Advances in
CLINICAL CHEMISTRY

VOLUME 22

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Advances in CLINICAL CHEMISTRY

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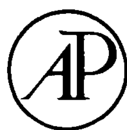

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PREFACE

In this volume, the Editors have compiled a group of articles that they feel represents a continuation of the aims of this publication to provide the clinical chemist with reviews of "state-of-the-art" methodology, newer areas of medicine and physiology that affect the clinical chemist, and those areas related to the latest information relating chemistry to disease. Brown, Kalow, Pilz, Whittaker, and Woronick present an article entitled "The Plasma Cholinesterases: A New Perspective." This is truly an international effort and is presented by the authors on behalf of the Commission on Toxicology of the Clinical Chemistry Division of the International Union of Pure and Applied Chemistry. It provides a superb review of human serum cholinesterase and its variants and a critical assessment of the physical and chemical properties of the enzyme.

Markert and Frei have reviewed "Biochemical Events Related to Phagocytosing Cells." The clinical chemist is required more and more to deal with isolation and measurement of components in leukocytes. These authors have considered the techniques for isolation of the cells and their subcellular components as well as the biochemical mechanisms involved in phagocytosis in both normal and pathological situations. Alkaline phosphatase has been of considerable interest to clinical chemists for more than 50 years; Posen and Doherty have reviewed "The Measurement of Serum Alkaline Phosphatase in Clinical Medicine" and, in a detailed fashion, have outlined available information regarding the significance of serum alkaline phosphatase in a variety of disease states as well as the many physiological and analytical factors affecting the activity of this enzyme.

Clark and Kricka have reviewed "High-Resolution Analytical Techniques for Proteins and Peptides and Their Applications in Clinical Chemistry" and include consideration of isotachophoresis, high-performance liquid chromatography, and high-resolution two-dimensional electrophoretic techniques for separation and analysis of complex protein mixtures. These techniques are not now widely used in clinical chemistry laboratories but represent the tools of the future, when laboratories will be required to measure gene products and the myriad proteins present, as in complex biologic fluids of significance in health and diseases.

The Editors wish to express their appreciation to the contributors and to the publisher for continuing superb efforts in the preparation and publication of this volume.

A. L. Latner
M. K. Schwartz

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THE PLASMA CHOLINESTERASES: A NEW PERSPECTIVE[1]

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[1]For the Commission on Toxicology, Clinical Chemistry Division, International Union of Pure and Applied Chemistry.

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1. Introduction

1.1 Historical Background and Terminology

In 1914, Dale discovered that the action of acetylcholine on the heart of the frog was short-lived, and he suggested that an enzyme was present in the blood which catalyzed the hydrolysis of choline esters (D1). In the 1920s, Engelhart and others, having shown that acetylcholine was produced by stimulating the vagus nerve of the frog, also found that the choline ester was destroyed by an enzyme, present in heart muscle extracts, which was inhibited by the alkaloid eserine.

By this time, esterases had been described in many animal tissues, and there seemed no reason to suppose that acetylcholine would be exceptionally resistant to their action. In 1932, Stedman *et al.* (S38) showed that liver esterases from the pig or cat were unable to catalyze the hydrolysis of acetylcholine, and a search was made for the enzyme responsible for the hydrolysis of this choline ester. They went on to describe and purify, for the first time, an enzyme present in horse serum which did catalyze the hydrolysis of acetylcholine. They called this enzyme "choline-esterase," and this name—with or without the hyphen—has persisted.

During the following years, the picture became confused since acetylcholine-hydrolyzing enzymes obtained from various organs were found to have optimal activity at different concentrations of substrate. An explanation for this was put forward by Alles and Hawes (A13). They demonstrated the existence of two choline-esterases: The first, whose activity was greatest at low concentrations and was inhibited by excess

substrate, was present in human erythrocytes; and the second, whose activity increased throughout the range of substrate concentrations studied, was present in human serum.

In later reports of work using purified enzymes, it was suggested that the erythrocyte enzyme was specific for choline esters and should be called "true cholinesterase," while the serum enzyme, which could also hydrolyze noncholine esters, should be called "pseudocholinesterase." In fact, both enzymes are to some degree nonspecific, and these names are not recommended by the Commission on Biochemical Nomenclature of the International Union of Pure and Applied Chemistry and the International Union of Biochemistry (I2). Instead, the trivial names "acetylcholinesterase" and "cholinesterase" should be used for the erythrocyte and serum enzymes (EC 3.1.1.7 and EC 3.1.1.8, respectively).[2]

There is a vast and continually growing literature on these two enzymes, but many papers have not distinguished clearly between them. The purpose of the present survey is to focus attention on human serum cholinesterase and its variants in the light of recent research, and to offer a critical assessment of reports of its physical and chemical properties and clinical and toxicological applications. The methodologies of cholinesterase assays and the older literature on the variant enzymes will not be considered in detail: Relevant references may be found in several authoritative reviews (B15, D5, F4, G13, G16, H5, K5, L10, L24, U1, W35). Acetylcholinesterase, also, has been extensively reviewed (N1, S22, S23, W29) and it will be discussed here only in order to make particular points of comparison with cholinesterase.

1.2. Clinical Background

The cholinesterases are generally accepted as being synthesized in the liver, and the assay of cholinesterase first became of interest to the clinician and to the clinical chemist as a test of liver function. Low serum cholinesterase activities are found in acute hepatitis, acute cirrhosis, and in liver metastases—that is, in those conditions where the hepatic synthesis of the protein is impaired. The synthesis of several other proteins is also reduced in such conditions, so that cholinesterase assay has been largely superseded as a test of liver function by measurements related to such proteins as albumin and prothrombin. Nevertheless, cholinesterase still has a place in the assessment of hepatic and other diseases, as discussed in Section 5.2.

[2] An earlier recommendation (I1) designated benzoylcholinesterase (EC 3.1.1.9) separately, but this is now considered (I2) to refer to a side reaction of cholinesterase.

Well-established toxicological and forensic applications of cholinesterase assays are related to the fact that some organophosphorus compounds, especially the insecticides and nerve gases, are notable for being extremely potent inhibitors of the cholinesterases. They owe their characteristic toxic effects to the specific inhibition of acetylcholinesterase at the neuromuscular junction. The assay of red cell acetylcholinesterase or serum cholinesterase provides a useful measure of adventitious exposure to these compounds, such as occurs in agricultural or horticultural practice. Although the actions of these enzymes are not directly related to the symptoms of organophosphorus poisoning, their inhibition undoubtedly reflects the situation in the tissues. Chronic exposure to organophosphorus compounds diminishes the activities of both cholinesterase and acetylcholinesterase. Development of the features of systemic poisoning is accompanied by rapid and substantial inhibition of the two enzymes. When exposure ceases, cholinesterase activity reverts to normal over a period of 3 to 6 weeks, as the enzyme is resynthesized by the liver. Acetylcholinesterase activity increases less rapidly and takes 13 to 17 weeks to return to normal as the red cells are turned over.

Certain organophosphorus compounds are used, infrequently, in the treatment of glaucoma, where they are administered as eyedrops to reduce intraocular pressure. There may be a markedly depressed level of serum cholinesterase activity in such patients, demonstrating significant systemic absorption of the compound.

The best known clinical application of cholinesterase assay concerns the abnormally prolonged effect of the muscle relaxant succinylcholine that is found in a small proportion of patients. This compound, which was introduced into clinical medicine in the early 1950s (B29, B41, T47), owes its relaxant action to competition with acetylcholine for the receptors at the neuromuscular junction; both cause depolarization of the muscle fibers, which contract. Acetylcholine is rapidly destroyed by acetylcholinesterase, so that repeated stimuli are applied to the muscle, causing a controlled contraction which persists as long as the nerve is stimulated. When, however, succinylcholine is administered, it is not destroyed by acetylcholinesterase, and its action persists until a large proportion of the dose has been hydrolyzed in the plasma. After initial contraction, the muscle fibers passively elongate to give the relaxation required by the anesthetist.

Succinylcholine is most often given during the induction of anesthesia. The drug is administered intravenously, with a dosage of about 1 mg/kg body weight, after the anesthetic has taken effect. It is usually given to relax the patient's laryngeal muscles to facilitate the passage of an endotracheal tube, through which anesthesia and ventilation can be main-

tained. In fact, the muscle relaxant action of succinylcholine is only moderately selective—most of the body muscles are relaxed, including those of chest wall and diaphragm, so that the patient stops breathing. However, the relaxant activity is normally short-lived, and endotracheal ventilation is maintained for the period of 2 to 10 minutes until the patient breathes spontaneously again. In a small proportion of patients, relaxation following the administration of succinylcholine persists for a period of many minutes or even hours. Paralysis of the patient's respiratory muscles for this period leads to prolonged apnea, and artificial ventilation must be carried out until spontaneous respiration returns. In the majority of cases, prolonged apnea is a rare and easily managed complication of anesthesia, but it remains an occasional cause of death, particularly when adequate facilities for artificial ventilation are not readily available.

The pharmacologists' and clinical chemists' interest in succinylcholine arose from the belief that the hydrolysis, and therefore inactivation, of the drug was brought about by the action of cholinesterase, and that in cases of prolonged response, the patient had a deficiency of the enzyme. The investigation of this thesis led to the discovery of the genetically determined variants of cholinesterase, which are described in Section 2.

2. The Cholinesterase Variants and Multiple Forms

Genes at two different loci are generally regarded as being responsible for controlling the inheritance of plasma cholinesterase variants in humans. All these enzyme types result from allelic genes, meaning that any two may be combined in a given subject, who is homozygous for cholinesterase when the two alleles are identical, and heterozygous when the two alleles are different. The available evidence, summarized in the following sections, indicates that at least eight genes, none of which appears to have dominance, are allelic at the first locus, and two genes are allelic at the second locus. The eight allelic genes can give rise to 36 different genotypes, 18 of which have been reported to occur in various families, some being very rare. Several other phenotypes which may be caused by genes allelic to the eight mentioned above have also been reported.

Other genes controlling the synthesis of plasma cholinesterase are suspected to exist, as evidenced by anomalous results obtained in studies of some individuals and families. Because all the cholinesterase genotypes have not yet been discovered, some of the genotypes assigned to certain individuals may eventually be found to be in error. The limited sen-

sitivities of the laboratory tests currently available for determining phenotypes makes it highly probable that some of the genotypes that have been assigned in the past will have to be reassigned to newly discovered ones.

2.1. Nomenclature

It is unfortunate that a standardized system of nomenclature has not been adopted by workers in this field. Systems of nomenclature have been proposed by Motulsky (M18), by Lehmann and Liddell (L22), and by Goedde and Baitsch (G19, G20). All three systems continue in use to varying degrees. However that proposed by Motulsky (M18) appears to be the most widely accepted and applied, and it is therefore used in this review.

In Motulsky's system of nomenclature, the first locus mentioned above is referred to as the E_1 locus (E for esterase) and the second is called the E_2 locus. The most thoroughly studied allelic genes of the E_1 locus are the E_1^u, E_1^a, E_1^f, and E_1^s genes. The E_1^u gene is responsible for the production of the most common form of serum cholinesterase, which is best designated the "usual" enzyme (G19). The E_1^a gene produces an enzyme—the "atypical" enzyme—which is relatively resistant to inhibition by dibucaine; the E_1^f gene produces an enzyme which is relatively resistant to inhibition by fluoride ion. The gene that is designated E_1^s—"silent"—in much of the literature is actually a group of allelic genes in which either no enzyme or only a small percentage of the usual amount of enzyme activity is produced.

