion of Pulmonary Parenchyma

- (B)  $PO_2$  N;  $PCO_2$   $\uparrow$   
 $O_2$  Therapy
- (C)  $PO_2$   $\uparrow$ ;  $PCO_2$   $\uparrow$   
 $O_2$  Therapy
- (A)-(D)  $PO_2$   $\downarrow$ ;  $PCO_2$  N  $\uparrow$   
 Collapse of Lung  
 Pneumonia
- (D)  $PO_2$   $\downarrow$ ;  $PCO_2$  N  
 "Age"  
 Atelectasis  
 Bronchitis, Early  
 Cardiac Failure  
 Congenital Heart Disease with R-L Shunt  
 Pneumonia  
 "Prematurity"
- (E)  $PO_2$  N;  $PCO_2$  N  
 Normal  
 "Non-Ventilatory Hypoxia"  
 Hemic Anoxia  
 Ischemic Anoxia  
 Histotoxic Anoxia
- (F)  $PO_2$   $\uparrow$ ;  $PCO_2$  N  
 $O_2$  Therapy
- (D)-(G)  $PO_2$   $\downarrow$ ;  $PCO_2$  N  $\downarrow$   
 Asbestosis  
 Idiopathic Fibrosis  
 Pulmonary Edema  
 Sarcoidosis
- (G)  $PO_2$   $\downarrow$ ;  $PCO_2$   $\downarrow$   
 Acute Pulmonary Inflammation  
 Alkylating Agents  
 Busulfan  
 Cyclophosphamide

TABLE VII

Conditions Associated with Blood Gas ImbalanceModified from Weisberg (1)

Drug Overdose  
 "Interstitial Pulmonary Fibrosis"  
 Myocardial Infarct  
 Post-Op  
 Pulmonary Oxygen Toxicity  
 Radiation Pneumonitis  
 "Shock Lung"  
     Hemorrhage  
     Thermal Injury  
 Trauma (e.g., Thoracic)  
 Viral Pneumonitis

(G)-(H)  $P_{O_2} \downarrow$ ;  $P_{CO_2} \downarrow$   
 Asthma, Early  
 Pulmonary Embolus  
 Shock, Late

(I)  $P_{O_2} \uparrow$ ;  $P_{CO_2} \downarrow$   
 Anxiety  
 Dehydration  
 Diabetic Acidosis  
 Hyperventilation Syndrome  
 Neurologic Disease  
 Shock, Early  
 Uremia

Literature Cited

1. Weisberg, H. F.; Interpretation of Electrolyte, Acid-Base, and Oxygen Imbalance, Private mimeographed printing, 4th ed., 1974.
2. Severinghaus, J. W.; Weiskopf, R. B.; Nishimura, M.; and Bradley, A. F.; Oxygen Electrode Errors Due to Polarographic Reduction of Halothane. *J. Appl. Physiol.* (1971), 31, 640-642.
3. Bates, M. L.; Feingold, A.; and Gold, M. I.; The Effects of Anesthesia on an In-vivo Oxygen Electrode. *Am. J. Clin. Path.* (1975), 64, 448 - 451.
4. Laver, M. B.; Personal Communication (1975).
5. Weiskopf, R. B.; Nishimura, M.; and Severinghaus, J. W.; The Absence of an Effect of Halothane on Blood Hemoglobin O<sub>2</sub> Equilibrium in Vitro. *Anesthesiology* (1971), 35, 579 - 581.
6. Raine, J.; and Bishop, J. M.; A-a Difference in O<sub>2</sub> Tension and Physiological Dead Space in Normal Man. *J. Appl. Physiol.* (1963), 18, 284 - 288.
7. Stephen, C. R.; and Talton, I.; Immediate Postoperative Care, with Particular Reference to Blood-gas Studies. *Can. Anesth. Soc. J.* (1964), 11, 586- 597.
8. Conway, C. M.; Payne, J. P.; and Tomlin, P. J.; Arterial Oxygen Tension of Patients Awaiting Surgery. *Br. J. Anaesth.* (1965), 37, 405 - 408.
9. Sorbini, C. A.; Grassi, V.; Solinas, E.; et al; Arterial Oxygen Tension in Relation to Age in Healthy Subjects. *Respiration* (1968), 25, 3 - 13.
10. Kitamura, H.; Sawa, T.; and Ikezono, E.; Postoperative Hypoxemia: The Contribution of Age to the Maldistribution of Ventilation. *Anesthesiology* (1972), 36, 244 - 252.
11. Neufeld, O.; Smith, J. R.; and Goldman, S. L.; Arterial Oxygen Tension in Relation to Age in Hospital Patients. *J. Am. Geriatr. Soc.* (1973), 21, 4 - 9.
12. Wahba, W. M.; Body Build and Preoperative Arterial Oxygen Tension. *Can. Anesth. Soc. J.* (1975), 22, 653 - 658.
13. Gordh, T.; Linderholm, H.; and Norlander, O.; Pulmonary Function in Relation to Anaesthesia and Surgery Evaluated by Analysis of Oxygen Tension of Arterial Blood. *Acta Anaesth. Scand.* (1958), 2, 15 - 26.
14. Palmer, K. N. V.; and Gardiner, A. J. S.; Effect of Partial Gastrectomy on Pulmonary Physiology. *Br. Med. J.* (1964), 1, 347 - 349.
15. Nunn, J. F.; Influence of Age and Other Factors on Hypoxia in the Postoperative Period. *Lancet* (1965), 2, 466 - 468.
16. Davis, A. G.; and Spence, A. A.; Postoperative Hypoxemia and Age. *Anesthesiology* (1972), 37, 663 - 664.
17. Marshall, B. E.; and Wyche, M. Q.; Hypoxaemia During and After Anesthesia. *Anesthesiology* (1972), 37, 178 - 209.

18. Consolazio, C. F.; Johnson, R. E.; and Pecora, L. J.; *Physiological Measurements of Metabolic Functions in Man*, McGraw-Hill, New York 1963.
19. Sahn, S. A.; Lakshminarayan, S.; and Petty, T. L.; Weaning from Mechanical Ventilation. *J.A.M.A.* (1976), 235, 2208 - 2212.
20. Kory, R. C.; Personal Communication (1970).
21. Audio Digest Fndn., Blood Gas Analysis, *Int. Med. Series* 19 (17), Sept. 1, 1972.
22. Comroe, J. H.; Forster, R. E.; DuBois, A. B.; et al.; *The Lung: Clinical Physiology and Pulmonary Function Tests*, Year Book, Chicago, 2nd ed., 1962.
23. Van Liere, E. J.; and Stickney, J. C.; *Hypoxia*, University Chicago Press, Chicago, 1963.
24. Dejours, P.; *Respiration*, Oxford, New York 1966.
25. Fishman, A. P.; Goldring, R. M.; and Turino, G. M.; General Alveolar Hypoventilation; A Syndrome of Respiratory and Cardiac Failure in Patients with Normal Lungs. *Quart. J. Med.* (1966), 35, 261 - 275.
26. Zilva, J. F.; and Pannall, P. R.; *Clinical Chemistry in Diagnosis and Treatment*, Year Book, Chicago 1972.
27. Snider, G. L.; Interpretation of the Arterial Oxygen and Carbon Dioxide Partial Pressures; A Simplified Approach for Bedside Use. *Chest* (1973), 63, 801 - 806.
28. Mays, E. E.; An Arterial Blood Gas Diagram for Clinical Use. *Chest* (1973) 63, 793 - 800.
29. West, J. B.; *Respiratory Physiology - The Essentials*, Williams & Wilkins, Baltimore, 1974.
30. Davis, H. L.; Acute Respiratory Insufficiency, in Stollerman, G. H. (ed), *Adv. Int. Med.* (1974), 19, 213 - 238.

## Analytical Aspects of Clinical Enzymology

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Clinical enzymology, born early this century with the demonstration that body fluids contain amylase and lipase, progressed slowly until the end of World War II by the addition of a few diagnostic tests such as the measurements of serum acid and alkaline phosphatase activity (1,2,3). Then as a result of the rapid growth during the past 30 years of protein biochemistry and genetics, the study of human enzymes has provided powerful insights into the etiology of a large number of diseases, has provided many new and useful diagnostic tests, and is now being exploited in the development of new analytical tools for the specific measurement of organic molecules. The development of enzyme measurements for the understanding, detection, diagnosis, and management of disease rests on a solid foundation of analytical validity provided by the pure reagents and solid-state electronic instruments now available.

### Nature of Enzymes

Enzymes are exceptionally efficient catalytic proteins which increase the speed of a chemical reaction without themselves undergoing a permanent change. Under optimal conditions, most enzymatic reactions proceed from  $10^8$  to  $10^{14}$  times more rapidly than the corresponding non-enzymatic reactions. For example, one molecule of catalase, the enzyme which converts hydrogen peroxide into water and atomic oxygen, is able to deal with approximately 5 million molecules of  $H_2O_2$  per minute. This represents an acceleration of at least  $10^{10}$  over the rate of the spontaneous reaction (4). Another striking and biochemically important aspect of enzyme catalyzed reactions is their specificity for the reaction catalyzed and the structure of the substrate utilized.

Urease, for instance, possesses exceptionally high specificity. It is very active in catalyzing the breakdown of urea to carbon dioxide and ammonia, but inactive when substituents such as methyl and other alkyl groups are attached to the  $\text{NH}_2$  groups of urea, or if the oxygen is replaced by sulfur. Virtually all intracellular processes essential to life including the biosynthesis of the enzyme themselves, are enzyme catalyzed. Structurally all enzymes are proteins, often with integral glycosidic moieties or attached metals, lipids, or a vitamin derived coenzyme essential for enzymatic activity. The environment is also critical, for enzymes are sensitive to heavy metals, detergents, changes of pH and ionic strength.

It is important to keep in mind that enzymes are recognized by their catalytic activity, and that this activity can be and often is shared by a number of closely related proteins, the isoenzymes. Isoenzymes can be differentiated by small physico-chemical differences in electrophoretic mobility, degree of inhibition by certain chemicals, resistance to thermal denaturation, etc. Isoenzymes differ from each other in their subunit composition when the enzyme molecule is an aggregate of different subunits. Biologically, the small differences in substrate specificity, product inhibition and regulator responsiveness between each "family" of isoenzymes may allow the body to modulate enzymatic activity according to local biochemical needs (5). Clinically, these differences are quite useful in locating the tissue which is releasing a particular isoenzyme in response to disease.

Classification of Enzymes. A systematic classification and nomenclature has been established by the Commission on Enzymes of the International Union of Biochemistry (6), which divides enzymes into six general groups:

1. Oxidoreductases - catalyzing oxidation-reduction reactions.
2. Transferases - catalyzing group transfer reactions.
3. Hydrolases - catalyzing hydrolytic reactions.
4. Lyases - catalyzing the addition of groups at the site of a double bond or vice versa.
5. Isomerases - catalyzing isomerization.
6. Ligases (synthetases) - catalyzing the condensation of 2 molecules coupled with the cleavage of a pyrophosphate bond of ATP or similar triphosphate.

To the above we may add:

Transcriptases - catalyzing the translation of information from one linear macromolecule into the structure of the nascent linear macromole.

### Instrumentation

Instruments that follow the conversion of a substrate into a product, must be capable of taking two or more readings during an accurate time base. Since the rate of enzyme catalysis may vary with time as a function of substrate or product concentration, an instrumental analog output (i.e., a recorder tracing) is highly useful. Modern digital electronic circuits enable instruments to print digital results - a highly convenient feature. Many enzyme assays require ultraviolet light for the measurement of the reaction (i.e., the change of NAD into NADH) catalyzed by the enzyme being measured or by additional enzyme reactions coupled to it. Consequently, the instrument should be equipped with a light source, monochromator and detector suitable for measurements in the near UV range (7).

Light Sources. The tungsten lamp is commonly used as a source of visible and near UV light. Other lamps used are hydrogen, quartz or glass-halogen and hollow cathode lamps. Miniature lamps of the last two types are now being used in some newer instruments to provide high intensity near - UV light.

Monochromators. Replica gratings and narrow band-pass filters are used commonly, more so than quartz prisms. Computer control of the monochromator is available in some instruments, so that optimum intensity at the desired wavelength or maximum absorption by the examined substance can be obtained. The recent availability of low cost microprocessors has brought the possibility of on-line control of all elements of the spectrophotometer, plus on-line data reduction into the realm of practicality.

Current instruments use complex and refined optical systems in order to radiate the cuvette with monochromatic light selected from the radiant spectrum of the light source. A radically different approach may be practical when tunable lasers become available at reasonable prices.

The photodetectors in current use, such as photomultiplier tubes, can respond to only one signal at a time, and hence the spectrum of light absorption or fluorescent emission by the sample can only be scanned by manipulating the monochromator. A complete spectrum of both emission and absorption can be obtained, however, by using a vidicon tube to monitor the entire light spectrum passing through the cuvette, without this light having been diffracted by a monochromator. The dramatic increase in information yield and in optical simplification should greatly enhance the power of spectrophotometric methods.

Time Base. Until recently, the only available way of measuring the duration of an enzyme reaction was through the



use of conventional mechanical escapement watches, with which the operator visually correlated the elapsed time with the instrumental readings or determined when to add a reaction-stopping reagent. This approach was obviously fraught with errors due to lapses in the operator's attention, lack of visual correlation of the time base with the instrumental readings, etc. An analog time base can monitor the speed of movement of a chart paper strip by an electric motor. While the analog time base still is highly useful, it is being complemented by the digital time base using oscillating quartz crystals. These digital clocks in turn can automatically control the digital printout of the instrumental reading at accurately predetermined time intervals, thereby eliminating errors due to operator-instrument interaction. Digital timers are also incorporated into computers or into microprocessors which in turn can control the entire instrument.

Cuvets. The cuvet in which an enzyme reaction proceeds can be of any size or shape, as long as it has optical surfaces for the entry and exit of light. The conventional 3 ml rectangular Beckman cuvet has been gradually replaced by cuvetts which require only a small volume, at most 1 ml of reaction mixture, and even by cuvetts which require only a few microliters. These cuvetts may have glass, quartz, or sapphire optical surfaces, with the other walls being made of any inert material, including Kel-F. Flow-through cuvetts maintain the light path constant and eliminate cuvet transport devices and optical variations between cuvetts (8). Flow-through cuvetts are now reliable and quite popular. For multiple cuvetts, manual transport devices are available for the accurate positioning of micro-cuvetts, and mechanized transport devices include mechanical dwell-cams, the manual transport employed in some spectrophotometers, and the rapidly spinning cuvetts used in the centrifugal fast analyzer (9) (Fig.1).

Specimen Transport. For many years the market was dominated by the continuous flow, bubble-interrupted, system of Skeggs (10). While this system has been invaluable to most clinical laboratories and was the first widely accepted mechanized system for handling large numbers of enzyme assays, it is now recognized that in its widely used Auto-Analyzer-I form it suffers from several limitations which include carry over from sample to sample, low sensitivity read-out instruments, and especially the single point read-outs. The miniature flow-through cuvetts used in the Auto-Analyzer-II and in the computer operated SMAC have reduced these defects to a negligible level. Of the many alternatives available, the two which at present appear to be the leading contenders are: a) the centrifugal fast analyzer, in which a rotor containing many samples spins rapidly past a single photometric station

(Fig. 2) and b) discrete sample processing using small test tubes, in order to ensure the physical separation of each enzyme assay (i.e., the LKB-8600) (Table I).

Temperature Control. While it was well known that enzyme catalysis is a direct function of temperature, little attention was paid to its control in kinetic enzyme assays until the pioneer work of Schneider and Willis (11). These workers showed that the temperature compartment of the Beckman DU spectrophotometer varied widely as a function of room temperature and of the number of times the cuvet compartment was opened. Thus, while most authors have assumed that they were conducting their assay at room temperature (i.e., a nominal 25<sup>o</sup>) direct measurements showed that the cuvette temperature was closer to 32<sup>o</sup> C. Schneider and Willis suggested that thermospacers, hollow plates adjacent to each side of the cuvette compartment through which water at a constant temperature is circulated, be used in order to standardize clinical enzyme assay temperatures. This arrangement works well if the room temperature can be kept reasonably close to that of the water bath. Otherwise, each time the cuvet compartment is opened a significant temperature fluctuation occurs. A better approach was introduced by R. J. Emary, who designed for the Gilford spectrophotometers a flow-through microcuvet which contains in its walls a temperature microsensor and an electric heater, all controlled by solid state electronic circuitry. This cuvette can be calibrated by removing one of its windows, and inserting a miniature thermistor into its cavity, in order to check the accuracy of the settings in the electronic circuitry. This arrangement is said to yield temperatures stable to within 0.1<sup>o</sup>C. The cuvet temperature fluctuations which occur in the centrifugal fast analyzers can be controlled by similar devices built into the rotor.

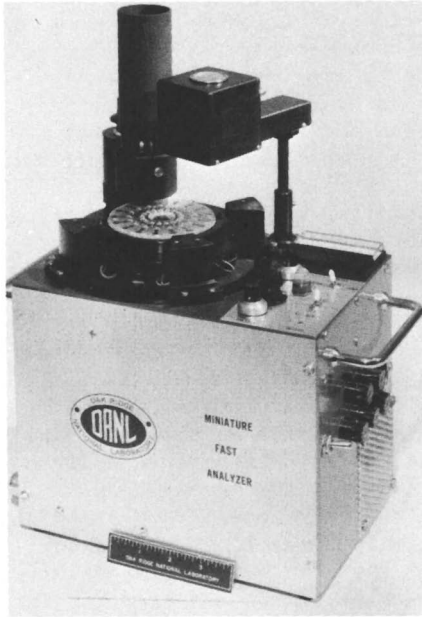
Kinetic Read Out. Of the many instruments dedicated to enzyme analyses only those which provide a continuous strip-chart recording of the enzyme kinetics or continuous monitoring with several digital print-outs of each enzyme reaction can be considered to be true kinetic analyzers. Equally acceptable is the fast centrifugal analyzer, in which each of the multiple cuvettes in the rotor is monitored several hundred times per minute by the stationary photodetector (Fig. 2). The remaining automated instruments rely on either one point or two point measurements, and hence cannot detect variations from zero-order kinetics (Table I).

Instrument Productivity. The number of samples that can be processed by one instrument in a given period of time is determined by the length of time each sample has to be read by the photodetector or other read-out device, or by the number of cuvetts per unit time which pass the detector. For example,

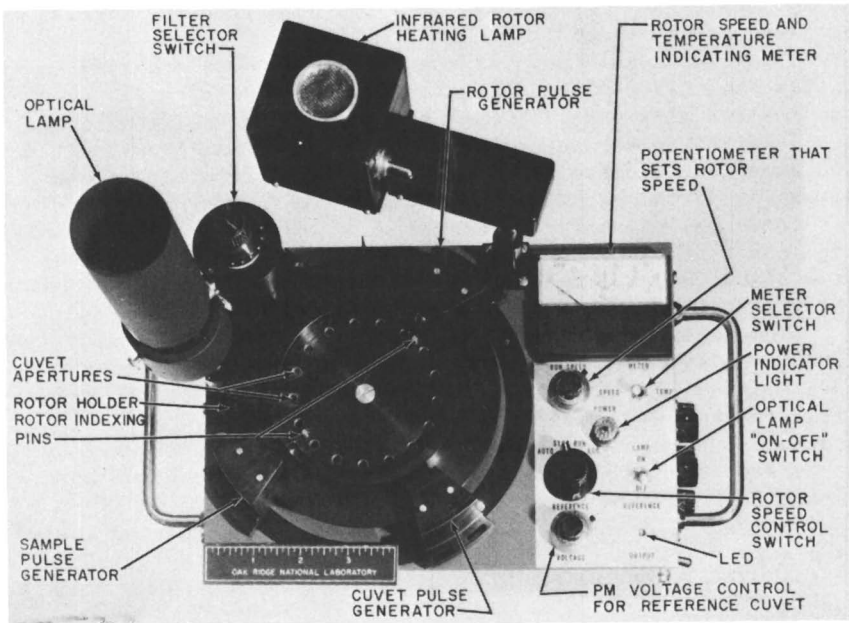
**TABLE I**  
**SOME AUTOMATED INSTRUMENTS FOR ENZYME ANALYSES**

| INSTRUMENT               | 1   | 2   | 3     | 4        | 5           | 6   | 7   | 8 | COST      |
|--------------------------|-----|-----|-------|----------|-------------|-----|-----|---|-----------|
| BECKMAN SYSTEM TR        | M   | 45  | MK    | F340+    | HAB25,30,37 | FQ  | D   | + | \$ 15,000 |
| PERKIN ELMER             | M   | 150 | MK    | F340     |             | FQ  | D   | + | ---       |
| GILFORD 300N             | M   | 60  | MPK   | M340-700 | E25,30,37   | FTQ | D+A | + | \$ 8,000  |
| GILFORD 3500             | M   | 60  | MPK   | M340-700 | E25,30,37   | FTQ | D   | + | \$ 25,000 |
| DUPONT ACA               | M   | 60  | 3PK   | FB340    | HAB 37      | PD  | D   | + | \$ 80,000 |
| LKB 8600+8200 PRINTER    | M   | 80  | MK    | F340     | HAB30,35,37 | PD  | D+A | + | \$ 22,000 |
| HYCEL MARK X             | 100 | 40  | EP    | F340     | WB37        | FQ  | D+A | + | \$ 90,000 |
| HYCEL MARK XVII.         | 100 | 40  | EP    | F340     | WB37        | FQ  | D+A | + | \$120,000 |
| ABBOTT BICHROMATIC A100  | M   | 90  | 3PK   | F34      | WB30,37     | PD  | D+A | + | \$ 24,000 |
| VICKERS 300              | M   | 300 | 2PKTS | F340     | HBL         | F   | D   | + | \$250,000 |
| ORTHO DIAG. ACCUCHEM     | M   |     | 2PKTS | F340     | OB37        | FTC | D   | + | \$180,000 |
| TECHNICON AAIL           | 250 | 60  | SP    | F340     | OB37        | FT  | D+A | + | \$ 18,000 |
| TECHNICON SMAC           | M   | 150 | 3PK   | F340     | OB37        | FT  | D+A | + | \$224,000 |
| U.CARBIDE CENTRIFICHEM   | M   | 200 | MPK   | F340     | HAB25,30,37 | FQ  | D+A | + | \$ 32,000 |
| ELECTRONUCLEONIC GEMSAEC | M   | 200 | MPK   | M340-700 | WB UP TO 50 | FQ  | D+A | + | \$ 40,000 |
| AMINCO ROTOICHEM         | M   |     | MPK   |          |             |     |     |   |           |

COLUMN NO. 1 = SAMPLE VOLUME, M=MICRO LESS THAN 100 MICRO LITERS  
 COLUMN NO. 2 = MAXIMUM NO. OF SAMPLES PER HOUR  
 COLUMN NO. 3 = PRINCIPLE OF MEASUREMENT, M=MULTIPLE, S=SINGLE, P=POINT, K=KINETIC  
 COLUMN NO. 4 = WAVE LENGTH, F=FILTER, M=MONOCHROMETER, G=GRATING  
 COLUMN NO. 5 = TEMPERATURE CONTROL, WB=WATER, OB=OIL BATH, HAB=HOT AIR BATH, E=ELECTRIC  
 COLUMN NO. 6 = CUVETTE, P=PLASTIC, D=DISPOSABLE, FT=FLOW THROUGH  
 COLUMN NO. 7 = DISPLAY, D=DIGITAL, A=ANALOG  
 COLUMN NO. 8 = COMPUTER AND DATA REDUCTION, +=YES  
 COST=APPROXIMATE PRICE FOR COMPLETE SYSTEM



*Figure 1. Front view of the miniature centrifugal analyzer. The mechanical, analytical, and optico-electronic components are in one compact unit. Digital data reduction is in another unit, not shown. (Courtesy of Dr. Carl A. Burtis, of the former Oak Ridge National Laboratory).*



*Figure 2. Top view of the miniature centrifugal analyzer. The sample disc, not shown, fits on the rotor holder and can process 16 samples and 1 reference simultaneously. (Courtesy of Dr. Carl A. Burtis).*

the miniature centrifugal fast analyzer can process 16 samples plus one reference cell past the photodetector several hundred times a minute, and reactions can be monitored in one or two minutes for all 16 samples. The rotor can be brought to full speed within a fraction of a second and can be stopped within an equally short period of time by a built-in clutch. Automatic mixing of sample and reagents occurs within one second. The remainder of the minute or two can then be used for monitoring the enzyme kinetics in the multiple cuvetts. Since all cuvetts are surveyed within the same time, the instrument yields truly comparable results. The fact that the instrument is controlled by a small computer or by a microprocessor allows for true automation. The centrifugal fast analyzer is in the authors' opinion the ideal instrument for monitoring enzyme kinetics. The rotors can be preloaded with lyophilized reagents, which can be dynamically dissolved by the addition of buffer to the spinning rotor. Multiple samples can then be introduced into each of the radial cuvettes, or a single sample can be dynamically apportioned between the multiple cuvettes, each of which contain reagents for a different enzyme reaction. Consequently, multiple samples can be monitored for the same enzyme activity, or several different enzyme activities can be measured for the same sample. The very fast data reduction offered by the on-line computer provides the operator with printed results as soon as the analysis is complete. This approach provides highly precise data (Table II).

Other analyzers such as the Gilford Automated Enzyme Analyzer and the LKB-8600 Reaction Rate Analyzer analyze discrete samples one at a time. These instruments provide kinetic analyses, digital data reduction at the time each sample is analyzed, and excellent electronic and optical characteristics. Recently, Atwood has developed kinetic enzyme analyzers which require only 9 seconds for measuring an enzyme activity, using highly stable and sensitive electronic circuits (12). This short read out time allows a large number of samples to be processed by one instrument in an automated mode.

The continuous flow methods used by the Auto Analyzer have traditionally relied on the single point measurement of a dialysate of the incubated mixture (10). More recently, the computerized sequential multiple analyzer (SMAC) provides two point analysis by electronically eliminating the bubble signal and by passing the same bubble segmented stream through two cuvetts separated by a time delay coil. Critical evaluations of this system are not available at this time. Other automated analyzers such as those produced by Hycel rely upon single point assays using colorimetric methods, and hence the results may be dubious.

An entirely different breed of instruments has been introduced by Beckman for measuring substrate concentrations using enzymes as the reagent and specific potentiometric electrodes

**TABLE II**  
**WITHIN-ROTOR VARIATION OF TWO MINIATURE ANALYTICAL SYSTEMS**

| ANALYTICAL RESULTS <sup>a</sup> |    |       |                       |                                   |
|---------------------------------|----|-------|-----------------------|-----------------------------------|
| LABORATORY                      | N  | MEAN  | STANDARD<br>DEVIATION | RELATIVE<br>STANDARD<br>DEVIATION |
| A                               | 16 | 59.30 | 0.4                   | 0.63                              |
| B                               | 16 | 60.10 | 0.4                   | 0.63                              |

<sup>a</sup>Data obtained from 16 replicate alkaline phosphatase assays that were measured within a single rotor. Results are given in I.U., 30° C.

Data courtesy of Dr. C. A. Burtis, Molecular Anatomy Program, Oak Ridge National Laboratory of the former Atomic Energy Commission.

as the detectors. Instruments for measuring glucose and urea with highly specific methods are currently available. This approach relies on the measurement of reaction rate as first described and recently reviewed by Malmstadt (13).

### Analytical Considerations

Available methods provide measurements of enzyme activity rather than of enzyme concentration. In order that the measured activity be proportional to enzyme concentration, the reaction conditions which include pH, temperature, initial substrate concentration, sample and total volume and reaction time must be held constant and be carefully controlled.

Under optimum conditions when substrate and coenzyme concentrations are at saturating levels, i.e., the concentration of substrates far exceeds the concentration of the enzyme, and other variables are optimized and maintained constant, the initial rate of an enzymatic reaction is directly proportional to the amount of enzyme present. If one of the reactants absorbs monochromatic light, a given enzymatic activity can often be monitored in a spectrophotometer by following the negative rate of change in absorbance of the substrate as it is consumed, or positive rate of change in absorbance of the product as it is formed. Methods based on rate of the reaction-kinetic methods - offer many advantages over single or end-point (usually colorimetric) techniques (14-18).

#### Advantages of Kinetic Methods

1. Visual proof of linear kinetics, making obvious the occurrence of undesirable conditions such as substrate depletion or lag phase non-linearity.
2. Visual display of changes in the reaction rate.
3. Maximum accuracy as the measurement can be made in the region of maximum linear velocity.
4. The method requires less time since the reaction velocity is measured directly.
5. Greater specificity since the rate of the reaction usually is not affected by interfering substances commonly found in serum, and since each sample serves as its own blank.
6. There is extensive clinical documentation of UV-kinetic enzyme results showing excellent correlation with confirmed diagnosis.
7. Measurements are feasible over a broad range of enzyme activities.
8. Kinetic methods are adaptable to most enzymes.

#### Disadvantages of End-Point Methods

1. The method of color development may be non-specific and therefore the reaction may be affected by many interfering factors commonly found in serum such as turbidity, hemoglobin and bilirubin.
2. Non-linearity during progression of enzyme reaction or an initial lag phase which is undetected and may result in significant error.
3. The chromogen is often not completely specific, and metabolites and drugs may give falsely elevated results.
4. End-point methods are often not based on kinetically optimum conditions. However, an end-point method is often the only convenient one available. In this case, the method should have been validated by showing that the catalysis of the substrate follows well defined kinetics, rate of reaction is proportional to enzyme concentration, blanks and interfering substances are corrected for, and that appropriate standards are available.

Analytical Criteria for Clinical Enzyme Methods. The great variety of methods available for certain of the clinically useful enzymes can be confusing in the absence of criteria for assessing these methods. These criteria should be similar to those used to judge other quantitative clinical chemistry methods, and should also take into account the kinetic nature of the reaction. Some years ago, Amador and Wacker (17) suggested the following criteria, which are now widely used:

1. The kinetics should be zero-order during the initial portion of the reaction, over a practical working range that includes the usually encountered enzyme activities.
2. The measured activity should be directly proportional to enzyme concentration over a practical working range.
3. The reaction mixture should be as simple as possible, preferably consisting of premixed reagents of known stability. Lyophilized reagents or the single premixed reagent for LD assays are good examples.
4. The concentrations of substrate, coenzyme, buffer, activators, pH, etc., should provide maximum enzyme activities.
5. Inhibitors should be removed from sample. An example is urinary phosphate, which can be removed by dialysis prior to measuring the urinary alkaline phosphatase activity (18).
6. Inhibitors should not be present in the reagent solution. Examples are the dehydrogenase inhib-



itors which form from NADH when it is stored improperly, and the use of glycine as a buffer for alkaline phosphatase.

7. The rate measurement should be standardized with the purified substrate or product of the enzyme. For example, glutamic-oxalacetic transaminase activity can be standardized with oxalacetate but not with "convenient" substances such as pyruvate (17). When reference serums are used, the label value should be confirmed at frequent intervals by the actual method being used routinely, as label values can be in error (19). Conversion factors to convert the results obtained by one method into those obtained by another can give erroneous results and should not be used.