The genes at the E_2 locus, which affect the isozyme pattern of the enzyme, are called E_2^- for the more common variant, and E_2^+ for the less common variant. Various means of designating the multiple forms of cholinesterase, which can be characterized from their electrophoretic properties, are considered in Section 2.6.

The different systems of nomenclature of the variants, and some of the properties of the cholinesterases present in the plasmas of individuals of the reported genotypes, are summarized in Table 1.

2.2. The Dibucaine- and Fluoride-Resistant Variants

A major advance in understanding the cause of hypersensitivity to succinylcholine came in 1957 when Kalow *et al.* (K11, K12) reported an *in vitro* method for detecting the presence of atypical serum cholinesterase in human plasma. Kalow and Staron (K15) reported that the inheritance of the atypical enzyme variant is caused by an autosomal gene without

dominance (E_1^a), which is allelic to the usual gene (E_1^u). The method of detecting the presence of the atypical enzyme is based upon the differential inhibition of the usual and atypical forms of the enzyme by dibucaine. The results obtained by this procedure are expressed as the dibucaine number (DN), which is the percentage of inhibition in the presence of 10^{-5} mol/liter dibucaine using 5×10^{-5} mol/liter benzoylcholine as substrate. Using this assay procedure, Kalow and Staron (K15) determined the dibucaine numbers of a large number of sera and found that the dibucaine numbers were distributed into three distinct groupings. Those with a mean DN of about 79 were homozygous for the usual enzyme ($E_1^uE_1^u$), those with a mean DN of about 62 were heterozygous for the usual and atypical enzymes ($E_1^uE_1^a$), and those with a DN below 20 were homozygous for the atypical enzyme ($E_1^aE_1^a$).

In 1961, Harris and Whittaker (H12) reported that the usual and dibucaine-resistant serum cholinesterases could also be differentiated on the basis of their inhibition by fluoride ion. The percentage of inhibition under standard conditions by fluoride ion was called the fluoride number (FN). However, several individuals who had been classified as having the usual phenotype on the basis of dibucaine number were found to have anomalously low fluoride numbers. There was a similar subgroup among those who had been designated as being usual–atypical heterozygotes based upon dibucaine number. Family studies demonstrated that the fluoride-resistant form of the enzyme was inherited, and it was postulated that a third gene, allelic to the usual gene at the E_1 locus, was segregating in these families (H12, H14). The individuals who had been misclassified as $E_1^uE_1^a$ were now reclassified as $E_1^aE_1^f$. Not long afterwards, Liddell *et al.* (L32) found an individual who was homozygous for the fluoride-resistant gene, $E_1^fE_1^f$.

2.3. E_1^j and E_1^k Variants

In 1976, Garry *et al.* (G6) reported a family in which segregation for the E_1^u, E_1^a, and E_1^f genes was occurring. Anomalous dibucaine numbers obtained with some of the sera led to the postulate of a new gene at the E_1 locus. Of the E_1^j variants studied, only $E_1^aE_1^j$ heterozygotes can be detected by use of inhibitors. The one thing that the proposed new phenotypes had in common was that the enzyme activities of the new variants were generally lower when an E_1^j gene replaced an E_1^u gene, but this was not useful for assigning genotypes. It was proposed that the usual enzyme, as produced by the E_1^u gene, was still being produced in these phenotypes, but that the level of the circulating enzyme was reduced by about 66%. Results obtained with radial immunodiffusion were consis-

TABLE 1. GENETIC VARIANTS

Genotype			Phenotype			With benzoylcholine substrate		
	Alternative nomenclature[b,c,d]			Alternative nomenclature[b]		Relative activity[e,f]	Dibucaine number[g,h]	Fluoride number[i]
$E_1^uE_1^u$	$Ch_1^UCh_1^U$	NN	U	U	Usual	100	76–87	55–70
$E_1^aE_1^a$	$Ch_1^DCh_1^D$	DD	A	A	Atypical	50	8–30	10–28
$E_1^fE_1^f$	$Ch_1^FCh_1^F$	FF	F	F	Fluoride	50	63–69	30–37
$E_1^sE_1^s$	$Ch_1^SCh_1^S$	SS	S	S_1[a]	Silent silent type I, silent type O	0	—	—
$E_1^tE_1^t$	—	$E_1^TE_1^T$[q]	T	S_2[a]	Silent, silent type II, silent type T	3[r]	—	—
$E_1^sE_1^t$	—	—	ST	—	—	2[q]	—	—
$E_1^uE_1^r$	—	—	UR	—	—	52[q,s]	—	—
$E_1^uE_1^a$	$Ch_1^UCh_1^D$	ND	UA	I	Intermediate	78	51–71	42–59
$E_1^uE_1^f$	$Ch_1^UCh_1^F$	NF	UF	UF	—	80	70–80	45–54
$E_1^uE_1^s$	$Ch_1^UCh_1^S$	NS	US	U	—	65	80[e]	64[e]
$E_1^aE_1^f$	$Ch_1^DCh_1^F$	DF	AF	IF	—	60	39–59	27–38
$E_1^aE_1^s$	$Ch_1^DCh_1^S$	DS	AS	A	—	20	21[e]	23[e]
$E_1^fE_1^s$	$Ch_1^FCh_1^S$	FS	FS	F	—	61[t]	67[u]	37[u]
$E_1^uE_1^j$	—	—	UJ	—	—	—	—	—
$E_1^aE_1^j$	—	—	AJ	—	—	—	—	—
$E_1^fE_1^j$	—	—	FJ	—	—	—	—	—
$E_1^uE_1^k$	—	—	UK	—	—	—	—	—
$E_1^aE_1^k$	—	—	AK	—	—	—	—	—

[a] Dietz *et al.* (D15, D16) except where noted.

[b] Columns 1 and 5 are based upon the nomenclature of Motulsky (M18) except where noted. In all cases where the $E_1^uE_1^s$, $E_1^aE_1^s$, and $E_1^fE_1^s$ heterozygotes have been studied, the subtype of the "silent" gene has not been determined.

[c] Column 2 lists the nomenclature proposed by Goedde and Baisch (G19).

[d] Column 3 lists the nomenclature proposed by Lehmann and Liddell (L22) except where noted.

[e] From Usdin (U1) or Simpson and Kalow (S29).

[f] Using the method of Kalow and Lindsay (K14).

[g] Using the method of Kalow and Genest (K11).

[h] Brown *et al.* (B39).

[i] Computer analysis by Brown *et al.* (B39) of published data using the method of Harris and Whittaker (H15).

[j] Symbols: −, not sensitive; ±, usually not sensitive; +, moderately sensitive; ++, very sensitive.

[k] Price and Brown (P14); mean values ± SD.

[l] Calculated from the data summarized by Propert and Brackenridge (P17).

AT THE E_1 LOCUS OF HUMAN SERUM CHOLINESTERASE

With propionylthiocholine substrate[a]				
Relative activity	Dibucaine number	Fluoride number	Approximate incidence (%) (Caucasians)	Sensitivity to succinylcholine[j]
100	83.6 ± 1.3 (84 ± 1.1[k])	79.7 ± 1.2 (76 ± 1.6[k])	96[l,m]	–
23 (31[k])	19.9 ± 2.7 (19 ± 2.3[k])	84.0 ± 1.8 (77 ± 4.0[k])	0.054[m]	+ +
42	71.8	53.6	0.00064[o]	+
0.4	5.3 ± 4.3	35.1 ± 6.1	0.00059[p]	+ +
2	67.6 ± 4.3	67.7 ± 1.7	—	+ +
—			Rare	+ +
—			Rare	?
69 (93[k])	72.7 ± 3.1 (73 ± 2.6[k])	80.0 ± 1.6 (75 ± 1.8[k])	3.6[m]	±
71 (91[k])	79.8 ± 1.2 (83 ± 1.1[k])	73.0 ± 1.7 (73 ± 1.9[k])	1.0[l]	±
55	84.4 ± 0.8	79.3 ± 1.4	0.48[p]	±
43 (48[k])	60.2 ± 3.1 (61 ± 5.5[k])	68.3 ± 1.0 (69 ± 5.9[k])	0.059[l]	+
21	20.7 ± 4.1	82.3 ± 3.4	0.0091[p]	+ +
41	76.7	64.9	0.00067[o]	+
56[v]	83.7 ± 2.2[v]	79.4 ± 1.4[v]	Rare	?
23[v]	57.0 ± 2.9[v]	82.0 ± 1.9[v]	Rare	?[w]
47[v]	76.9[v]	68.2[v]	Rare	?
89[x]	84.3 ± 0.9[x]	79.9 ● 1.8[x]	Rare	?
33[x]	63.6 ± 2.7[x]	79.7 ± 1.6[x]	Rare	?

[m] Steegmuller (S39).

[o] Whittaker (W15).

[p] Data for Americans of unspecified race (S32). The incidence figure is for all "silent" gene subtypes combined.

[q] Scott and Wright (S17).

[r] Determined at 30°C (S12).

[s] Scott and Wright (S17) have described one individual with a presumed $E_1^fE_1^t$ or $E_1^fE_1^s$ genotype. The relative activity was found to be less than 10% of that found for the usual enzyme. They reported the DN and FN values for this individual to be "within the range found for the usual enzyme."

[t] Whittaker (W7).

[u] Whittaker and Berry (W16).

[v] From the data of Garry *et al.* (G6).

[w] Garry *et al.* (G6) speculate that prolonged apnea might occur with this genotype.

[x] From the data of Rubinstein *et al.* (R12).

tent with a reduction in the number of circulating enzyme molecules (R13). However, no evidence was presented that the type of enzyme present in these individuals was identical to the E_1^u enzyme product. The authors considered it unlikely that a new enzyme molecule with lower enzymatic and immunologic activity was being produced (R13). The possibility that a suppressor gene present on the same chromosome as the E_1 gene was responsible for these observations was not considered.

It was suggested that the unusual activity and inheritance pattern (including lower than usual activity) reported in a family by Lehmann *et al.* (L26) could be explained on the basis of the presence of the E_1^j gene in the family. However, Whittaker (W9) suggested that this family might be an example of a butanol variant (see below). None of the families proposed as carriers of the E_1^j gene has been studied with butanol as a differentiating agent. Bonderman and Bonderman (B26) also claim to have found two individuals who are of the proposed phenotype of Lehmann *et al.* (L26), although there appear to be some inconsistencies with the previous data.

In other studies, two families segregating for a new cholinesterase gene designated E_1^k were discovered by Rubinstein *et al.* (R13). The presence of a new variant[3] was deduced by the same methods that were used to characterize the E_1^j variant, and it was proposed that the E_1^k variant produces the E_1^u enzyme, but that the serum level is reduced by about 33%. So far, only the $E_1^aE_1^k$ variant has been distinguishable by use of inhibitors. The criticisms applied to the E_1^j variant apply to this variant as well.

2.4. Chloride, Butanol, and Other Possible Variants

In examining the effect of sodium chloride on cholinesterase variants, Whittaker (W10, W11) reported the probable existence of two new phenotypes that differ in their sensitivity to inhibition by chloride ion. Two individuals (DN = 80–81, FN = 60–63, and chloride number = 25) from the first group of nine individuals were found to be hypersensitive to succinylcholine. The second group consisted of two individuals with the following approximate respective values: DN = 39 and chloride number = 46, and DN = 53 and chloride number = 46. No family studies of these two probable phenotypes have been reported, although two individuals in the larger group are siblings.

King and Dixon (K24) reported a family in which another possible phenotype was detected by means of chloride inhibition. Six out of seven family members had DN and FN values typical of the $E_1^uE_1^u$ or $E_1^uE_1^f$

[3] The E_1^k variant was so designated in honor of Dr. Werner Kalow (Eds.).

genotypes. However, the chloride number at 25°C was zero for these individuals. This seems to be the only case in which complete resistance to inhibition by chloride ion has been reported.

While studying the effect of *n*-butanol on various phenotypes, Whittaker (W9) found what appeared to be two new phenotypes on the basis of their butanol and dibucaine numbers. Limited family studies suggested that there might be a genetic basis for these observations. One of the individuals in one of the two new groups had DN = 40, FN = 36, butanol number = 96, and a low activity. A family in which Lehmann *et al.* (L26) could not adequately explain inheritance patterns had four children with DN of about 40, FN of about 36, and a low activity (see W9). Whittaker (W9) suggested that this family might be an example of one of the butanol phenotypes she had detected, while Garry *et al.* (G6) felt that this family might contain individuals who are heterozygous for the E_1^j gene. The present reviewers consider that all three groups may have studied either the same or similar variants.

Agarwal *et al.* (A4) investigated a group of 21 patients with puzzling characteristics. These patients had prolonged apnea upon administration of succinylcholine, but all had the usual amount of enzyme activity when assayed with benzoylcholine, and all had dibucaine and fluoride numbers characteristic of the usual phenotype when measured with benzoylcholine as substrate. The sera of these patients were examined using a colorimetric procedure (A3) with succinylcholine as substrate. In this procedure, the choline produced by hydrolysis is oxidized by cytochrome c, using rat liver mitochondria as a source of choline oxidase, and the reaction is followed by measuring the change in absorbance at 550 nm. It was found that only six of these patients had activities and dibucaine numbers (measured by the choline oxidase procedure) characteristic of the $E_1^uE_1^u$ genotype. These six patients may actually be of the usual genotype, since there are many other causes for prolonged apnea with succinylcholine (Section 3.1).

Nine of the remaining patients had dibucaine numbers (measured by the choline oxidase procedure) characteristic of the $E_1^aE_1^a$ genotype and the sera of six had no activity with succinylcholine (Section 3.2). These 15 patients had cholinesterases whose properties appear to be quite different from those of any other variants that have been reported, with one possible exception. The six patients whose serum exhibited no activity with succinylcholine but did show the usual level of activity with benzoylcholine, represent a probable new phenotype. The nine patients who had DN values characteristic of the usual phenotype when using benzoylcholine as substrate but DN values characteristic of the atypical enzyme when using succinylcholine, must also be classified as representing

a probable new phenotype. This group of 15 patients has several characteristics in common with a group of nine individuals selected by means of chloride inhibition by Whittaker (W10, W11): All nine had DN and FN values characteristic of the usual phenotype, but had chloride numbers which were not. In addition, two members of the group had prolonged apnea with succinylcholine, but in contrast to the Agarwal's group of patients (A4), they also had decreased levels of enzyme activity (W11). Unfortunately, nothing has been reported about the enzyme levels of the other seven members of Whittaker's group of patients (W10).

The present reviewers suggest that one—or perhaps only some—of the members of Agarwal's group (A4) belongs to one of the proposed chloride phenotypes (W10, W11). It seems probable that one or more new genes for cholinesterase has been detected by these methods, but more family studies are needed to demonstrate this. It also seems probable that both laboratories have studied carriers of the same proposed new gene, but in a number of heterozygous genotypes.

Raj *et al.* (R1) also reported three unusual cases of prolonged apnea following administration of succinylcholine. The patients had DN values characteristic of the usual phenotype, but had succinylcholine numbers characteristic of the usual-atypical phenotype (Section 6.3.2.1).

Peck (P8) studied a family in which one member had a 62% decreased enzyme activity with benzoylcholine, along with a DN of 27 and an FN of 16. The author concluded that this was an $E_1^uE_1^a$ individual in whom the usual cholinesterase accounted for only 20% of the total cholinesterase activity, but from these data, the genotype is likely to have been $E_1^aE_1^s$. However, this may be another example of a carrier state for one of the poorly characterized genes which determine the quantitative measure of cholinesterase activity.

Irwin and Hein (I3) reported a family in which DN and FN values indicated that the father was of the usual phenotype, the mother the atypical phenotype, and the two sons the intermediate phenotype. However, one of the sons had about 25% more cholinesterase activity with benzoylcholine as substrate than did his father. Furthermore, the son's cholinesterase activity was greater than that of his father when tested with several other substrates. The pattern of K_m values for this son indicated that his cholinesterase was qualitatively different from that of anyone else in his family. An unidentified gene that affects the amount of enzyme activity, as well as the qualitative properties of cholinesterase, might exist in this family. However, the findings may have been complicated by a physiologically high level of cholinesterase activity which is found in some children, and which decreases to normal adult values at puberty.

Whittaker and Vickers (W23) reported a family in which the silent gene appeared to be segregating, but in which the amount of enzyme activity in some of the members was about half of that expected for the $E_1^uE_1^s$ genotype. All members in two generations had DN and FN values which were characteristic of the usual phenotype.

Baker *et al.* (B2) reported a family in which the usual, atypical and silent genes seemed to be segregating. However, some of the assigned genotypes appear to be anomalous. One individual, who was assigned the homozygous silent genotype, had a DN of 12 and an enzyme activity using acetylcholine of 1.12 μmol/minute/milliliter. This is an exceedingly high value for this genotype, in view of the range of 3.0 to 7.4 μmol/minute/milliliter reported for the homozygous usual genotype, and a range of 2.0 to 3.0 μmol/minute/milliliter reported for the $E_a^uE_1^s$ genotype by these same authors (B2). For other reports of activity measurements under similar conditions, see references B26 and B27.

Fishtal *et al.* (F2) and Bonderman and Bonderman (B27) have also reported studies on individuals with anomalous measured parameters.

Singh *et al.* (S30) and King and Griffin (K25) have both suggested that the large standard deviations of the inhibition numbers observed for some groups indicate that the different phenotypes studied are heterogenous with regard to genotype, but there are many components of variance to account for in making such comparisons.

It should be noted that in performing family studies, it may, in some cases, be necessary to perform paternity testing in order to explain results which appear to be anomalous (A1, G22, M13).

2.5. Silent Variants

The discovery of an individual having no detectable serum cholinesterase activity was reported by Liddell *et al.* (L33) in 1962. Such individuals are said to possess a "silent" gene for cholinesterase, and to date, about 105 subjects have been reported to be homozygous for "silent" cholinesterase. Simpson and Kalow (S27) presented evidence that the "silent" cholinesterase gene is allelic to the usual cholinesterase gene.

However, the situation concerning silent cholinesterase variants is rather complex, and some conclusions that have been drawn may be incorrect. Some reports have stated that no enzyme activity and no cholinesterase-like protein could be detected in the "silent" sera studied, but it is clear that whether or not any cholinesterase activity is observed depends on a number of factors. If the gene and all of its alleles are absent, no protein product will be produced, and the phenotype will be silent under all assay conditions. The same holds true if a gene is present

but is truly silent in the sense that no protein product is produced. However, if a protein product is produced, the phenotype may still be silent under all assay conditions if the protein has no enzyme activity toward any substrate under any conditions. For example, a protein molecule in which an essential part of the active site has been deleted may be present in the serum. Likewise, an incompletely synthesized or an incompletely assembled molecule may be secreted into the serum. Another possibility that has not been considered is that serum cholinesterase may be synthesized as a pro-cholinesterase which must be activated by reacting with, say, a hydrolytic enzyme before it is secreted. In this case, the expression of what is called the "silent" gene may reflect the absence of the activating enzyme, but this hypothesis would be difficult to reconcile with the observation of allelism. Finally, silent cholinesterase sera may contain a protein in which the cholinesterase-active site has been permanently blocked by steric hindrance caused by an unfavorable conformation of the variant molecule, or in which the active site has been blocked by a "lethal synthesis" type of reaction with a small molecule *in vivo.* In many of these cases, it may be possible to detect the presence of an enzymatically inactive cholinesterase-like protein molecule by employing highly specific and highly sensitive affinity or immunologic techniques. It would not be surprising if a radioimmunoassay procedure capable of specifically detecting and measuring a serum cholinesterase-like protein in most sera currently classified as silent were developed. As has previously been suggested, in many cases it may be that the E_1 gene is not silent, but the product of the E_1 gene is (S39). The problem of inherited deficiencies of proteins in humans has been discussed by Schultze and Heremans (S11).

If an assay procedure fails to detect any enzyme activity, changing the procedure to make it more sensitive, or changing to an inherently more sensitive one may demonstrate that some enzyme activity is indeed present. The particular silent serum may contain an enzyme variant which has a very large Michaelis constant for the substrate employed in the assay, in which case a higher substrate concentration may be able to demonstrate the existence of enzyme activity. On the other hand, the silent serum may contain an enzyme variant which truly has no hydrolytic activity toward the substrate employed in the assay, but which may have readily detectable hydrolytic activity toward another choline-containing substrate. Several examples will be discussed below.

2.5.1. *Heterogeneity of "Silent" Cholinesterase Phenotypes*

In 1965, Goedde *et al.* (G23, G24) reported studies on two sera which were reported to be silent when assayed by the spectrophotometric ben-

zoylcholine procedure of Kalow *et al.* (K11, K12). When these sera were examined using a micromanometric technique in which the benzoylcholine concentration was increased 20-fold to 10^{-3} mol/liter, it was found that both silent sera contained cholinesterase activity, but at a value that was 2–3% that of usual serum under identical conditions. It was also demonstrated that the cholinesterase activity in the two silent sera could be precipitated by antiserum to usual serum cholinesterase. It was estimated that the silent sera contained a substantial amount of protein capable of reacting with the cholinesterase antibodies in the antiserum. Of five other cases studied by Goedde *et al.* (G17, G25), two had no detectable enzyme as assayed by catalytic activity and immunotitration. These latter cases may be examples of type I silent cholinesterase described below.

A rather large number of individuals who are homozygous for silent serum cholinesterase has been detected among the Eskimos of western Alaska (G33, S17). Among approximately 5000 Eskimos studied, 39 were reported to have no detectable enzyme activity, and 24 had 2–10% of usual activity when assayed with benzoylcholine. However, when some of these same sera were studied by Rubinstein *et al.* (R11), all the silent sera contained some enzymatic activity when acetylthiocholine was used as substrate. Again, however, two groups were recognized. One group, called type I, had about 1% of usual activity, and the other group, called type II, had about 3% of usual activity. The inhibition patterns using quinidine, dibucaine, and Mytelase were different for each group, and both types were different from usual cholinesterase. On acrylamide gel electrophoresis, type I serum produced two bands of activity, one of which was faster than the fastest band found in usual serum. With type II sera, five cholinesterase bands were found, and all of these corresponded with those found in usual serum (L10). When tested with antiserum to usual serum cholinesterase, no reaction was detected with type I serum, whereas type II serum did react with the antibodies. Upon chromatography on DEAE-cellulose, type I serum produced only one peak of activity when assayed with acetylthiocholine. DEAE-cellulose chromatography of usual serum produced three peaks of activity, the first of which contained only 0.6% of the total activity, and corresponded in position to the first peak found with types I and II sera. The amount of activity found in this peak for usual serum was sufficient to account for all the activity found in type I sera. The second and third peaks contained 96% and 3.4% of the activity, and corresponded in position to the second and third peaks found in type II sera.

Thus, type I sera appear to be quantitatively and qualitatively different from type II sera and usual sera, while type II sera appear to be

quantitatively different from usual sera. Rubinstein *et al.* (R11) stated that the cholinesterase in type I sera has many similarities to the acetylcholinesterase of erythrocyte ghosts. They also reported a "homozygous" silent serum which seemed to be intermediate between types I and II in many of its properties.