Clinical Criteria for Clinical Enzyme Methods. While it is possible to measure the activity of several thousand enzymes in body samples, in most cases it is practical to measure only a few while obtaining clinically useful results. In order to select the enzyme measurements which will be most useful, the following guidelines are suggested:

1. Increases or decreases in enzyme activity produced by disease should be sufficiently large so that confusion with the normal range does not result. For example, the elevated serum GPT seen in acute viral hepatitis is useful because it is many times greater than the normal range.
2. The normal range should be established with samples obtained from an adequate sample of healthy persons of specified age and sex. The effect of physiological variables such as activity, eating, menstruation and pregnancy should be known. The confidence limits should be determined with the appropriate statistical tools. Normal ranges determined with hospital patients should be rejected (20, 21).
3. The correlation between altered enzyme activities and a given disease, i.e., the diagnostic accuracy should be based on accepted symptoms, and laboratory changes. Tissue diagnoses should be used whenever possible as a standard of reference (22).
4. The diagnostic specificity for a given disease should be determined by examining patients with other diseases of the same organ, and diseases of several organs known to contain that enzyme. Ideally, the proposed test should clearly differentiate either between diseases of the same organ or between affected organs. For instance, the CK

isoenzyme pattern may be useful in distinguishing heart from other organ damage, but the alkaline phosphatase isoenzymes yield ambiguous data when biliary versus liver versus other tissue damage is being differentiated (23).

5. The diagnostic usefulness should be established by appropriate retrospective or prospective studies to see if the test in fact improves the diagnostic accuracy. For inherited enzyme diseases, the measurement of the enzyme activity can be diagnostically specific and highly useful. For otherwise obvious diseases such as metastatic prostatic carcinoma, enzyme tests may be of little value (24).

**Reagents.** The measurement of enzyme activities requires rigid control of the analytical conditions, including accurate measurement of reagent and sample volumes, and careful control of temperature, pH and reagent stability.

**Buffers.** pH is usually maintained within a range of 0.1 pH unit through the use of commonly available buffers with a pK close to the pH desired. The commonly employed buffers and some of their characteristics are listed in Table III. The "good" buffers described by Cleland have not improved the clinical assay of enzymes, while the commonly available relatively simple buffers have (25). Barbitol is a commonly used buffer, but has little to offer because it is quite weak and therefore does not maintain pH adequately. Other than serving as a buffer for electrophoresis, barbitol should probably be abandoned. Glycine is also a commonly used buffer, but has the disadvantage of being a chelating agent, and is a well known and strong inhibitor of alkaline phosphatase. Surprisingly, glycine is still commonly used to buffer alkaline phosphatase activity assays, but its use should definitely be discontinued (25). Tris (THAM) buffer is also commonly used, but it should be recognized that the pH of its solution is significantly affected by variations in temperature. Sodium or potassium phosphate and pyrophosphate have excellent buffering capacities. Phosphate tends to interact with NADH and slowly degrades it over a period of weeks (14). Pyrophosphate has good buffering capacity over the broad pH range used for most clinical enzyme assays, is inexpensive, and accordingly is an excellent buffer for the enzyme reaction or for color developer solutions which when added to the reaction abruptly change the pH to stop the enzyme reaction and develop color. The organic acids such as lactic, acetic, and citric acids are also excellent buffers in the lower pH ranges (26), but citrate is a chelating agent of the divalent metals necessary for some enzyme activities.

**Substrates.** Many of the substrates used for enzyme reac-



tions are artificial, and accordingly the measured activity may not necessarily be an accurate reflection of the physiological activity found in the organism. This consideration becomes particularly important when it is necessary to know the activity of a suspected mutant enzyme in order to detect an inherited metabolic disease, such as Tay-Sachs disease. In these instances, if an abnormally low activity is found with an artificial substrate, the measurement should be repeated using the natural substrate whenever possible (27).

When the substrate is available in either the d- or l-racemic form, it is preferable to use the appropriate isomer rather than its mixture. In a case of transaminase assays for GOT and GPT activity, for example, the initial assays used the d-l amino acid as substrate, and a marked improvement in activity and linearity was found by Henry and co-workers when they used l-aspartate or l-alanine, respectively (28).

Exogenous Coenzymes and Enzymes. Even when the coenzyme is known to be tightly bound to the apoenzyme, it is desirable to determine if addition of pure coenzyme will further activate the reaction mixture. Prolonged incubation may be necessary to obtain complete binding of the added coenzyme to the apoenzyme (29). Also, some of the commercial reference sera which have been prepared by extensive dialysis may be coenzyme deficient, and hence may show marked activation by added coenzyme, as in the case of commercial sera containing GOT which is markedly activated by added pyridoxal-pyridoxamine phosphates (26) (Fig. 3). When linked enzyme assays are being used, the exogenous enzymes and coenzymes must be present in large excess in order to insure very fast turn-over of the products of the primary enzyme reactions, and provide linearity over an adequate working range with a minimal lag phase. While it is possible to use two or even three or more linked enzyme reactions to measure an initial enzymatic reaction, it is now recognized that at most two exogenous enzymes should be used, as otherwise marked limitations in working range and low primary enzyme activity will result (30). Some coenzymes, such as NAD, are prepared by the manufacturers using ethanol. The purified NAD may therefore contain trace amounts of ethanol which may serve as a substrate for serum alcohol dehydrogenase in the absence of other substrates, leading to troublesome "blanks" sometimes called "nothing" dehydrogenase.

When linked enzyme assays are used, the exogenous added enzymes may also be contaminated with small traces of the primary enzyme whose activity is measured, thereby leading to falsely high activities. In this instance it is also desirable to make certain that the added enzymes are free of any undesirable activity, i.e., pig heart malic dehydrogenase should be free of GOT activity when used for GOT assays (17).

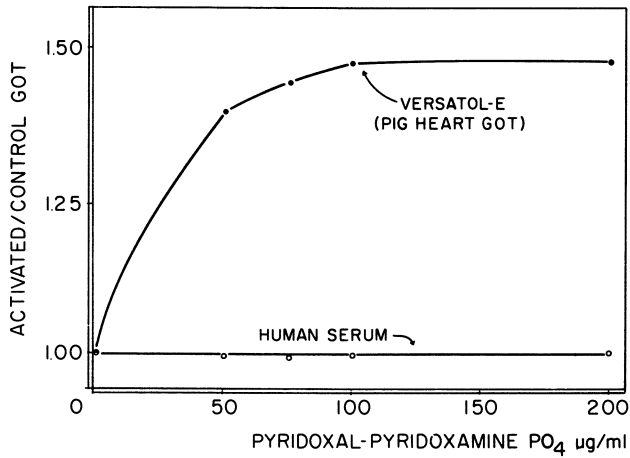
**Product Acceptors.** Many enzyme assays use acceptors, as for instance 2-ethylaminoethanol and other aminated alcohols which act as acceptors for the phosphoryl product of the reaction catalyzed by alkaline phosphatase (25) (Fig. 4). Hydroxylamine can act as an acceptor for the hydroxyacetone produced by enolase and semicarbazide can act as an acceptor for the pyruvate produced by LD. It is necessary to optimize the concentration of such an acceptor before using it routinely as often what may be a theoretically desirable acceptor is in practice superfluous.

**Enzyme Reference Serums.** Several companies sell lyophilized or stabilized reference serums for the calibration of instruments and for quality control. The label values given for the enzymatic activity of these serums should never be taken at face value, as at times they may be quite erroneous (19,33). Also, these values should only be used for the assay with which they were standardized, as interconversion of activity from one method to another for the same enzyme may often lead to marked errors. For instance, it is not recommended that alkaline phosphatase expressed in Bodansky units be multiplied by a factor to convert it to the units of the King-Armstrong method, or any other method for that matter.

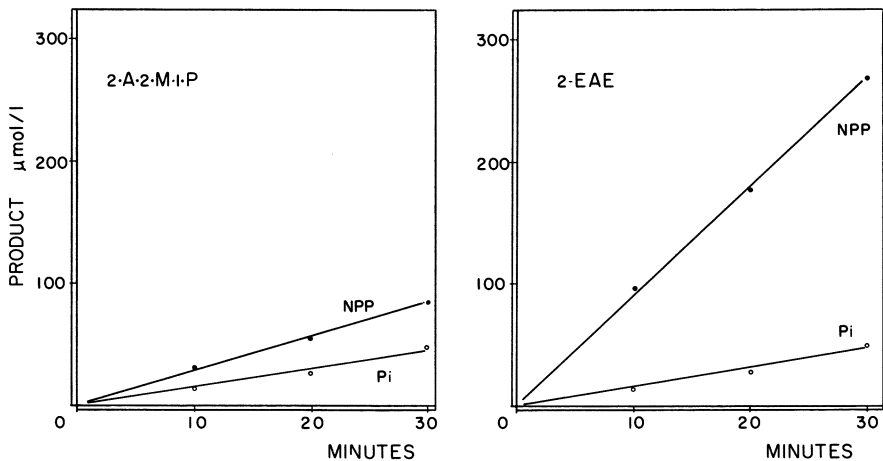
**Optimization of Substrate Concentrations.** Computer analyses of enzyme kinetics may be very useful for the calculation of enzyme constants, eliminating the tedium associated with manual calculations. Recently, computer models for optimizing reagent concentrations have been described but these models require so many experimental points that the model rests on the experimental data rather than having predictive usefulness (31).

The experimental verification of published results with newer buffers, more sensitive instruments or better reagents has led to a significant improvement, and often simplification of the method (32). Consequently, newly described methods should always be checked out point by point before using them routinely.

**Sample Collection and Enzyme Stability.** Serum samples are collected with chemically clean, sterile glassware. Blood is allowed to clot at room temperature, the clot is gently separated from the test tube with an applicator stick, and the blood is centrifuged for 10 minutes at 1,000 g. If the red cells are known to contain the enzymes whose activity is being measured, as in the case of LD, even slightly hemolyzed serums must be discarded. When acid phosphatase is to be measured, the serum should be placed immediately in ice and processed as soon as possible, or it should be acidified by the addition of a small amount of sodium citrate. Anticoagulants such as EDTA, fluoride and oxalate inhibit some serum enzymes. However, heparin activates serum lipoprotein lipase.



**Figure 3.** Addition of the GOT coenzymes pyridoxal and pyridoxamine phosphates in concentrations up to 200  $\mu\text{g/ml}$  has no effect on human serum GOT but activates by 45% the pig heart GOT activity of Versatol-E, a commercial reference serum



**Figure 4.** The release of nitrophenol (NP) and inorganic phosphate (Pi) is zero order in 2-amino-2-methyl-1-propanol and in 2-ethylaminoethanol (2-A-2-M-1-P and 2-EAE). About as much Pi is released in both buffers, but the amount of NP released is 2.6 times greater in 2-EAE. The difference between the NP and Pi slopes represents the amount of transphosphorylation, which is also greater in 2-EAE.

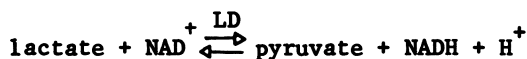
Urine may be collected for assays of enzyme activities following cleansing of the genitalia with mild antiseptic soap followed by rinsing with water. The urine is collected in a chemically clean container with no preservative. As the activity of urinary enzymes is a function of the volume of the specimen it is important to time the collection accurately. A collection period of 8 hours is quite adequate, and the use of longer periods is not desirable because enzyme activities can rapidly decrease in the relatively hostile medium of the urine. The urine should be refrigerated and transferred promptly to the laboratory, where it should also be processed promptly. Delays of more than two hours after the collection is completed may lead to markedly erroneous results. Urines with bacterial overgrowth, hemolyzed blood, or those obtained following any type of instrumental examination of the urinary tract may also lead to erroneous results, and should not be used for assays of enzyme activities. The stability of some clinically important enzymes in the various body fluids is given in Table IV.

White blood cells, red blood cells and cultured fibroblasts are commonly used to measure enzyme activities, especially for the diagnosis of inherited enzyme abnormalities. Leukocytes may be collected by sedimentation in viscous media such as Fycol. The collection of red cells presents no problem following centrifugation of anticoagulated blood. The assay of enzymes and fibroblasts requires appropriate tissue culture facilities and extensive experience in dealing with cultured human cells.

### Recommended Methods for Diagnostically Useful Enzymes

Many enzyme activities have been proposed for diagnosis, in serum, urine, cerebrospinal fluid and other body fluids, and the proposed methods have been reviewed elsewhere (17). Here we will confine ourselves to the tried and most commonly used enzyme activities which yield useful information in ambulatory or hospital patients.

Lactic Dehydrogenase (LD). LD catalyzes the reversible conversion of lactate to pyruvate in the presence of NAD:



The reaction may be followed by measuring the rate of appearance or disappearance of NADH at 340 nm. In some methods a coupled reaction using diaphorase or phenazine methosulfate plus an indicator dye are used. The amount of pyruvate formed can also be measured colorimetrically. With the general avail-

ability of UV spectrophotometers, the LD kinetic method (340 nm) is now widely used. The reaction employing pyruvate and NADH requires preincubation to eliminate other substrates found in serum which are consumed by endogenous serum enzymes in the presence of NADH. While the lactate and NAD reaction does not require this preincubation step, it is 2.5 times slower than the pyruvate reaction. This drawback can easily be corrected by increasing the proportion of serum in the final reaction mixture. The precision of either method is excellent and standardized methods are available (34). The colorimetric methods for measuring LD activity are either cumbersome, require expensive reagents such as phenazine methosulfate and nitro-tetrazolium blue as indicator dye, or else are unreliable as in the case of the dinitrophenylhydrazine colorimetric method which suffers from large blanks, non-zero order kinetics, and other significant systematic errors (15,16).

LD is well known to be the combined activity of 5 isoenzymes (35) which in turn are thought to result from the combination in varying proportions of two basic subunits, heart (H) and muscle (M) subunits, to form a tetramer (36). Additional isoenzymes in skin and testicle have been described, and it may be that the LD isoenzyme spectrum ultimately will turn out to be complex.

Myocardium is characterized by a predominance of LD-1 ( $H_4$  tetramer) whereas LD-5 ( $M_4$  tetramer) is the dominant species in liver and skeletal muscle. The heart isoenzyme LD-1 is heat stable whereas liver and skeletal muscle LD-5 is heat labile. The LD-1 is less than 30% of total LD in normal serum, whereas in patients with an acute myocardial infarction it is between 50-90% of total LD activity. The ratio of LD-1/LD-2 is usually greater than one in acute myocardial infarction (37).

Electrophoretic methods of separation of LD isoenzymes have become routine in clinical laboratories. Efforts are now being made to standardize the methodologies used for LD isoenzymes, particularly by Rosalki (38). The preferred methods are based on electrophoresis on a solid medium, so that the several bands may be scanned instrumentally. Differential isoenzyme inhibition with urea or other inhibitors is based on the fact that the heart LD isoenzyme is more resistant to inhibition than other isoenzymes. However, the analyst then has the problem of allocating the observed degree of inhibition between the different isoenzymes of a given sample, a problem that has not been resolved satisfactorily thus far. Hence, differential inhibition is not as reliable for isoenzyme separation as is electrophoresis.

$\alpha$ -Hydroxy-butyric Dehydrogenase (HBD). HBD reflects the activity of LD-1 (heart) towards  $\alpha$ -hydroxy-butyrate which it oxidizes to oxo-butyrate in the presence of NADH. Measurement of this serum activity has been found to correlate well with



**TABLE IV**  
**STABILITY OF SOME CLINICAL ENZYMES IN HUMAN BODY FLUIDS**

| EC NO.   | TRIVIAL NAME                          | ABBREVIATION | SPEC | 25°C | 4°C | -10°C |
|--|---------------------------------------|--------------|------|------|-----|-------|
| <b>OXIDOREDUCTASES--CATALYZING OXIDATION-REDUCTION REACTIONS</b> |                                       |              |      |      |     |       |
| 1.1.1.1  | ALCOHOL DEHYDROGENASE                 | AD           | S    | 7D   | 7D  | 1M    |
| 1.1.1.27   | LACTATE DEHYDROGENASE                 | LD           | S    | 24H  | 24H | UN-S  |
|  |                                       |              | U-D  | 6H   | 12H | UN-S  |
|  |                                       |              | U    | 4H   | 14H |       |
|  |                                       |              | CSF  | 5D   | 5D  | 10D   |
| 1.1.1.27   | HYDROXYBUTYRATE DEHYDROGENASE         | HBD          | S    | 5H   | 15D | 21D   |
| 1.1.1.42   | ISOCITRATE DEHYDROGENASE              | ICD          | S    | 7D   | 7D  | 7D    |
| 1.1.1.49   | GLUCOSE-6-PHOSPHATE DEHYDROGENASE     | GPD          | RBC  | 7D   | 7D  | 8D    |
| 1.6.4.2  | GLUTATHIONE REDUCTASE                 | GTD          | S    |      |     |       |
| <b>TRANSFERASES--CATALYZING GROUP-TRANSFER REACTIONS</b>         |                                       |              |      |      |     |       |
| 2.1.3.3.   | ORNITHINE CARBAMYL TRANSFERASE        | OCT          | S    | 3D   | 1W  | 1Y    |
| 2.3.2.1.   | GLUTAMYLTRANSFERASE (GCT)             | GMT          | S    | 2D   | 30D | 1Y    |
|  |                                       |              | U    |      |     | UN-S  |
|  |                                       |              | U-D  |      |     | 5W    |
| 2.6.1.1.   | ASPARTATE TRANSAMINASE (GOT)          | AST          | S    | 2D   | 14D | 1M    |
|  |                                       |              | U    | 5H   |     | 2D    |
|  |                                       |              | CSF  | 6H   | 14D |       |
| 2.6.1.2.   | ALANINE TRANSAMINASE (GPT)            | ALT          | S    | 2D   | 1W  | UN-S  |
| 2.7.7.12   | HEXOSE-1-PHOSPHATE URIDYL TRANSFERASE | HUT          | RBC  | 14D  | 14D | 1W    |
| 2.7.3.2  | CREATINE KINASE                       | CK           | S    | 2D   | 1W  | 1M    |

**TABLE IV (CONTINUED)**  
**STABILITY OF SOME CLINICAL ENZYMES IN HUMAN BODY FLUIDS**

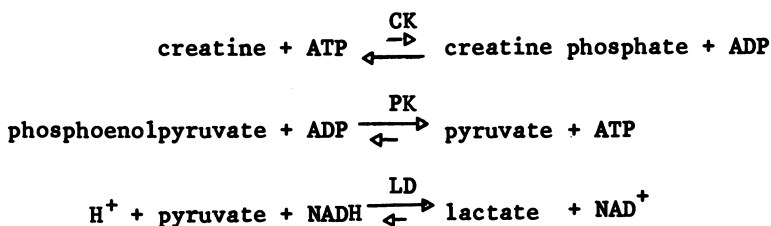
| EC NO.  | TRIVIAL NAME                | ABBREVIATION | SPEC | 25 °C       | 4 °C | -10 °C |
|---|-----------------------------|--------------|------|-------------|------|--------|
| <b>HYDROLASES CATALYZING HYDROLYTIC REACTIONS</b>   |                             |              |      |             |      |        |
| 3.1.1.3   | LIPASE                      | LPS          | S    | 7D          | 21D  |        |
| 3.1.1.8   | CHOLINESTERASE              | CHS          | S    | 10D         | 7D   | S-M    |
|   |                             |              | RBC  |             | 3D   |        |
| 3.1.3.1.  | ALKALINE PHOSPHATASE        | ALP          | S    | 2D          | 3D   | 1M     |
|   |                             |              | U    | 6H          | 6H   | UN-S   |
|   |                             |              | U-D  | 1D          | 1D   | UN-S   |
| 3.1.3.2.  | ACID PHOSPHATASE            | ACP          | S    | 4H          | 14D  | 115D   |
|   |                             |              | U    | 7D          | 15D  | UN-S   |
| 3.1.3.5   | 5-NUCLEOTIDASE              | NTP          | S    | 4D          | 4D   | 5M     |
| 3.2.1.1   | AMYLASE                     | AMS          | S    | 27D         | 7M   | 2M     |
| 3.2.1.31  | B-GLUCURONIDASE             | GRS          | S    | 1D          | 7D   | 7D     |
| 3.4.1.1   | LEUCINE AMINOPEPTIDASE      | LAS          | S    | 7D          | 7D   | 7D     |
| 3.4.4.1   | PEPSIN                      | PPS          | S    |             | 21D  |        |
| 3.4.4.4   | TRYPSIN                     | TPS          | DF   | V-UN-STABLE |      |        |
|   |                             |              | FEC  |             | 8D   |        |
| 3.5.4.3   | GUANINE DEAMINASE           | GDS          | S    | 3D          | 2W   | 10M    |
| 3.5.4.4   | ADENINE DEAMINASE           | ADS          | S    | 3D          | 2W   | 6M     |
| <b>LYASES CATALYZING THE ADDITION OF GROUPS TO DOUBLE BONDS OR VICE VERSA</b>   |                             |              |      |             |      |        |
| 4.1.2.13  | ALDOLASE                    | ALS          | S    | 5H          | 5D   | 15D    |
| <b>ISOMERASES CATALYZING ISOMERIZATIONS</b>   |                             |              |      |             |      |        |
| 5.3.1.1   | TRIOSEPHOSPHATE ISOMERASE   | TPI          | RBC  | 2D          | 6D   | 3.5W   |
| 5.3.1.9   | GLUCOSE PHOSPHATE ISOMERASE | GPI          |      |             |      |        |
| SPEC=SPECIMEN, S=SERUM, U=URINE, U-D=URINE DIALYZED, CSF=CEREBROSPINAL FLUID, DF=DUODENAL FLUID, RBC=ERYTHROCYTES, H=HOUR, D=DAY, W=WEEK, M=MONTH, UN-S=UNSTABLE. |                             |              |      |             |      |        |

measurements of the activity of the heart LD isoenzyme in serum. The method of Wilkinson as modified by Rosalki appears to be the method of choice (39).

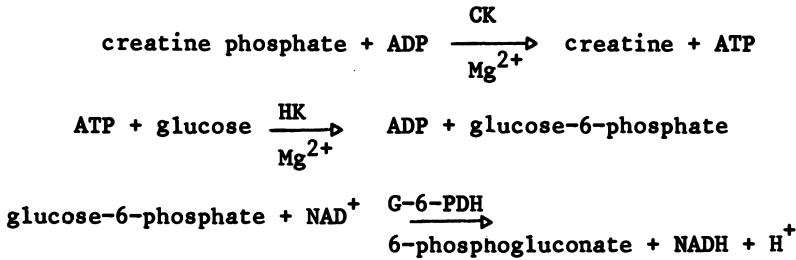
HBD is a biochemical rather than electrophoretic assessment of the LD isoenzyme which is associated with heart. All five isoenzymes of LD exhibit some activity toward  $\alpha$ -hydroxybutyrate as substrate, but heart LD shows the greatest activity. Serum HBD measurement is not as valuable as the electrophoretic determination of heart LD isoenzyme. High HBD activity has also been found in diseases of the liver. Rises associated with the hepatic effects of congestive heart failure can be disconcerting in the differential diagnosis of myocardial infarction. Wilkinson has used the serum HBD/LD ratio for the differentiation of myocardial disease from other disorders in which HBD activity is elevated, whereas Rosalki has not found the ratio to be helpful (39).

### Creatine Kinase (CK)

CK catalyzes the reversible phosphorylation of creatine in the presence of ATP and magnesium. When creatine phosphate is the substrate, the resulting creatine can be measured as the ninhydrin fluorescent compound, as in the continuous flow Auto Analyzer method. Kinetic methods based on coupled enzymatic reactions are also popular. Tanzer and Gilvarg (40) developed a kinetic method using the two exogenous enzymes pyruvate kinase and lactate dehydrogenase to measure the CK rate by following the oxidation of NADH. In this procedure the main reaction is run in a less favorable direction.



A kinetic procedure employing the reverse reaction is coupled to the enzymes hexokinase and glucose-6-phosphate dehydrogenase, as used by Nielson and Ludvigson after the method of Oliver (41). This procedure was later modified and optimized by Rosalki (38).



The ATP formed by CK is used by exogenous hexokinase to phosphorylate glucose. The resulting glucose-6-phosphate is then oxidized by exogenous glucose-6-phosphate dehydrogenase and NADP to 6-phosphogluconic acid and NADPH, a reaction measured kinetically at 340 nm. Several refinements of this procedure have been reported which include optimization of pH, substrate and exogenous enzyme concentrations, and CK activators such as sulfhydryl compounds (cysteine, glutathione, etc.). Adenosine monophosphate is included in order to inhibit any myokinase in the sample, as this enzyme also has CK-like activity. An interesting alternative is Worthington Biochemical's method, using an NADH dependent G-6-PD obtained from microbial sources. These coupled enzyme methods must employ a large excess of the exogenous enzymes, substrates and coenzymes in order to keep pace with the initial CK reaction, as otherwise spuriously low CK activities will be recorded. For this reason, the use of ninhydrin to monitor fluorimetrically the appearance of creatine appears to be the simplest and most accurate method (30).

CK has a dimeric structure composed of two subunits designated M and B (43), M for muscle and B for brain, with the isoenzymes being designated MM, MB and BB. The MM dimer is found in skeletal muscle and minimally in smooth and cardiac muscle. The BB dimer is found in brain and minimally in lung and smooth muscle (42). The MB isoenzyme of intermediate electrophoretic mobility is found chiefly in myocardium (43) and very little in skeletal muscle and smooth muscle.

The isoenzymes can be separated by electrophoresis on cellulose acetate, and Roberts, et al (44) have described a method whereby the separated isoenzymes are eluted and then assayed kinetically.

CK isoenzymes have been separated by electrophoresis on cellulose acetate, agar gel, agarose and polyacrylamide gel. Often these methods are not sensitive enough for quantitation of different CK isoenzymes. Konttinen and Somer (45) used electrophoresis on agarose to obtain sharper bands and increased sensitivity. Staining with nitro-blue tetrazolium was found to

be superior to other tetrazolium salts. Nerenberg et. al used electrophoresis on agar gel, and quantitated the separated bands by measuring the UV fluorescence of NADPH. This method has been slightly modified by Roe et. al (43) and Wagner et. al (46), who found it to be reproducible and easy but required a densitometer, fluorometer and one hour incubation time. Takahashi (47) developed a method for separating CK isoenzymes using a DEAE-Sephadex A-50 ion exchange column and a gradient elution buffer system. Mercer (48) modified this column procedure using a stepwise gradient elution. Worthington Corp. has packaged a method based on a minicolumn so that it is simple enough for routine use. The elution time (2-4 hours) usually associated with column chromatography has been shortened by the use of minicolumns and stepwise elution techniques. Need for pretreatment of sample (usually dialysis) has also been eliminated by equating the column's exchangeable chloride concentration to the chloride concentration of the serum. Results using these techniques are comparable to the results obtained with electrophoretic techniques. Henry, Roberts and Sobel (49) used batch absorption on "DEAE glycoPhase" glass beads instead of minicolumns, in order to use very small samples of serum. All separative steps are performed in a single test tube and can be completed in a few minutes. This method has been compared to their previously reported electrophoretic method and found to be comparable. Jockers-Wreton and Pfleiderer (50) have described another technique for the quantitation of CK isoenzymes based on specific antisera against the pure CK-MM and CK-BB isoenzymes from human tissue. They report the method is highly specific and sensitive and comparatively less time consuming, but these antisera are not available commercially.

Rao et. al recently published another method for the quantitation of the MB fraction. The method is based on the selective activating capacity of dithiothreitol on CK isoenzyme MB, after isoenzyme MM is activated by glutathione (51). Apparently the MB isoenzyme is not activated by glutathione but is activated by dithiothreitol. The difference between CK activities obtained in the presence of glutathione alone and those obtained with both glutathione and dithiothreitol represent MB activity. The correlation is excellent ( $r = 0.998$ ) between the activity of MB in the isoenzyme mixture determined by this method, and the activity of isolated MB.

CK is distributed in various organs with highest concentrations in skeletal muscle, myocardium, and brain and lesser amounts in the gastrointestinal tract, uterus, urinary bladder, and kidney (42). The CK content of liver and red blood cells is negligible so that diseases of these tissues are unlikely to increase the serum CK activity. The serum CK level begins to increase in 2-4 hours after myocardial infarction and reaches a peak in 24-36 hours and returns to normal in about 3 days. The maximum average elevation is about 7 times the upper limit

of normal. Compared to serum GOT, LD, and HBD, CK activity rises sooner, achieves a higher peak and returns to normal more rapidly. The degree of elevation is related to the magnitude of tissue necrosis and correlates well with the clinical course and prognosis. This makes the serum CK one of the most sensitive tests for myocardial infarction. The sensitivity and early elevation of serum CK following myocardial infarction may be helpful in the prompt selection of the patients with chest distress requiring the services of an intensive care unit. The transient nature of the CK elevation following infarction is a disadvantage and thus the timing of the blood samples is important. Although elevated serum CK activity is a sensitive indicator of myocardial necrosis, abnormal values may occur in a variety of extracardiac disorders such as muscle disorders, neurologic and psychiatric disorders, hypokalemia, pulmonary disease, hypothyroidism, prolonged coma, eclampsia, hemorrhagic pancreatitis, gangrene of the gallbladder, pancreatic carcinoma, diabetic ketoacidosis, electric countershock, urologic procedures, radiotherapy, sleep deprivation, and dissecting aneurysm. Increased physical activity and intramuscular injections should be considered as potential etiologic factors of abnormal CK levels in patients with chest pain, particularly when the ECG and other serum enzymes are not confirmatory. Galen et. al (52) studied the serum levels of GOT, HBD, CK and LD as well as isoenzymes of LD and CK in a large number of patients with and without myocardial infarction. They concluded that total GOT, HBD and CK activity in serum did not discriminate between two groups of patients with and without myocardial infarction well enough to be of diagnostic value in problem cases. On the other hand, the separation and identification of CK and LD isoenzymes provided the best discrimination between the two groups. Isoenzyme studies provide the only laboratory method currently available to predict accurately the presence or absence of an acute myocardial infarction. Combined CK and LD isoenzyme studies permit identification of the tissue source of an elevated total enzyme level, and in acute myocardial infarction it is possible to identify the myocardium as the specific source.

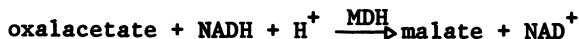
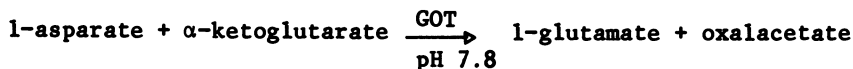
The only other condition which causes (MB) to appear in serum is muscular dystrophy. The MB isoenzyme appears in the serum of the patient with acute myocardial infarction within 6 hours and remains elevated for approximately 48-60 hours. As the acute episode resolves, the MB fraction decreases rapidly and is usually undetectable after 72 hours. Although (MM) remains elevated and the total CK activity may be above the normal range, it is important to recognize that the majority of the MM is also derived from myocardium. The LD and CK total activities and isoenzyme patterns are frequently normal up to six hours after onset of symptoms. Between 6 and 12 hours postinfarction there is usually an increased LD activity but normal isoenzyme pattern, while (MB) has already appeared. An

LD isoenzyme abnormality does not precede the appearance of the (MB) in the serum following myocardial infarction. The reappearance of (MB) is interpreted as diagnostic of reinfarction, and correlates with new electrocardiographic changes and an altered clinical course.

Wagner et. al (46) studied 376 patients to evaluate the importance of identification of the myocardial-specific MB isoenzyme in the diagnosis of acute myocardial infarction. An attempt was made to determine the incidence of falsely positive (MB). No acute infarction was diagnosed in all patients in whom neither total CK nor the isoenzymes of LD indicated myocardial necrosis. Incidence of falsely negative (MB) was zero in 55 patients. They concluded that determination of the isoenzymes of CK provides both a sensitive and specific indication of acute myocardial infarction.