Scott (S12) purified usual serum cholinesterase and cholinesterase from a homozygous silent individual having about 2% of usual activity, which corresponds to silent type II cholinesterase. In most respects, the properties of the two purified cholinesterases were similar but not identical. The greatest differences noted were that the silent enzyme was more heat stable, and that, with it, there was less substrate inhibition by excess benzoylcholine.

Statistical analysis of familial data by Scott (S13) led to the conclusion that the type I and type II silent cholinesterases (referred to by Scott as type O and type T, respectively) are allelic to one another and, by extension, to the E_1^u gene (S16). As indicated above, and in Section 2.7, some populations have an unexpectedly high incidence of a silent cholinesterase gene (A17, G33, P1, S17).

Additional evidence concerning the heterogeneity of "homozygous" silent sera has been reported by Lubin *et al.* (L36), who studied two patients whose silent cholinesterases were different from one another and were unlike any of those reported by Rubinstein *et al.* (R11). Two other patients with dissimilar silent cholinesterases were studied by Das (D2). Arnason *et al.* (A17) have also reported on a possible "nearly silent" cholinesterase variant. It is difficult to compare these results with those of previous workers because of differences in experimental procedures.

Another nearly silent variant has been described by Scott and Wright (S17). This variant has less than 10% of usual activity, and a major band that migrates slightly faster than the major band of usual cholinesterase on starch gel electrophoresis. The properties of this new variant are also different from those of the type I and type II silent variants. Family studies indicate that this variant is allelic to the E_1 locus, and the gene has been designated E_1^r. Several $E_1^uE_1^r$ individuals with about half the enzyme activity of usual homozygotes were detected. An individual who was either $E_1^rE_1^s$ or $E_1^rE_1^t$ had less than 10% of usual activity. No $E_1^rE_1^r$ homozygotes were detected.

In this context, it may be noted that Evans and Magill (E20) studied a family in which an unusual pattern of inheritance occurred, which was interpreted as being the result of a mutation in the parents. Two of the siblings were found to be homozygous for a silent gene; two were homozygous for the usual gene; and two were heterozygous for the usual

and atypical genes. It was not possible to determine the genotypes of the parents because both were deceased. Lymphocyte and red cell antigen studies indicated that there was less than a 1/500 chance that the children were nonconsanguineous. The presence of a suppressor gene was considered unlikely. The possibility that the silent gene in question was nonallelic to the E_1 locus was dismissed. Scott (S12, S13) raised the question of whether one of the silent genes might be nonallelic to the E_1 locus, but concluded it was improbable. Evans and Magill (E20) suggested that the most likely explanation for the distribution of genotypes in this family is the occurrence of a mutation in one of the parents at a late stage in his or her reproductive life. However, Simpson (S26) questioned this hypothesis and suggested that the parents were of the $E_1^uE_1^s$ and $E_1^uE_1^a$ genotypes, and that a C_5^+ gene (Section 2.6.1) may also be present in the family. Evans and Magill (E21) disputed this, and have asserted that the laboratory findings were confirmed by one of the present reviewers (M.W.), who also found no evidence of the presence of the C_5^+ enzyme. Although it is possible that a mutation was observed in this family, in the absence of direct studies of the parents, it would seem that this possibility is remote. Alternative explanations should be considered, but it is likely that the true explanation may never be uncovered.

2.5.2. *Other Possible "Silent" Variants*

As mentioned previously, whether or not an enzyme is classified as being silent partially depends on assay conditions. A good example of this phenomenon was reported by Agarwal *et al.* (A4) to occur among a group of 21 patients who had prolonged apnea. All the patients had usual levels of enzyme activity when assayed using benzoylcholine, and had DN and FN values corresponding to the usual phenotype. However, when assayed by the choline oxidase procedure of Agarwal *et al.* (A3) (Section 2.4), six of the patients' sera had no measurable activity. These patients appear to represent a new phenotype.

These cases bring up some interesting and important points. Classification as to phenotype may depend on the assay used. Six of these cases were classified as being of the "usual" phenotype on the basis of activity with benzoylcholine, DN, and FN. However, on the basis of activity with succinylcholine, the same patients would be classified as being of the silent phenotype. In the cases under discussion, we know that the gene is not silent, although we may say that the enzyme is silent under these assay conditions. These six patients do not belong to that group of individuals whose phenotype has been defined as being "usual," because all of their enzyme characteristics do not correspond to those of the usual

phenotype. They may be considered as a subclass of the group of phenotypes called silent because the enzyme appears to be silent with some substrates, although not with others. However, at this stage, classification as a silent subtype would be undesirable because this suspected new variant has not been fully characterized. For example, the succinylcholine concentration used in the assay was 0.64 mmol/liter. If this concentration is much less than the value of the Michaelis constant for this substrate with this particular variant, no enzyme activity would be detected under the conditions of the assay. Therefore, the concentration should be varied to determine whether activity is present at much higher concentrations. The fact that succinylcholine is not a substrate, or at best a poor one, for this suspected variant, suggests that decamethonium and other dicationic compounds would also be poor substrates or inhibitors. This possibility could be exploited as a basis for differentiating this suspected variant from other variants, using more convenient and more commonly used assay procedures to determine a decamethonium number. Other possible methods for differentiating this suspected variant are outlined in Section 2.4.

A similar situation regarding a patient classified as being of the atypical phenotype has been reported by Simpson (S24). This patient's serum was found to have no activity toward acetylcholine, although it had the expected amount of activity with benzoylcholine.

In summary, the situation concerning the silent or near silent cholinesterase variants is complex. There are at least three cholinesterase variants which are most importantly characterized by a severe quantitative deficiency in cholinesterase activity. Family studies indicate that these three variants are alleles of the gene for usual cholinesterase, E_1^u, although more studies are needed in this area. There are indications that a variety of other near-silent cholinesterase subtypes exist. Cases of "true silence," because the gene is absent or because it produces no protein product, appear to be very rare. Many cases reported to have no cholinesterase activity have subsequently been found to have at least a trace of activity when studied under more sensitive assay conditions or with a different substrate.

2.6. Multiple Forms

2.6.1. *Genes at the E_2 Locus*

Harris *et al.* (H6, H7) reported that some sera, upon starch gel electrophoresis at pH 6, exhibited an additional, slow-migrating cholin-

esterase band, which was designated the C_5 band. Familial studies indicated that the presence of the extra band was inherited. Individuals who lack this band are called the C_5^- phenotype and those with the band are called C_5^+. The $E_1^uE_1^u$ genotype has about 30% more cholinesterase activity in the presence of the C_5^+ phenotype than it has in the presence of C_5^- phenotype. Assay by radial immunodiffusion also indicates that the C_5^+ phenotype has more cholinesterase (A14).

However, these methods cannot be used to distinguish the two phenotypes because there appears to be considerable overlap between the relevant cholinesterase activities. Harris *et al.* (H10) and Robson and Harris (R7) presented evidence that the gene responsible for producing the extra band is not allelic to the E_1 locus. The gene that codes for the extra band has been designated the E_2^+ gene, whereas its allele is called the E_2^- gene (M18). The incidence of the C_5^+ phenotype appears to be quite variable among different populations, with values of 0 to 29% reported (R7, S31, S39). It has been stated (S25, S31) that the pattern of inheritance of the E_2^+ gene seems to be autosomal dominant in certain cases, although there are numerous reports of C_5^+ children being born to C_5^- parents (H7, R7). A possible explanation for these discrepancies is that the starch gel electrophoretic methods used by most authors are simply not sensitive enough to detect all cases of the C_5^+ phenotypes. Simpson (S25) found that a polyacrylamide gel electrophoretic system (2.5% acrylamide spacer gel, pH 6.7; 7% separating gel, pH 8.9) was able to detect about 25% more C_5^+ individuals than were detected by a 15% starch gel system at pH 5.3. However, Singh *et al.* (S31) denied that the acrylamide gel system is any better than the starch gel system. It must be concluded that present methods for detecting the C_5^+ phenotype are not completely reliable, and may produce false-negative results. With the greater versatility of polyacrylamide gel, it would seem that a more sensitive and reliable polyacrylamide gel system could be developed to overcome these problems.

Scott and Powers (S15) partially purified the slow-migrating cholinesterase from usual-C_5^+ serum, as well as the major cholinesterase isoenzyme from usual-C_5^- serum. Determination of various kinetic parameters showed that the two forms of enzyme are different. It has been reported that the dibucaine-resistance of the extra C_5^+ band (H10) and of the purified C_5^+ enzyme (S15), is determined by the type of gene present at the E_1 locus. In support of this, Drew and Rundle (D24) found that the $E_1^uE_1^u$-$E_2^+E_2^+$ and $E_1^uE_1^u$-$E_2^-E_2^-$genotypes are indistinguishable on the basis of dibucaine, fluoride, or Ro2–0683 (Roche) inhibitor numbers. However, the C_5^+ phenotype has thus far been found only in association with the

$E_1^uE_1^u$ and $E_1^uE_1^a$ genotypes. As a result of these observations, it has been suggested that the extra band is a hybrid or complex of the enzyme protein from the E_1 locus with a second protein from the E_2 locus (M21, S15).

Gallango and Arends (G2) reported that the C_5^+ variant was found in 22% of a group of multiple myeloma patients, as compared with 8.5% of a normal Venezuelan population. No family studies were made, but it seems likely that this is an acquired characteristic rather than a genetic characteristic.

2.6.2. *E Cynthiana and Other Possible Variants at Unidentified Loci*

In 1966, Neitlich (N6) reported a family in which four members had cholinesterase activities that were several times normal, the elevated values being associated with an extra, slow migrating band on starch gel electrophoresis. When challenged with a small dose of succinylcholine, one of the family members was found to be resistant to its effects. The extra band was found to be slower migrating than the C_5^+ band coded at the E_2 locus (R4, Y3). Yoshida and Motulsky (Y3) named this variant "Cynthiana" after its place of origin. These authors found that the specific activity of the Cynthiana variant did not differ from that of the usual variant, as determined by immunologic tests and diisopropyl fluorophosphate titrations. Electrophoresis in starch gel under various conditions indicated that the extra band was composed of a large-sized aggregate of subunits, as compared with the major band of usual cholinesterase. After partial purification of the Cynthiana component, the C_5^+ component, and the major usual component from the appropriate sera, the relative electrophoretic differences still remained (R4). It has been inferred (Y3) that the increased activity of Cynthiana serum is caused by the overproduction of variant molecules. These variant molecules also form unusually large aggregates. A new variant studied by Klein *et al.* (K29) may be identical to the Cynthiana variant. The locus of the Cynthiana gene has not been determined.

There are reports that still other types of cholinesterase variants can be identified by electrophoretic studies. In most cases, it has not been determined whether these are genetically determined variants or acquired variants. Most of these variants have been detected by electrophoresis in a sieving gel such as starch or polyacrylamide. However, it must always be kept in mind that two new slow-migrating bands are detectable after performing two-dimensional electrophoresis on serum that has been stored (H6). The first band appears after storage for about 10 days at either 4°C

or $-20^{\circ}C$, while the second band appears after several weeks or more of storage.

In some adult sera, traces of an additional cholinesterase band were found near the major cholinesterase band after two-dimensional electrophoresis (H6). This band, which is much more prominent when cord blood or fetal blood is analyzed, is called the fetal band. Its significance is unknown.

Van Ros and Druet (V2) reported the discovery of a slow-migrating cholinesterase variant, which they named C_6, in the sera of four African subjects. Although this may be a hereditary trait, its familial nature has not been demonstrated. These same authors also studied by two-dimensional electrophoresis two other Africans who each had a pair of additional slow bands which were called C_{7a} and C_{7b}. Again, the possibility that these may be genetic in origin has not been demonstrated. None of these three bands is identical to those which were identified as the fetal band or storage bands by Harris *et al.* (H6).