#### Glutamic-oxalacetic Transaminase (GOT).

GOT (AST is the more recent abbreviation) catalyzes the transamination of l-aspartic acid in the presence of  $\alpha$ -ketoglutaric acid, with pyridoxal phosphate being a required co-enzyme. The reaction is:

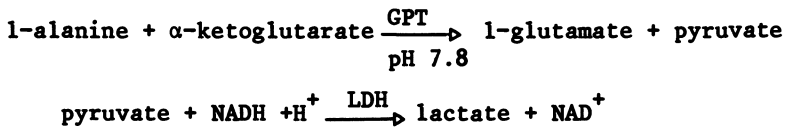


The product of oxalacetic acid was first measured by Karmen using a coupled enzyme method, with exogenous malic dehydrogenase and NADH. The decrease in absorbance at 340 nm is a measurement of the GOT activity when the exogenous enzyme and co-factor are present in large excess. This method, with some technical revisions (14,28) is still the method of choice. Colorimetric measurements of the product oxalacetate have been made using dinitrophenylhydrazine, but these methods have been shown to suffer from numerous systematic drawbacks which can markedly effect accuracy (14). For example, there are variable serum blanks, the substrate concentrations employed are far from optimal, the method is standardized with pyruvate, a substance which has no role in the transamination reaction, the kinetics are not zero order, and the measured absorbances are not proportional to increasing enzyme activity. Consequently, the dinitrophenylhydrazine methods cannot be recommended for general use. Methods

based on the azoene dyes Ponceau Fast L and Violet B have been described by Babson and co-workers and Klein and co-workers. The methods have been modified by Sax and co-workers and by Amador and Salvatore, who stabilized the final color by the addition of appropriate buffers at pH 3, optimized the substrate concentrations, and showed that acceptable correlation existed with the revised kinetic method (26). Automated continuous flow methods for these dyes, as well as for the kinetic method are now available (26).

### Glutamic-pyruvic Transaminase (GPT)

GPT (or ALT) catalyzes the following reaction:



The resulting pyruvate may be measured by coupling the reaction with lactic dehydrogenase and NADH, or chemically by using dinitrophenylhydrazine. The kinetic method has been revised by Henry and co-workers to optimize the analytical conditions, and still is the method of choice (28).

### Glutamyl Transferase (GMT)

This enzyme, also known as  $\gamma$ -glutamyl transpeptidase, catalyzes the transfer of the  $\gamma$ -glutamyl radical from a  $\gamma$ -glutamyl donor to a variety of acceptors, which may be amino acids or peptides, or many compounds such as glutathione, S-pyruvoyl, S-acetophenone and S-propanone. Derivatives of glutathione, dinitropropionitrile,  $\gamma$ -glutamyl aniline,  $\gamma$ -glutamyl -  $\alpha$ - naphthylamide, and  $\gamma$ -glutamyl- $\beta$ -naphthylamide, have been used as substrates. However, as some of these substrates are not sufficiently soluble to yield optimum substrate concentration, are carcinogenic (glutamyl aniline) or require long incubation time and many procedural steps to convert the enzymatic product into colored compound for colorimetric procedures, they have been abandoned. Orlawski introduced  $\gamma$ -L-glutamyl-p-nitroanilide as the substrate which is now widely used. It was also shown that addition of glycyl-glycine as a co-substrate in the GMT test provided an activating and buffering agent in the system. In 1969 Szasz (53) published a kinetic photometric method for serum GMT



in which he used both  $\gamma$ -glutamyl-p-nitroanilide and glycyglycine. The enzyme liberates p-nitroanilide with an absorption maximum around 405 nm. The GMT activity is directly proportional to the amount of p-nitroaniline liberated per unit time. This kinetic method is said to be accurate, rapid and precise and seems to be the method of choice.

The GMT in human serum reacts most rapidly with  $\gamma$ -glutamyl-p-nitroanilide at pH 8.2. The same activity is found in 2-amino-2-methylpropane-1:3 diol, diethanolamine, triethanolamine and tris buffers. Magnesium ions have no effect on the activity but favor the solubilization of the substrate. Bondar and Moss (54) found that free glutamate, due to elevated serum glutamate concentrations or glutamate released by substrate breakdown, increases the apparent GMT activity. They concluded that the assay should be performed in the presence of 1.0 mM/l glutamate in order to reduce the possibility of falsely elevated results. This was not observed by others. Rowe and co-workers have indicated that certain batches of p-nitroanilide substrate contain impurities which may reduce GMT activity and increase the  $K_m$  values (55). Huesby and Stromme (56) confirmed the presence of such impurities and recommended pyridine extraction for substrate purification.

In automated instruments such as the LKB the reaction is started by the addition of glycyglycine after preincubation of enzyme with substrate. But the substrate may undergo hydrolysis or internal transpeptidation during such preincubation, resulting in substrate concentration variation from sample to sample. Seiffert and Chaves (57) incubated the sample and substrate at pH 6.0, because at this pH the substrate is stable. When the glycyglycine is added the pH becomes optimal and the reaction starts. The substrate  $\gamma$ -glutamyl-nitroanilide is poorly soluble in many buffers and solvents. This has caused difficulty in attempts to adapt the enzyme reaction to automated or semiautomated enzyme analyzers (such as the LKB 8600 reaction rate analyzer) in which enzyme reaction must be started by a small volume of concentrated substrate solution. Such difficulties are more pronounced at 37°C, for at this temperature the substrate  $K_m$  is almost double that at 25°C and its optimal concentration is significantly greater. Rosalki (58) observed that  $\gamma$ -glutamyl-p-anilide is highly soluble in HCl. The enzyme reaction is initiated using a concentrated substrate solution in 0.5 mM HCl, after preincubation of samples at 37°C with Tris-glycyglycine buffer at a pH adjusted to yield optimal pH upon the addition of the acid substrate solution. With this procedure, it is possible to carry out the reaction at much higher substrate and acceptor concentrations.

### Alkaline Phosphatase (ALP)

Alkaline phosphatase catalyzes the dephosphorylation of a number of artificial substrates (2) including  $\beta$ -glycerophosphate, phenylphosphate, p-nitrophenylphosphate, thymolphthalein phosphate, and phenolphthalein phosphate. In addition, as shown recently for bacterial and human enzymes, alkaline phosphatase simultaneously catalyzes the transphosphorylation of a suitable substance which accepts the phosphoryl radical, thereby preventing the accumulation of phosphate in the reaction medium (25).

This acceptor is absent in most of the earlier assays, but commencing with the introduction for clinical use of the kinetic p-nitrophenylphosphate method buffered by 2-amino-2-methyl-1-propanol in 1963 (18), the use of a phosphoryl acceptor as the buffering agent has gained wide acceptance, leading to the introduction of diethylamine (DEA), and of 2-ethyl-amino-ethanol (EAE) (25) as the activating, phosphorylatable buffers. The degree of phosphorylation of the buffer correlates well with the degree of activation of the alkaline phosphatase activity (Table V). Relatively high concentrations of buffer (1-2M) and non-physiological pH's (pH 10) are necessary (Fig. 5). In the human body, alkaline phosphatase must handle an unknown substrate, probably at a much lower turnover, and may transfer the liberated phosphoryl radical to another as yet unidentified physiological acceptor, possibly ethanolamine.

As in the case of acid phosphatase, the analyst may measure the liberated phosphate or the liberated organic radical. It is easier to measure the liberated organic radical, particularly if it is chromogenic, as in the case of p-nitrophenol, phenolphthalein, or thymolphthalein. These latter two substrates, while analytically valid, suffer from the defect of high cost and proprietary ownership. p-Nitrophenyl phosphate, the most chromogenic substrate, is widely available, and hence methods based on it are the methods of choice, particularly when an activating phosphorylatable buffer is employed. Magnesium ion has been said to activate alkaline phosphatase, but this reviewer has repeatedly observed no activation by this ion (Fig. 6). Surprisingly, the recently reported high concentrations of substrates which leads to maximum velocity, (20mM/l) of either phenylphosphate or nitrophenylphosphate are considerably higher than those initially reported (25). No ready explanation is available for this discrepancy, but it is possible that modern substrate preparations are free of impurities such as phosphate or phenol, which are known inhibitors of the enzyme.

When phenyl phosphate is the substrate, the released phenol is conveniently measured using the reaction described by Powell and Kind and King based on 4-amino antipyrine (4-AAP) and ferricyanide. It is possible to incorporate the 4-AAP directly into the buffered substrate mixture, so that color can be developed

**TABLE V**  
**BUFFER ACTIVATION OF SERUM ALKALINE PHOSPHATASE**

| <b><u>BUFFER</u></b>        | <b><u>RELATIVE ACTIVITY</u></b>          |
|-----------------------------|--|
|                             | <b>Phenyl Phosphate Substrate</b>        |
| Carbonate                   | 1.0                                      |
| 2-Amino-2-Methyl-1-Propanol | 2.51 $\pm$ 0.24                          |
| Diethanolamine              | 6.65 $\pm$ 0.41                          |
| 2-Ethylaminoethanol         | 7.03 $\pm$ 0.41                          |
| <hr/>                       |  |
|                             | <b>p-Nitrophenyl Phosphate Substrate</b> |
| Glycine                     | 1.0                                      |
| 2-Amino-2-Methyl-1-Propanol | 3.13 $\pm$ 0.04                          |
| Diethanolamine              | 7.21 $\pm$ 0.17                          |
| 2-Ethanolamine              | 9.37 $\pm$ 0.12                          |

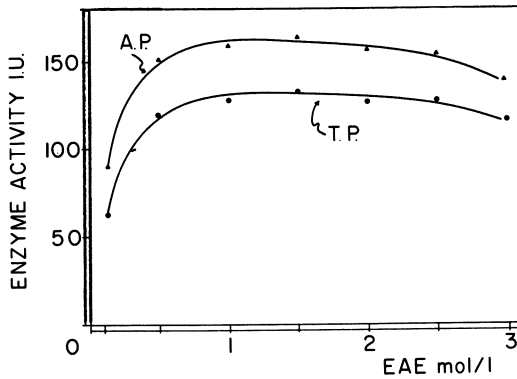


Figure 5. The increase in serum alkaline phosphatase activity (ALP) parallels the increase in transphosphorylase activity (TP) produced by increasing the molarity of 2-ethylaminoethanol (2-EAE).

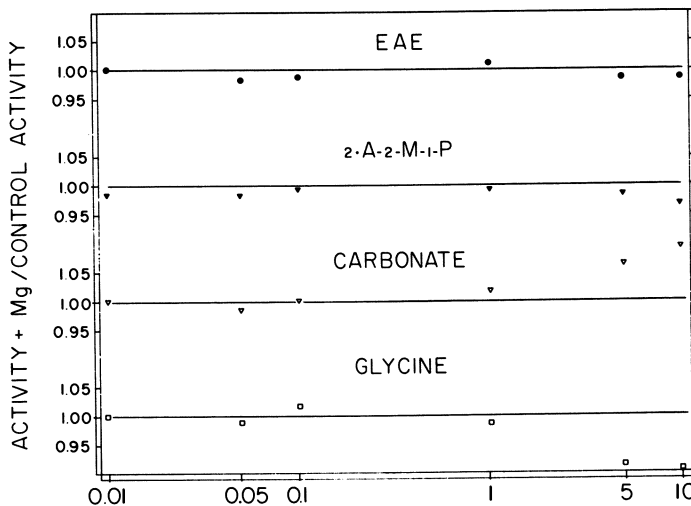


Figure 6. Magnesium in concentrations of 0.001–10 mM/l. produces no significant activation of alkaline phosphatase in EAE, DEA, or 2A2M1P buffers. It activates the enzyme in carbonate buffer but inhibits it in glycine buffer.

by the simple addition of ferricyanide solution (Table VI).

Alkaline phosphatase assays based on  $\beta$ -glycerophosphate now appears to be obsolete, and methods buffered by either glycine or barbital are also obsolete as these buffers inhibit ALP or are poor buffers. Serum alkaline phosphatase is known to be composed of several isoenzymes which presumably arise from bone, liver, intestine, and placenta. The placental alkaline phosphatase is known to be much more resistant to heat denaturation than the other isoenzymes, and this resistance provides a simple test for it (5). The other enzymes can be separated through the differential inhibition by phenylalanine, by electrophoresis and by specific antibodies. However, the clinical usefulness of the results obtained is in doubt (23).

### Diseases of the Liver

Serum enzymes have been used extensively in the detection and differential diagnosis of diseases of the liver and biliary tract as the latter are often curable by surgery, while diseases of the liver usually are not, the differential diagnosis between these two groups of diseases is of considerable clinical importance. The task is aided somewhat because the liver cells are different in their enzymatic content from the cells lining the bile duct. However, pathologic processes often involve both type of cells, thereby obscuring the diagnostic boundaries.

The enzymes found in liver cells (Group I enzymes) include more than a dozen enzymes used in diagnostic laboratories, but those used most commonly are the transaminases (GOT and GPT), which continue to be the most widely used indicators of liver cell integrity. Enzymes found in the biliary cells (Group II) include alkaline phosphatase, glutamyl-transferase, leucine aminopeptidase and 5-nucleotidase.

### Liver Cell Enzymes

In liver cells the activity of GOT is higher than that of GPT, but most of the GPT activity is located in the cytoplasm and therefore leaks more readily into the blood stream with minor or reversible cell damage. Enzymes located in the mitochondria, such as one of the GOT isoenzymes appear in serum only when there has been more severe liver cell injury including cell death. While considerable amounts of both GOT and GPT are found in cardiac muscle, skeletal muscle and kidney, differential diagnosis is aided by the fact that the liver shows a much higher total GPT activity. An important clinical application of measurements of transaminase activity is the detection and diagnosis of viral

**TABLE VI**  
**EVOLUTION OF SERUM ALKALINE PHENYL PHOSPHATASE METHODS**

| <u>STEP</u> | <u>KING-ARMSTRONG</u>                | <u>KIND-KING</u>                     | <u>ROOS</u>                          | <u>HANSEN</u>                                   | <u>AMADOR-URBAN</u>                                 |
|-------------|--------------------------------------|--------------------------------------|--------------------------------------|---|---|
| 1.          | ADD SERUM TO CARB BUFFERED SUBSTRATE | ADD SERUM TO CARB BUFFERED SUBSTRATE | ADD SERUM TO CARB BUFFERED SUBSTRATE | ADD SERUM TO CARB BUFFERED SUBSTRATE WITH 4-AAP | ADD 20uL SERUM TO EAE BUFFERED SUBSTRATE WITH 4-AAP |
| 2.          | INCUBATE 37° 30MIN                   | INCUBATE 15MIN                       | INCUBATE 22MIN                       | INCUBATE 15MIN                                  | INCUBATE 10MIN                                      |
| 3.          | ADD PHENOL REAGENT                   | ADD NaOH                             | ADD 4-AAP                            | ADD FERRICYANIDE                                | ADD BUFFERED FERRICYANIDE                           |
| 4.          | FILTER                               | ADD NaHCO <sub>3</sub>               | ADD FERRICYANDIE                     | READ ABSORBANCE                                 | READ ABSORBANCE                                     |
| 5.          | ADD FILTRATE TO SODIUM CARBONATE     | ADD 4-AAP                            | READ ABSORBANCE                      |   |   |
| 6.          | INCUBATE AT 37° FOR 5MIN             | ADD FERRICYANIDE                     |                                      |   |   |
| 7.          | READ ABSORBANCE                      | READ ABSORBANCE                      | REAGENT BLANK ONLY                   | REAGENT BLANK ONLY                              | REAGENT BLANK ONLY                                  |

4-AAP = 4-AMINO ANTIPIRINE, CARB = CARBONATE-BICARBONATE,  
EAE = ETHYL-AMINO-ETHANOL (OR 2-DIETHANOLAMINE) AS PHOSPHORYLATABLE BUFFER  
(BASED ON REFERENCES 2,25,32)

hepatitis, as the marked elevations of GOT and GPT activities reflect the clinical course of the disease. Persons with sub-clinical viral hepatitis often have mild elevations of the serum GPT activity.

Another common liver disease, alcoholic liver damage produced by moderate to heavy alcoholic intake, is also reflected by an elevation of the serum GOT and GPT activities. The serum glutamyl transferase activity is reported to be a sensitive index of alcoholic intake and can serve to monitor persons on alcoholic withdrawal programs (60). The LD-5 isoenzyme arises mainly from liver tissue, but has a short half-life (61), which is about 1/5 and 1/2 of the half life of the transaminases, GPT and GOT respectively. Some authors consider that a normal LD-5 isoenzyme activity in a jaundiced patient is sufficient evidence to exclude primary liver disease and that obstruction is probably responsible for the jaundice (62). In hemolytic jaundice the LDH-1 and 2 isoenzymes are elevated.

The Group II (biliary tract) enzymes are abnormal usually when the serum bilirubin concentration is also abnormal. Most commonly used is alkaline phosphatase which is a highly sensitive indicator of biliary tract obstruction, perhaps because the enzyme is synthesized as an induced response to obstruction of even small bile ducts. Most techniques used to identify the origin of an elevated serum alkaline phosphatase are not very useful from a clinical viewpoint (23). The simultaneous measurement of GMT activity has been found to be useful in differentiating between the hepatic and bony origin of alkaline phosphatase. An increased GMT activity in a patient with an increased ALP activity is a good indication that there is biliary biliary tract disease (62,63).

Solberg and co-workers have applied discriminate analysis of clinical laboratory tests combined with careful clinical and anatomic diagnoses of liver disease in order to determine which combinations of the many dozen liver diagnostic tests available are the best (64). These authors found that the measurement of GPT, GMT, GOT, ALP and ceruloplasmin were the most useful enzymatic tests, when combined with other non-enzymatic tests such as the measurement of bilirubin, cholesterol, hepatitis-B associated Australian antigen, etc. Another group of highly useful enzymes, not discussed in this review, are those clotting factors and the enzyme cholinesterase which are synthesized by the liver cells.

Reduction in liver cell mass or diffuse liver cell damage often results in a decreased activity of blood clotting enzymes in the blood plasma. The lack of quantitative methods resulting from the lack of chromogenic substrates and rate reaction methods for the measurement of the clotting factors, together with the lability of many of these clotting factors has restricted up to now the ready diagnostic application of the measurement of most clotting factor activities.

### Amylase (AMS)

Amylase catalyzes the splitting of starch and similar polysaccharide macromolecules into smaller polysaccharides, finally leading to maltose (65). It has the distinction of being the first enzyme activity in body fluids used for the diagnosis of disease, and its activity has been measured clinically during most of this century (1). All the traditional methods use starch solubilized through heat and suspended in a buffered solution. The iodometric method is classic for measuring the starch remaining after enzymatic attack. Starch solubilization is said to be enhanced by dimethylsulfoxide. In recent years, a variety of other polysaccharide substrates coupled to dyes have been used, thus as the amylase hydrolyses the substrates, dye is released into solution. The unhydrolyzed substrate is precipitated or separated by centrifugation, and the amount of dye liberated into solution is then measured.

The voluminous literature concerning the assay of amylase activity reflects general dissatisfaction with available methods. The major problem associated with measuring amylase activity is standardization of a substrate of known and reproducible composition and concentration. Starches are heterogenous in both molecular weight and degree of branching, and both can profoundly affect the susceptibility of the starches to amylolytic attack. Starches in water form a colloidal suspension whose micelles vary in size and deteriorate rather rapidly with mold contamination. At lower temperatures starch in solution forms large micelles which are less susceptible to amylase hydrolysis. A wide variety of methods for the estimation of amylase has been employed based mainly on the measurement of decrease in viscosity of a starch solution, decrease in turbidity of a starch suspension, decrease in starch iodine color (amylolytic), or increase in the reducing power of the starch solution (saccharogenic). Amylolytic assays are reproducible and simple, but some sera and urines contain material that inhibits formation of the starch iodine color so that it is not always a true estimate of the starch actually present.

The saccharogenic procedure of Somogyi modified by Henry and Chiamori (65) still serve as the reference method, but suffers from lack of standards, poor precision with normal activities, poor sensitivity and inadequate substrate stability. Recently Shipe and Savory (66) have used a stable liquid starch in which the optical brightener is absent. This suspension of oxidized corn starch contains antifoam (dimethylpolysiloxane) lubricant, sodium tetraborate as the preservative, and Tris buffer. Shipe and Savory measured the light scattering with a fluorometer whose kinetic reading was recorded at 37° C for 12 minutes. The method was precise with day to day variation of 3.4%. The method correlated well with the Phadebas TM amylase test which will be discussed later. The



key to the nephelometric measurement of starch was the preparation of a stable colloidal suspension that does not settle out during the test run. Zinterhofer using a similar approach has used known formazan turbidity standards to establish the amount of light scattering thereby avoiding secondary enzyme standards (67). The rate of decrease in turbidity which is expressed in formazan turbidity units (FTU) per min. per ml of sample under specified conditions is proportional to the enzyme activity. The method measures amylase in serum or urine in 2 to 4 minutes using 50 ul of sample. The substrate is amylopectin dissolved in dimethylsulfoxide. Amylase activity expressed in FTU is approximately half that when expressed as Somogyi units per 100 ml. The method is linear up to 600 FTU. According to Seligson's group this is a fast method for emergency diagnoses.

In the late 1960's a new series of methods was introduced for the determination of amylase, involving the use of a starch-dye complex. Dyes such as Renazol brilliant blue (68) Reactive Red 2B (69) (used in the substrate Dy-Amyl, General Diagnostic Division, Warner-Chilcott Laboratories), Cibachrom Blue (70) (used in Amylochrome, Roche Diagnostic Division of Hoffman-La Roche Inc.) and other dyes (71) are bound to amylopectin or amylose. Most of these substrates are available commercially and many are being used on a routine basis.

In the Phadebas TM amylase test (72) (Pharmacia Labs) the substrate was a water insoluble cross-linked blue starch in tablet form which also contains some inert ingredients, sodium and potassium phosphate buffer salts and sodium chloride. This polymer was hydrolyzed by amylase into water soluble blue starch fragments. After centrifugation the absorbance of the blue supernatant was proportional to the activity of amylase present in the test samples. The day to day variation on a quality control serum had a coefficient of variation of 2.7% based on 30 days of data in our laboratory. The method is simple, reproducible and uses microquantities of serum.

Clinically, measurements of amylase in serum and urine, and measurements of lipase in serum, have been the most useful tests in the assessment of pancreatic disease, particularly in acute pancreatitis and in the recurrent attacks of chronic pancreatitis. The highest concentration of human amylase is found in the parotid (salivary) gland and pancreas. Liver, oviduct mucosa, skeletal muscle, gastric and small intestinal mucosa and red blood cells also have some amylase activity. The amylase in normal serum is of heterogeneous origin, and the major isoenzymes are from pancreas and parotid gland. Gamklon and Schersten (73) showed human liver tissue contains only traces of amylase activity. Hence the increased serum activity occasionally found in cases with liver or bile duct disease probably originates from other sites.

Since pancreatic and salivary isoamylases differ from one

another in electrophoretic mobility, determination of isoenzymes of amylase in serum or urine may disclose the origin of an elevated serum amylase activity. Electrophoretic separation has been performed on agar, agarose, poly-acrylamide gel and cellulose acetate and earlier also on paper. Skude (74) has developed an electrophoretic separation technique on agarose as a supporting media which he claimed to be the media of choice, because of its high resolving power and ease of handling. The method is claimed to be simple and can be performed routinely in a clinical laboratory. Takeuchi et. al (75) separated isoenzymes of amylase in human serum, urine, pancreatic juice and saliva by electrofocusing. The amylases in serum were separated into two major isoenzymes with isoelectric points of pH 6.4-6.5 (saliva) and pH 6.9-7.0 (pancreas) respectively, and one minor isoenzyme with an isoelectric point of pH 5.9 of unknown origin.

Amylase enters the blood largely via the lymphatics. An increase in hydrostatic pressure in the pancreatic ducts leads to a fairly prompt rise in the amylase concentration of the blood. Neither an increase in volume flow of pancreatic juice nor stimulation of pancreatic enzyme production will cause an increase in serum enzyme concentration. Elevation of intraductal pressure is the important determinant. Stimulation of flow in the face of obstruction can, however, augment the entry of amylase into the blood, as can disruption of acinar cells and ducts. A functional pancreas must be present for the serum amylase to rise. Serum amylase determination is indicated in acute pancreatitis, in patients with acute abdominal pain where the clinical findings are not typical of other diseases such as appendicitis, cholecystitis, peptic ulcer, vascular disease or intestinal obstruction. In acute pancreatitis, the serum amylase starts to rise within a few hours simultaneously with the onset of symptoms and remains elevated for 2 to 3 days after which it returns to normal. The peak level is reached within 24 hours. Absence of increase in serum amylase in first 24 hours after the onset of symptoms is evidence against a diagnosis of acute pancreatitis (76).

Various conditions such as perforated peptic ulcer, cholecystitis, common bile duct and intestinal obstruction, trauma to the abdomen inducing pancreatitis and ruptured ectopic pregnancy may cause an elevated serum amylase but the levels are usually not as high as those found in acute pancreatitis. Mumps and bacterial parotitis, which block the secretion of salivary amylase are associated with mild elevations of serum amylase.

Opiates and other narcotics and analgesic drugs may unpredictably elevate the serum amylase. The elevation may last up to 24 hours. Therefore it is important that blood for amylase determination be drawn before giving the patient analgesic drugs for pain. Elevation of the enzyme in peritoneal fluid is strong

evidence of acute pancreatitis (77).

The rate of urine amylase excretion is a sensitive reflection of the amylase released into the blood. The urine amylase remains abnormal 1-2 weeks after the serum returns to normal because the renal clearance of amylase rises 3-fold in acute pancreatitis and takes 1-2 weeks to return to normal. In pancreatitis, a number of investigators have reported a higher percentage of urinary amylase elevations, as contrasted with serum amylase elevations, particularly when the urinary amylase output over an interval is measured. Random urine collections for one, two and 24 hours are 792-4264 (2926±1074 S.D.) units per 24 hours. However, the wide range of normals make interpretation of results difficult. Of 107 patients with elevated serum or urine amylases, 16 were found to have a normal pancreas at operation (78).

The study of the mechanism of urinary excretion of amylase and the amylase clearance has been the subject of many studies in recent years. Levitt et. al (79) studied the renal clearance of amylase in renal insufficiency, acute pancreatitis and macro-amylasemia. In acute pancreatitis, the kidney cleared amylase at a markedly increased rate. The ratio of the amylase clearance rate to the creatinine clearance rate ( $C_{am}/C_{Cr}$ ) averaged 3 times normal early in the course of acute pancreatitis, and this elevation could persist after the serum amylase returned to normal. Comparison of amylase clearance to creatinine clearance was to minimize irrelevant changes due to variation in renal function. The increased clearance of amylase makes the urinary amylase a more sensitive indicator of pancreatitis. The ratio of  $C_{am}/C_{Cr}$  can be determined on a random urine sample and thus avoids the time, labor, and errors involved in collecting the timed urine specimen required for the urinary amylase determination.

Possible causes of increased ratio of  $C_{am}/C_{Cr}$  in pancreatitis can be liberation of a more readily excretable form of amylase, a protein with a molecular weight of 55,000 or less which can be easily filtered via the glomerulus and undergoes no appreciable tubular reabsorption.

Warshaw and Fuller (80) studied the specificity of increased renal clearance of amylase in acute pancreatitis. They established  $3.1\% \pm 1.1$  S.D. for the  $C_{am}/C_{Cr}$  ratio in normal persons. In patients with acute pancreatitis this ratio rises regularly and significantly to  $9.8 \pm 3.5$  S.D. They suggested that renal permeability to amylase is altered in acute pancreatitis and provides the basis for using the  $C_{am}/C_{Cr}$  ratio in the differential diagnosis of hyperamylasemia. Levine et. al (81) observed an increased clearance ratio in situations other than acute pancreatitis both with and without elevated serum amylase activity. They indicated that this ratio may be increased in patients with diabetic ketoacidosis or extensive burns who also present no clinical or other evidence of associated acute pan-

creatitis. Levitt and Cooperband (82) in an editorial suggested that marked elevations of this ratio perhaps result from an increase in glomerular permeability, and that small changes in glomerular permeability result in large changes in renal amylase clearances.

### Lipase (LPS)

Numerous workers have found that measurements of serum lipase activity are useful in the diagnosis of pancreatitis (83, 84, 85). Despite this, serum lipase determinations are not usually performed in clinical laboratories, probably due to inherent problems associated with the conventional methods, based on an emulsified lipid substrate. The methods are also not very suitable for manual batch analysis nor for automation due to laborious post incubation procedures.

Cherry and Crandall in 1932 (86) used olive oil as substrate with gum acacia as the emulsifier. This method has served as the basis for a number of modifications that increased the stability of the emulsion, decreased incubation time and gave better precision. When a serum sample is incubated with a stabilized olive oil emulsion, lipase acts at the interface of substrate and water to hydrolyze olive oil into fatty acid plus diglycerides, and to a small extent to monoglycerides and glycerol. The bile salt sodium deoxycholate activates the reaction. These methods measure the liberated fatty acids by titration with a standardized NaOH solution. An indicator such as phenolphthalein, thymolphthalein or methyl red or a pH meter are used to detect the end point.

Lippi et. al (87) and Dirstine (88) circumvented titration by converting the liberated fatty acids into copper salts, which after extraction in chloroform are reacted with diethyldithiocarbamate to form a colored complex which is measured photometrically. While the end point appears to be more sensitive than the pH end point determination, the advantages are outweighed by the additional steps of solvent extraction, centrifugation and incomplete extraction when low concentrations of copper salts are present. Other substrates used for the measurement of lipase activity have been tributyrin (89), phenyl laurate (90), p-nitrophenyl-stearate and  $\beta$ -naphthyl laurate (91). It has been shown that these substrates are hydrolyzed by esterases and thus lack specificity for lipase. Studies on patients with pancreatitis indicate olive oil emulsion is definitely superior to water soluble esters as substrates for measuring serum lipase activity.

Several turbidimetric methods (92, 93) have been described specifically for the kinetic determination of lipase in serum but these methods suffer from lack of linearity with increasing enzyme concentration, and instability of emulsions.

Recently Shihabi and Bishop (93) described a refinement in the preparation of a stable substrate and demonstrated the feasibility monitoring the reaction kinetically. This procedure has been evaluated by Lifton et. al. (94), who found that this method correlated well ( $r = 0.914$ ) with the copper soap-lipase method of Dirstine. They concluded that the method was rapid (less than 5 min. per sample), accurate, precise and linear over a clinically useful range. Its simplicity allows its application as an emergency procedure. Attempts to use this assay for urine lipase activity were unsuccessful.

Seligson's group (95) has published a similar turbidimetric procedure but used nephelometry to measure continuously the effect of lipase on the light scattering of an olive oil emulsion. The instrumentation and approach is the same as that described above for the nephelometric determination of amylase. The method according to the authors is fast and precise with good specificity and sensitivity. The short time required for analysis makes it suitable for emergency use. The technical simplicity permits this method to be easily automated, and it appears to be the lipase method of choice.

### Acid Phosphatase (ACP)

Acid phosphatase catalyzes the dephosphorylation of an artificial organic substrate, such as B-glycerophosphate, phenylphosphate or thymolphthalein monophosphate. The analyst may measure either the phosphate or the organic radical liberated. In the older  $\beta$ -glycerophosphate method, phosphate was measured, but in all other methods the organic radical is measured. This has the advantage of considerably simplifying the methodology, increasing sensitivity and improving specificity. At present, the method which uses sodium thymolphthalein phosphate as a substrate, described by Roy and co-workers, appears to be the best documented and most specific for prostatic disease (96). After incubation of serum and substrate at a slightly acid pH, the reaction is stopped and the product color is brought out by the simple addition of an NaOH solution.

The adult male prostate contains abundant acid phosphatase which it secretes into the semen. The production of this enzyme is governed by the circulating levels of androgenic hormones. Castration or estrogen administration markedly reduces the prostatic urinary acid phosphatase of males. Other organs such as the liver, kidney, spleen, red cells and platelets also contain significant amounts of acid phosphatase.

Measurement of serum acid phosphatase activities as an aid in the diagnosis and treatment of advanced prostatic carcinoma is based on the observation by Gutman and Gutman that the activity is elevated by skeletal metastases (3). Many workers have

confirmed that the serum acid phosphatase activity is elevated in 50 to 75% of patients with skeletal metastases, in about 30 to 40% of patients with soft tissue invasion, and in about 10 to 20% of patients with carcinoma localized within the prostatic capsule. Remissions in metastatic growth, such as those produced by estrogen therapy, are usually but not always reflected by a fall in this serum activity. Schwartz, Greenberg, and Bodansky on the basis of studies of four patients over several months suggested that elevated activities of serum isocitric dehydrogenase and phosphohexose isomerase correlate better with metastatic growth than those of serum acid or alkaline phosphatase (97).

Nodular hyperplasia of the prostate is usually associated with a normal serum acid phosphatase activity. Complications such as acute urinary obstruction or prostatic infarction will elevate this serum activity for several days as will cystoscopy and catheterization (98). Digital palpation of the prostate may result in an elevation which subsides within a few hours. The substrate phenyl phosphate, which is hydrolyzed by the serum acid phosphatases originating from many tissues, has been used in most of the published studies. Total serum acid phenylphosphatase is elevated in diseases of the liver, disease of bone such as Paget's disease, and several blood dyscrasias, especially those involving platelets (99,100).

Red blood cells also contain sufficient acid phenylphosphatase for mild hemolysis to cause false elevations. Therefore, inhibitors such as ethanol, formaldehyde, copper sulfate, and l-tartrate have been used to inhibit selectively the enzyme of one or more tissues and enhance the specificity of the test (101). Ethanol is unsuitable because it inhibits the enzyme from erythrocytes and prostate simultaneously, and because it yields serum activities which correlate poorly with prostatic disease. Formaldehyde inhibits the erythrocytic enzyme and has been said to yield clinically satisfactory results. The copper resistant acid phosphatase of serum is elevated by metastatic carcinoma of the breast, as well as by other metastatic cancers, and is also elevated by a wide variety of non-cancerous diseases.

l-Tartrate is a most widely used inhibitor of prostatic acid phenylphosphatase activity. With this inhibitor Bonner and associates detected five cases of unsuspected carcinoma of the prostate in 221 hospital patients and clarified the diagnoses in another four patients (98). Nonetheless, the diagnostic specificity of an elevated l-tartrate inhibited activity is not absolute, as Fishman et. al found it elevated in 48 of 1,190 males without cancer. Whitmore and associates also observed this activity to be elevated in 3 of 20 patients with uncomplicated nodular hyperplasia (102). Hill compared the total versus the "prostatic" serum activities in 20 patients with localized untreated carcinoma of the prostate and observed

no significant difference in the incidence of elevated activities as measured by two methods (103). Langley also observed a significant incidence of false positive activities (99).

$\beta$ -glycerophosphate has been reported to be a specific substrate for an elevated serum acid phosphatase activity of prostatic origin. The diagnostic usefulness of this method had not been examined critically until Marshall and Amador tested it in 117 patients with carcinoma and in 63 patients with nodular hyperplasia of the prostate (100). The  $\beta$ -glycerophosphatase activity was elevated in 46% of patients with intracapsular carcinoma and in 56% with extracapsular invasion or soft tissue metastases. In 82% with skeletal metastases, the activity was elevated. In all of the cases, the initial clinical diagnosis of prostatic carcinoma had been made on the basis of rectal examination, x-ray findings or histologic examination of resected prostatic tissue. Surprisingly, in no case was prostatic carcinoma first disclosed by elevated activity of serum acid phosphatase. Moreover, the normal activity in a significant proportion of patients with skeletal metastases did not appear to hinder the establishment of a correct diagnosis by other means.

An elevated activity of serum acid  $\beta$ -glycerophosphatase was not specific for carcinoma, as Marshall and Amador found it in 8% of patients with uncomplicated prostatic hyperplasia, in some patients with bone fractures, carcinoma involving the liver, and other diseases of bone and liver. This activity has also been reported to be elevated in 3 of 12 patients with Gaucher's disease.

Babson proposed  $\alpha$ -naphthyl phosphate as an essentially specific substrate for the activity of prostatic acid phosphatase in serum (104). However Marshall, Price, and Amador found that this substrate is not specific for the prostatic enzyme because urine of human females contain 50 times more acid  $\alpha$ -naphthyl phosphatase than male serum and 50% as much activity as male urine. Platelets have significant activity and the serum activity can increase to abnormal values following clotting. These workers also observed elevated activities in females with skeletal metastases of the breast. In 50 hospitalized male patients who had no evidence of prostatic cancer and 25 hospitalized female patients, the incidence of false positive results was 12%, a magnitude sufficient to preclude meaningful clinical interpretation (105).

## Renin

Renin is released by the juxta-glomerular cells when kidney perfusion is impaired. Reduction of the blood volume markedly stimulates release of this enzyme into the blood. The diuretic drug furosemide also markedly stimulates the release of renin,

through unknown mechanisms, with the renin activity reaching a peak in peripheral blood five hours after drug administration. The adrenal hormone aldosterone markedly inhibits the release of renin. Based on these observations, it has been proposed that a standardized test for patients with arterial hypertension be the measurement of serum renin activity 5 hours after administration of furosemide. Initial studies have shown that patients with aldosterone secreting tumors have extremely low or zero stimulated renin activities; patients with other corticosteroid secreting adrenal tumors have low renin activities, patients with idiopathic hypertension have normal activities, and patients with renal vascular hypertension have elevated renin activities. The renin activities were measured using the radio-immunoassay method of Haber, in which the amount of angiotensin I released from angiotensinogen is measured after a suitable period of incubation. The converting enzyme is inhibited to prevent the conversion of angiotensin I into angiotensin II (106). This method is widely accepted and is commercially available.

#### Glucose-6-phosphate Dehydrogenase (GPD)

GPD in presence of NADP catalyzes oxidation of glucose-6-phosphate to 6-phosphogluconolactone plus NADPH which is quickly converted to 6-phosphogluconate. Human serum and RBC also contain variable amounts of phosphogluconate dehydrogenase (PGD) which in presence of NADP catalyzes the conversion of 6-phosphogluconate to ribulose-5-phosphate with the production of another mole of NADPH. Therefore, with appropriate conditions a single mole of glucose-6-phosphate may result in the production of 2 moles of NADPH. The activity of GPD, has been measured by three approaches:

1. Based on the difference in the rate of NADPH production with both glucose-6-phosphate and 6-phosphogluconolactone as substrates and the rate of NADPH production with only 6-phosphogluconolactone as its substrate (107).
2. Based on the inhibition of PGD by 2,3-diphosphoglycerate (108).
3. An excess of PGD is added to the reaction mixture containing glucose 6-phosphate and NADP to assure that 2 moles of NADPH are produced per mole of glucose-6-phosphate oxidized (109). This method has been improved by Nicholson et. al (110) to make it micro, accurate and rapid. Catalano and co-workers (111) have compared the above three approaches and concluded that in terms



of relative reagent cost the third approach is more economical.

The multiple human variants of G-6-PD and the relationship of their kinetic properties to the presence of hemolytic anemia under conditions which closely simulate the erythrocyte internal environment have been elegantly studied by Yoshida (112). This author found that those enzyme variants with significantly reduced physiological activity are those associated with hemolytic anemia.

### Literature Cited

1. Wohlgenuth, J.; Beitrag zur funktionellen Diagnostik des Pankreas. Berliner klin. Wschr. (1910), 47, 92-98.
2. King, E. J.; and Armstrong, A. R.; A convenient method for determining serum and bile phosphatase activity. Canad. Med. Ass. J. (1934), 31, 376-381.
3. Gutman, A. B.; and Gutman, E. B.; An "acid" phosphatase occurring in the serum of patients with metastasizing carcinoma of the prostate gland. J. Clin. Invest. (1938), 17, 473-478.
4. Calvin M.; Chemical Evolution. Oxford University Press. New York, 1969.
5. Stadtman, E. R.; The role of multiple enzymes in the regulation of branched metabolic pathways. Ann. N.Y. Acad. Sci. (1968), 151, 516-530.
6. International Union of Biochemistry: Enzyme Nomenclature. Elsevier, Amsterdam. 1965, p.7.
7. Warburg, O.; Christian, W.; Giese, A.; Wasserstoffubertragendes co-ferment, seine zusammensetzung und wirkungsweise. Biochem. Z. (1935), 282, 157-205.
8. Amador, E.; Pippert, J. A.; Emary, R. J.; Serum creatinine and glucose analyses by an automated rapid sampling digital system. Clin. Chem. (1971), 17, 655.
9. Scott, C. D.; Burtis, C. A.; A miniature fast analyzer system. Anal. Chem. (1973), 45 (3), 327A- 340A.
10. Skeggs, L. T.; New dimensions in medical diagnosis. Anal. Chem. (1966), 38 (5), 31A-44A.
11. Schneider, A. J.; Willis, M. J.; Sources of variation in a standardized and semi-micro procedure for serum glutamic oxalacetic transaminase. Clin. Chem. (1958), 4, 392-408.
12. Atwood, J. G.; DiCesare, J. L.; Making enzymatic methods optimum for measuring compounds with a kinetic analyzer. Clin. Chem. (1975), 21, 1263-1269.
13. Malmstadt, H. V.; Cordos, E. A.; Delaney, C. J.; Automated reaction rate methods of analysis. Anal. Chem. (1972), 44 (12), 26A- 41A.

14. Amador, E.; and Wacker, W. E. C.; Serum glutamic-oxaloacetic transaminase activity. A new modification and an analytical assessment of current assay technics. *Clin. Chem.* (1962), 8, 343-350.
15. Amador, E.; Dorfman, L. E.; and Wacker, W. E. C.; Serum lactic dehydrogenase activity. An analytical assessment of current assays. *Clin. Chem.* (1963), 9, 391-399.
16. Amador, E., Reinstein, H.; and Benotti, N.; Precision and accuracy of lactic dehydrogenase assays. *Am. J. Clin. Path.* (1965), 44, 62-69.
17. Amador, E.; and Wacker, W. E. C.; Enzymatic methods used for diagnosis, in *Method Of Biochemical Analysis*, vol. 13, edited by Glick, D. Interscience Publishers, New York, pp. 265 - 356, 1965.
18. Amador, E.; Zimmerman, T. S.; and Wacker, W. E. C.; Urinary alkaline phosphatase activity. II. An analytical validation of the assay method. *JAMA* (1963), 185, 953-957.
19. Dobrow, D. A.; and Amador, E.; The accuracy of commercial enzyme reference sera. *Am. J. Clin. Path.* (1970), 53 60-67.
20. Amador, E.; and Hsi, B. P.; Indirect methods for estimating the normal range. *Amer. J. Clin. Path.* (1969), 52, 538-546.
21. Amador, E.; and Masod, M. F.; Characterization of the normal serum glutamic-oxalacetic transaminase activity of healthy adults. *Am. J. Clin. Path.* (1967), 47, 3-8.
22. Amador, E., Franey, R. J.; and Massod, M. F.; Serum glutamic-oxaloacetic transaminase activity; Diagnostic accuracy of the revised spectrophotometric and the dinitrophenylhydrazine methods. *Clin. Chem.* (1966), 12, 475-481.
23. Winkelman, J.; Nadler, S.; Demetrio, J.; and Pileggi, V. J.; Clinical usefulness of alkaline phosphatase isoenzyme determinations. *Am. J. Clin. Path.* (1972), 57, 625-634.
24. Marshall, G.; and Amador, E.; Diagnostic usefulness of serum acid  $\beta$ -glycerophosphatase activities in prostatic disease. *Am. J. Clin. Path* (1969), 51, 551-554.
25. Amador, E.; and Urban, J.; Transphosphorylation by human alkaline phosphatases. *Am. J. Clin. Path.* (1972), 57 167-172.
26. Amador, E.; Salvatore, A. C.; Serum glutamic-oxalacetic transaminase activity. Revised manual and automated methods using diazonium dyes. *Am. J. Clin. Path.* (1971), 55, 686-697.
27. Dreyfus, J. C.; Poenaru, L.; and Svennerholdm, L.; Absence of hexosaminidase A and B in a normal adult. *New Engl. J. Med.* (1975), 292 61-63.
28. Henry, R. J.; Chiamori, N.; Golub, O. J.; and Berkman, S.;

- Revised spectrophotometric methods for the determination of glutamic-oxaloacetic transaminase, glutamic-pyruvic transaminase and lactic acid dehydrogenase. *Am. J. Clin. Path.* (1960), 34, 381-398.
29. Rej. R.; and Vanderlinde, R. E.; Effects of buffers on asparate aminotransferase activity and association of the enzyme with pyridoxal phosphate. *Clin. Chem.* (1975), 21, 1585-1591.
  30. Dinovo, E. C.; Miyada, D. S.; and Nakamura, R. M.; Evaluation of direct and indirect coupled enzyme assay systems for measurements of creatine kinase activity. *Clin. Chem.* (1973), 19, 994-997.
  31. Russell, C. D.; Cotlove, E.; Serum glutamic-oxaloacetic transaminase: Evaluation of a coupled-reaction enzyme assay by means of kinetic theory. *Clin. Chem.* (1971), 17, 1114-1122.
  32. Roos, K.; A simplified method for the determination of serum alkaline phosphatase activity. *Scand. J. Clin. & Lab. Invest.* 15, Suppl. (1963), 69, 233-243.
  33. Bowers, G. N., Jr.; Kelley, M. L.; and McComb, R. B.; Precision estimates in clinical chemistry. I. Variability of analytic results in a survey reference sample related to the use of a non-human serum alkaline phosphatase. *Clin. Chem.* (1967), 14, 595-607.
  34. Gay, R. J.; McComb, R. B.; and Bowers, G. N.; Optimum reaction conditions for human lactate dehydrogenase isoenzymes as they affect total lactate dehydrogenase activity. *Clin. Chem.* (1968), 14, 740-753.
  35. Vessel, E. S., and Bearn, A. G.; Observations on the heterogeneity of malic and lactic dehydrogenase in human serum and red blood cells. *J. Clin. Invest.* (1958), 37, 672-677.
  36. Cahn, R. D.; Kaplan, N. O.; and Levine, L.; et. al.: Nature and development of lactic dehydrogenase. *Science* (1962), 136, 962-969.
  37. Wroblewski, F.; and Gregory, K. F.; Lactate dehydrogenase isoenzymes and their distribution in normal tissues and plasma and in disease states. *Ann. N.Y., Acad. Sci.* (1961), 94, 912-932.
  38. Rosalki, S. B.; Standardization of isoenzyme assays with special reference to lactate dehydrogenase isoenzyme electrophoresis. *Clin. Biochem.* (1974), 7, 29-40.
  39. Rosalki, S. B.; Serum hydroxybutyrate dehydrogenase. A new test for myocardial infarction. *Brit. Heart J.* (1963), 25, 795-802.
  40. Tanzer, M. L.; and Gilvarg, C.; Creatine phosphokinase measurements. *J. Biol. Chem.* (1959), 234, 3201-3204.
  41. Oliver, T. T.; A spectrophotometric method for the determination of creatine phosphokinase and myokinase. *Biochem. J.* (1955), 61, 116-122.

42. Dawson, D. M.; and Fine, I. H.; Creatine Kinase in human tissues. *Arch. Neurol.* (1967), 16, 175-180
43. Roe, C. R.; Limbird, L. E.; Wagner, G. S.; and Nerenberg, S. T.; Combined isoenzyme analysis in the diagnosis of myocardial injury: Application of electrophoretic methods for the detection and quantitation of the creatine phosphokinase MB isoenzyme. *J. Lab. Clin. Med.* (1972), 80, 577-590.
44. Roberts, R.; Henry, P. D.; Witteveen, S.A.G.J.; and Sobel, B. E.; Quantification of serum creatine phosphokinase isoenzyme activity. *Am. J. Cardiol.* (1974), 33, 650-654.
45. Konttinen, A.; and Somer, H.; Specificity of serum creatine kinase isoenzymes in diagnosis of acute myocardial infarction. *Br. Med. J.* (1973), 1, 386-389.
46. Wagner, G. S.; Roe, C. R.; Limbird, L. E.; Rosati, R.A.; and Wallace, A. G.; Importance of identification of the myocardial-specific isoenzyme of creatine phosphokinase (MB form) in the diagnosis of acute myocardial infarction. *Circulation* (1973), 47, 263-269.
47. Takahashi, K.; Ushikubo, S.; Oimomi, M.; and Shinki, T.; Creatine phosphokinase isoenzymes of human heart muscle and skeletal muscle. *Clin. Chim. Acta* (1972), 38, 285-290.
48. Mercer, D. W.; Separation of tissue and serum creatine kinase isoenzymes by ion-exchange column chromatography. *Clin. Chem.* (1974), 20, 36-40.
49. Henry, P. D.; Roberts, R.; and Sobel, B. E.; Rapid separation of plasma creatine kinase isoenzymes by batch absorption on glass beads. *Clin. Chem.* (1975), 21, 884-849.
50. Jockers, W.; Wretouy, E.; Pfleiderer, G.; Quantitation of creatine kinase isoenzymes in human tissues and sera by an immunological method. *Clin. Chim. Acta.* (1975), 58, 223-232.
51. Rao, P. S.; Lukes, J. J.; Ayres, S. M.; and Mueller, H.; New manual and automated method for determining activity of creatine kinase isoenzyme MB by use of dithiothreitol: Clinical applications. *Clin. Chem.* (1975), 21, 1612-1618.
52. Galen, R. S.; Reiffel, J. A.; and Gambino, S. R.; Diagnosis of acute myocardial infarction. *JAMA* (1975), 232, 145-147.
53. Szasz, G.; A kinetic photometric method for serum  $\gamma$ -glutamyl transpeptidase. *Clin. Chem.* (1969), 15, 124-136.
54. Bondar, R. J. L.; and Moss, G. A.; Enhancing effect of glutamate on apparent serum  $\gamma$ -glutamyl transpeptidase activity. *Clin. Chem.* (1974), 20, 317-319.
55. Rowe, J. A.; Tarlow, D.; and Rosalki, S. B.; Impurity in  $\gamma$ -glutamyl p-nitroanilide used as substrate for

- D-glutamyl transferase. *Clin. Chem.* (1973), 19, 435-436.
56. Huseby, N. E.; and Stromme, J. H.; Practical points regarding routine determination of  $\gamma$ -glutamyl transferase ( $\gamma$ -GT) in serum with a kinetic method at 37° C. *Scand. J. Clin. Lab. Invest.* (1974), 34, 357-363.
  57. Seiffert, U. B.; and Chaves, M.; Zur messung der aktivitat der  $\gamma$ -glutamyl-transpeptidase mit automaten. *Clin. Chim. Acta* (1973), 48, 237-239.
  58. Rosalki, S. B.; and Tarlow, D.; Optimized determination of  $\gamma$ -glutamyltransferase by reaction-rate analysis. *Clin. Chem.* (1974), 20, 1121-1124.
  59. Posen, S.; Neale, F. C.; and Chubb, J. S.; Heat inactivation in the study of human alkaline phosphatases. *Ann. Internal Med.* (1965), 62, 1234-1243.
  60. Rosalki, S. B.; Rau, D.; Serum  $\gamma$ -glutamyl-transpeptidase activity in alcoholism. *Clin. Chim. Acta* (1972), 39, 41-47.
  61. Schmidt, E.; and Schmidt, F. W.; Clinical enzymology. *FEBS letters* (1976), 62, E62-E79.
  62. Batsakis, G.; Sodeman, T. A.; and Deegan, M. J.; Enzymatic evaluation of hepatobiliary disease. *Laboratory Med.* (1974), 5, 33-50.
  63. Betro, M. G.; Oon, R. C. S.; and Edwards, J. B.; Gammaglutamyl transpeptidase in diseases of liver and bone. *Am. J. Clin. Path.* (1973), 60, 672-678.
  64. Solberg, H. E.; Skrede, S.; and Blomhoff, J. P.; Diagnosis of liver diseases by laboratory results and discriminant analysis. Identification of best combinations of laboratory tests. *Scand. J. Clin. Lab. Invest.* (1975), 35, 713-721.
  65. Henry, R. J.; and Chiamori, N.; Study of the saccharogenic method for the determination of serum and urine amylase. *Clin. Chem.* (1960), 6, 434-452.
  66. Shipe, J. R.; and Savory, J.; Kinetic nephelometric procedure for measurement of amylase activity in serum. *Clin. Chem.* (1972), 18, 1323-1325.
  67. Zinterhofer, L.; Wardlaw, S.; Jatlow, P.; and Seligson, D.; Nephelometric determination of pancreatic enzymes I. amylase. *Clin. Chim. Acta* (1973), 43, 5-12.
  68. Rinderknecht, H.; Wilding, P.; and Haverback, B. J.; A new method for the determination of  $\alpha$ -amylase. *Experientia* (1967), 23, 805.
  69. Babson, A. L.; Kleinman, N. M.; and Megraw, R. E.; A new substrate for serum amylase determination. *Clin. Chem.* (1968), 14 802-803.
  70. Klein, B.; Foreman, J. A.; and Searcy, R. L.; New chromogenic substrate for determination of serum amylase activity. *Clin. Chem.* (1970), 16, 32-38.
  71. Sax, M. S.; Bridwater, A. B.; and Moore, J. J.; Determination of serum and urine amylase with use of

- Procion Brilliant Red M-2BS amylopectin. *Clin. Chem* (1971), 17, 311-315.
72. Ceska, M.; Birath, K.; and Brown, B.; A new and rapid method for the clinical determination of amylase activities in human serum and urine. Optimum conditions. *Clin. Chim. Acta* (1969), 26, 437-444.
  73. Gamklon, R.; and Schersten, T.; Activity of  $\alpha$ -amylase and  $\alpha$ -1,4-glucosidase in human liver tissue. *Scand. J. Clin. Lab. Invest.* (1972), 30, 201-207.
  74. Skude, G.; Electrophoretic separation, detection, and variation of amylase isoenzymes. *Scand. J. Clin. Lab. Invest.* (1975), 35, 41-47.
  75. Takeucki, T.; Matsushima, T.; and Sugimura, T.; Separation of human  $\alpha$ -amylase isoenzymes by electrofocusing and their immunological properties. *Clin. Chim. Acta* (1975), 60, 207-213.
  76. Adams, J. T.; Libertino, J. A.; and Schwartz, S. I.; Significance of an elevated serum amylase. *Surgery* (1968), 63, 877-884.
  77. Keith, L. M., Jr.; Zollinger, R. M.; and McCleary, R. S.; Peritoneal fluid amylase determinations as an aid in the diagnosis of acute pancreatitis. *Arch. Surg.* (1950), 61, 930-936.
  78. Brooks, F. P.; Current Concepts: Testing pancreatic function. *N. Eng. J. Med.* (1972), 286, 300-303.
  79. Levitt, M. D.; Rapoport, M.; and Cooperband, S. R.; The renal clearance of amylase in renal insufficiency, acute pancreatitis and macroamylase. *Ann. Int. Med.* (1969), 71, 919-925.
  80. Warshaw, A. L.; and Fuller, J. A. F.; Specificity of increased renal clearance of amylase in diagnosis of acute pancreatitis. *New Eng. J. Med.* (1975), 292, 325-328.
  81. Levine, R. I.; Glanser, F. L.; and Berk, J. E.; Enhancement of the amylase creatinine clearance ratio in disorders other than acute pancreatitis. *New Eng. J. Med.* (1975), 292, 329-332.
  82. Levitt, M.; and Cooperband, S. R.; Increased renal clearance of amylase in pancreatitis. *New Eng. J. Med.* (1975), 292, 364-365.
  83. Roe, J. H.; Ticktin, H. E.; and Schneider, M.; Determination and clinical significance of serum lipase. *Enzymol. Biol. Clin.* (1966), 7, 731.
  84. Song, H.; Tietz, N. W.; and Tan, C.; Usefulness of serum lipase, esterase and amylase estimation in the diagnosis of pancreatitis - a comparison. *Clin. Chem.* (1970), 16, 264-268.
  85. Ticktin, H. E.; Trujillo, N. P.; Evans, P. F.; and Roe, J. H.; Diagnostic value of a new serum lipase method. *Gastroent.* (1965), 48, 12-17.

86. Cherry, I. S.; and Crandall, L. A.; The specificity of pancreatic lipase: Its appearance in the blood after pancreatic injury. *Am. J. Physiol.* (1932), 100, 266-273.
87. Lippi, U.; Stevanato, G.; and Guidi, G.; A rapid photometric micromethod for serum lipase determination. *Clin. Chim. Acta* (1972), 37, 199-202.
88. Dirstine, P. H.; Sobel, C.; and Henry, R. J.; A new rapid method for the determination of serum lipase. *Clin. Chem.* (1968), 14, 1097-1106.
89. Goldstein, N. P.; Epstein, J. H.; and Roe, H. J.; Studies of pancreatic function IV. A simplified method for determination of pancreatic lipase using aqueous tributyrin as substrate, with one hundred normal values by this method. *J. Lab. Clin. Med.* (1948), 33, 1047-1051.
90. Saifer, A.; and Perle, G.; Photometric micro determination of serum lipase with a phenyl laurate substrate. *Clin. Chem.* (1961), 7, 178-184.
91. Yang, J. S.; and Biggs, H. G.; Rapid, reliable method for measuring serum lipase activity. *Clin. Chem.* (1971), 17, 512-518.
92. Vogel, W. C.; and Zieve, L.; A rapid and sensitive turbidimetric method for serum lipase based upon differences between lipases of normal and pancreatitis serum. *Clin. Chem.* (1963), 9, 168-181.
93. Shihabi, Z. K.; and Bishop, C.; Simplified turbidimetric assay for lipase activity. *Clin. Chem.* (1971), 17, 1150-1153.
94. Lifton, L. J.; Slicker, K. A.; Pragy, D. A. and Katz, L.A.; Pancreatitis and lipase, a re-evaluation with a five minute turbidimetric lipase determination. *JAMA* (1974), 229, 47.
95. Zinterhofer, L.; Wardlaw, S.; Jatlow, P.; and Seligson, D.; Nephelometric determination of pancreatic enzymes. II lipase. *Clin. Chim. Acta* (1973), 44, 173-178.
96. Roy, A. V.; Brown, M. E.; and Hayden, J. E.; Sodium thymolphthalein monophosphate, a new acid phosphatase substrate with greater specificity for the prostatic enzyme in serum. *Clin. Chem.* (1971), 17, 1093-1102.
97. Schwartz, M. K.; Greenberg, E.; and Bodansky, O.; Comparative values of phosphatases and other serum enzymes in following patients with prostatic carcinoma. *Cancer* (1963), 16, 583-594.
98. Bonner, C. D.; Hamburger, F.; and Fishman, W. H.; Some factors other than neoplasms altering the prostatic fraction of acid phosphatase in the serum. *Surg. Gyn. Obst.* (1954), 99, 179-183.
99. Langley, J. R.; The deceptive acid phosphatases. *Calif. Med.* (1965), 103, 343-340.
100. Marshall, G.; and Amador, E.; Diagnostic usefulness of serum acid  $\beta$ -glycerophosphatase activities in prostatic

- disease. *Amer. J. Clin. Path.* (1969), 51, 551-554.
101. Mathes, G.; Richmond, S. G.; and Sprunt, D. H.; Use of l-tartrate in determining "prostatic" serum acid phosphatase. Report of 514 cases. *J. Urol.* (1956), 75, 143-150.
  102. Whitmore, W. F.; Bodansky, O.; Schwartz, M. K.; Ying, S. H.; and Day, E.; Serum prostatic acid phosphatase levels in proved cases of carcinoma or benign hypertrophy of the prostate. *Cancer* (1956), 9, 228-233.
  103. Hill, J. H.; Prostatic serum acid phosphatase in patients with localized prostatic cancer. *Am. J. Clin. Path.* (1956), 26, 120-130.
  104. Babson, A. L.; and Read, P. A.; A new assay for prostatic acid phosphatase in serum. *Am. J. Clin. Path.* (1959), 32, 6-9.
  105. Marshall, G.; Price, W. A.; and Amador, E.; Serum  $\alpha$ -naphthyl phosphatase activity. *Am. J. Clin. Path.* (1969), 51, 202-206.
  106. Latta, J.; Improved measurement of plasma renin activity and concentration. *Clin. Chem.* (1975), 21, 1344-1345.
  107. Bishop, C.; Assay of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase in red cell. *J. Lab. Clin. Med.* (1966), 68, 149-155.
  108. Yoshida, A.; and Lin, M.; Regulation of glucose-6-phosphate dehydrogenase activity in red blood cells from hemolytic and non hemolytic variant subjects, *Blood* (1973), 41, 877-891.
  109. Dror, Y.; Sasson, H. F.; Watson, J. J.; and Johnson, B. C.; Glucose-6-phosphate dehydrogenase assay in liver and blood. *Clin. Chim. Acta* (1970), 28, 291-298.
  110. Nicholson, John F.; Bodourian, Selma, H.; and Pesce, M. A.; Measurement of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase activities in erythrocytes by use of a centrifugal analyzer. *Clin. Chem.* (1974), 20, 1349-1352.
  111. Catalano, Edward W.; Johnson, George F.; and Solomon, Harvey, M.; Measurement of erythrocytes glucose-6-phosphate dehydrogenase activity with a centrifugal analyzer. *Clin. Chem.* (1975), 21, 134-138.
  112. Yoshida, A.; Hemolytic anemia and G6PD deficiency. *Science* (1973), 179, 532-537.



# 8

## Modern Liquid Chromatography in Clinical Chemistry

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Over the past few years one of the most rapid growing fields of chemical analysis has been high performance column liquid chromatography (HPLC). Whereas classical LC was characterized as a relatively slow and inefficient process, HPLC approaches the separation speeds obtained in gas chromatography. Accompanying the developments in design of high performance columns has been the introduction of modern instrumentation (e.g. pumps, detectors). Today, one can purchase from instrument companies complete HPLC packages or modular components. HPLC is rapidly becoming a standard tool in a variety of applications, including pharmaceutical analysis, biochemical research and natural products research. Complete texts on HPLC, as well as extensive reviews, are available (e.g. 1-6).

With notable exceptions, the application of HPLC to clinical chemistry has not as yet been extensive. This is somewhat surprising in view of the potential the method has for this area. This potential arises, in part, from the fact that HPLC is well suited to the types of substances that must be analyzed in the biomedical field. Ionic, relatively polar species can be directly chromatographed, without the need to make volatile derivatives as in gas chromatography. Typically, columns are operated at room temperature so that thermally labile substances can be separated. Finally, certain modes of HPLC allow fractionation of high molecular weight species, such as biopolymers.

The purpose of this article is to present HPLC and to summarize some typical applications in the clinical laboratory. The directions which HPLC will take in the future will also be suggested. It is hoped that this review will develop interest in HPLC by clinical chemists.

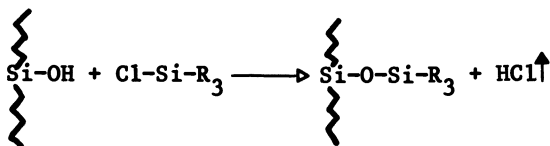
Overview of HPLC

**General Description.** Liquid chromatography encompasses any chromatographic method in which the mobile phase is a liquid (c.f. gas chromatography). A variety of stationary phases and retention mechanisms are available such that a broad range of modes of separation are possible. It is worthwhile to briefly describe the important modes that find use in clinical chemistry.

Adsorption and ion exchange chromatography are well-known methods of LC. In adsorption, one frequently selects either silica or alumina as stationary phase for separation of non-ionic, moderately polar substances (e.g. alcohols, aromatic heterocycles, etc.). This mode works best in the fractionation of classes of compounds and the resolution of isomeric substances (7). Ion exchange, on the other hand, is applicable to the separation of ionic substances. As to be discussed later, this mode has been well developed as a tool for analysis of urine constituents (8).