Ashton and Simpson (A19) also reported the presence of an extra, slow-migrating, electrophoretic band (called C_6) in a Brazilian population. The same, or a similar, variant was observed in a Venezuelan population by Gallango and Arends (G2), who also found a band between C_6 and C_5^+, which they called the C_{56} band. The C_{56} band was found only in multiple myeloma patients (see Sections 4.3.5 and 4.3.7).

Simpson (S25) identified an extra, slow-migrating band in an American Indian woman when her serum was studied by polyacrylamide gel electrophoresis. A slightly different band was found among several Eskimo sera.

In most cases, it has definitely been shown that the extra bands are not storage bands or the C_5^+ band. However, in most cases, the methods used to demonstrate the existence of a band have been sufficiently different from those used by other investigators that it is usually not possible to determine whether a "new" band is different from any of those reported previously.

At the present time, it seems that the two-dimensional technique of Harris *et al.* (H6) has been used to identify more electrophoretic variants than has any other method, and is capable of greater resolution than are the other methods that have been employed. Using this method on all the new electrophoretic variants that have been described may make it possible to determine whether they are in fact all different from one another. Probably the best resolution currently available is isoelectric focusing in a gel medium in the first direction, followed by electrophoresis in a polyacrylamide gradient gel in the second direction (A15, W36). There

are no reports, as far as the reviewers are aware, that this has ever been attempted with any of the serum cholinesterases.

2.6.3. *Proposed Epigenetic Mechanism for Formation of Electrophoretic Variants*

Ogita (O3) reported an unusual case in which a patient with a leiomyoma had prolonged apnea following the administration of succinylcholine. When investigation showed that the patient had only about 2% of usual activity with butyrylcholine and no detectable activity with acetylcholine, the patient was suspected of being homozygous for the silent gene. However, family studies did not confirm this. For about 6 months, the patient's serum continued to exhibit only traces of cholinesterase activity. Then, the activity of the enzyme in the serum began to increase slowly during the next 6 months. Acrylamide gel electrophoresis during the early part of this second stage demonstrated the presence of only slow-migrating bands which were named C_6, $C_{7a,b}$, $C_{8a,b}$, and C_{10}. During the latter part of this period, the slow-migrating bands continued to be present, and the major band usually present in serum also appeared. When the patient's serum from the first 6-month period was incubated with the purified major component of usual cholinesterase, electrophoresis demonstrated the presence of the major cholinesterase component, together with slow-migrating bands corresponding to those found in the patient's serum at later stages and a slow-migrating component called C_9. The patient's serum used in this experiment produced no cholinesterase bands at all. It was postulated that the serum at this stage contained two factors: one factor (X) responsible for altering the electrophoretic mobility of cholinesterase, and a second factor (Y) responsible for reducing the activity of cholinesterase. It was proposed that factor X is a neuraminidase-like enzyme which alters the electrophoretic mobility by cleaving various amounts of sialic acid residues from cholinesterase. In confirmation of this hypothesis, it was found that incubating the patient's serum with normal serum resulted in the release of free sialic acid. It was suggested that factor Y might be a proteolytic enzyme.

The hypothesis was advanced that the neuraminidase-like enzyme is genetically controlled but is not expressed except under appropriate physiological or pathological conditions. The gene responsible for the presence of the neuraminidase-like enzyme is tentatively called N^R, whereas its allele is called N^U and is associated with the absence of the enzyme in the serum under any conditions. The proposed mechanism by which the slow-migrating cholinesterase forms are produced is called the "epigenetic modification" hypothesis. Ogita (O3) has suggested that most of the slow-migrating electrophoretic variants described by other authors

may be the result of expression of the proposed N^R gene. He further suggested that the C_5^+ phenotype may be divided into 2 types: Type I controlled by genes at the E_1 and E_2 loci, and Type II controlled by genes at the E_1 and N loci. This could explain why the expression of the C_5^+ phenotype is so variable, because the gene at the N locus may not always be expressed. Several relatives of the propositus studied by Ogita (O3) were found to have a C_5^+-like component in their sera.

2.7. Population Studies

Studies of the distribution of serum cholinesterase variants within different human populations have been performed by numerous investigators. The $E_1^uE_1^u$ genotype is present in about 97% of most populations. It has been found that the distribution of any of the nonusual genotypes varies considerably among different races, tribes, and other groupings of people. Variation between geographically separated groups with the same ethnic background has also been reported.

The E_1^a gene has not been detected among certain groups of Negroes and East Asians; the frequency of this gene is highest among Caucasian peoples. The E_1^f gene, which is rare in most populations, was found with a high frequency among a group of Punjabis and among certain Bantu tribes. The "silent" cholinesterase genes, which are also rare among most populations, occur with a high frequency among various groups of Alaskan Eskimos (Section 2.5.1). Roughly 0.5% of a healthy Caucasian population is heterozygous for the usual and silent esterase ($E_1^uE_1^s$), and tends to show 50 to 60% of the average esterase activity, but there is no simple test which allows these subjects to be recognized with certainty. They can be recognized best through family studies which show that their low activities are inherited, or through the demonstration of constancy of their activities over long periods of time.

The E_1^u gene is the most common cholinesterase gene among all populations studied. Summaries of cholinesterase gene frequencies among various human populations have been published (B15, G5, P16, P17, S31, S39, W19, W20), but although several of these reports contain extensive tables of data, none of them contains a complete listing of all reported gene frequencies. In consulting these tables, it should be kept in mind that in some of these reports, the gene frequencies for the E_1^u and E_1^a alleles may be slightly in error because no attempt was made to detect carriers of the E_1^f or E_1^s genes. Furthermore, in some studies, inefficient screening tests were applied to the samples, and only those which reacted abnormally were studied in detail.

Estimates of the distribution of the E_2^+ allele also reveal large dif-

ferences among different populations, but the literature on the frequency of the E_2^+ allele is much sparser than that of the E_1 alleles. Several summaries of E_2^+ allele distributions have been published (S31, S39, W19).

3. Succinylcholine Apnea and the Cholinesterase Variants Coded for by Genes at the E_1 Locus

As indicated in Section 1.2, the whole field of cholinesterase polymorphism, and much of pharmacogenetics, was opened up by the clinical observation that in certain patients receiving succinylcholine, prolonged apnea developed. The first hypersensitive patients were reported to have diminished serum cholinesterase activities (B29, E19). Not long afterward, it was recognized that in some patients, the decreased enzyme activities associated with apnea were inherited (F5, L25). Later, it was discovered that some of the susceptible patients had an atypical variety of serum cholinesterase, and that this atypical cholinesterase was inherited (H13, K15, L33). Then it was realized that other, noninherited factors that reduce the cholinesterase activity of the plasma make an individual more susceptible to prolonged apnea when succinylcholine is administered (B5). This section therefore begins with a discussion of the pharmacological and biochemical relationships among serum cholinesterase, succinylcholine, and prolonged apnea (C14, G27, L23-24). Evidence for established, new, and unidentified variants of serum cholinesterase is then presented, together with the types of procedures by which these variants may be detected.

Recommended procedures for collecting and storing plasma and serum specimens and laboratory procedures used for distinguishing the more common serum cholinesterase variants are discussed in Section 6.

3.1. Pharmacological Aspects of Succinylcholine Apnea

Among the drugs hydrolyzed by plasma cholinesterase (Section 4.1), succinylcholine has a special status for two reasons. First, it is generally administered by intravenous injection so that there is initially a relatively large amount and high concentration of the drug exposed to attack by the plasma enzyme. Second, the paralysis which it causes is a dramatic event, usually with unmistakable onset and termination.

The most widely held theory to explain the mode of action of succinylcholine is as follows. When the drug is injected into the blood stream of a patient in the usual dosage, most of it is probably hydrolyzed by the plasma cholinesterase within the first minute (B31, K5). A small fraction

of the succinylcholine reaches the postjunctional membrane at the neuromuscular junction and produces a depolarizing neuromuscular block, during which the muscle becomes unresponsive to further neural stimulation, and apnea ensues. The drug acts directly on the postjunctional membrane in a manner analogous to acetylcholine or, less importantly, as a weak inhibitor of acetylcholinesterase, thereby allowing the local concentration of acetylcholine to increase, since acetylcholinesterase is not capable of hydrolyzing succinylcholine at an appreciable rate. Within a few minutes, the neuromuscular blockade is terminated as succinylcholine diffuses away into the extracellular fluid. In those individuals having decreased levels of plasma cholinesterase, or having one of several variant forms of the enzyme, the rate of hydrolysis of succinylcholine is slow. In these cases, the motor endplate is overwhelmed by an almost continually maintained, relatively high concentration of the drug. The resulting prolonged apnea ceases only when the succinylcholine concentration has been reduced by a combination of the low activity of serum cholinesterase—if it is present—and other mechanisms. Direct evidence that plasma cholinesterase is necessary to prevent succinylcholine from producing prolonged apnea is contained in several reports (H4, S6, S7, S49) that prolonged apnea can be rapidly terminated by administration of a commercially available preparation of human serum cholinesterase. In most cases, intravenous injection of 90 mg of purified enzyme terminated the apnea in less than 15 minutes.

There is a precise and extensive set of clinical data (K13) describing the relationships among duration of succinylcholine action and cholinesterase activity, body weight, and age. These were compiled when succinylcholine was still a relatively new drug, and when it was appropriate at the time to test a wide range of doses. Because of the breadth of testing, these data firmly established a linear relationship between the logarithm of the dose and the duration of complete paralysis, with any other relationship being ruled out on the basis of statistical probability. The weight of this evidence has not always been recognized by subsequent investigators.

This logarithmic relationship between dose and duration of action is a fundamental one which is unaffected by the functional presence or absence of cholinesterase. That is, individual logarithmic dose-duration curves are parallel straight lines for subjects with different esterase activities. The explanation can only be that the cholinesterase activity chiefly determines the initial loading of the muscle endplate; in other words, that it hydrolyzes a substantial portion of succinylcholine immediately after injection into the blood stream, and thereby regulates the fraction available for receptor occupation in the endplate. A more recent

publication (H41) indicates that the fraction initially hydrolyzed is not quite as large as was originally envisaged, obviously because a substantial part of the dose quickly enters the extracellular fluid compartment, thereby partly escaping the immediate, most rapid phase of cholinesterase action. The net effect is a dampening of the initial esterase action upon succinylcholine (H41).

The actual termination of action of succinylcholine most likely involves its displacement from the receptors by a process which is akin to ion exchange and which is, therefore, vulnerable to changes of electrolyte composition in the vicinity (L15); also the character of the neuromuscular block produced by succinylcholine can change with time (L17). These observations may explain many of the frequent inconsistencies between clinical observation and laboratory assessment of the cholinesterase status, as for instance reported by the Danish Cholinesterase Research Unit (V5) and by Peck (P8). There are several studies in which 30% to 40% of all patients showing prolonged apnea were found to have the usual enzyme as determined by dibucaine number and in some cases, other procedures (B5, D4, K7, L23, T5, W23). Conditions associated with prolonged apnea include (W15) malnutrition, liver diseases, various types of metastatic cancer, chronic anemia and blood dyscrasias, late pregnancy, uremia, severe acute infections, radiation treatment, administration of certain phenothiazines, treatment of glaucoma with echothiopate iodide eyedrops, and exposure to anticholinesterase insecticides. Hence, it would be wrong to assume that all prolonged effects of succinylcholine must be due to some failure of cholinesterase function (B5, B17, K8, W23). More importantly, these concepts allow of the postulate that events at the site of succinylcholine action in muscle may have an exaggerated effect on the duration of action of the drug when the esterase function is marginal.

Before it can be unequivocally stated that apnea is caused by a prolonged peripheral neuromuscular block, the existence of such a block should be directly demonstrated by a peripheral nerve stimulator (B5). This is often not done, and therefore in many cases it is not possible to determine the true cause of the apnea, especially when there is some delay in following up the event.

3.2. Biochemical Aspects of Succinylcholine Apnea

The available data suggest a simple relationship between the rate of enzymic hydrolysis of succinylcholine and the duration of its action. Suppose that a patient with the usual type of plasma cholinesterase in average concentration receives a standard dose of succinylcholine which

causes complete paralysis of 2 minutes' duration. If esterase activity were to be halved, the duration of action would be expected to double, i.e., to be of the order of 4 minutes—a prolongation which is clinically unimportant (S8). This is the situation for most $E_1^uE_1^a$ heterozygotes. A reduction of esterase activity to 25% of normal would cause the drug action to last approximately 8 minutes (D23). By the same token, a succinylcholine paralysis lasting 1 hour would imply that the level of enzyme activity is 3% of normal or less. However, even the relatively mild reduction of esterase activity to 25% of normal is hardly compatible with life if it occurs as a consequence of a 75% reduction of protein synthesis (H48, L9, T1). It follows that any complete apnea lasting more than 10 minutes or so cannot be due to a simple reduction of esterase concentration (K33). If the undue event is not at the neuromuscular endplate, that is, if a substantial prolongation of action is due to a failure of cholinesterase action, it can only be due to enzyme inhibition (E6) or to the presence of genetic variants of the enzymes which do not hydrolyze succinylcholine. Thus, if cholinesterase tests are to be helpful in assessing succinylcholine action, determination of the genetic type is much more important than measurement of enzyme activity (E6, S41) with any of the appropriate substrates.

Attempts to explain the prolonged apnea occurring in some patients who are carriers of certain cholinesterase variant genes were made by studying the hydrolysis of succinylcholine by usual and variant human sera *in vitro* and by purified cholinesterase preparations. Goedde *et al.* (G26) used two different methods to study the kinetics of the hydrolysis of succinylcholine and of succinylmonocholine. One of these methods used radioactive succinylcholine and involved high-voltage electrophoresis to separate the reactants from the products. The second method was an indirect one in which the succinylcholine remaining in a given reaction mixture was quantitated by adding a standard amount of benzoylcholine to the reaction mixture, and determining the percentage of inhibition of the rate of hydrolysis of benzoylcholine. In other experiments, the K_i values of succinylcholine and -monocholine acting as competitive substrates for the hydrolysis of benzoylcholine were determined (Table 2). Similar experiments with succinylcholine as a competitive substrate for the hydrolysis of butyrylcholine were reported by Hobbiger and Peck (H34) and by Peck (P8), and are reported in Table 2. Hersh *et al.* (H27) studied the hydrolysis of succinylthiocholine by the $E_1^uE_1^u$, $E_1^uE_1^a$, and $E_1^aE_1^a$ forms of the enzyme, the results of which are also reported in Table 2.

It was found that the usual enzyme, in contrast to the atypical enzyme, has a greater affinity for succinylcholine, succinylmonocholine, and succinylthiocholine as judged from K_i and K_m values. It was also found that

TABLE 2
SUCCINYLCHOLINE, SUCCINYLMONOCHOLINE, AND SUCCINYLDITHIOCHOLINE AS SUBSTRATES AND/OR INHIBITORS OF USUAL AND ATYPICAL CHOLINESTERASE VARIANTS

Genotype	Substrate[a]	K_m of substrate (mol/liter)	Inhibitor[a]	K_I (mol/liter)	pH	Temperature (°C)	Reference
$E_1^uE_1^u$	SDC	4.0×10^{-5}	—	—	7.4	37	G26
$E_1^uE_1^u$	SMC	8.4×10^{-3}	—	—	5.7	37	G26
$E_1^aE_1^a$	SMC	0.17	—	—	5.7	37	G26
$E_1^uE_1^u$	BzCh	2.4×10^{-5}[b,c]	SDC	2.2×10^{-5}	7.4	26	G26
$E_1^uE_1^u$	BzCh	4.2×10^{-6}	SDC	6.6×10^{-6}	7.4	26	R1
$E_1^uE_1^u$	BzCh[d]	4.0×10^{-6}	—	—	7.4	26	D10
$E_1^aE_1^a$	BzCh[d]	2.2×10^{-5}	—	—	7.4	26	D10
$E_1^aE_1^a$	BzCh	3.0×10^{-5}[c]	SDC	3.1×10^{-3}	7.4	26	G26
$E_1^aE_1^a$	BzCh	4.1×10^{-5}	SDC	2.4×10^{-4}	7.4	26	R1
$E_1^uE_1^u$	BzCh	—	SMC	4.4×10^{-3}	7.4	26	G26
$E_1^aE_1^a$	BzCh	—	SMC	5.4×10^{-2}[e]	7.4	26	G26
$E_1^uE_1^u$	BuCh	6.0×10^{-4}	SDC	1.3×10^{-4}	7.45	37	H34
$E_1^aE_1^a$	BuCh	2.3×10^{-3}	SDC	7.4×10^{-3}	7.45	37	H34
$E_1^uE_1^u$	SDTC	3.5×10^{-5}	—	—	7.4	30	H27
$E_1^uE_1^a$	SDTC	9.7×10^{-5}	—	—	7.4	30	H27
$E_1^aE_1^a$	SDTC	1.1×10^{-3}	—	—	7.4	30	H27

[a] Abbreviations used: SDC, succinylcholine; SMC, succinylmonocholine; BzCh, benzoylcholine; BuCh, butyrylcholine; SDTC, succinyldithiocholine.

[b] Note that this K_m value is about 5 times larger than the value reported in Reference L34.

[c] Calculated from the data reported in Reference G26.

[d] Findings of K_m and V_{max} for a series of *n*-acylcholine esters are reported in Reference D10, and inhibition data for 16 inhibitors, including SDC, are reported in Reference K10.

[e] Calculated from the relationship $K_I = I_{50}(1 + S/K_m)^{-1}$.

with both enzymes, succinylcholine had a greater affinity for the enzyme than did succinylmonocholine. Although it was not possible to determine the K_m value for succinylcholine using the atypical enzyme (G26), it may be estimated from the data in Table 2 that the K_m value at pH 7.4 and 37°C would be about 6×10^{-3} mol/liter. Hence, the Michaelis constant for succinylcholine is larger than normal by two orders of magnitude (K5)—that is, there is an extremely low affinity between atypical esterase and succinylcholine so that no hydrolysis takes place *in vivo*, regardless of the enzyme concentration or its activity toward other substrates. Quite recently, another variant has been described which does not hydrolyze succinylcholine, but which otherwise appears to be normal and cannot be detected by dibucaine or other differential inhibition tests (A4, G15). There is as yet no formal confirmation of this observation, but a study using succinyldithiocholine as substrate yielded enzyme distribution curves

which strongly support the contention of there being a variant with selective failure of succinylcholine hydrolysis (B5).

The nature of the dose-response curves for succinylcholine in subjects with atypical esterase has one important consequence for the understanding of abnormal clinical events. A usual dose of succinylcholine (50 to 100 mg) injected intravenously into a subject lacking cholinesterase activity causes complete paralysis of the respiratory muscles for 1 hour or so; in clinical practice, this means that as much as 2 hours may elapse before adequate spontaneous respiration is restored—perhaps in exceptional cases, even 3 hours. However, an effect which lasts still longer than this period cannot be explained solely as a consequence of cholinesterase dysfunction, and the fault is likely to be in muscle. The elimination of the drug in subjects lacking esterase function is probably due to renal excretion and nonenzymatic hydrolysis.

Hence, it is tempting to speculate that the occurrence of prolonged apnea is associated with a low affinity of the variant enzyme for succinylcholine, and it is generally agreed that the most common cause of prolonged apnea is the presence of a susceptible cholinesterase genetic variant in the plasma of the patient. Prolonged apnea can occur with any of the known genetic variants, although, as already mentioned, the apnea may be unrelated to the variant of cholinesterase present. However, in the presence of certain of the cholinesterase variants, the incidence of prolonged apnea following commonly administered doses of succinylcholine is very high—as much as 100% for some variants. The genotypes that are most susceptible are $E_1^aE_1^a$, $E_1^aE_1^f$, $E_1^aE_1^s$, $E_1^fE_1^f$, $E_1^fE_1^s$, and $E_1^sE_1^s$. In general, individuals who are heterozygotes with one E_1^u gene are usually not overly sensitive to the effects of succinylcholine. Some of the susceptible individuals who have been classified as the $E_1^uE_1^u$ genotype have undoubtedly been misclassified. As discussed in Section 2, there is ample evidence that unidentified and poorly characterized phenotypes exist, and that some of these presumed phenotypes are associated with prolonged succinylcholine apnea. In some cases, there have been insufficient family studies to unambiguously establish the genetic nature of the presumed phenotypes; in others, there has been a lack of suitable testing procedures to completely differentiate the presumed new variants from the other variants which have been more thoroughly investigated.

It is sometimes recommended that if it is necessary to administer succinylcholine to a patient who is known to be overly susceptible, very small doses be used. Lee-Son *et al.* (L21) reported an interesting case in which the drug was administered on a number of occasions to a patient who had a dibucaine number (Section 2.2) of 23, and who may have been homozygous for the atypical gene, or heterozygous for the atypical

and silent genes. The patient was titrated with 1-mg doses of succinylcholine while a nerve stimulator–muscle twitch recorder system was used to monitor neuromuscular blockade. From these measurements, it was determined that the ED_{50} for succinylcholine (presumably as the chloride salt) was 68 ± 4 (SE) μg/kg body weight. On a molar basis, this amounts to 1.0×10^{-7} mol/kg. It was found that a total dose of 6 to 8 mg of succinylcholine was sufficient to depress the twitch response by more than 90%. Spontaneous respiration resumed 1 to 10 minutes after the last dose. Similar measurements made with decamethonium iodide yielded an ED_{50} value of 49 ± 5 μg/kg body weight, or 0.96×10^{-7} mol/kg. A total dose of 4 to 6 mg of decamethonium iodide was sufficient to depress the twitch response by greater than 90%, and spontaneous respiration resumed within 5 minutes of the last dose. Decamethonium is not hydrolyzed by serum, and its action is most likely reversed by diffusion away from the motor endplate. The similarity of the magnitudes of the ED_{50} values of decamethonium and succinylcholine and the rapid rate of recovery when a similar small dose of either drug is administered suggest that the mechanisms of action of both compounds as well as the mechanisms of recovery are very similar, if not identical.

Finally, it deserves to be emphasized that the relationship found in man between duration of succinylcholine action and plasma cholinesterase activity is not present in many animals; in most species, the drug is hydrolyzed at a comparatively slow rate by some esterase, presumably mostly in the liver (H3, H35, L4). Because of this lack of rapid destruction, succinylcholine has been used, for instance, in dart guns to subdue wild animals. The plasma cholinesterase tests for the study of succinylcholine have no use except in man.