Other modes of LC operation include liquid-liquid partition chromatography (LLC) and bonded phase chromatography. In the former, a stationary liquid phase which is immiscible with the mobile phase is coated on a porous support, with separation based on partition equilibrium differences of components between the two liquid phases. This mode offers an alternative to ion exchange in the fractionation of polar, water soluble substances. While quite useful, the danger exists in LLC that the stationary phase can be stripped from the column, if proper precautions are not taken. Hence, it is typical to pre-equilibrate carefully the mobile and stationary phases and to use a forecolumn, heavily loaded with stationary phase (9).

Recently, the use of chemically bonded phases has become of great interest. In the typical case an organochlorosilane is reacted with dry porous silica to produce a surface that consists of an organic matrix (10).



By far the most popular phase system at the present time is the one in which R = n-octadecyl. Since the stationary phase is hydrophobic and nonpolar, while the mobile phase is relatively polar, this mode of operation is frequently called reverse phase

chromatography. This mode finds use in the separation of substances of weak to moderate polarity. Moreover, since the phase is covalently bonded to the surface, there is no need for a forecolumn. Another feature of this phase system is the rapid equilibration of the column with solvent change-over. Other bonded phases employ functional groups such as amino, hydroxyl and ether. In addition, chemically bonded ion exchangers are now commercially available. We shall discuss the usefulness of these phase systems later in this paper.

Finally, two other column LC modes should be mentioned — exclusion chromatography and affinity chromatography. Exclusion chromatography (i.e. gel filtration or gel permeation) (11) is a high molecular weight separation method based on size differences of molecules. One uses a porous support of a well controlled pore structure (e.g. dextran gel or porous glass) for the fractionation of biopolymers. Very large species are unable to penetrate the porous matrix and hence elute first. Small substances travel fully through the pore structure and elute last. Those species of intermediate size elute between the two extremes. Gel filtration finds some use in the analysis and especially the preparative separation of biopolymers.

Affinity chromatography (12) has become an important tool in the isolation of purified fractions of such substances as enzymes. Advantage is taken of specific interactions such as antigen-antibody interactions. One substance of the pair (e.g. antigen) is bonded to a support. When a mixture is passed through the column, the specific interaction retains the corresponding antibody relative to other substances. A change of mobile phase conditions then elutes the pure antibody. This method has a real potential for analysis of specific proteins in body fluids.

Classical vs. High Performance LC. Most workers are familiar with classical LC, a tool that has been predominantly used for preparative scale clean-up of samples. In order to appreciate more fully HPLC, Table I compares some of the column characteristics of classical vs. HPLC.

We first note the very large differences in column performance for the two methods. Effective plates per second represents the speed characteristics of a column (e.g., the number of plates that can be generated in a given time interval) (13). As can be seen, HPLC is 100 to 1000 times faster than classical LC. (We shall discuss the differences between PLB and PB in the next section.) This improved performance arises mainly from the use of significantly smaller particle sizes in HPLC. Moreover, in classical LC, the mobile phase is delivered to the column by gravity feed, hence, the very low mobile phase velocities. In HPLC, it is desirable to improve performance

by operating at velocities 10 to 100 times larger. The use of smaller particle sizes and higher velocities in HPLC requires high pressure pumping systems to deliver the mobile phase through the column. In a sense, pressure is the price that one must pay for the high performance. On the other hand, it is worth pointing out that in the pressure ranges used in HPLC, liquids are not very compressible so that there is no danger from explosions (as would be the case in gas chromatography at these pressures).

Below the dotted line in Table I we list less fundamental differences between the two methods. Column lengths tend to be somewhat shorter in HPLC using small particle PB as a consequence of the high efficiencies that can be generated with the smaller particle sizes. For analytical scale HPLC, tube diameters of 3-4 mm are selected; however, for preparative scale, tube diameters of 1 cm or above are not uncommon.

Besides differences in column design, we need to note that instrumentation is quite different for HPLC relative to the classical mode. Pumps, injectors and detectors are all important components in the achievement of high performance. In addition, automated injection is available which could be important in the clinical chemistry field.

### Column Packings in HPLC

We have seen that particle sizes of 100-150  $\mu$  in classical LC lead to slow and inefficient columns. The reason for this is related to the slow diffusion of molecules in the liquid state (e.g., gaseous diffusion coefficient  $\approx 10^5$  liquid diffusion coefficients).

Figure 1A illustrates a porous particle with the flow stream of mobile phase indicated by the arrows. If we consider, for simplicity, the particle as an adsorbent, then the pores will be filled with "stagnant" mobile phase. Molecules that enter the pores must diffuse through this liquid in order to be released again into the flow stream. (Adsorption-desorption rates are assumed to be rapid.). The time it takes to travel through the particle will be a function of the diffusion coefficient and the square of the pore depth. Slow mass transfer rates through the particles will mean that there will be excessive losses in efficiency (i.e., broad bands) at reasonable mobile phase velocities in the flowing stream, as solute molecules inside the pores will be unable to keep up with molecules in the flowing system. The obvious solution to improved separation speeds is to decrease pore depths, so that the distance over which diffusion in the stagnant mobile phase must occur is reduced.

Two approaches have been taken to achieve faster analysis

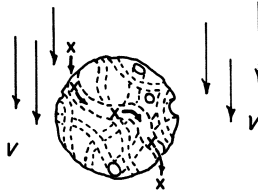
**TABLE I**  
**CLASSICAL vs. HIGH PERFORMANCE LIQUID CHROMATOGRAPHY**

| <u>Parameter</u>            | <u>Classical</u> | <u>HPLC</u>                         |
|-----------------------------|------------------|-------------------------------------|
| effective plates per second | .01              | 2-5 PLB*<br>~ 25 PB**               |
| particle diameter           | 100-150 $\mu$    | 30-40 $\mu$ PLB*<br>5-15 $\mu$ PB** |
| mobile phase velocity       | .01 cm/sec       | .1-1 cm/sec                         |
| pressure drop               | 15 psi           | ~ 500-3000 psi                      |
| - - - - -                   |                  |                                     |
| column length               | 1 meter          | 1 meter PLB<br>25 cm PB             |
| column diameter             | 1 cm             | 4 mm                                |

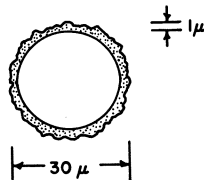
\* PLB = porous layer bead

\*\* PB = porous bead

A. STAGNANT MOBILE PHASE MASS TRANSFER



B. POROUS LAYER BEAD



*Figure 1. A, porous particle used to illustrate slow mass transfer due to diffusion in the stagnant mobile phase within the particle. B, illustration of a porous layer bead.*

times -- porous layer beads (PLB) and small particle porous beads (PB). Porous layer beads, as illustrated in Figure 1B, consists of spherical beads of  $\sim 40 \mu$  diameter upon which is deposited a thin ( $\sim 1 \mu$ ) porous layer. The small porous layers allow fast mass transfer in and out of the pores, in comparison to the fully porous  $150 \mu$ . A variety of PLB are commercially available (2) both as packing itself and in the form of prepacked columns. Many porous surfaces can be used as a stationary phases, including silica, alumina and chemically bonded phases.

While PLB were introduced first (14,15) more recently small PB ( $\sim 5-15 \mu$  diameter) have become of major interest. This is a result of the higher separation speeds found with such particles. Not only is the "stagnant" mobile phase mass transfer problem reduced, as in PLB, but solute mixing in the flowing stream is enhanced as a result of the smaller distance between the particles. The performances achieved with the small particle columns are equivalent to those obtained with capillary columns in gas chromatography (13). Examples illustrating the separation speed of such columns will be presented in the applications section of this paper.

The question arises as to which column packing to use in practice. Table II compares PLB and PB from this point of view. Besides producing higher column performance PB also have high sample capacities by virtue of the larger stationary phase content per column volume (recall that most of a PLB particle is simply a spherical bead). Counterbalancing these advantages is the fact that the small particle columns are much more difficult to pack reproducibly. While PLB can be dry packed into a column, PB must use special slurry packing techniques (16), in order to prevent particle agglomeration in the column. It is recommended that commercially packed small particle PB columns be used by anyone not well experienced in the field. In addition, the narrow bands produced with such columns require care in terms of extra column effects (e.g., injections, connections, detector). For a recent discussion on the practical handling of such columns, the reader is referred to (17).

From Table II, we can reach the following important conclusions. First, for simple separations of only a few components and not great demands on analysis time (e.g.,  $\sim 15-30$  minutes), PLB are the logical choice. As the number of components increase, as their relative retentions become smaller, or as the time demands increase, one ought to use small particle PB. With respect to clinical chemistry, many problems can now be successfully solved with PLB. Ultimately because of the faster analysis potential, small particle PB will be column of choice. However extra care must be exercised in using such columns. As experience grows with small particle PB, it is clear that they will become simpler to work with.

## EQUIPMENT

Major advances have occurred over the past few years in equipment for HPLC. Figure 2 presents a block diagram of a basic LC instrument. We shall now discuss individual components.

The solvent reservoir provides a source of mobile phase for the column. For the most part, this component is separate from the pump; however, in a few cases, it is part of the pumping system (e.g., gas displacement pump). Depending on the flow rate or the time of operation, the reservoir may consist of a few hundred mls or several liters. Obviously care must be exercised that solvent evaporation is minimized, so that the composition of mixed solvents does not vary with time. Moreover, since the water content of the mobile phase can greatly affect retention in adsorption chromatography (7), control of the water vapor above the solvent can be important. A further precaution is degassing of solvents (1). If sufficient quantities of gases are dissolved in the mobile phase, it is possible that gas bubbles will be released in the detector cell and thus cause excessive noise and loss of signal. Generally, solvents are first heated or ultrasonically treated prior to use. This is especially necessary when aqueous solutions are employed.

The flow stream then typically travels through a filter (preferably less than 2  $\mu$  pore size) which may or may not be part of the pumping system. This component is necessary to remove particles that could damage the check valves of most pumps. Since microorganisms can grow in water, it is required that aqueous solutions be filtered before use.

A central component of the LC instrument is the pump, for its characteristics can strongly influence an analysis (e.g., flow reproducibility). The three principal types of systems--pneumatic, syringe type and reciprocating piston--have been reviewed in the literature (18-20).

In the pneumatic pumping system, the pressure (and not the flow rate) is maintained constant as variations in chromatographic conditions occur. Thus, a change in mobile phase viscosity (e.g. gradient elution) or column back pressure will result in a change in flow rate for these types of pumps. The gas displacement pump in which a solvent is delivered to the column by gas pressure is an example of such a pneumatic pump. The gas displacement system is among the least expensive pumps available and is found in several low cost instruments. While the pump is nonpulsating and hence, produces low noise levels with the detectors in current use, its flow stability and reproducibility are only adequate. In addition, its upper pressure limit is only  $\sim$  2000 psi which may be too low in certain applications.

The second type of constant pressure pumping system is the

TABLE II  
COMPARISON OF PLB AND PB COLUMNS

| <u>Characteristic</u> | <u>PLB</u>         | <u>PB</u>             |
|-----------------------|--------------------|-----------------------|
| speed                 | fast               | faster                |
| capacity              | low                | good                  |
| packing procedure     | easy               | difficult             |
| instrument demands    | moderate           | high                  |
| applicability         | simple separations | demanding separations |

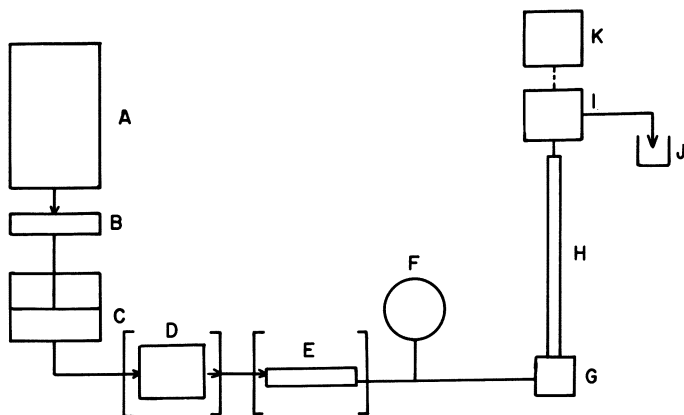


Figure 2. Block diagram of a liquid chromatograph. A, solvent reservoir; B, filter; C, pump; D, pulse dampener (optional); E, pre-column (used only in liquid-liquid chromatography); F, pressure gauge; G, injector; H, column; I, detector; J, fraction collector; K, recorder or other readout device.



pneumatic amplifier pump, in which gas pressure is amplified to higher liquid pressures by the use of pistons of different areas. This pump is nonpulsating and is capable of reasonably high flow rates which can be important in preparative scale LC.

The syringe type pump consists of mechanically driven piston emptying a large volume chamber ( $\sim 250$  ml). In some variations, two large piston chambers are used so that as one empties the other fills. These pumps tend to maintain fairly constant flow rate and are pulseless. However, solvent change-over is not rapid and can be wasteful of solvent. While quite satisfactory, these pumps tend to be expensive.

Finally, the dual reciprocating piston pumps consist of two small volume chambers ( $\sim 0.1$ - $0.4$  ml) that alternately empty and fill. The two pistons travel in a phase and variable speed relationship such that pulsations are minimized. Moreover, pressure or flow detection downstream is part of a feedback loop to maintain flow constant by variation in the speed of the piston movements. These pumps allow easy changeover of solvents and simple variation in mobile phase flow rate. Other pumping systems can be found in the articles that have been referenced.

The next device is a pulsation dampener which is placed in brackets in Figure 2 to indicate that its inclusion will be a function of whether the pump produces pulsations and whether the detector senses these pulsations as noise. The pulsation dampener could simply consist of a coil of tubing or a pressure gauge Bourdon tube. Following this device is a forecolumn which is again placed in brackets since it is only used in the case of liquid-liquid chromatography. The forecolumn consists of a short column of large particle packing heavily loaded with the stationary liquid phase. The forecolumn thus provides protection that the mobile phase is pre-equilibrated with the stationary phase before entering the column. If not, then bleeding of stationary phase from the column can take place with the ensuing change in retention. The next component, a pressure gauge, is typically inserted into the system in order to determine the pressure drop in the column.

The injection device is also an important component in the LC system and has been discussed elsewhere (2,18). One type of injector is analogous to sample delivery in gas chromatography, namely syringe injection through a self-sealing septum. While this injection procedure can lead to good column efficiency, it generally is pressure limited, and the septum material can be attacked by the mobile phase solvent. For higher pressures, stopped flow injection has been used in which a valve releases the pressure in the column to atmosphere, at which point syringe injection at the top of the column (without septum) takes place. There has, however, been a trend in recent years to use valve injectors because of their generally high pressure capabilities and their ability to deliver large

volumes of sample (in excess of 100  $\mu\text{l}$ ) rapidly to the column. In clinical chemistry, the selection of valve-loop injectors makes further sense, since the reproducibility of sample volume is higher than can be obtained with a syringe, and automatic injection is possible.

The final component to be discussed is the detection device of which much has already been written (2,21,22). Table 3 lists the most popular detectors at the present time with an indication of the detection limits obtainable for favorable substances.

By far, the most frequently used device is the ultraviolet detector. Three types are employed (23) -- single wavelength with low pressure mercury source, multiwavelength filter photometer with medium pressure mercury source, and spectrophotometer. In all cases, low volume detector cells ( $\sim 8 \mu\text{l}$ ) are employed in order to limit extra band broadening.

In the single wavelength detector, 254 nm and 280 nm are strong lines from the mercury source and hence, are selected in most commercial instruments. Of the three types of U.V. detectors, this one gives the lowest noise level (down to 0.00002 O.D.), but, of course, some flexibility is lost in not being able to work at other wavelengths. Nevertheless, when one purchases an instrument, this is the first detector selected.

The filter photometer provides the ability to detect at wavelengths above 280 nm and, indeed into the visible region. However its noise level is 2-5 times higher than the single wavelength low pressure mercury source. Finally, the major advantage that the spectrophotometer provides is the capability to detect in the 200-250 nm region. Substances that are not ordinarily considered to be U.V. active (e.g., alkyl bromides) can be analyzed with this detector. The noise level with this detector is 5-20 times larger than with the single wavelength device. Examples of the usefulness of the three detectors can be found in the references (21,22).

The second most widely used detector in HPLC is the differential refractometer (RI). Being a bulk property detector, the RI responds to all substances. As noted in Table 3 the detection limits are several orders of magnitude higher than obtained with the UV detector. Thus, one turns to the RI detector in those cases in which substances are non-UV active, e.g. lipids, prostaglandins. In addition, the RI detector finds use in preparative scale operation. Finally, relative to the UV detector, the RI is significantly more temperature and flow sensitive and cannot be used in gradient elution.

In some respects, the detector comparable to the RI is the moving wire system. Here, the solution eluting from the column drips on to a wire, with roughly 3% remaining on the wire. The wire transports the deposited material to an oven where the mobile phase is removed by evaporation. Further transport

brings the deposited sample to a combustion chamber where the substance is brought into the vapor state and reduced to methane. The methane is finally swept into a flame ionization detector for final analysis. As can be seen in Table III, the detection levels are roughly equivalent to the RI detector, both detectors responding to concentration. The moving wire detector finds value in the analysis of non-UV active substances in which gradient elution must be employed. However, the moving wire detector has not found great acceptance in the U.S., undoubtedly because of its sophisticated operation and high price.

Fluorescence detection is becoming more popular, especially in the analysis of biochemical substances. In favorable cases, detection levels as low if not lower than the UV detector can be achieved. Of course, it must be recognized that some solvents can strongly influence the detection limits through such processes as quenching. The key advantage of fluorescence, however, is in its high selectivity. Another aspect of fluorescence detection is the use of fluorescent derivatives to enhance selectivity and detection levels. A good example of this is the use of fluorescamine with primary amines such as amino acids, peptides, catecholamines, etc. (24).

The final detector to be discussed is based on electrochemical processes. The use of a polarographic detector has been well documented in the literature (25). More recently, advanced electrochemical systems have been selected for detection at trace levels (26). In essence, the redox reaction is performed in thin layers between the electrodes, and thus, essentially all material is electrolyzed to produce high current efficiencies. In the right set of circumstances the detector is outstanding; for example, it can be used to analyze picogram levels of catecholamines. The detector, however, is not a general one as the potential at which, for example, oxidation occurs must be relatively low in order that many other substances will not be oxidized. In addition, electrode surfaces can be altered during operation which can markedly affect reproducibility. Nevertheless, much interest exists with this detector and its future is certainly bright.

### Analytical Capability

Successful use of modern liquid chromatography in the clinical laboratory requires an appreciation of the method's analytical characteristics. The quantitative reproducibility with respect to peak height or peak area is quite good. With a sample loop injector relative standard deviations better than 1% are to be expected. The variability of syringe injection (3-4% relative standard deviation) requires the use of an internal standard to reach the 1% level (2,27).

**TABLE III**  
**Detectors for HPLC**

| <u>Detector</u> | <u>Lowest Detection Levels*, gm/ml</u> |
|-----------------|--|
| UV              | $5 \times 10^{-10}$                    |
| RI              | $5 \times 10^{-7}$                     |
| Moving Wire     | $5 \times 10^{-7}$                     |
| Fluorescence    | $5 \times 10^{-10}$                    |
| Electrochemical | $\sim 10^{-10}$                        |

\*A function of sample type in most cases

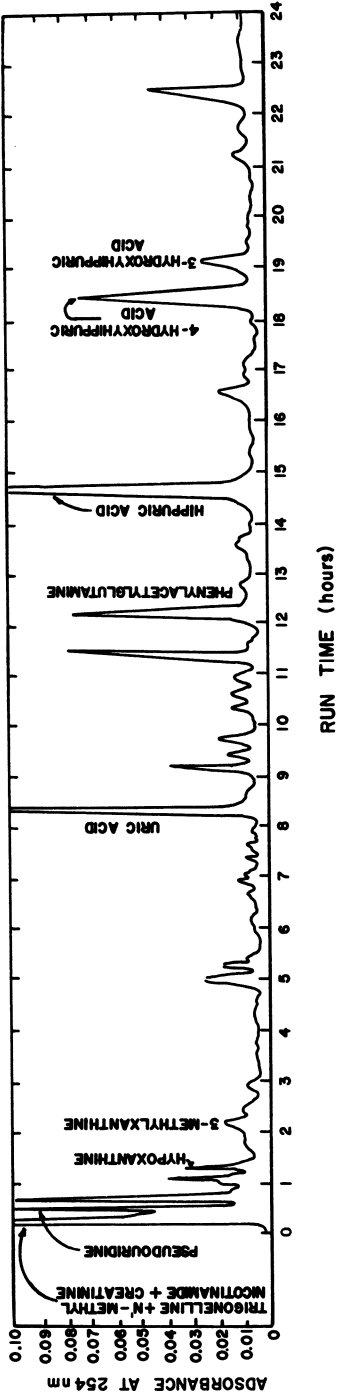
The precision in retention from injection to injection will often be better than 1%. Over longer periods of time such precision requires the following (a) good flow control from the pump (b) constant mobile and stationary phases and (c) temperature control of the column. The critical question of reproducibility from column to column is still a matter of concern, especially when dealing with the more sophisticated packing materials, e.g. small porous particles, bonded phases. While frequently this reproducibility is quite good, workers should recognize that care must be exercised to achieve and/or maintain reproducible columns. Undoubtedly, with experience, this need not be a severe problem.

Finally, some comments on trace analysis are in order (28,29). It needs to be recognized that large volumes of liquid samples can be injected into the column. This is contrary to gas chromatography in which a phase change (i.e. vaporization) must occur upon injection. The large sample volumes mean that column dilution in liquid chromatography can be significantly lower than gas chromatography. In the right set of circumstances subnanogram detection levels can be achieved. Of course, the type of detector also plays an important role, as well as the use of specific derivatives to enhance detection (30).

### Applications

A number of applications of modern LC of relevance to clinical chemistry have appeared in the literature. It is obviously not possible to mention all examples; rather, we shall try to select applications which illustrate the efforts to date.

One area of extensive development has been the use of small particle ion exchange resins for the separation of complex biological mixtures. Originally studied by Paul Hamilton (31), C.D. Scott and colleagues at Oak Ridge National Laboratory have extensively applied this methodology to the analysis of constituents of urine (8). Using gradient elution in conjunction with anion and cation exchange columns in series, a large number of components can be detected from direct urine injection. Most analyses involve U.V. detection; however, post column reactions are sometimes employed, e.g. fluorescence detection of organic acids and other oxidizable substances from Ce(IV) to Ce(III) (fluorescent) (32). At present over 120 U.V. active substances and 18 carbohydrates (33) have been identified. Diseased states can be correlated with elevated levels of certain components. A typical complex urine chromatogram is shown in Figure 3. Note that this chromatogram is essentially obtained with no clean-up of the urine.



*Figure 3. Typical chromatogram of UV-absorbing constituents of a 0.15-ml sample of urine. Conditions: 150-cm anion exchange column (12-15  $\mu$ , Aminex A-27); buffer, acetate at pH 4.4; gradient, buffer concentration from 0.015M to 6M; flow rate, 10.5 ml/hr, column temperature, 25°C for first 5 hr and 60°C thereafter (34).*

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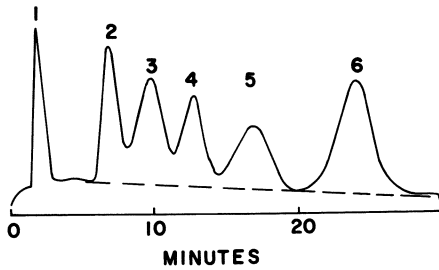
Ion exchange chromatography has also been extensively applied to the analysis of nucleic acid components (3,14,35). A wide variety of bases, nucleosides and nucleotides have been separated using porous layer bead ion exchangers. A representative chromatogram of the separation of ribonucleoside monophosphoric acids from the work of Smukler (36) is shown in Figure 4. Recently, ion exchangers chemically bonded to small particle diameter ( $\sim 10 \mu\text{m}$ ) silica have been successfully applied to the separation of nucleic acid constituents (37). The rapid separations using such supports undoubtedly mean that they will find increasing use in the future.

Adsorption chromatography using small particle silica or alumina has also been employed in the separation of biologically meaningful substances. Phospholipids, for example, have been separated on silica (38). One of the big problems for such substances is detection, since many of the compounds are not U.V. active. Generally, the refractive index detector is employed for isocratic operation, and the moving wire detector for gradient operation. Formation of U.V.-active derivatives is also possible (39).

Another example of the use of small particle silica is in the analysis of theophylline in plasma, as shown in Figure 5 (40). The clean-up procedure is simply a single extraction of the plasma with an organic solvent. This analysis has also been achieved by reverse phase chromatography (41), and this points out the fact that in some separations (e.g. with components of moderate polarity) either the adsorption or reverse phase mode can be used.

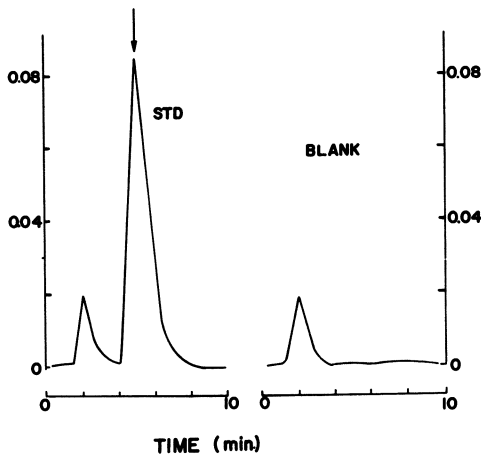
Reverse phase chromatography is finding increasing use in modern LC. For example, steroids (42) and fat soluble vitamins (43) are appropriately separated by this mode. Reverse phase with a chemically bonded stationary phase is popular because mobile phase conditions can be quickly found which produce reasonable retention. (In reverse phase LC the mobile phase is typically a water-organic solvent mixture.) Rapid solvent changeover also allows easy operation in gradient elution. Many examples of reverse phase separations can be found in the literature of the various instrument companies.

While most of the high speed-high pressure LC work to date has been with small molecules, i.e.  $\text{MW} < 1000$ , it is clear that the high performance techniques will play a role in the chromatographic separation of higher molecular weight species, e.g. proteins. While the LC separation of biopolymers is well established (11), many of the supports currently in use are unable to withstand the pressures necessary for high performance. We can anticipate the application of porous layer bead and especially small particle supports, capable of withstanding pressure, to the separation of biopolymers in the near future. Such materials will have an impact on clinical analysis.



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**Figure 4.** Separation of ribonucleoside monophosphoric acids. Conditions: 250-cm anion exchange column; gradient, 0.01M  $\text{KH}_2\text{PO}_4$  containing  $\text{H}_3\text{PO}_4$  (pH 2.6) to 0.15M  $\text{KH}_2\text{PO}_4$  in 30 min; column temperature, 70°C; detector, UV at 254 nm. 1, cytidine-5'-monophosphoric acid; 2, uridine-5'-monophosphoric acid; 3, adenosine-5'-monophosphoric acid; 4, inosine-5'-monophosphoric acid; 5, 3',5'-cyclic adenosine monophosphoric acid; 6, guanosine-5'-monophosphoric acid (36).



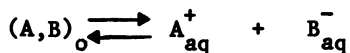
Clinical Chemistry

**Figure 5.** Chromatograms for theophylline in plasma extracts. Arrow indicates theophylline peak. Conditions: 50 cm  $\times$  3 mm (i.d.) column with 10  $\mu\text{m}$  silica gel (Micropak Si 10; Varian); mobile phase, 84/15/1 chloroform/isopropanol/acetic acid; flow rate, 40 ml/hr; detector, UV, 273 nm (40).



As an example, consider the separation of the creatine kinase isoenzymes, MM, MB, and BB. Mercer has used classical ion-exchange chromatography (DEAE - Sephadex - A50) for the resolution of these three isoenzymes (44). To speed up the separation and ultimately to allow an automated analysis, Toren has used porous layer bead ion exchangers (45). Separation of the MM and BB isoenzymes was achieved in less than 10 min, as seen in Figure 6. Interestingly, the MB isoenzyme was strongly retained on the support (presumably through adsorption on exposed silanol groups). Through better chemical bonding technology, however, it is possible to substantially reduce the activity of the silica surface (46). Such chemically bonded phase systems for exclusion and ion exchange chromatography will prove to be quite useful.

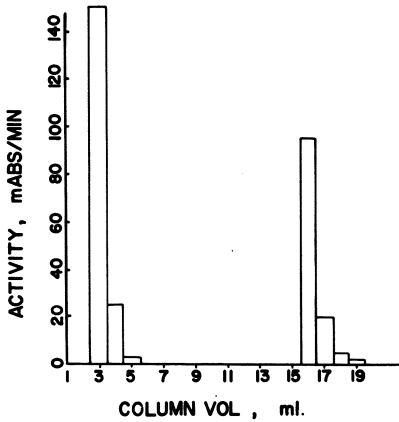
It is useful to conclude with several examples from our laboratory. We have applied the procedure of ion pair partition chromatography to the separation of biochemically important mixtures (47,48). In this method of liquid-liquid partition, the basic distribution process can be written as



where  $(A,B)_o$  is the ion pair in the organic phase and  $A_{aq}^+$  and  $B_{aq}^-$  are oppositely charged ions in the aqueous phase. In a typical experiment, e.g. the separation of amines, the stationary phase would consist of an acidified aqueous solution containing a high concentration ( $\sim 0.1M - 1M$ ) of counterion, e.g.  $ClO_4^-$ . This phase would be coated on small particle ( $\sim 5 - 10\mu$ ) porous silica. The mobile phase would consist of an immiscible organic solvent mixture, e.g. butanol/heptane.

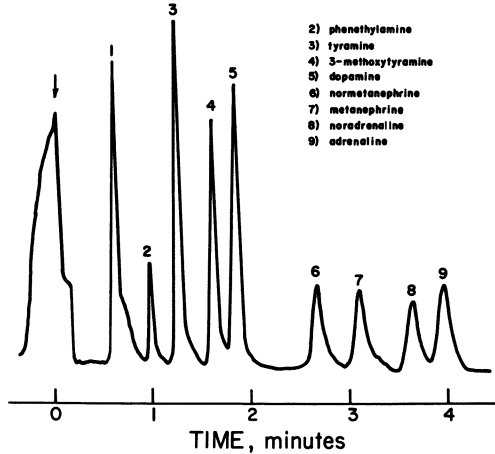
Because a chemical step is imposed on top of the physical distribution process of partition, there is a great potential for selectivity, as noted by Schill et al. (49,50). Such factors as pH, type and composition of the organic phase, and ionic strength of the aqueous phase can be used to control relative retention. The concentration and type of counterion mainly control the absolute retention.

Taking advantage of the high performance conditions of the silica columns, we have separated a variety of mixtures including thyroid hormones, sulfa drugs and catecholamines. Figure 7 shows the rapid separation of eight catecholamines using perchlorate as counterion and the organic phase listed in the figure. It is to be noted that reverse phase ion pair partition using  $C_{18}$  chemically bonded phases and an aqueous mobile phase can also be successfully employed (51). The ion



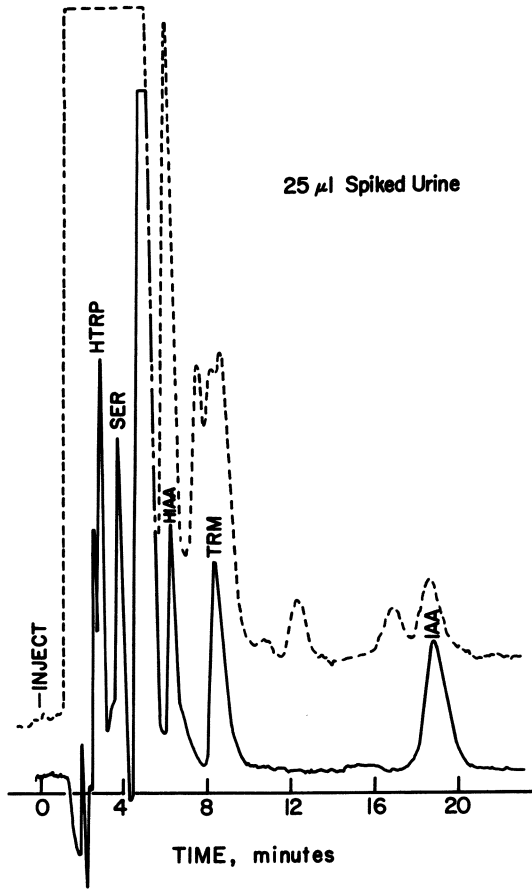
Clinical Chemistry

**Figure 6.** High pressure liquid chromatogram of creatine kinase isoenzymes. First peak, MM; second peak, BB. Conditions: 50 cm × 4.6 mm (i.d.) column with "Vydac" porous layer bead anion exchange; mobile phase, step gradient; Solvent A, 10 mmol/liter Tris buffer, pH 8.3; solvent B, 10 mmol/liter Tris buffer, pH 7.0, 0.5 mol KCl; flow rate, 2 ml/min; detection, collected fractions assayed (45).



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**Figure 7.** Separation of eight biogenic amines using ion pair partition liquid chromatography. Conditions: 30 cm column with 4 μm silica; stationary phase, 0.1M HClO<sub>4</sub>/0.9M NaClO<sub>4</sub>; mobile phase, ethylacetate/tributyl phosphate/hexane (72.5/10/17.5); velocity, 0.8 cm/sec (47).



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**Figure 8.** Chromatograms of ultraviolet (····) and fluorescence (—) response of deproteinized urine doped with 0.4 µmoles of standard indoles. Conditions: 30-cm column; stationary phase, µ Bondapak C<sub>18</sub>; mobile phase, 10/90 methanol in 0.01M NaAc, pH = 4.6; flow rate, 2.5 ml/min. HTRP, 5-hydroxytryptophan; SER, serine; HIAA, 5-hydroxyindole acetic acid; TRM, tryptamine; IAA, indoleacetic acid (52).

pair method should prove of value in the clinical laboratory.

A second example from our work involves the analysis of indole compounds in urine using high performance reverse phase liquid chromatography with fluorescence detection (52). By control of pH and methanol composition the separation of a series of standard indole derivatives was achieved. The selective detection afforded by fluorescence permitted a very simple clean-up of the urine sample. The urinary proteins are first precipitated from a 10 ml urine sample. Using a spiked sample of  $\sim 1$  nanomole each, 25  $\mu$ l of urine after precipitation is injected directly into the liquid chromatograph and the resulting chromatogram is shown in Figure 8. The dotted line represents the UV detection which, as can be seen, contains a large number of components that prevent analysis of the components of interest. On the other hand, five indoles can be easily detected and the three chemically important 5-hydroxyindoles can be analyzed in less than eight minutes. Detection limits are  $\sim 5$  nanogram using a filter fluorimeter, a level more than adequate for diseased states.

### Conclusion

This introduction to modern liquid chromatography should suggest some of the roles LC might play in clinical analysis. An area worthy of study is the development of systems of increasing sample throughput beyond the single column operation. Scott has introduced a prototype multicolumn system based on the centrifugal analyzer principle (53). In this set-up a series of LC columns is rotated on a disc, with sample delivery at the center of the disc and elution and spectrophotometric analysis on the outside. He has suggested using affinity columns for rapid serum protein analysis by this approach. Of course, other principles, such as segmented flow, could be envisioned in an automated LC system as well. Undoubtedly, we can expect to see the availability of such systems in the next few years.

### Literature Cited

1. Kirkland, J. J., ed.; "Modern Practice of Liquid Chromatography", John Wiley & Sons, New York, 1971.
2. Snyder, L.R.; and Kirkland, J.J.; "Introduction to Modern Liquid Chromatography", Interscience, New York, 1974.
3. Brown, P.R.; "High Pressure Liquid Chromatography, Biochemical and Biomedical Applications", Academic Press, New York, 1973.
4. Hadden, N., et al.; "Basic Liquid Chromatography", Varian Aerograph, Walnut Creek, California, 1972.

5. Done, J. N.; Knox, J. H.; and Loheac, J.; "Applications of High-Speed Liquid Chromatography", John Wiley and Sons, New York, 1974.
6. Michaelis, A. F.; Cornish, D. W.; and Vivilecchia, R; J. Pharm. Sci. (1973), 62, 1399.
7. Snyder, L. R.; "Principles of Adsorption Chromatography", Marcel Dekker, New York, 1968.
8. Scott, C. D.; Science (1974), 186, 226.
9. Snyder, L. R.; and Kirkland, J. J.; Chapter 7, op cit.
10. Pryde, A.; J. Chromatog. Sci. (1974), 12, 486.
11. Determann, H.; "Gel Chromatography", Springer-Verlag, New York, 1968.
12. Lowe, C.R.; and Dean, P. D. G.; "Affinity Chromatography", John Wiley and Sons, New York, 1974.
13. Karger, B. L.; "Modern Practice of Liquid Chromatography", Kirkland, J. J., ed.; John Wiley and Sons, New York, 1971.
14. Horvath, C.; Preiss, B.; and Lipsky, S. R.; Anal. Chem. (1967), 39, 1422.
15. Kirkland, J. J.; J. Chromatog. Sci. (1969), 7, 7.
16. Majors, R. E.; Anal. Chem. (1972), 44, 1822.
17. Rabel, F. M.; American Laboratory (May, 1974), 7, 53.
18. Berry, L. V.; and Karger, B. L.; Anal. Chem. (1973), 45, 819A.
19. Bombaugh, K. J.; Amer. Lab. (1973), 5, 69.
20. Jackson, M. T.; and Henry, R. A.; Amer. Lab. (1974), October.
21. Veening, H.; J. Chem. Ed. (1970), 47, A-675.
22. Schomberg, G.; Z. Anal. Chem. (1975), 277, 275.
23. Baker, P. R.; Williams, R. C.; and Steichen, Y. C.; J. Chromatog. Sci. (1974), 12, 499.
24. Udenfriend, S.; Stein, S.; Bohlen, P.; Dairman, W.; Leimgruber, W.; and Weigele, M.; Science (1972), 178, 871.
25. Koen, J. G.; Huber, J. F. K.; Poppe, H.; and du Boef, G.; J. Chromatog. Sci. (1970), 8, 192.
26. Kissinger, P. T.; Felice, L. J.; Riggin, R. M.; Pachla, L. A.; and Wenke, D. C.; Clin. Chem. (1974), 20, 992.
27. Barth, H.; Dallmeier, E.; Courtois, G.; Keller, H. E.; and Karger, B. L.; J. Chromatog. (1973), 83, 289.
28. Kirkland, J. J.; Analyst (1974), 99, 859.
29. Karger, B. L.; Martin, M.; and Guiochon, G.; Anal. Chem. (1974), 46, 1640.
30. Frei, R. W.; and Santi, W.; Z. Anal. Chem. (1975), 277, 303.
31. Hamilton, P.B.; "Handbook of Biochemistry, Selected Data for Molecular Biology", Sober, H. A. Ed.; Chemical Rubber Co., Cleveland, 1968, p.B43.
32. Katz, S.; and Pitt, W.W.; Anal. Lett. (1972), 5, 177.
33. Pitt, W. W.; Scott, C. D.; Johnson, W. F.; and Jones, G.;

- Clin. Chem. (1970), 16, 657.
34. Scott, C. D.; Chilcote, D. D.; and Lee, N.E.; Anal. Chem. (1972), 44, 85.
35. Burtis, C. A.; and Gere, D. R.; "Nucleic Acid Constituents by Liquid Chromatography", Varian Aerograph, California, 1970.
36. Shmukler, H. W.; J. Chromatog. Sci. (1972), 10, 137.
37. Hartwick, R. A.; and Brown, P. R.; J. Chromatog. (1975), 112, 651.
38. Aitzetmuller, K.; Chromatog. Rev. (1975), 113, 231.
39. Jungalwala, F. B.; Turel, R. J.; Evans, J. E.; and McCluer, R.H.; Biochem. J. (1975), 145, 517.
40. Sitar, D. S.; Piafoky, K. M.; Rangno, R.E.; and Ogilvie, R.I.; Clin. Chem. (1974), 99, 35.
41. Manion, C. V.; Shoeman, D. W.; and Azarnoff, D. L.; J. Chromatog. (1974), 101, 169.
42. Fitzpatrick, F. A.; Siggia, S.; and Dingman, J. Sr.; Anal. Chem. (1972), 44, 2211.
43. Williams, R. C.; Schmidt, J. A.; and Henry, R. A.; J. Chromatog. Sci. (1972), 10, 494.
44. Mercer, D.W.; Clin. Chem. (1974), 20, 36.
45. Kudirka, P. J.; Busby, M. G.; Carey, R. N.; and Toren, E. C. Jr.; Clin. Chem. (1975), 21, 250.
46. Regnier, F.; Purdue University, unpublished results.
47. Persson, B. A.; and Karger, B. L.; J. Chromatog. Sci. (1974), 12, 521.
48. Karger, B. L.; Su, S.; Marchese, S.; and Persson, B. A.; J. Chromatog. Sci. (1974), 12, 678.
49. Eksborg, S.; and Schill, G.; Anal. Chem. (1973), 45, 2092.
50. Schill, G.; "Advances in Ion Exchange and Solvent Extraction, Vol. 6", Marinsky, J. A.; and Marcus, Y., eds.; Marcel Dekker, 1974.
51. Knox, J.; and Jurand, J.; J. Chromatog. (1975), 110, 103.
52. Graffeo, A.P.; and Karger, B.L.; Clin Chem. (1976), 22, 184.
53. Scott, C. D.; Pitt, W. W.; and Johnson, W. F.; J. Chromatog. (1974), 99, 35.

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# 9

## Electrothermal Atomic Absorption Spectrometry of Trace Metals in Biological Fluids

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As a consequence of the recent increase of scientific knowledge regarding the metabolism, biochemical pathology and toxicology of trace metals, there has arisen widespread clinical interest in measurements of trace metals in body fluids, tissues and excreta (33,41,65,77). Laboratories in many university medical centers and governmental institutions have become involved in determinations of copper, zinc, lithium, gold, lead, cadmium, mercury, manganese, nickel and other metals in biological materials. Increasing attention has been focused upon clinical applications of electrothermal atomic absorption spectrometry, since this relatively new technique has appeared to provide the requisite analytical sensitivity, specificity and convenience (33,41,65,77). Regrettably, this technique is subject to pitfalls, interferences, and sources of imprecision that have hindered its clinical use. As will be discussed in this review, progressive refinements of electrothermal atomizers and of optical systems for background compensation have largely overcome these obstacles. It now appears probable that electrothermal atomic absorption spectrometry will be adopted by clinical chemistry laboratories as a routine method for measurements of trace metals in body fluids.

The scope of this review is limited to electrothermal atomic absorption spectrometry, with emphasis upon its clinical applications. This article is intended to supplement the recent treatises on the basic technique which have been written by Aggett and Sprott (1), Ingle (29), Kirkbright (34), Price (63), and Woodruff (83). This resume does not consider various related topics, such as (a) atomic fluorescence or emission spectrometry; (b) non-flame atomization devices which employ direct current

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arcs, radio-frequency plasma jets, microwave excitation fields, or demountable hollow-cathode sputtering cells; (c) relatively low-temperature atomic absorption spectrometry of Hg vapor; or (d) atomic absorption spectrometry of As and Se following volatilization by chemical reduction to their respective gaseous hydrides.

### Electrothermal Atomizers

In 1961, L'vov (44) described the first electrothermal atomizer for atomic absorption analysis of trace metals. L'vov's atomizer consisted of a graphite plug which fitted into a socket in an electrically heated graphite tube. A liquid sample was dried on the plug, which was then inserted into the tube. An alternating current arc was used to discharge the sample into an argon atmosphere within the graphite tube. Atomic recombination was retarded by the high temperature and the inert gas, and atomic absorption spectrometry of the atom cloud was performed by an optical beam along the axis of the tube (44). Numerous ingenious variations of the electrothermal atomizer have been developed, including use of graphite tubes, rods and braided filaments (2,3,8,12,22,42,50,51,52,54,55,56,81,83,84), as well as metallic ribbons, cups and filaments constructed of tungsten, tantalum, molybdenum or platinum (12,26,27,28,48,82). In this discussion, attention is focused primarily upon the three designs of electrothermal atomizers that have been most widely used for analyses of trace metals in biological materials.

Massmann (51,52) devised the graphite tube furnace which has served as the prototype of commercial graphite furnaces that have been manufactured by Perkin-Elmer Co. (50), Beckman Instruments GmbH. (Germany), and Jarrell-Ash Division of Fisher Scientific Co. A liquid sample with a volume from 5 to 50  $\mu$ l is introduced into a hollow graphite tube by means of a micropipet. The graphite tube is heated electrically by electrode connections at both ends of the tube. The graphite tube assembly is encased within a water-cooled jacket, and the cavity of the tube is slowly flushed with an inert gas such as argon or nitrogen. During each analysis, the temperature of the graphite tube is increased in a stepwise fashion so that (a) the sample is dried at 100-125°C, (b) the sample is pyrolyzed at 500-1200°C, so that organic constituents are destroyed, and (c) the metals in the sample are vaporized and atomized at 1500-3000°C. During this programmed sequence, the slow stream of inert gas flows through the cavity of the graphite tube, successively sweeping away (a) the water or solvent vapors that are released during drying, (b) the smoke and fumes that are generated during pyrolysis, and (c) the atom cloud that is liberated during atomization. The light path of the atomic absorption spectrometer traverses the length of the graphite tube. During the atomization phase, atomic absorption of the analyte in the atom cloud is measured by the spectrometer and is recorded with a strip-chart recorder. The



peak atomic concentration, "N", (atoms/ml) in the cavity of the graphite furnace is related to the analyte concentration, "C", (mol/liter of solution injected into the graphite tube by a micropipet) by the following equation:  $N = 6 \times 10^{20} VCeB/V_c$ , where V = volume of analyte (ml), e = vaporization efficiency, B = atomization efficiency, and  $V_c$  = volume of the cavity of the graphite tube (ml), provided that the analyte diffuses slowly out of the cavity (26).

West and Williams (81) and Amos *et al* (2) have employed an alternative design for an electrothermal atomizer, which has served as the prototype of the graphite rod atomizers manufactured by Varian-Techtron Pty, Ltd. (53). The sample (0.5 to 5  $\mu$ l) is dried within a small cavity located at the mid-length of a thin graphite rod, which is supported between two electrodes. The graphite rod is unenclosed, but is protected by a laminar stream of an inert gas such as argon or nitrogen. Optionally, the upward current of inert gas may be encircled by a concentric laminar stream of hydrogen. The elevated temperature of the heated graphite rod ignites the hydrogen, which forms a barrier that (a) entrains atmospheric oxygen, hindering oxidation of the atom cloud, and (b) assists in the reduction of metallic oxides to free metal atoms (64). During a brief atomization phase, the sample is suddenly vaporized, and the atom cloud is measured by its attenuation of a transverse optical beam that passes through an aperture at right angles to the length of the graphite rod. This simple, economical design has the apparent disadvantages of relatively limited sample volume and relatively short path-length of light in the atom cloud. However, Amos *et al* (2) have found that this atomizer provides analytical sensitivity that rivals that obtained with Massmann's graphite tube furnace.

A third design concept for an electrothermal atomizer uses a metallic or graphite boat, as described by Donega and Burgess (12). This configuration has been adapted by Hwang *et al* (26, 27, 28) in the form of the tantalum ribbon atomizers which are manufactured by Instrumentation Laboratories, Inc. and by Jarrell-Ash Division of Fisher Scientific Co. The apparatus (26) consists of a thin tantalum strip that is shaped to serve as a combination sample boat and heating element. The ends of the tantalum strip are clamped onto the tops of two electrodes, and the assembly is enclosed within an argon-purged chamber. The optical beam traverses quartz windows at the sides of the argon-purged chamber, and passes immediately over the sample cavity in the tantalum strip. A hinged door at the front of the chamber permits access for introduction of the sample. By use of different tantalum strips with sample cavities of various sizes, the atomizer can accommodate sample volumes from 1 to 100  $\mu$ l. When it is necessary to use atomization temperatures in the range from 2500 to 3000°C, a graphite cuvet can be substituted for the tantalum strip.

Torsi and Tessari (78) studied the influence of heating rate upon analytical sensitivities in electrothermal atomic absorption. They observed that in the graphite tube furnace the residence time of the atom cloud in the optical path is relatively long (5 to 20 sec), compared to the transitory burst of atoms (*ca* 0.5 sec) which occurs during the sudden heating of the graphite rod atomizer. The residence time of the atom cloud in the lightpath of the tantalum ribbon atomizer is presumably intermediate between that achieved with the other two basic designs.

### Inert Atmospheres for Electrothermal Atomization

According to Reeves *et al* (64), the inert atmosphere of the electrothermal atomizer serves the dual purposes of (a) protecting the graphite tube or rod against oxidation, and (b) retarding the degradation of analyte atoms in the atom cloud by recombination (*e.g.* with O<sub>2</sub>). From theoretical viewpoints, argon is the most suitable inert gas for these purposes. Thus, Maessen and Posma (46) showed that the diffusion coefficients of gold atoms in nitrogen and argon atmospheres are, respectively, one-fourth and one-fifth of that observed in a helium atmosphere. Hwang *et al* (26) reported that the specific heat of argon is lower than that of nitrogen and helium. Moreover, with use of nitrogen as the atmosphere, there is a possibility of forming a toxic gas, cyanogen, which has an absorption band in the ultraviolet spectrum. Despite these theoretical advantages of argon, many analysts prefer to employ nitrogen, which is less expensive than argon. Cruz and van Loon (10) discounted the possibility of generating cyanogen from nitrogen at 3000°C, and stated that cyanogen formation requires a higher temperature (*ca* 5000°C). From a practical viewpoint, there appears to be little difference between the use of argon and nitrogen atmospheres for most biological applications of electrothermal atomic absorption spectrometry. For greatest analytical sensitivity, the flow-rate of the inert gas should be adjusted to the minimum that is necessary to sweep away smoke and other interfering vapors, in order that the atom cloud be subjected to the least possible dilution (3). With some graphite tube atomizers, the flow of inert gas can be interrupted during the atomization cycle of the programmed sequence of changes in furnace temperature.

### Characteristics of Graphite Tubes

After a brief period of use, the graphite tubes and rods that are commonly employed in electrothermal atomizers begin to deteriorate, and their electrical characteristics become subject to drift (7,9,47). This is one of the most troublesome sources of analytical variability. Maessen *et al* (47) demonstrated that the properties of graphite (*e.g.* porosity and conductivity)

change appreciably during heating. Alteration of the electrical conductivity brings about changes in the rate of heating as well as in the ultimate temperature. Changes of porosity of graphite affect the deposition of liquid samples and also influence the diffusion rate of sample vapors into the graphite. Substitution of pyrolytic graphite for standard graphite has been an important innovation in electrothermal atomic absorption spectrometry. According to Aspila *et al* (3), pyrolytic graphite has the following advantages over standard graphite: (a) lower permeability to liquids and gases; (b) higher thermal conductivity; (c) higher resistance to oxidation; and (d) higher sublimation point (ca 3700°C). Clyburn *et al* (9) has introduced a simple technique for depositing a layer of pyrolytic graphite upon the surfaces of graphite heating elements. A mixture of methane and inert gas (argon or nitrogen) is introduced into the graphite atomizer system during operation at a temperature in excess of 2000°C. The layer of pyrolytic graphite which is thus generated is extremely dense, non-porous and resistant to oxidation. According to Clyburn *et al* (9) this treatment extends the effective lifetime of the graphite elements and improves analytical precision.

#### Temperature Calibration and Regulation

Electrothermal apparatuses for atomic absorption spectrometry are currently sold by most instrument manufacturers with "factory calibrations" of temperature scales. These temperature calibrations may be grossly inaccurate under the conditions which actually exist in analytical laboratories, owing to (a) local variations in electrical current, (b) fluctuations in pressure and temperature of coolant water, (c) variations in electrical resistance of batches of graphite tubes and rods or metallic ribbons or boats, and (d) changes in thermal response which occur during repeated heating of atomization devices. Findlay *et al* (18) observed that temperatures within a graphite furnace were substantially lower than were indicated by the manufacturer's calibration, and that large temperature variations occurred within the furnace, both axially and radially. Lundgren *et al* (43) reported similar findings, and concluded that factory calibrations cannot be relied upon for estimation of atomization temperatures. Based upon the findings of Findlay *et al* (18) and Lundgren *et al* (43), temperature calibrations should be verified in each laboratory by use of a thermocouple, an optical pyrometer, and/or by observations of the melting and boiling points of reference materials.

Inadequate regulation of atomizer temperature is a major source of imprecision in electrothermal atomic absorption spectrometry. The programmed heating of electrothermal atomizers can be achieved by five different methods, depending upon the electrical or physical parameters which are monitored during

the heating steps (55). The first and most common method is (a) to utilize a two- or three-stage electrical current program for heating the atomizer. Montaser and Crouch (55) noted that programmed heating can also be accomplished by monitoring and controlling (b) the voltage across the atomizer, (c) the power dissipated by the atomizer, (d) the infrared radiation emitted by the atomizer, and (e) the actual atomizer temperature, as measured by a thermocouple. Montaser and Crouch (55) found that thermal control by the radiation monitoring method is the most satisfactory means of achieving stable plateaus of temperature. They showed that the infrared radiation which is emitted by the atomizer is directly related to the atomizer temperature, and that a radiation feed-back control system can effectively compensate for changes in atomizer properties and other fluctuations which influence the temperature produced by electrothermal systems. Similarly, Lundgren and Johansson (42) described a graphite tube furnace in which a photodiode detector for infrared radiation is used as feed-back input for the temperature controller. They demonstrated that it is feasible to keep the atomizer temperature constant within  $\pm 10^{\circ}\text{C}$  (42,43). Such improved methods for temperature regulation should soon be adopted by instrument manufacturers, and will undoubtedly improve the accuracy of trace metal analyses.

During analyses of protein-containing biological materials, steadily progressive increases of temperature may be essential during the drying and ashing steps, in order to avoid foaming of the sample or sudden discharge of particles of ash, with attendant loss of analyte. Such gradual increments in temperature (*i.e.* a "ramp-mode") have been recommended by Fuchs *et al* (20) for analysis of serum aluminum, by Grafflage *et al* (23) for analyses of serum chromium and manganese, and by Pekarek *et al* (61) for analysis of serum chromium. The "ramp-mode" for drying and ashing has also proven to be necessary for measurements of serum nickel, using a direct electrothermal technique which has recently been developed in the author's laboratory. The "ramp-mode" can be achieved by careful manual adjustments of the atomizer controls, but this procedure is tedious and imprecise. Therefore, instruments which provide a "ramp-mode" for programmed increase of temperature during the drying and ashing cycles are advantageous for biological applications. The Perkin-Elmer Co. has recently introduced a "ramp-mode" accessory for their Model HGA 2100 graphite tube furnace. It is desirable that a sudden increase in temperature occur at the end of the ashing cycle, in order to reach the high-temperature plateau of the atomization cycle. This abrupt increase in temperature is necessary in order to achieve optimal analytical sensitivity (78). A brief (5 to 10 sec) "after-burn" cycle at the upper thermal limit of the atomizer is oftentimes necessary during analyses of biological materials, in order (a) to minimize the accumulation of ash, and (b) to prevent the "memory" effects which are

discussed later in this review. Unfortunately, the "after-burn" cycle shortens the lifetime of the heating elements.

### Sampling and Contamination Problems

Micro-pipetting instruments such as the "Eppendorf" or "Oxford" pipettors with disposable plastic cone tips are customarily employed to dispense the liquid samples into electrothermal atomizers. Sampling problems which are associated with the use of these pipettors are among the troublesome aspects of electrothermal atomic absorption spectrometry (67,75). The plastic cone-tips are frequently contaminated with metals, and they should invariably be cleaned before use by soaking in dilute "ultra pure" nitric acid, followed by multiple rinses with demineralized water which has been distilled in a quartz still. Each cone-tip should be then prepared by repeatedly pipetting and discarding the specimen which is to be analyzed. Particular care must be exercised to avoid contamination of the cone-tip by allowing it to touch any portion of the graphite tube or rod or of the metallic boat. The delivery of the sample into the atomizer must be done meticulously in order to avoid irregular splattering of droplets within the sample cavity (67). After repeated use, the porosity of graphite tubes becomes variable at different locations within the sample cavity. Hence, failure to deposit successive samples at the identical site can be a source of variability in analytical results. Measurements of trace metals in body fluids should invariably be performed in duplicate or triplicate, in order to minimize imprecision and to detect sporadic contamination. The tedious attention that must be directed to manual sampling makes electrothermal analysis unacceptable to some technologists in clinical chemistry laboratories. For this reason it would be desirable to employ automatic sampling devices such as are used in automated systems for gas chromatography. An automatic sampler for electrothermal atomic absorption spectrometry has recently been designed by Maessen *et al* (47), and may be a harbinger of commercial developments in this field. Techniques for sampling of solid materials for electrothermal atomic absorption spectrometry have been described by Barnett and Kahn (4) and by Lundgren and Johansson (42), and a method for continuous sample introduction into an electrothermal atomizer has been introduced by Kantor *et al* (38). Perhaps the most promising device for automation of electrothermal atomic absorption is the "graphite yarn thermal atomizer" that has been developed by Applied Research Laboratories Division of Bausch and Lomb Co. Graphite yarn is dispensed from a spool and is automatically unreeled through (a) a sampling chamber where the sample is pipetted onto the yarn, and (b) an electric furnace for desolvation and vaporization.

Sweat from the fingers and palms of the hands is rich in trace metals (25) and is a common source of contamination in

trace metal analysis. The graphite tubes, tantalum ribbons, plastic cone-tips and other components of electrothermal atomization systems should not be handled without use of plastic forceps or disposable plastic gloves. In electrothermal analyses of biological materials, it is necessary to remove the carbon ash from the sample cavity after almost every analysis. The lens paper which is customarily used for this purpose should be handled only with forceps or gloves in order to minimize contamination from sweat. In the design of electrothermal atomizers, it would be desirable to include an automatic system for ash removal, (such as vigorous flushing of the sample cavity with a jet of inert gas).

#### Methods for Standardization and Computation

Aqueous standard solutions are a source of certain difficulties in electrothermal atomic absorption spectrometry of trace metals in biological fluids. The viscosities and surface tensions of aqueous standard solutions are substantially less than the viscosities and surface tensions of serum, blood and other protein-containing fluids. These factors introduce volumetric disparities in pipetting of standard solutions and body fluids, and also cause differences in penetration of these liquids into porous graphite tubes or rods. Preliminary treatment of porous graphite with xylene may help to minimize the differences of liquid penetration (53,67). A more satisfactory solution of this problem is preparation of standards in aqueous solutions of metal-free dextran (50-60 g/liter), as first proposed by Pekarek *et al* (61) for the standardization of serum chromium analyses. This practice has been used successfully by the present author for standardization of analyses of serum nickel. The standard solutions which are prepared in aqueous dextran resemble serum in regard to viscosity and surface tension. Introduction of dextran-containing standard solutions is an important contribution to electrothermal atomic absorption analysis of trace metals in body fluids.

The "method of standard additions" has been employed as a technique for standardization of atomic absorption analyses of metals in biological fluids (13,21). In this procedure, several concentrations of standard analyte are added to samples of the biological fluid to be analyzed. The calibration curve which is obtained after additions of the standard analyte to the biological fluid should parallel that obtained when aqueous standards are analyzed. Extrapolation of the standard additions curve back to a negative intercept on the abscissa furnishes an estimate of the concentration of the analyte in the original sample (21). This technique is helpful in assessing the validity of methods of trace metal analysis (11,13,58). However, in the author's opinion, the "method of standard additions" is neither practical nor reliable as a routine method for standardization

of trace metal analyses in body fluids. Cruz and van Loon (10) and Fuller (21) noted that non-linearity of the calibration curves obtained by electrothermal atomic absorption spectrometry limits the reliability of the "method of standard additions". Moreover, the dilution of the sample which this technique entails may mask matrix interferences.

Atomic absorption spectrometers with dual monochromators are commercially available from Jarrell-Ash Division of Fisher Scientific Co., and from Instrumentation Laboratories, Inc. These instruments with dual wavelength monitoring capability permit the use of "internal standards". In this technique, the atomic absorption of the analyte is measured as a proportion of the atomic absorption at a reference wavelength of a known amount of another metal that has been added to the sample. This arrangement can be very useful in compensating (a) for volumetric differences in sampling, (b) for variations in atomizer temperature, and (c) to some extent for background compensation, as discussed subsequently. However, care must be exercised in selecting as the internal standard a metal with melting point, vapor pressure, free energy of oxide dissociation, and resonance wavelength which closely resemble those of the analyte (1).

Computations of the results of electrothermal atomic absorption analyses are usually based upon measurements of the peak heights of the recorder tracings. This technique has the advantages of simplicity and convenience, but it may be responsible for analytical errors. Maessen and Posma (46) showed that the time-constant for response of the measurement system has a critical influence upon the analytical signals. When they employed a measurement and recording system with a time-constant of 0.01 sec, undistorted signals were obtained by the strip-chart recorder. In contrast, when the time-constant was 0.3 sec (such as provided by common strip-chart recorders), highly distorted signals were obtained, and the peak heights were reduced to one-half or less of the undistorted peaks (46). Schramel (73) concluded from a careful study of matrix effects upon the curve-form of recorded absorption signals that the peak-height method of computation is inaccurate and inadequate. Integration of the output signal during the atomization phase furnishes a measure of peak area, which leads to more accurate and more precise analytical results. Schramel (73) emphasized that peak area (not peak height) is the correct index of analyte atoms released into the atomic cloud during electrothermal atomization. Sarbeck and Landgraf (70) employed a mini-computer system for automated integration of absorption signals which are detected during electrothermal atomization of trace metals. Such a system is marketed by Jarrell-Ash Division of Fisher Scientific Co.

### Sources of Interference

In order to understand matrix interferences in electrother-

mal atomization, it is necessary to examine the mechanisms of thermal vaporization of metals. Aggett and Sprott (1) showed that some metals, such as silver, gold and copper vaporize at temperatures that are considerably below their boiling points, while other metals, such as calcium and magnesium, require temperatures in excess of their boiling points for vaporization. From a penetrating study of the mechanisms of atomization, Aggett and Sprott (1) inferred that the higher the temperature of vaporization of a metal relative to its boiling point, the greater is the influence of the metallic oxide upon the atomization process. The free energy levels that are required for the dissociation of oxides of silver, gold and copper are negligible or very low, and these oxides do not play a significant role in the atomization process. On the other hand, the free energy levels that are required for dissociation of calcium and magnesium are relatively very high. Hence, the thermal dissociation of salts of calcium and magnesium (and various other metals) involves intermediate formation of metallic oxides prior to vaporization of the metal atoms. When graphite is used as the electrothermal atomizer, the graphite may reduce metallic oxides to the metals, with attendant liberation of carbon monoxide. Aggett and Sprott (1) found that the minimum temperatures for vaporization of Co, Fe, Ni and Zn from a graphite rod were significantly lower than from a tantalum filament, suggesting that these free metal atoms can be liberated by chemical reduction of their respective oxides, rather than by direct thermal dissociation. Findlay *et al* (19) emphasized the hazards of preatomization losses of trace metals in electrothermal atomic absorption spectrometry, when the ashing temperature is permitted to exceed the minimum temperature for vaporization of the analyte.