4. Chemical and Biochemical Properties of Cholinesterases

4.1 Substrate Specificity

The cholinesterases, as indicated by the name, hydrolyze the esters of choline. However, the specificity for choline esters is not absolute, as evidenced by the hydrolysis of other esters, albeit at a slower rate. While acetylcholinesterase has high specificity not only for acetylcholine—$CH_3COO(CH_2)_2N^+(CH_3)_3$—but also for acetylthiocholine and other acetyl esters, most plasma cholinesterases catalyze the hydrolysis of butyrylcholine, butyrylthiocholine, and other butyryl esters at faster rates than the corresponding acetyl derivatives. With some homologous esters of acetylcholine, it was shown early on (D10) that butyrylcholine

was the optimal substrate for the human plasma (usual) enzyme, whereas pentanoylcholine showed the highest V_{max} for the atypical enzyme. In other studies, the order of the relative rates of hydrolysis by the human plasma enzyme was butyryl > valeryl > propionyl > acetyl > α-ethylbutyryl > α-methylbutyryl (S18), whereas for the rat or mouse enzyme, the order was propionyl > butyryl > acetyl (M25).

The introduction of a double bond at the α,β position in the alkyl group of choline esters reduces the rate of hydrolysis of the ester compared with that of the corresponding saturated alkyl ester. A double bond at any other position appears to have a less predictable effect, e.g., 4-pentenoylcholine—$CH_2=CH(CH_2)_2COO(CH_2)_2N^+(CH_3)_3$—is hydrolyzed faster than is vinylacetylcholine—$CH_2=CHCH_2COO(CH_2)_2N^+(CH_3)_3$. Substitution of an alkyl group at the α-position in the unsaturated acid moieties of choline esters causes an additional reduction in the rate of hydrolysis of these compounds (S18), as with the saturated analogs.

Beckett and his co-workers (B14) examined the hydrolysis by purified horse serum cholinesterase of a number of analogs of butyrylcholine. The introduction of an α-methyl group into the choline moiety of the butyryl ester was found to decrease the rate of enzymic hydrolysis only slightly, whereas a drastic reduction was found for β-methyl substitution. The substitution of various functional groups into the butyryl moiety of butyrylcholine modified the affinity of the substrate for the enzyme. These results are summarized in Table 3. Since K_m values can be assumed to be inversely proportional to affinity values, the results shown in this table indicate that β-hydroxyl substitution increases the affinity of the substrate for the enzyme, and α,β-unsaturation effects even greater affinity. Acetoacetylcholine has a greater affinity for the enzyme surface

TABLE 3
COMPARISON OF THE MICHAELIS CONSTANTS K_m AND V_{max} FOR THE HYDROLYSIS OF BUTYRYLCHOLINE AND SOME ANALOGS BY PURIFIED HORSE SERUM CHOLINESTERASE[a]

Substrate	$K_m \times 10^3$ (mol/liter)	V_{max} (μmol/30 minutes)
Butyrylcholine	1.52	4.44
Acetoacetylcholine	0.29	1.45
β-Hydroxybutyrylcholine	1.22	0.57
Crotonylcholine	0.76	0.22

[a] Data of Beckett *et al.* (B14).

than even crotonylcholine and is hydrolyzed more rapidly than β-hydroxybutyrylcholine.

The kinetics of hydrolysis of lactoylcholine—$CH_3CH(OH)COO(CH_2)_2N^+(CH_3)_3$—and its isomers were studied by Sastrey and White (S3). The rationale for this study stemmed from the possible formation of lactoylcholine from propionylcholine—$CH_3CH_2COO(CH_2)_2N^+(CH_3)_3$—or acrylylcholine—$CH_2=CHCOO(CH_2)_2N^+(CH_3)_3$—both of which have been isolated from animal tissues (B3, W24). DL-Lactoylcholine was found to be hydrolyzed by cholinesterase at a rate three to four times faster than acetylcholine, but in contrast to acetylcholinesterase, the plasma enzyme did not differentiate between the D- and L-enantiomorphs of lactoylcholine.

The foregoing studies have dealt chiefly with model substrates *in vitro*. Several of the early papers by Augustinsson, referred to in Section 4.1.1, considered substrate specificity from the viewpoint of species variations. It is also important to recognize that plasma cholinesterase may be associated with the hydrolysis, *in vivo*, of a large number of drugs (K4, L1, L4) that contain ester bonds susceptible to enzymic hydrolysis. Apart from succinylcholine (Section 3.1), cholinesterase is known to be responsible in man for the hydrolysis of cocaine (S40), procaine (K2), and other esters with local anesthetic properties. Whether enzymatic hydrolysis terminates the pharmacologic effect depends on the whole mechanism of action of the particular drug.

4.1.1. *Species Variation*

In spite of an immense literature on the substrate specificity of cholinesterase, the significance of its variation from one species to another (e.g., A21, A27, M25, E2) remains obscure. Even such closely related species as the Macaque and Mangabey monkeys show striking differences in the substrate specificities of their plasma cholinesterases (A27). There is, however, little variation in substrate specificity for the enzyme when it occurs in different tissues within a species. The complications in this field were indicated early on by Kalow and his associates, who showed that the substrate affinity of the usual human plasma cholinesterase is quite different from that of the atypical enzyme (D10). It is therefore highly desirable to state the source of the enzyme, as well as the substrate used, when describing work on plasma cholinesterase.

Horse and human plasma cholinesterases have been found to have similar substrate specificity profiles (M25), for example, in respect to the relative rates of hydrolysis of some isomeric substrates (B14), as summarized in Table 4. It can be assumed that the areas in the regions of the

TABLE 4
RATES OF HYDROLYSIS OF SOME ISOMERIC BUTYRYLCHOLINE ANALOGS BY HORSE AND HUMAN SERUM CHOLINESTERASES RELATIVE TO ACETYLCHOLINE AND BENZOYLCHOLINE[a]

Substrate (6×10^{-2} mol/liter)	Purified horse cholinesterase	Horse cholinesterase	Human cholinesterase
Acetylcholine	1.00	1.00	1.00
Benzoylcholine	0.34	0.28	0.33
Butyrylcholine	2.27	2.22	2.13
L-Butyryl-α-methylcholine	1.84	2.02	1.97
D-Butyryl-α-methylcholine	1.50	1.70	1.68
L-Butyryl-β-methylcholine	0.10	0.12	0.11
D-Butyryl-β-methylcholine	0	0	0

[a] After Beckett *et al.* (B13).

active sites of human and horse serum cholinesterases are very similar, but the enzymes do differ in their catalytic properties toward certain substrates, such as procaine.

At high substrate concentrations, neither human nor horse plasma cholinesterase shows substrate inhibition with either acetyl- or butyrylcholine, but substrate inhibition is observed with halogenoacetylcholines (S2), acetylsalicylcholine (A21), benzoylcholine (A21), and lactoylcholine (S3).

Benzoylcholine has been shown to be hydrolyzed by the plasma cholinesterases from many species, but not to be hydrolyzed by acetylcholinesterase. It is important to check whether the plasma of a given species does hydrolyze benzoylcholine before assuming that the plasma of that species has negligible enzymic activity in general. Acetyl-β-methylcholine is, in contrast, readily hydrolyzed by acetylcholinesterase but very slowly by the plasma enzyme (M12). These two substrates may therefore be used in complementary ways to characterize a given enzyme.

Horse serum butyrylcholinesterase isoenzymes have been resolved by electrophoresis on polyacrylamide gel, and the differential reactivity of the various isoenzymes toward butyrylthiocholine has been measured simultaneously in a single gel (C7). The results are shown in Table 5. Only 63% of the total enzymic activity was found to be associated with the major component (C_4), compared with 80% found by previous investigators (S49).

TABLE 5
MICHAELIS CONSTANTS K_m AND V_{max} FOR THE HYDROLYSIS OF BUTYRYLTHIOCHOLINE BY HORSE SERUM CHOLINESTERASE ISOENZYMES USING GEL SCANNING[a]

Isoenzyme	$K_m \times 10^4$ (mol/liter)	V_{max} (arbitrary units)	Activity[b] (%)	Relative mobility[c]
I	2.46	2.96	63	0.25
II	7.62	1.24	20	0.30
III	6.21	0.77	12	0.36
IV	12.14	0.38	3	0.45

[a] After Chui *et al.* (C7).
[b] Calculated on the basis of the relative absorbance from the stained zymogram using 1×10^{-3} mol/liter butyrylthiocholine.
[c] The relative mobility of each isoenzyme was calculated from its migration in comparison with the tracking dye.

4.2 ENZYME PURIFICATION

Augustinsson (A25) studied the cholinesterase activities of the plasmas of many vertebrate species. Subsequent workers have confirmed that the highest activity of the enzyme is found in equine and human plasma or serum. These sources have been used consequently by most workers as starting materials for the purification of cholinesterase. Outdated plasma from national blood banks is valuable for large-scale preparations of the human enzyme.

Various techniques (Table 6) have been used in successive attempts at purification. Early workers employed fractional precipitation with ammonium sulfate and, in spite of the inherent limitations, achieved a remarkable degree of purification. This approach is therefore commonly used as the first stage of purification of the enzyme. Advances in separation techniques, particularly ion exchange chromatography on diethylaminoethyl cellulose, have provided more efficient means of removing the bulk of contaminating proteins. Unfortunately, some workers have paid little regard to the rationale of this particular method, and have thus failed to realize optimal purification. Clearly, in a buffer of higher pH than its isoelectric point, a protein bears a negative charge and can be bound to an anion exchange resin. Most contaminating proteins which are more acidic than the enzyme in question can be eluted with a suitable buffer solution. The enzyme may then be selectively eluted by buffer containing a positively charged substrate analog if the concentration of the total anion in the buffer is identical with or lower than that used in the preliminary washing. The pH of the chromatography buffer should be

TABLE 6
EXAMPLES OF PURIFICATION OF CHOLINESTERASES FROM DIFFERENT SOURCES[a]

Source	Precipitation	Electrophoresis	Other techniques	Gel filtration	Ion exchange	Purification factor	Reference
Horse serum	Ammonium sulfate	—	—	—	—	5,000	S45
Human serum	Alcohol	—	—	—	—	3,400	S46
Horse serum	Ammonium sulfate (I)	Zone (II)		—	Sephadex 2A (III)	not stated	H24
Horse serum	Ammonium sulfate (I)	Zone (III)	Ultracentrifugation (II)	—	—	14,000	J3
Human serum	Rivanol-ammonium sulfate (I)	Zone (III)	Alumina chromatography (II)	Sephadex G200 (IV)	—	10,000	H18
Human serum	Ammonium sulfate (I)	—	—	—	Whatman DE32 (II)	480	G1
Human serum	Calcium chloride (I)	Electrofocusing (III)	—	Sephadex G200 (IV)	Whatman DE52 (II)	13,000	D7
Human serum	—	—	Calcium phosphate gel chromatography (I)	—	Whatman DE52 (II)	>3000	Y2
Horse serum	Ammonium sulfate (I)	Zone (III)	Ultracentifugation (II)	Sephadex G200 (V)	Whatman DE52 (IV)	150 (after precipitation)	L19
Horse serum	Ammonium sulfate (I)	Preparative (IV)	Sepharose 6B (III)	Sephadex G200 (II)	—	10,000	M7
Horse serum	Ammonium sulfate (I)	Preparative (III)	—	—	Sephadex A50 (II)	19,000	M6
Horse serum	Ammonium sulfate (I)	Zone (III)	Ultracentrifugation (II)	Sephadex G200 (V)	Whatman DE52 (IV)	not stated	T3
Human serum	Calcium chloride (I)	Preparative (III)	—	—	Whatman DE52 (II)	8,000	M20
Human serum	—	—	Affinity chromatography on CH Sepharose 4B (II)	—	Whatman DE52 (I)	not stated	L34
Human serum	—	—	Affinity chromatography (I)	Sepharose 6B (III)	DEAE Cellulose (II)	10,000	P10
P. polycolor	Ammonium sulfate (I)	—	Hydroxyapapite chromatography (III and V)	Sephadex G150 (IV)	CM Sephadex (II)	9,270	N2
Dog pancreas	Ammonium sulfate (I)	—	Kieselguhr adsorption (II)	—	—	2,000	M11
Porcine parotid gland	Ammonium sulfate (I)	—	—	Sephadex G200 (III)	CM Sephadex (II)	3,220	T9
Plaice body muscle	Ammonium sulfate (II)	—	Autolysis with bacteria (I)	Sephadex G200 (III)	—	2,000	L37

[a] Roman numerals indicate the sequence of purification stages.

between the isoelectric point of the enzyme and that of the substrate analog. Electrostatic affinity is the major factor determining protein–ion exchange, so that the partial neutralization of negatively charged enzyme by its positively charged substrate analog could effect the specific elution of the enzyme from the anion exchanger. Yoshida (Y2) used this principle successfully by absorbing partially purified human cholinesterase onto DEAE-cellulose and eluting with a linear gradient of choline chloride, as substrate analog, in sodium chloride–acetate buffer. The elution profile so obtained of human plasma cholinesterase is shown in Fig. 1. In a similar way, Main *et al.* (M6) purified horse serum cholinesterase, using Sephadex A50, which in the present authors' experience gives better results than does Whatman DE52, preferred by other workers (see Table 6).