Interferences by matrix components in electrothermal atomic absorption spectrometry have been categorized by Aggett and Sprott (1) as either (a) "vapor phase", or (b) "surface phase". Vapor-phase interference is usually attributed to condensation of gaseous atoms in a manner so that analyte atoms are occluded in clusters of condensing interferant atoms. Interfering metal atoms tend to combine with traces of oxygen and to condense as the oxides. Vapor phase interference can be identified by the fact that the suppressive effect of the interferant upon the atomic absorbance of the analyte increases as a function of distance from the graphite or tantalum surface (1). Machata and Binder (45) have shown that addition of lanthanum salts to biological fluids effectively suppresses certain vapor phase interferences in trace metal analysis. Surface-phase interference is independent of the distance from the graphite or tantalum surface, and is attributed to changes in the thermal conductivity of the graphite or tantalum which are induced by the matrix (1). Surface phase interference results in broadening of the absorbance signal, and can be reduced by measurements of peak areas rather than by peak heights (73). Moreover, surface-phase



interference can be minimized by use of the infrared radiation-monitoring technique of temperature regulation, as previously discussed.

Spectral overlap of emission and absorption wavelengths is a potential cause of interference in atomic absorption spectrometry (57). Thus, (a) the emission line of Fe at 352.424 nm is close to the resonance line of Ni at 352.454, (b) the emission line of Sb at 217.023 nm is close to the resonance line of Pb at 216.999 nm, and (c) the emission line of As at 228.812 nm is close to the resonance line of Cd at 228.802 (57). To date, these practically coincident spectral lines have not been reported to be of practical importance as sources of analytical interference in atomic absorption analyses of biological materials.

Interference owing to sample "carry-over" or "memory effect" can be a serious problem in electrothermal atomic absorption spectrometry (77). The memory effect may be caused by condensation of analyte in a cooler region of the atomizer, and subsequent revaporization of analyte during the next atomization cycle (3). Another cause of memory effect is occlusion of the analyte within porous graphite, owing to the presence of other metals and carbon ash. Memory effect can be minimized by (a) increasing the flow-rate of inert gas, (b) increasing the temperature and duration of the atomization cycle, (c) using an "after-burn" cycle, as previously mentioned, and (d) substituting pyrolytic graphite for common graphite.

Interference from molecular absorption bands may be troublesome in analyses of relatively volatile metals such as cadmium and bismuth, which vaporize within the temperature ranges that are necessary for destruction of organic constituents of biological materials (80). Another cause of interference in electrothermal atomic absorption spectrometry of body fluids is nonspecific light-scattering owing to smoke and combustion vapors. Interferences from molecular absorption bands and light scattering can usually be ameliorated by the use of optical systems for background compensation, as will be described subsequently. In order to understand the various background correction systems, it is necessary to appreciate the optical maneuvers which are used to prevent interference by light that is emitted by electrothermal atomizers during the atomization phase.

In electrothermal atomic absorption spectrometry, the vaporized metal atoms within the atom cloud are predominantly in the ground state and are capable of absorbing discreet wavelengths of incident light. These specific resonance wavelengths are provided as monochromatic emission lines by a lamp with a hollow cathode containing the metal to be analyzed. The beam of light is passed through the atom cloud and is focused upon the entrance slit of a monochromator. Light at the selected wavelength is detected by a photomultiplier tube. In order to avoid interference by the intense light which is emitted from the

electrothermal atomizer, it is customary to modulate the incident light beam with a mechanical or electronic chopper, and to tune the photomultiplier detector to the same frequency of modulation. Under these conditions, the photomultiplier detector circuit should respond only to the pulsed signal from the light beam, and should not respond to the continuous signal produced by light emission from the electrothermal atomizer. The alternating current from the photomultiplier detector circuit is amplified and recorded. Owing to idiosyncrasies in the design of optical systems, light from the electrothermal atomizer can sometimes be reflected from the surfaces of a mechanical chopper, resulting in a pulsed wide-band signal which is registered by the photodetector and recorded as a shift of base-line absorption during the atomization cycle. A system of optical baffles and apertures can be installed in recent models of the atomic absorption spectrometers which are manufactured by the Perkin-Elmer Co. The baffle and aperture system minimizes interference by pulsed reflection of light which is emitted from the graphite tube furnace (31). Accurate installation of this baffle and aperture system is necessary in order to achieve reliable measurements of trace metals at concentrations near the detection limits.

#### Background Correction Systems

When biological materials are vaporized in an electrothermal atomizer, there is atomic absorption at the specific resonance wavelength of the analyte, and there is also nonspecific continuum absorption owing to molecular absorption bands and light-scattering by smoke and combustion vapors. In order to measure the specific absorption of the analyte, it is essential to correct or compensate for the nonspecific background absorption. Several ingenious optical systems have been developed for this purpose (8,27,49,85). For example, Hwang *et al* (27) described a hydrogen background correction system for use with Instrumentation Laboratories' Model 353 atomic absorption spectrometer. This system incorporates two hollow cathode lamps. One of the lamps, which contains a hollow cathode constructed of an alloy of the analyte, is electronically pulsed at a frequency of 0.5 kilohertz. The reference lamp is a hydrogen discharge tube which is pulsed at 1 kilohertz. Coincident beams from the two lamps are passed through the electrothermal atomizer, and a specific resonance wavelength of light is selected by the monochromator. The absorbance at this wavelength that emanates from the hydrogen lamp is electronically subtracted from that which emanates from the analyte lamp, in order to generate an automatically compensated readout.

As an alternative approach, Koirttyohann *et al* (35) employed a mechanical chopper system for deuterium-background correction similar to the one that is marketed by Perkin-Elmer Co. (49). One-half of the rotating chopper is transparent and the second-

**TABLE I**  
**Electrothermal Atomic Absorption Spectrometry of Trace Metals**  
**in Body Fluids, Tissues and Excreta**

| Trace Metal | Boiling Point (1)<br>°C | Minimum Vaporisation Temp. from Graphite Rod (1)<br>°C | Suitable Wavelengths for Atomic Absorption (63)<br>nm | Suitable Wavelengths for Background Compensation (13, 63)<br>nm | References to Biological Applications |                      |                         |
|-------------|-------------------------|--|---|---|---------------------------------------|----------------------|-------------------------|
|             |                         |  |   |   | Whole Blood, Serum, or Plasma         | Urine or Sweat       | Tissues, Feces, or Hair |
| Al          | 2467                    | 1550   | 309.3   |   | <u>20</u>                             |                      | <u>72</u>               |
| Au          | 2966                    | 1080   | 242.8   |   | <u>46, 47</u>                         |                      |                         |
| Bi          | 1560                    | 860  | 223.1   |   | <u>6</u>                              | <u>6</u>             |                         |
| Cd          | 765                     | 270  | 228.8   | 226.8   | <u>6, 45, 62, 74</u>                  | <u>6, 36, 45, 62</u> | <u>14, 72</u>           |
| Co          | 2900                    | 1300   | 240.7   | 239.3   | <u>47</u>                             |                      |                         |
| Cr          | 2482                    | 1380   | 357.9   |   | <u>11, 23, 60, 61</u>                 | <u>11, 69, 72</u>    | <u>71</u>               |
| Cu          | 2595                    | 1160   | 324.8   | 296.1   | <u>6, 16, 22, 53, 79</u>              | <u>6</u>             | <u>4, 14, 72, 76</u>    |
| Fe          | 3000                    | 1230   | 248.3   |   | <u>53, 59, 86</u>                     |                      | <u>72</u>               |
| Li          |                         |  | 670.8   |   | <u>47</u>                             |                      |                         |

TABLE I - Continued

| Trace Metal | Boiling Point(1)<br>°C | Minimum Vaporisation Temp. from Graphite Rod(1)<br>°C | Suitable Wave Lengths for Atomic Absorption(63)<br>nm | Suitable Wave Lengths for Background Compensation(13, 63)<br>nm | References to Biological Applications                                |                   |                            |
|-------------|------------------------|---|---|---|--|-------------------|----------------------------|
|             |                        |   |   |   | Whole Blood, Serum, or Plasma  | Urine or Sweat    | Tissues, Feeces, or Hair   |
| Mn          | 2097                   | 1210  | 279.5   |   | <u>5, 6, 23, 68</u>  | <u>6</u>          | <u>72</u>                  |
| Ni          | 2732                   | 1420  | 232.0   | 231.6   | <u>60, 77, 87</u>  | <u>87</u>         |                            |
| Pb          | 1744                   | 860   | 217.0, 283.3  | 220.4, 282.3  | <u>6, 10, 13, 15, 17, 24, 27, 28, 32, 37, 38, 39, 45, 53, 58, 67</u> | <u>6, 36</u>      | <u>24, 66, 72</u>          |
| Sr          |                        |   | 460.7   |   | <u>5</u>   |                   |                            |
| Tl          |                        |   | 276.8   |   | <u>24, 45</u>  | <u>24, 45</u>     | <u>24, 36</u>              |
| Zn          | 907                    | 800   | 213.9   | 210.4   | <u>7, 24, 40, 45, 53</u>   | <u>34, 40, 45</u> | <u>14, 24</u><br><u>45</u> |

half is mirrored. During one phase of the rotation of the chopper, light from the hollow cathode lamp passes through the transparent sector of the chopper and is passed through the electrothermal atomizer. During the second phase of rotation of the chopper, light from a deuterium continuum source is reflected from the mirrored sector of the chopper and is passed through the electrothermal atomizer. Both beams of light are focused on the spectrometer slit, and the signal from the photomultiplier tube is amplified by a phase-sensitive lock-in amplifier. When the two light beams are balanced, the amplifier output is zero. Continuum absorption owing to light-scattering or molecular bands reduces the intensity from both beams equally, causing no change in amplifier output. On the other hand, free atoms of the analyte absorb the narrow spectral band from the hollow cathode lamp much more efficiently than the broad spectral band from the continuum source. The two half-cycles are then of unequal energy, and the unbalance appears as the amplifier output signal.

Cruz and van Loon (10) pointed out the limitations of background correction systems that employ hydrogen or deuterium continuum sources. Such systems are limited to relatively low background absorption levels and to wavelengths where the  $H_2$  or  $D_2$  emissions are intense. Cruz and van Loon (10) recommend background correction by measuring the attenuation of a nearby non-resonance line of the analyte. This technique was employed by Ealy *et al* (13) for electrothermal atomic absorption of blood lead with the Jarrell-Ash Model 810 dualmonochromator spectrometer. Ealy *et al* (13) performed simultaneous measurements of lead at the 283.3 nm resonance line and at the nearby 282.3 nm non-resonance line. The specific absorbance owing to lead was automatically computed as the difference between the two absorbance measurements. Similarly, Kilroe-Smith (32) performed background compensation in electrothermal analyses of blood lead by measurements of the absorbance at 283.3 nm (resonance wavelength) and at 287.3 (non-resonance wavelength). Suitable pairs of resonance and non-resonance lines of several trace metals which are of clinical interest are listed in Table I.

Two additional optical systems for background correction deserve mention: Woodruff and Shrader (85) devised a system in which a light beam from a hollow cathode lamp is polarized horizontally, while a reference light beam from a hydrogen lamp is polarized vertically. After passing through the electrothermal atomizer and monochromator, the two perpendicularly polarized beams are separated by a polarizing prism and are measured by two photomultiplier tubes. In the system described by Church *et al* (8), a hollow cathode discharge lamp is placed in a uniform magnetic field (20 to 30 gauss) which splits the resonance line into three Zeeman components. One of the Zeeman lines lies within the absorption profile of the analyte atoms, and the other two Zeeman lines are symmetrically displaced on

either side of the absorption profile. The attenuation of the lines which lie outside the absorption profile of the analyte represents non-specific absorption, whereas the attenuation of the central line which lies within the absorption profile represents specific plus non-specific absorption. By difference, the specific absorption of the analyte can be measured. According to studies cited by Church *et al* (8), this method of background correction is applicable to every element that can be measured by atomic absorption spectrometry. A Zeeman-effect background correction system is sold by Arthur H. Thomas Co.

### Applications to Biological Materials

In Table I are listed comprehensive citations of published methods for analyses of trace metals in body fluids and other clinical specimens by means of electrothermal atomic absorption spectrometry. Readers are cautioned that many of the early methods that are cited in Table I have become outmoded, owing to improvements in instrumentation for electrothermal atomic absorption spectrometry. All of the published methods need to be critically evaluated in the prospective analyst's laboratory before they can be confidently employed for diagnostic measurements of trace metals in body fluids. Despite these caveats, the author believes that Table I should be helpful as a guide to the growing literature on clinical and biological applications of electrothermal atomic absorption spectrometry.

### Abstract

Electrothermal atomic absorption spectrometry has five major advantages for measurements of trace metals in biological materials, in comparison to measurements by conventional flame atomic absorption analysis: (1) Trace metal contamination is minimized by avoidance of preliminary chemical extractions and additions of reagents. (2) Sample volumes are small (1 to 50  $\mu$ l). The entire sample is vaporized in electrothermal atomization, whereas in most flame nebulization systems only a small fraction of the sample enters the flame. (3) Atoms are released in higher concentrations. In electrothermal atomization, the atom cloud is released into a relatively small volume of gas. In contrast, in flame atomization, the atom cloud is diluted by the high flow rate of gases and by expansion of gases during combustion. (4) Molecular recombination of atoms is retarded by the atmosphere of inert gas which restricts chemical reactions (*e.g.* oxidation). In contrast, in flame atomization, oxidation occurs very rapidly. (5) By programmed increments in temperature, electrothermal atomization permits organic constituents to be pyrolyzed prior to vaporization and atomization of metals. Despite these advantages, electrothermal atomic absorption is particularly subject to interferences and sources of imprecision

which necessitate critical evaluations in the prospective analyst's laboratory before an electrothermal method can be confidently employed for diagnostic measurements of a specific trace metal in body fluids.

#### Literature Cited

1. Aggett, J. and Sprott, A. J.; "Non-Flame Atomization in Atomic Absorption Spectrometry". *Anal. Chim. Acta* (1974), 72, 49-56.
2. Amos, M.D., Bennett, P.A., Brodie, K. G., Lung, P. W. Y., and Matousek, J. P.; "Carbon Rod Atomizer in Atomic Absorption and Fluorescence Spectrometry and its Clinical Application". *Anal. Chem.* (1971), 43, 211-215.
3. Aspila, K. I., Chakrabarti, C. L., and Bratzel, M. P., Jr.; "Pyrolytic Graphite-Tube Micro-Furnace for Trace Analysis by Atomic Absorption Spectrometry". *Anal. Chem.* (1972), 44, 1718-1720.
4. Barnett, W. B. and Kahn, H. L.; "Determination of Copper in Fingernails by Atomic Absorption with the Graphite Furnace". *Clin. Chem.* (1972), 18, 923-927.
5. Bek, F., Janouskova, J., and Moldan, B.; "Direct Determination of Manganese and Strontium in Human Blood Serum by Means of the Graphite Furnace, Perkin-Elmer HGA-70". *Chem. Listy* (1972), 66, 867-875.
6. Bourdon, R., Galliot, M., and Prouillet, F.; "Dosage du cuivre, du plomb, du manganèse, du bismuth, du cadmium et de l'or dans les liquides biologiques par spectrométrie d'absorption atomique sans flamme". *Ann. Biol. Clin.* (1974), 32, 413-422.
7. Chooi, M. K., Todd, J. K., and Boyd, N. D.; "Effect of Carbon Cup Aging on Plasma Zinc Determination by Flameless Atomic Absorption Spectrometry". *Clin. Chem.* (1975), 21, 632-634.
8. Church, D. A., Hadeishi, T., Leong, L., McLaughlin, R. D., and Zak, B. D.; "Two-Chamber Furnace for Flameless Atomic Absorption Spectrometry". *Anal. Chem.* (1974), 46, 1352-1355.
9. Clyburn, S. A., Kantor, T., and Veillon, C.; "Pyrolysis Treatment for Graphite Atomization Systems". *Anal. Chem.* (1974), 46, 2213-2215.
10. Cruz, R. B. and Loon, J. C. van; "A Critical Study of the Application of Graphite-Furnace Non-Flame Atomic Absorption Spectrometry to the Determination of Trace Base Metals in Complex Heavy-Matrix Sample Solutions". *Anal. Chim. Acta* (1974), 72, 231-243.
11. Davidson, I. W. F. and Secrest, W. L.; "Determination of Chromium in Biological Materials by Atomic Absorption Spectrometry Using a Graphite Furnace Atomizer". *Anal. Chem.* (1972), 44, 1808-1813.

12. Donega, H. M. and Burgess, T. E.; "Atomic Absorption Analysis by Flameless Atomization in a Controlled Atmosphere". *Anal. Chem.* (1970), 42, 1521-1524.
13. Ealy, J. A., Bolton, N. E., McElheny, R. J., and Morrow, R. W.; "Determination of Lead in Whole Blood by Graphite Furnace Atomic Absorption Spectrophotometry". *Amer. Ind. Hyg. Assoc. J.* (1974), 35, 566-570.
14. Evenson, M. A. and Anderson, C. T., Jr.; "Ultramicro Analysis for Copper, Cadmium and Zinc in Human Liver Tissue by Use of Atomic Absorption Spectrophotometry and the Heated Graphite Tube Atomizer". *Clin. Chem.* (1975), 21, 537-543.
15. Evenson, M. A. and Pendergast, D. D.; "Rapid Ultramicro Direct Determination of Erythrocyte Lead Concentration by Atomic Absorption Spectrophotometry with Use of a Graphite Tube Furnace". *Clin. Chem.* (1974), 20, 163-171.
16. Evenson, M. A. and Warren, B. L.; "Determination of Serum Copper by Atomic Absorption, With Use of the Graphite Cuvette". *Clin. Chem.* (1975), 21, 619-625.
17. Fernandez, F. J.; "Micromethod for Lead Determination in Whole Blood by Atomic Absorption, With Use of the Graphite Furnace". *Clin. Chem.* (1975), 21, 558-561.
18. Findlay, W. J., Zdrojewski, A., and Quickert, N.; "Temperature Measurements of a Graphite Furnace Used in Flameless Atomic Absorption". *Spectrosc. Lett.* (1974), 7, 63-72.
19. Findlay, W. J., Zdrojewski, A., and Quickert, N.; "Pre-atomization Losses in Flameless Atomic Absorption Spectroscopy". *Spectrosc. Lett.* (1974), 7, 355-364.
20. Fuchs, C., Brasche, M., Paschen, K., Norbeck, H., and Quellhorst, E.; "Aluminium-Bestimmung im Serum mit Flammenloser Atomabsorption". *Clin. Chim Acta* (1974), 52, 71-80.
21. Fuller, C. W.; "A Simple Standards Additions Technique Using the Model 306 Atomic Absorption Spectrophotometer". *At. Absorp. Newsl.* (1972), 11, 65-66.
22. Glenn, M., Savory, J., Hart, L., Glenn, T., and Winefordner, J.; "Determination of Copper in Serum With a Graphite Rod Atomizer for Atomic Absorption Spectrophotometry". *Anal. Chim. Acta* (1971), 57, 263-269.
23. Grafflage, B., Buttgereit, G., Kubler, W., and Mertens, H. M.; "Die Messung der Spurenelemente Chrom und Mangan im Serum mittels flammenloser Atomabsorption". *Z. Klin. Chem. Klin. Biochem.* (1974), 12, 287-293.
24. Hauck, G.; "Erfahrungen mit der flammenlosen Atomabsorption bei der Untersuchung biologischen Materials auf Spuren von Schwermetallen". *Z. Anal. Chem.* (1973), 267, 337-341.
25. Hohnadel, D. C., Sunderman, F. W., Jr., Nechay, M. W., and McNeely, M. D.; "Atomic Absorption Spectrometry of Nickel, Copper, Zinc, and Lead in Sweat from Healthy Subjects during Sauna Bathing". *Clin. Chem.* (1973), 19, 1288-1292.
26. Hwang, J. Y., Mokeler, C. J., and Ullucci, P. A.; "Maximization of Sensitivities in Tantalum Ribbon Flameless Atomic



10. Boué, J.; Boué, A.; and Lazar, P.; "The Epidemiology of Human Spontaneous Abortions With Chromosomal Abnormalities". *Aging Gametes, Int. Symp.* Seattle, (1975), 330-348.
11. Fuchs, F.; and Riis, P.; "Antenatal Sex Determination". *Nature (Lond.)*, (1956), 177, 330.
12. Steele, M. W.; and Breg, W. T.; "Chromosome Analysis of Human Amniotic Fluid Cells". *Lancet*, (1966), 1, 383-385.
13. Jacobson, C. B.; and Barter, R. H.; "Intrauterine Diagnosis and Management of Genetic Defects". *Am. J. Obstet. Gynecol.* (1967), 99, 796-807.
14. Nadler, H. L.; "Antenatal Detection of Hereditary Disorders". *Pediatrics*, (1968), 42, 912.
15. Levine, M. D.; McNeil, D. E.; Kaback, M. M.; Frazer, R. E.; Okada, D. M.; and Hobel, C. J.; "Second-Trimester Fetoscopy and Fetal Blood Sampling: Current Limitations and Problems." *Amer. J. Obstet. Gynecol.*, (1974), 120, 937-943.
16. Valenti, C.; "Antenatal Detection of Hemoglobinopathies". *Am. J. Obstet. Gynecol.*, (1973), 115, 851-853.
17. Hobbins, J. C.; and Mohoney, M. J.; "Fetal Blood Drawing". *Lancet*, (1975), 2, 107-109.
18. Omenn, G. S.; Figley, M. M.; Graham, C. B.; and Heinrichs, W. L.; "Prospects for Radiographic Intrauterine Diagnosis - The Syndrome of Thrombocytopenia With Absent Radii". *N. Engl. J. Med.*, (1973), 288, 777-778.
19. Campbell, S.; Johnston, F. D.; Holt, E. M.; and May, P.; "Anencephaly: Early Ultrasonic Diagnosis and Active Management". *Lancet*, (1972), 2, 1226-1227.
20. Campbell, S.; "The Prenatal Detection of Fetal Abnormality by Ultrasonic Diagnosis". *Birth Defects, Proc. 4th Int. Conf.*, Motulsky, A. G. and Lenz, W., ed., Excerpta Medica, Amsterdam, 1974, 234-239.
21. Kan, Y. W.; Valenti, C.; Guidotti, R.; Carnazza, V.; and Rieder, R. F.; "Fetal Blood Sampling in Utero". *Lancet*, (1974), 1, 79-80.
22. Kan, Y. W.; Golbus, M. S.; Klein, P.; and Dozy, M.; "Successful Application of Prenatal Diagnosis in a Pregnancy at Risk for Homozygous  $\beta$ -thalassemia". *New Engl. J. Med.*, (1975), 292, 1096-1099.
23. Hollenberg, M. D.; Kaback, M. M.; and Kazazian, H. H., Jr.; "Adult Hemoglobin Synthesis by Reticulocytes From the Human Fetus at Mid-Trimester". *Science*, (1971), 174, 698-702.
24. Kadotani, T.; Sato, H.; Ohama, K.; and Takahara, H.; "A Technical Note on the Antenatal Chromosome Analysis by Transabdominal Fetal Skin Biopsy". *Jap. Jour. Human Genet.*, (1972), 16, 42-47.
25. Hahnemann, N.; "Early Prenatal Diagnosis; A Study of Biopsy Techniques and Cell Culturing From Extraembryonic Membranes". *Clin. Genet.*, (1974), 6, 294-306.
26. Lind, T.; and Hytten, F. E.; "Relation of Amniotic Fluid Volume to Fetal Weight in the First Half of Pregnancy".

- Absorption Spectrometry". *Anal. Chem.* (1972), 44, 2018-2121.
27. Hwang, J. Y., Ullucci, P. A., and Mokeler, C. J.; "Direct Flameless Atomic Absorption Determination of Lead in Blood". *Anal. Chem.* (1973), 45, 795-798.
  28. Hwang, J. Y., Ullucci, P. A., Smith, S. B., Jr., and Malenfant, A. L.; "Microdetermination of Lead in Blood by Flameless Atomic Absorption Spectrometry". *Anal. Chem.* (1971), 43, 1319-1321.
  29. Ingle, J. D., Jr.; "Precision of Atomic Absorption Spectrometric Measurements". *Anal. Chem.* (1974), 46, 2161-2171.
  30. Kantor, T., Clyburn, S. A., and Veillon, C.; "Continuous Sample Introduction with Graphite Atomization Systems for Atomic Absorption Spectrometry". *Anal. Chem.* (1974), 46, 2205-2213.
  31. Kerber, J. D., Russo, A. J., Peterson, G. E., and Ediger, R. D.; "Performance Improvements with the Graphite Furnace". *At. Absorp. Newsl.* (1973), 12, 106-108.
  32. Kilroe-Smith, T. A.; "Linear Working Graphs in Blood Lead Determinations with the Beckman Flameless Atomic Absorption Cuvet". *Clin. Chem.* (1975), 21, 630-632.
  33. King, J. S.; "Editorial: A Burgeoning Branch of Clinical Analysis". *Clin. Chem.* (1975), 21, 467.
  34. Kirkbright, G. F.; "The Application of Non-Flame Atom Cells in Atomic Absorption and Atomic Fluorescence Spectroscopy. A Review". *Analyst (London)* (1971), 96, 609-623.
  35. Koirtjohann, S. R., Sievers, H., and Pickett, E. E.; "The Electrically Heated Furnace in Atomic Absorption" In "Trace Substances in Environmental Health - V". (D. D. Hemphill, Ed.), University of Missouri Press, Columbia, Mo., 1972, pp. 463-471.
  36. Kubasik, N. P. and Volosin, M. T.; "A Simplified Determination of Urinary Cadmium, Lead, and Thallium, with Use of Carbon Rod Atomization and Atomic Absorption Spectrophotometry". *Clin. Chem.* (1973), 19, 954-958.
  37. Kubasik, N. P. and Volosin, M. T.; "Use of the Carbon Rod Atomizer for Direct Analysis of Lead in Blood". *Clin. Chem.* (1974), 20, 300-301.
  38. Kubasik, N. P., Volosin, M. T., and Murray, M. H.; "A Quantitative Micro Technique for the Analysis of Lead in Blood by Carbon Rod Atomization and Atomic Absorption Spectrophotometry". *Clin. Biochem.* (1972), 5, 266-270.
  39. Kubasik, N. P., Volosin, M. T., and Murray, M. H.; "Carbon Rod Atomizer Applied to Measurement of Lead in Whole Blood by Atomic Absorption Spectrophotometry". *Clin. Chem.* (1972), 18, 410-412.
  40. Kurz, D., Roach, J., and Eyring, E. J.; "Determination of Zinc by Flameless Atomic Absorption Spectrophotometry". *Anal. Biochem.* (1973), 53, 586-593.
  41. Lisk, D. J.; "Recent Developments in the Analysis of Toxic

- Elements". *Science* (1974), 184, 1137-1141.
42. Lundgren, G. and Johansson, G.; "A Temperature-Controlled Graphite Tube Furnace for the Determination of Trace Metals in Solid Biological Tissue". *Talanta* (1974), 21, 257-264.
  43. Lundgren, G., Lundmark, L., and Johansson, G.; "Temperature Controlled Heating of the Graphite Tube Atomizer in Flameless Atomic Absorption Spectrometry". *Anal. Chem.* (1974), 46, 1028-1031.
  44. L'vov, B. V.; "The Analytical Use of Atomic Absorption Spectra". *Spectrochim. Acta* (1961), 17, 761-770.
  45. Machata, G. and Binder, R.; "The Determination of Lead, Thallium, Zinc and Cadmium Traces in Biological Material with Flameless Atomic Absorption". *Z. Rechtsmed.* (1973), 73, 29-34.
  46. Maessen, F. J. M. J., and Posma, F. D.; "Fundamental Aspects of Flameless Atomic Absorption Using the Mini-Massmann Carbon Rod Atomizer". *Anal. Chem.* (1974), 46, 1439-1444.
  47. Maessen, F. J. M. J., Posma, F. D., and Balke, J.; "Direct Determination of Gold, Cobalt, and Lithium in Blood Plasma Using the Mini-Massmann Carbon Rod Atomizer". *Anal. Chem.* (1974), 46, 1445-1449.
  48. McIntyre, N. S., Cook M. G., and Boase, D. G.; "Flameless Atomic Absorption Determination of Cobalt, Nickel, and Copper - A Comparison of Tantalum and Molybdenum Evaporation Surfaces". *Anal. Chem.* (1974), 46, 1983-1987.
  49. Manning, D. C.; "Using the Perkin-Elmer Deuterium Background Correction System". *At. Absorp. Newsl.* (1972), 11, 112-113.
  50. Manning, D. C. and Fernandez, F.; "Atomization for Atomic Absorption Using a Heated Graphite Tube". *At. Absorp. Newsl.* (1970), 9, 65-70.
  51. Massmann, H.; "Heutiger Stand der Atomabsorptionsspektrometrie". *Chimia* (1967), 21, 217-226.
  52. Massmann, H.; "Vergleich von Atomabsorption und Atomfluoreszenz in der Graphitküvette". *Spectrochim. Acta* (Part B) (1968), 23B, 215-226.
  53. Matousek, J. P. and Stevens, B. J.; "Biological Applications of the Carbon Rod Atomizer in Atomic Absorption Spectroscopy". *Clin. Chem.* (1971), 17, 363-368.
  54. Montaser, A. and Crouch, S. R.; "Analytical Applications of the Graphite Braid Nonflame Atomizer". *Anal. Chem.* (1974), 46, 1817-1820.
  55. Montaser, A. and Crouch, S. R.; "New Methods for Programmed Heating of Electrically Heated Nonflame Atomic Vapor Cells". *Anal. Chem.* (1975), 47, 38-45.
  56. Montaser, A., Goode, S. R., and Crouch, S. R.; "Graphite Braid Atomizer for Atomic Absorption and Atomic Fluorescence Spectrometry". *Anal. Chem.* (1974), 46, 599-601.
  57. Norris, J. D. and West, T. S.; "Some Applications of Spectral Overlap in Atomic Absorption Spectrometry". *Anal. Chem.* (1974), 46, 1423-1425.

58. Norval, E. and Butler, L. R. P.; "The Determination of Lead in Blood by Atomic Absorption with the High-Temperature Graphite Tube". *Anal. Chim. Acta* (1972), 58, 47-56.
59. Olsen, E. D., Jatlow, P. I., Fernandez, F. J., and Kahn, H. L.; "Ultramicro Method for Determination of Iron in Serum with the Graphite Furnace". *Clin. Chem.* (1973), 19, 326-329.
60. Pekarek, R. S. and Hauer, E. C.; "Direct Determination of Serum Chromium and Nickel by an Atomic Absorption Spectrophotometer with a Heated Graphite Furnace". *Fed. Proc.* (1972), 31, 700 Abs.
61. Pekarek, R. S., Hauer, E. C., Wannemacher, R. W., Jr., and Beisel, W. R.; "The Direct Determination of Serum Chromium by an Atomic Absorption Spectrophotometer with a Heated Graphite Atomizer". *Anal. Biochem.* (1974), 59, 283-292.
62. Perry, E. F., Koirtzohann, S. R., and Perry, H. M., Jr.; "Determination of Cadmium in Blood and Urine by Graphite Furnace Atomic Absorption Spectrophotometry". *Clin. Chem.* (1975), 21, 626-629.
63. Price, W. J.; "Analytical Atomic Absorption Spectrometry". Heyden and Son, Ltd., London, 1972, pp. 1-239.
64. Reeves, R. D., Patel, B. M., Molnar, C. J., and Winefordner, J. D.; "Decay of Atom Populations Following Graphite Rod Atomization in Atomic Absorption Spectrometry". *Anal. Chem.* (1973), 45, 246-249.
65. Reinhold, J. G.; "Trace Elements - A Selective Survey". *Clin. Chem.* (1975), 21, 476-500.
66. Renshaw, G. D., Pounds, C. A., and Pearson, E. F.; "Variation in Lead Concentration Along Single Hairs as Measured by Non-Flame Atomic Absorption Spectrophotometry". *Nature* (1972), 238, 162-163.
67. Rosen, J. F. and Trinidad, E. E.; "The Microdetermination of Blood Lead in Children by Flameless Atomic Absorption: The Carbon Rod Atomizer". *J. Lab. Clin. Med.* (1972), 80, 567-576.
68. Ross, R. T. and Gonzalez, J. G.; "Direct Determination of Trace Quantities of Manganese in Blood and Serum Samples Using Selective Volatilization and Graphite Tube Reservoir Atomic Absorption Spectrophotometry". *Bull. Environ. Contam. Toxicol.* (1974), 12, 470-474.
69. Ross, R. T., Gonzalez, J. G., and Segar, D. A.; "The Direct Determination of Chromium in Urine by Selective Volatilization with Atom Reservoir Atomic Absorption". *Anal. Chim. Acta* (1973), 63, 205-209.
70. Sarbeck, J. R. and Landgraf, W. C.; "Automated Peak Discrimination and Integration for Nonflame Atomic Absorption Analysis at Nanogram Levels". *J. Pharm. Sci* (1974), 63, 929-930.
71. Schaller, K. H., Essing, H. G., Valentin, H., and Schäcke, G.; "Quantitative Chrombestimmung im Harn mit flammenloser Atomabsorptions-Spektrometrie". *Z. Klin. Chem. Klin. Biochem.*

- (1972), 10, 434-437.
72. Schramel, P.; "Determination of Eight Metals in the International Biological Standard by Flameless Atomic Absorption Spectrometry". *Anal. Chim. Acta* (1973), 67, 69-77.
  73. Schramel, P.; "The Application of Peak Integration in Flameless Atomic Absorption Spectrometry". *Anal. Chim. Acta* (1974), 72, 414-418.
  74. Schumacher, E. and Umland, F.; "Verbesserte, schnelle Aufschlussmethode zur Cadmiumbestimmung in Körperflüssigkeiten mit Hilfe der Graphitrohrküvette". *Z. Anal. Chem.* (1974), 270, 285-286.
  75. Sommerfeld, M. R., Love, T. D., and Olsen, R. D.; "Trace Metal Contamination of Disposable Pipet Tips". *At. Absorp. Newsl.* (1975), 14, 31-32.
  76. Stevens, B. J.; "Biological Applications of the Carbon Rod Atomizer in Atomic Absorption Spectroscopy. 2. Determination of Copper in Small Samples of Tissue". *Clin. Chem.* (1972), 18, 1379-1384.
  77. Sunderman, F. W., Jr.; "Atomic Absorption Spectrometry of Trace Metals in Clinical Pathology". *Hum. Pathol.* (1973), 4, 549-582.
  78. Torsi, G. and Tessari, G.; "Influence of Heating Rate on Analytical Response in Flameless Atomic Absorption Spectrometry". *Anal. Chem.* (1973), 45, 1812-1816.
  79. Welz, B. and Wiedeking, E.; "Bestimmung von Spurenelementen im Serum und Urin mit flammenloser Atomisierung". *Z. Anal. Chem.* (1970), 252, 111-117.
  80. West, C. D.; "Relative Effect of Molecular Absorption on Atomic Absorption and Atomic Fluorescence". *Anal. Chem.* (1974), 46, 797-799.
  81. West, T. S. and Williams, X. K.; "Atomic Absorption and Fluorescence Spectroscopy with a Carbon Filament Atom Reservoir". *Anal. Chim. Acta* (1969), 45, 27-41.
  82. Williams, M. and Piepmeier, E. H.; "Commercial Tungsten Filament Atomizer for Analytical Atomic Spectrometry". *Anal. Chem.* (1972), 44, 1342-1344.
  83. Woodriff, R.; "Atomization Chambers for Atomic Absorption Spectrochemical Analysis: A Review". *Appl. Spectrosc.* (1974), 28, 413-416.
  84. Woodriff, R. and Ramelow, G.; "Atomic Absorption Spectroscopy with a High-Temperature Furnace". *Spectrochim. Acta (Part B)* (1968), 23B, 665-671.
  85. Woodriff, R. and Shrader, D.; "Furnace Atomic Absorption with Reference Channel". *Anal. Chem.* (1971), 43, 1918-1920.
  86. Yeh, Y.-Y., and Zee, P.; "Micromethod for Determining Total Iron-Binding Capacity by Flameless Atomic Absorption Spectrophotometry". *Clin. Chem.* (1974), 20, 360-364.
  87. Zachariasen, H., Andersen, I., Kostol, C., and Barton, R.; "Technique for Determining Nickel in Blood by Flameless Atomic Absorption Spectrophotometry". *Clin. Chem.* (1975), 21, 562-567.

## Drug Interference in Laboratory Testing

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Over the past decades, the practice of medicine has undergone considerable sophistication. Potent new drugs in practically every therapeutic category are available. Whenever a patient receives a drug, there is a possibility of drug-test interferences. When more than one drug is administered there may also be drug-drug interactions. These developments have created new problems for the laboratory scientist. A characteristic problem is the interpretation of laboratory data when a drug or its metabolite may interfere with a laboratory procedure. One drug may affect the action of another given at the same time, thus changing the value of a test result. Numerous attempts have been made by a number of compilations and articles to document these effects. In 1962, Caraway (2) published a comprehensive article on the chemical and diagnostic specificity of laboratory tests. In 1964, Borushek and Gold (1) published an article containing a list of drugs interfering with routine endocrine procedures. Wirth and Thompson (19) in 1965 published a list of substances and conditions which affect the results of laboratory procedures. They included such factors as temperature, light and drugs. Other review articles (8,11,14,17) have been of great value in helping to define this problem. One of the most ambitious approaches to this problem has been the development of a 9000-entry computerfile based on a compilation of 1030 literature references (20). This computerized compilation of effects of drugs on laboratory tests has been used to assist in the interpretation of unusual test results in the clinical chemistry laboratories of the National Institutes of Health and the Evanston Hospital of Northwestern University Medical Center, as well as in some other centers.

A major problem facing users of the various collations of laboratory test interferences is the relationship between drug dosage and diagnostic test interference and the possible synergistic effect of several drugs. The metabolic and clinical states

of the patient also affect the degree of laboratory interference. If an individual has normal kidney and liver function, he will respond differently to a drug dosage than the patient who is unable to detoxify or excrete the compound.

In order to cope with the problem of drug induced modifications of laboratory test values, laboratory scientists should possess an understanding of the mechanisms involved in these interferences. The purpose of this article is to consider these mechanisms and discuss the role of the computerized drug-test interference file in assisting in the prediction as well as interpretation of apparent test results.

### Mechanisms of Interference

There are four distinct mechanisms of interference in laboratory testing. Physical, chemical, pharmacological and drug-drug interactions are of special interest to the laboratory scientist. The mechanisms of the interferences will be briefly reviewed and wherever possible, solutions suggested.

#### Physical Effects

Drugs may interfere with colorimetric, photometric or fluorometric analyses by imparting a characteristic color to the specimen. Table I describes some unusually colored urines and their relationship to drug administration or metabolic disorders. This type of interference can be detected visually by laboratory personnel and drug interferences may be circumvented by collecting the specimen after an appropriate length of time during which the drug in question has been discontinued. There are many varieties of drugs and foods that can cause this type of interference. These should be considered first as possible causes of any unusually colored urine.

Some drugs act as indicators (e.g. phenolphthalein, vegetable laxatives) and affect tests carried out at a particular pH. The presence of sulfobromophthalein dye (BSP) in serum will interfere with serum protein determined by the biuret method. The dye is colorless at the pH of blood, but purple when made alkaline during the assay.

#### Chemical Effects

Drugs may be measured as analytes, either because of their similarity in chemical structure or because they contain a component that is an analyte. Patients who have received radiographic contrast media containing iodine will have an altered protein-bound iodine, although thyroxine is not affected when determined by protein binding methods. The most serious and long-lasting interferences are those caused by intravenously administered iodinated radiopaque agents (e.g. acetrizoate and

TABLE I  
COLORED URINES DUE TO DRUGS OR METABOLIC DISORDERS

| <u>Color of Urine</u> | <u>Cause</u>                                   | <u>Disorder</u>   |
|-----------------------|--|---|
| Orange-Red            | Phenazopyridine<br>Phenindione<br>Phensuximide | Drug Related  |
| Red                   | Porphyrins                                     | Congenital Porphyria<br>Acute Intermittant<br>Porphyria |
| Red-Brown             | Hemoglobin and<br>Derivatives                  | Crush Syndrome  |
| Red-Brown             | Urobilin                                       | Hemolytic Anemia  |
| Yellow                | Chloroquine                                    | Drug Related  |
| Green-Yellow          | Bile Pigments                                  | Regurgitative Jaundice                                  |
| Blue                  | Methylene Blue<br>Triamterene                  | Drug Related  |
| Blue                  | Indigo Compounds                               | Intestinal Disease<br>(Indicanemia)                     |
| Blue-Green            | Amitriptylene                                  | Drug Related  |
| Brown-Black           | Melanin<br>Homogentisic Acid                   | Malignant Melanoma<br>Alkaptonuria                      |



adipiodone). The drugs orabilex and dionsil have a negligible effect on the analysis of protein bound iodine at a concentration no greater than 100  $\mu\text{g}/\text{dl}$ , but seriously affect the procedure at 1000  $\mu\text{g}/\text{dl}$ .

Serum potassium concentration is increased by the concurrent administration of intravenous potassium penicillin G. The penicillin preparation contains 1.7 mmol of potassium per million units. Thus, a patient receiving 10 million units of the antibiotic receives 17 mmol (mEq.) of potassium.

A chemical interference may also result when the substance being determined reacts *in vivo* with a drug administered prior to the collection of blood for analysis. For example, serum calcium determinations may be affected by the administration of the chelating agent ethylenediaminetetraacetate (EDTA) (3). In certain conditions such as lead poisoning and collagen disease, EDTA has been used therapeutically to reduce the lead and calcium concentrations, respectively. Less well recognized is the fact that various chelating agents are also used as preservatives to prevent oxidation reactions in drug preparations. In patients on EDTA therapy, calcium cannot be determined by the indirect colorimetric or fluorometric methods based on the chelation of a calcium - EDTA complex. However, in calcium determinations by atomic-absorption spectroscopy, the complexing agent is destroyed in the flame and the direct concentration of calcium can be determined.

Drugs can also interfere with laboratory results by negating certain nonspecific oxidation and reduction reactions essential for the chemical assay. Penicillin, streptomycin and ascorbic acid are known to react with cupric ion; thus, false positive results for glucose may occur if a copper reduction method is used. If the specific enzymatic glucose-oxidase method is employed, ascorbic acid can cause a false negative result by preventing the oxidation of a specific chromogen in the reaction.

Increased transaminase activity has been observed when the diazocolorimetric method is used. Unusually increased activity has been reported in patients with ketosis and also in patients receiving erythromycin (13) or p-aminosalicylic acid (9). These test interferences can be obviated by employing ultraviolet kinetic procedures.

Colorimetric procedures used in steroid assays are often subject to drug interference. In the determination of 17-Ketosteroids by the Zimmerman reaction, drugs with the 17-Keto basic structure such as ascorbic acid, morphine and reserpine will cause increased values. In the determination of 17,21 - dihydroxysteroids by the Porter-Silber reaction the dihydroxy-acetone chain is the reactive unit. Drugs like meprobamate, chloral hydrate, chlorpromazine and potassium iodide will interfere with this reaction and cause elevated values. In the colorimetric determination of vanillylmandelic acid (VMA) by a diazo reaction, drugs like methocarbamol and methyl dopa cause

falsely elevated results.

Where specific drugs have been demonstrated to interfere with chemical reactions, patients should be maintained free of these drugs for at least 72 hours before collecting the specimen. Other analytical techniques, e.g. column chromatography and radioimmunoassay procedures, can also be substituted for an affected method.

### Pharmacological Effects

This type of interference usually results from the pharmacological or toxic activity of drugs but may also arise from therapeutic quantities of drugs. An example of this type is the increase in prothrombin time which results from the administration of a therapeutic amount of sodium warfarin. However, unexpected interferences resulting from side effects or adverse reactions may create problems in the interpretation of laboratory data. Disturbances in fluid and electrolyte balance and hypopotassemia are common. Thiazide diuretics and large doses of glucocorticosteroids may severely reduce the concentration of plasma potassium. Acetazolamide and ethoxazolamide may cause metabolic acidosis, a raised serum uric acid, and a lowered urinary excretion of uric acid. A rise in serum uric acid has also been reported following the administration of levodopa (4). The thiazides can also have a hyperglycemic activity and diabetics may require more insulin or oral antidiabetic drugs. Patients with latent diabetes occasionally develop an abnormal glucose tolerance curve. Salicylates have also been reported to reduce hyperglycemia in diabetics and produce hypoglycemia in normal children. Tables II and III list various drugs capable of increasing or decreasing blood glucose in non-diabetic subjects.

Hydantoin anticonvulsants may cause the appearance of positive L.E. cells and methemoglobinemia. Chlorpromazine has been implicated in causing a syndrome like systemic lupus erythematosus with accompanying positive tests for L.E. cells and antinuclear antibodies (6).

In many instances administered drugs alter a metabolic pathway and directly produce changes in biochemical values. Insulin administration results in changes in blood glucose, potassium, phosphorus and fatty acids. Allopurinol reduces uric acid by inhibiting the enzyme, xanthine oxidase; other drugs e.g. azoserine and methotrexate do so by preventing its biosynthesis. Hydrochlorothiazide increases uric acid retention by decreasing its tubular excretion. Oral contraceptives have a marked effect on plasma glucose, fatty acids and growth hormone levels (16). Estrogen-progestin oral contraceptives produce increases in serum iron, iron-binding capacity, transferrin, ceruloplasmin and copper (18). These effects on endocrine functions are quite marked as evidenced by measurement of the related biochemical constituents (Table IV) (12).

TABLE II

## DRUGS CAPABLE OF INCREASING BLOOD GLUCOSE

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|                   |                        |
|-------------------|------------------------|
| Dextrothyroxine   | Nicotinic Acid         |
| Diazoxide         | Phenothiazines         |
| Diphenylhydantoin | Steroids               |
|                   | Adrenocorticosteroids  |
| Diuretics         | Estrogens              |
|                   | Sympathomimetic Amines |

TABLE III

## DRUGS CAPABLE OF DECREASING BLOOD GLUCOSE

---

|                              |                 |
|------------------------------|-----------------|
| Alcohol                      | Propoxyphene    |
| Asparaginase                 | Propranolol     |
| Caffeine                     | Anabolic Agents |
| Haloperidol                  |                 |
| Monoamine Oxidase Inhibitors |                 |

Some drugs induce unexpected side effects unrelated to their therapeutic actions. Inheritance of hemoglobins differing in structure from normal hemoglobin A is well described. All types of hemoglobin are oxidized to methemoglobin by a wide range of agents and about 1% of hemoglobin present in the blood of normal people is in this form. Many drugs slightly increase the amount of methemoglobin, even in normal people, but induce a striking and sometimes fatal methemoglobinemia in susceptible patients. These drugs include chlorates, sulfonamides, nitrites, quinones, acetanilid and acetophenetidin. A similar phenomenon occurs with primaquine sensitivity. Susceptible patients have an inherited defect in red cell glucose 6-phosphate dehydrogenase (G6PD) and develop a severe hemolytic anemia when exposed to primaquine. Another abnormal drug response is porphyria. Acute porphyria on exposure to drugs can be caused by sudden massive induction of deltaamino levulinic acid synthetase in hepatic mitochondria; which results in uncontrolled formation of porphobilinogen and its metabolites. Susceptibility is inherited as an autosomal dominant trait and occurs even in heterozygotes. The nature of the molecular defect is unclear and presumably lies in the repression mechanism for the gene controlling formation of the enzyme protein. Exposure to any of the drugs listed in Table V results in further marked de-repression of enzyme synthesis and severe porphyria.

Table VI lists several drugs inducing hepatic microsomal enzymes (5). These enzymes can metabolize the drug as well as other substrates. Barbiturates, griseofulvin, and glutethimide induce enzymes which metabolize coumarin and phenindione derivatives and thus reduce their anticoagulant activity. Diphenylhydantoin and phenylbutazone stimulate cortisol hydroxylase activity and increase the urinary excretion of B-hydroxy cortisol and decrease the concentration of cortisol in the plasma.

Adverse side reactions may also occur with pharmacologically active drugs. Many drugs have been associated with inducing abnormal liver, renal and pulmonary function (10). These drug-test effects are unexpected and should be recognized. Drugs in this group include methyltestosterone, reserpine, phenacemide, oxyphenylbutazone, anesthetics and a variety of cancer chemotherapeutic agents.

### Drug-Drug Interactions

Drugs which independently have little effect on laboratory tests may interact to produce a significant alteration of test values. Table VII presents several drug interactions in diabetic human subjects which can affect an apparent test result (15).

The administration of clofibrate to a patient taking warfarin will potentiate the anticoagulant effect of warfarin by displacing it from its protein binding site (7). This interaction will cause

TABLE IV  
HORMONE LEVELS IN WOMEN ON ORAL CONTRACEPTIVES\*

| <u>Hormone</u>                | <u>Level in Menstruating Women</u> | <u>Level in Women on Oral Contraceptives</u> |
|-------------------------------|------------------------------------|--|
| Plasma Cortisol (8 AM)        | 15.0 ± 4.0 µg/dl                   | 40.0 ± 11 µg/dl                              |
| Serum Thyroxine               | 6.6 ± 1.4 µg/dl                    | 9.4 ± 1.7 µg/dl                              |
| Urinary 17-OH Corticosteroids | 4.6 ± 1.1 mg/d                     | 2.6 ± 0.9 mg/d                               |
| Estrogens, Total (Urine)      | 40.0 ± 18 µg/d                     | 26.0 ± 7.4 µg/d                              |
| Vanilylmandelic Acid (Urine)  | 3.5 ± 1.6 mg/d                     | 3.3 ± 1.6 mg/d                               |

\* from Lucis, O.J. and Lucis, R.; Bull. W.H.O. (1972), 46, 443-450.

TABLE V  
DRUGS INDUCING MARKED  
DE-REPRESSION OF ENZYME SYNTHESIS

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Chloroquine  
Aminopyrine  
Sulfonamides  
Diallylbarbiturate  
Allylisopropylacetylurea  
Hexachlorobenzene

TABLE VI

## DRUGS INDUCING HEPATIC MICROSOMAL ENZYMES

---

|                   |                  |
|-------------------|------------------|
| Phenylbutazone    | Barbiturates     |
| Cortisone         | Glutethimide     |
| Aminopyrine       | Chlordiazepoxide |
| Diphenylhydantoin | Testosterone     |
| Carbutamide       | Norethynodrel    |

TABLE VII

## DRUG INTERACTIONS REPORTED IN DIABETIC SUBJECTS

| <u>Drug Interaction</u> | <u>Hypoglycemic Drug</u> | <u>Reaction</u>   |
|-------------------------|--------------------------|---|
| Alcohol                 | Insulin                  | Inhibition of Gluconeogenesis                             |
|                         | Sulfonylurea             |   |
| Bishydroxycoumarin      | Phenformin               | Lactic Acidosis   |
|                         | Chlorpropamide           | Decrease in Excretion or Metabolism of Hypoglycemic Agent |
| Guanethidine            | Insulin                  | Decrease in Insulin Dosage Needed to Control Patient      |
| Oxytetracycline         | Insulin                  | Decrease in Insulin Dosage Needed to Control Patient      |
| Phenothiazine           | Insulin                  | Increase in Insulin Dosage Needed to Control Subject      |
| Propranolol             | Insulin                  | Decrease in Insulin Dosage Needed to Control Subject      |

a significant increase in the prothrombin time test used to regulate warfarin administration. Displacement of drugs from their binding sites is a common mechanism by which drug-drug interactions result in unexpected laboratory data. After absorption, about 50 to 80 percent of salicylate is bound to serum albumin, from which it displaces other substances such as bilirubin and thyroxine. Other drugs such as coumarin may also be displaced with a sudden change in prothrombin time.

Drug-drug interactions may also cause enzyme induction. Ethchlorvynol, glutethimide and haloperidol are known to inhibit the effectiveness of warfarin therapy by this mechanism (7). These drug-drug interactions may result in a reduced prothrombin time. Enzyme inhibition is another result of drug-drug interaction and the impairment of the hepatic glucuronyl transferase system by phenothiazine derivatives is a good example. Whenever several drugs are administered concurrently to a patient, their combined effect on laboratory results should be carefully considered.

#### Effectiveness of Computerized Drug Interference File

The computerized drug interference file has been used in two modes. In one, possible interactions are listed in response to direct queries (the role for which it was originally designed). In the other, the file has been used to provide automatic interpretation of effects of drugs on tests. In this mode, the file is used to provide a report alerting a physician to a possible interaction between a drug and a test (Figure 1). Without a computerized hospital information system, which could be accessed to provide information about a patient's clinical state and non therapeutic-drug procedures, the drug file cannot be completely effective in providing explanations for changes in laboratory data. Its role must be that of a warning system to highlight possible causes of changes in test values which can be accepted or rejected by the patient's physician on the basis of his clinical judgement.

Our experience in a University teaching hospital, in which over 10,000 patient days were monitored, indicated that the drug file provided the correct explanation for changes in laboratory data in approximately 21 percent of all the occasions in which a report was produced (21). A report, similar in content and format to an entry in the master file (20), was generated when an abnormal test result was produced and one of the administered drugs had been described as causing this effect. The yield of correct explanations would undoubtedly be increased by elimination of effects that were unlikely to arise in hospital practice, e.g. effects due to overdoses or toxic effects of drugs. To make it appropriate for on-line use in any particular hospital, the file should be modified to eliminate these effects. The only methodological interferences that should be included are those involving the procedures in use in the hospital laboratory. The file is

| DRUG: HEPARIN  |                          |                                   |
|--|--------------------------|-----------------------------------|
| -----  |                          |                                   |
| CLOTTING TIME INC<br>CONCENTRATION RELATED EFFECT              | BLOOD (WHOLE)            | PHYSIOLOGICAL EFFECT<br>REF: 0704 |
| FACTOR IX DEC<br>REPORTED EFFECT                               | BLOOD (WHOLE)            | PHYSIOLOGICAL EFFECT<br>REF: 0384 |
| FACTOR V DEC<br>CONCENTRATION RELATED EFFECT                   | BLOOD (WHOLE)            | PHYSIOLOGICAL EFFECT<br>REF: 0384 |
| FACTOR XI DEC<br>CONCENTRATION RELATED EFFECT                  | BLOOD (WHOLE)            | PHYSIOLOGICAL EFFECT<br>REF: 0384 |
| PLATELET COUNT DEC<br>REPORTED EFFECT FOLLOWING I.V. INFUSIONS | BLOOD (WHOLE)            | PHYSIOLOGICAL EFFECT<br>REF: 0656 |
| PTT INC<br>CONCENTRATION RELATED EFFECT                        | BLOOD (WHOLE)            | PHYSIOLOGICAL EFFECT<br>REF: 0704 |
| THROMBIN TIME INC<br>RELATED TO CONC OF CIRCULATING HEPARIN    | BLOOD (WHOLE)            | PHYSIOLOGICAL EFFECT<br>REF: 0704 |
| THROMBOPLASTIN GEN DEC<br>ABNORMAL RESPONSE (INHIBITION)       | BLOOD (WHOLE)            | PHYSIOLOGICAL EFFECT<br>REF: 0384 |
| AMMONIA INC<br>CONTAINS VARIABLE AMOUNTS OF                    | PLASMA<br>AMMONIUM SALTS | ANALYTICAL EFFECT<br>REF: 0181    |
| CORTICOSTEROIDS INC<br>IF CONTAMINATED BY IMPURITIES           | PLASMA                   | ANALYTICAL EFFECT<br>REF: 0524    |
| INSULIN DEC<br>EFFECT IN HEPARINIZED PLASMA AND SERUM          | PLASMA                   | ANALYTICAL EFFECT<br>REF: 0709    |
| INSULIN INC<br>SPURIOUSLY HIGH VALUES REPORTED FOR IMMUNOASSAY | PLASMA                   | ANALYTICAL EFFECT<br>REF: 0064    |
| PROTHROMBIN TIME INC<br>CONCENTRATION RELATED EFFECT           | PLASMA                   | PHYSIOLOGICAL EFFECT<br>REF: 0704 |
| TSH DEC<br>INTERFERES WITH THYROXINE BINDING TO PROTEIN        | PLASMA                   | PHYSIOLOGICAL EFFECT<br>REF: 0502 |

**Figure 1.** Report alerting physician to drug interference in laboratory testing



being modified for use in the automatic warning role in a community hospital to determine if it can provide a useful service for physicians with a different orientation from those in a University hospital.

In the off-line mode of use of the drug file the data-base, stored in a time-shared computer, is interrogated through a remote device such as a cathode ray terminal. The questions that may be asked include all the affects of a drug, all the drugs that affect a test, or more specifically does a particular drug affect a particular test procedure by a particular mechanism. In each of these situations it is possible to list the appropriate literature reference. The search procedure has been designed in an interactive mode so that even an individual who has never used it may obtain an explanation for a particular problem, but the experienced searcher can by-pass the longer interactive search routine. This mode of operation is in use only at the National Institutes of Health and a simpler procedure is used in Evanston Hospital.

The most important use of a computer in setting up the CLAUDE (computer listing of abnormal and usual drug effects) concept may be that of a text editor and sorter. Scanning a printout is much more efficient than searching the file in the computer except for any information that is not yet available on a printout. It is our intention to reproduce printouts of the file in a journal with a wide circulation to the appropriate audience when a significant amount of new information has been added to the master-file. The second edition has recently been published (22). It includes some information on the probabilities of certain effects occurring as well as an indication of the concentration of drug at which the effects occur, at least for the methodological effects.

The file is used routinely in the laboratory at the National Institutes of Health in an attempt to explain abnormal test results. The resident physicians affiliated with the Clinical Chemistry Service discuss the results with the patient-care physicians and determine if the results were due to the patient's clinical state or to a drug effect. This close monitoring of test results has led to recognition of deficiencies in what is believed are specific enzymatic procedures for the measurement of glucose and uric acid. Likewise, the guaiac procedure for occult blood in feces was found to yield false negative results under certain circumstances. This has prompted the development of a more specific procedure (Jaffe et al. unpublished).

The file may be queried in response to questions from clinical staff at the National Institutes of Health and physicians and laboratory scientists throughout the country who submit their problems by letter or telephone. The file has been used to provide information for a pharmaceutical manufacturing company preparing a new drug application to the Food and Drug Administration. For the latter organization, the file has been

used as a background resource for product class standards.

On many occasions the file has provided information about effects of drugs of which the user was previously unaware. Undoubtedly this has helped the care of some patients. We are aware of several cases in which prolonged workups of unusual test values were avoided because of the simple explanations provided by the file. We expect to expand the application of the file by introducing it in a revised form into several hospitals for on-line interpretation of data. Also, discussions have been held with several different institutions overseas to set up national centers for dissemination of information and to provide a better monitoring of foreign language publications to augment the content of the file.

### Literature Cited

1. Borushek, S. and Gold, J.; "Commonly Used Medications That Interfere With Routine Endocrine Laboratory Procedures". *Clin. Chem.* (1964), 10, 41-52.
2. Caraway, W.T.; "Chemical and Diagnostic Specificity of Laboratory Tests". *Amer. J. Clin. Pathol.* (1962), 37, 445-464.
3. Carr, M.H. and Frank, H.A.; "Calcium Analysis in Patients Being Treated With EDTA". *Clin. Chem.* (1957), 3, 20-21.
4. Cohon, M.S.; "Drug Interactions Involving Levodopa". *Rev. Drug Interactions* (1974), 1, 45-62.
5. Connery, A.H.; "Pharmacological Implications of Microsomal Enzyme Induction". *Pharm. Rev.* (1967), 19, 317-366.
6. Dubois, E.L.; Tallman, E. and Wonka, R.A.; "Chlorpromazine-Induced Systemic Lupus Erythematosus". *J.A.M.A.* (1972), 221, 595-596.
7. Ebert, R.; "Oral Anticoagulants and Drug Interactions". *Arch. Intern. Med.* (1968), 121, 373-374.
8. Elkins, M.P. and Kabat, H.F.; "Drug Induced Modifications of Laboratory Test Values". *Amer. J. Hosp. Pharm.* (1968), 25, 484-519.
9. Glynn, K.P.; Carfaro, A.F.; Fowler, C.W. and Stead, W.W.; "False Elevations of Serum Glutamic Oxaloacetic Transaminase Due to Paraaminosalicylic Acid". *Ann. Intern. Med.* (1970), 72, 525-527.
10. Little, D.M. and Eststone, H.J.; "Anaesthesia and the Liver". *Anaesthesiology* (1964), 25, 815-853.
11. Lubran, M.; "The Effects of Drugs on Laboratory Values". *Med. Clin. N. Amer.* (1969), 53, 211-222.
12. Lucis, O.J. and Lucis, R.; "Oral Contraceptives and Endocrine Changes". *Bull. W. H. O.* (1972), 46, 443-450.
13. Sabath, L.D.; Gerstein, D.A. and Finland, M.; "Serum Glutamic-Oxalacetic Transaminase. False Elevations During Administration of Erythromycin". *New Engl. J. Med.* (1968), 279, 1137-1139.

14. Schwartz, M.K.; "Interferences in Diagnostic Biochemical Procedures". Advances in Clinical Chemistry, pp. 1-45, Vol. 16, Bodansky, O. and Latner, A.L., Editors, Academic Press, New York, 1973.
15. Solomon, H.M. and Schrogie, J.J.; "Effect of Phenylramidol and Bishydroxycoumarin on the Metabolism of Tolbutamide in Human Subjects". Metabolism (1967), 16, 1029-1033.
16. Spellacy, W.N.; Buhl, W.C.; Spellacy, C.E.; Moses, L.E. and Golzieher, J.W.; "Glucose, Insulin and Growth Hormone Studies in Long Term Uses of Oral Contraceptives". Amer. J. Obstet. Gynecol. (1970), 106, 173-176.
17. Sunderman, F.W., Jr.; "Drug Interference in Clinical Biochemistry". Crit. Rev. Clin. Lab. Sci. (1970), 1, 427-449.
18. Sunderman, F.W., Jr.; "Effects of Drugs Upon Hematological Tests". Ann. Clin. Lab. Sci. (1972), 2, 2-11.
19. Wirth, W.A. and Thompson, R.L.; "The Effect of Various Conditions and Substances on the Results of Laboratory Procedures". Amer. J. Clin. Path. (1965), 43, 579-590.
20. Young, D.S.; Thomas, D.W.; Friedman, R.B. and Pestaner, L.C.; "Effects of Drugs on Clinical Laboratory Tests". Clin. Chem. (1972), 18, 1041-1301.
21. Young, D.S.; Friedman, R.B. and Pestaner, L.C.; "Automatic Monitoring of Drug-Laboratory Test Interactions" Drug Interactions, pp. 393-401, Morselli, P.L., Garattini, S., and Cohen, S.N., Editors, Raven Press, New York, 1974.
22. Young, D.S.; Pestaner, L.C. and Gibberman, V.; "Effects of Drugs on Clinical Laboratory Tests". Clin. Chem (1975), 21, 1D-432D.