Affinity chromatography is one of the most effective procedures for enzyme purification and yet, with the notable exceptions of Picard (P10) and of La Du and his co-workers (L2), it has received scant attention for the purification of cholinesterase. Its value in purifying acetylcholinesterase from both fish electric organ and erythrocyte membrane is discussed in an excellent review by Rosenberry (R8). In affinity

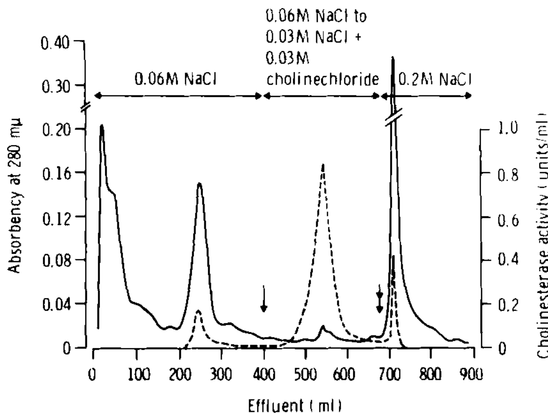


FIG. 1. Elution pattern of human plasma cholinesterase from DEAE-cellulose column: solid line, absorbance at 280 nm; broken line, enzyme activity. Partially purified enzyme preparation was placed on a DEAE-cellulose column (1.2 × 10 cm) and equilibrated with 0.02 mol/liter acetate buffer, pH 6.0. The column was washed with the same buffer containing 0.06 mmol/liter sodium chloride (up to 400 ml of effluent). Then (at single arrow) the enzyme was eluted with a linear gradient of sodium chloride and choline chloride ranging in concentration from 0.06 mmol/liter NaCl to 0.03 mmol/liter NaCl + 0.03 mol/liter choline chloride. At the double arrow, the buffer was changed to 0.02 mol/liter acetate buffer, pH 6.0, containing 0.2 mol/liter NaCl: flow rate 25 ml per hour. (After Yoshida, Y2.)

chromatography, the enzyme is specifically adsorbed onto an immobilized substrate, substrate analog, or coenzyme. After preliminary washing to remove contaminants, the enzyme is eluted either by a change in the physiochemical environment (e.g., of the ionic strength) or by competitive binding to a soluble substrate, substrate analog, or coenzyme in the elution buffer. Lockridge and La Du (L34) used procainamide on CH Sepharose B as an affinity substrate in the purification of some of the human plasma cholinesterase variants. With the atypical enzyme, an additional purification stage involving chromatography on BioGel A 1.5m was introduced to remove contaminants of lower molecular mass, but even then, the specific activity which was achieved was less than that realized with the usual enzyme.

Affinity chromatography offers the advantage of being a potentially single-stage process, with speedy and effective column regeneration (C4). In the present authors' opinion, a combination of ion exchange chromatography followed by affinity chromatography is currently the best procedure for the purification of the enzyme and its variants. Thus a large scale purification of human cholinesterase was reported by Picard (P10), using affinity chromatography on *m*-aminophenylmethylammonium agarose. Specific elution of the enzyme by 9-amino-10-methylacridinium from the affinity gel was followed by ion exchange chromatography on DEAE-cellulose and molecular filtration on Sepharose 6B. Additionally, with horse cholinesterase and using a procainamide affinity column, Main (private communication) achieved a 3000-fold purification when 10 liters of serum was passed through 100-ml gel columns. One further column resulted in electrophoretically pure enzyme with an overall yield of 75%.

Affinity chromatography is also valuable for the qualitative fractionation of the heterozygous cholinesterase variants. La Du and Choi (L2) used a diaminocaproylphenyltrimethylammonium Sepharose 4B affinity column to demonstrate that the elution pattern of the cholinesterase from a heterozygote $E_1^uE_1^a$ is quite different from that obtained from a mixture of the cholinesterases from homozygotes $E_1^uE_1^u$ and $E_1^aE_1^a$. Whereas two distinct peaks of enzymic activity were obtained for the elution pattern of the mixture (Fig. 2), the natural heterozygote $E_1^uE_1^a$ separated as a series of esterases with progressively higher dibucaine numbers. These results were explained by postulating that for $E_1^uE_1^a$ serum, the enzyme exists in five different forms, each being a tetramer produced by the random combination of usual (U) and atypical (A) esterase subunits. Thus the first chromatographic fractions contained a tetramer of the atypical enzyme, and the last fractions, a tetramer of the usual enzyme, with the in-

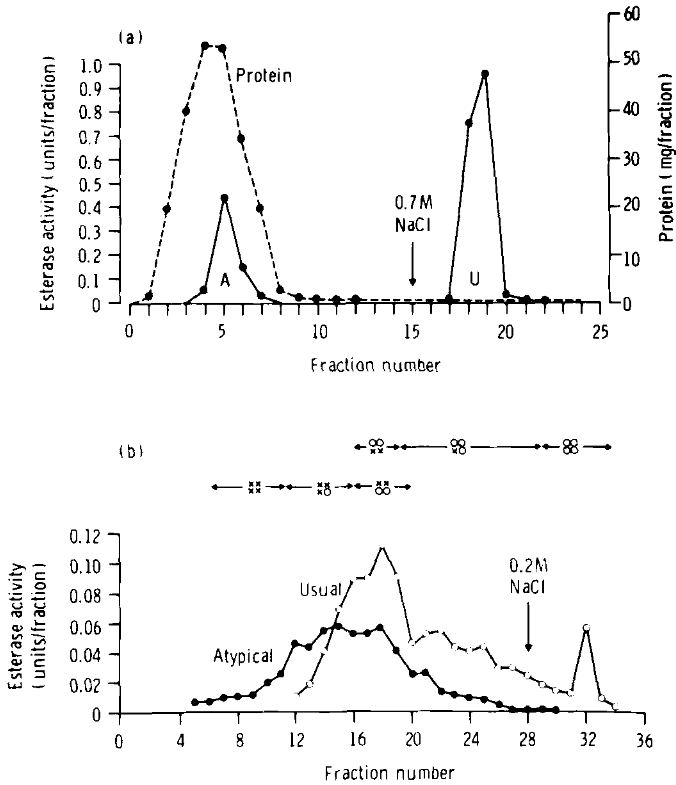


FIG. 2. Elution pattern of cholinesterase isoenzymes from: (a) a mixture of usual ($E_1^uE_1^u$) and atypical ($E_1^aE_1^a$) dialyzed sera; (b) dialyzed serum from an $E_1^uE_1^a$ individual after affinity chromatography. (After La Du and Choi, L2.)

termediate fractions being three different tetrameric hybrids made up of various proportions of usual and atypical subunits. Experimental support for this hypothesis was achieved by rechromatography of pooled fractions which were believed to contain the tetramer A_2U_2, when a single peak with dibucaine number 60 was obtained. Affinity chromatography of a large sample of $E_1^uE_1^a$ serum gave the separation of components illustrated in Fig. 3. Ion exchange chromatography on DEAE-cellulose at pH 4 has been claimed (D3) to resolve the heterozygote serum $E_1^uE_1^f$ into two major components corresponding to the homozygotes, but others have been less successful in using this technique. It is relevant to note that in plasma or serum at or below pH 4, the enzyme is rather labile, but it can be stabilized by desalting with a suitable dextran gel.

The $E_1^aE_1^a$ and $E_1^fE_1^f$ variants have been purified approximately

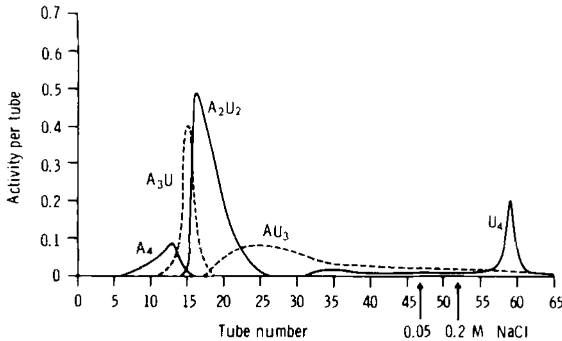


FIG. 3. Pattern of elution of tetramer from 10 ml of the same AU-heterozygous serum as was used in the experiment of Fig. 2, with a longer affinity chromatography column (1 × 30 cm). Assignment of the tetrameric composition of each fraction was based upon the dibucaine number and total activity, and the calculated dibucaine numbers for each possible tetramer. The homologous tetramers at the beginning and end, A4 and U4, had dibucaine numbers of 15 and 78, respectively. (After La Du and Choi, L2.)

2000-and 5000-fold, albeit in low yield (∼14%), by Das (D7), using the procedure already mentioned (D3).

The purification factors reported in Table 6 are not readily compared because of different means of determining the protein content of the products at various stages of purification, and the difficulty of drying specimens to constant weight after lyophilization. Apart from conventional colorimetric methods of assay, the concentration of cholinesterase can be estimated by measuring the absorbance at 280 nm (H18, T3). Moreover, in spite of the high purification factors claimed by some workers, little evidence of the homogeneity of their preparations, by means of ultracentrifugation or other criteria, has been presented.

Bacterial cholinesterase, which is present in some species belonging to the fluorescent group of *Pseudomonas* and *P. polycolor*, has been purified more than 9000-fold by Nagasawa *et al.* (N2). A cell-free extract was obtained by ultrasonic disruption followed by centrifugation. The enzyme was purified by ammonium sulfate fractionation followed by successive stages of column chromatography, and its homogeneity was demonstrated by disc gel electrophoresis and by analytical ultracentrifugation. Substrate specificity showed the enzyme to be butyrylcholine esterase.

Laing *et al.* (L6) purified a bacterial cholinesterase from *P. fluorescens* (Goldstein strain) by fractionation on a CM Sephadex column following ammonium sulfate fractional precipitation. However, acetylcholine was the only substrate used with the purified enzyme, so that it is uncertain whether this microbiol cholinesterase is a butyrylcholine esterase or an acetylcholine esterase similar to that isolated from *P. aeruginosa* (T2).

4.3. Physical Properties

4.3.1. *Sedimentation Coefficient*

Sedimentation coefficients—S_{20W}—representing the velocity of sedimentation in unit gravitational field in water at 20°C, may be used, together with diffusion constants, to calculate the relative molecular masses of purified enzyme preparations. Reported values of S_{20W} for cholinesterase isolated from human or equine serum are given in Table 7. Furthermore, the S_{20W} value for highly purified butyrylcholine esterase of porcine parotid gland is 9.7 units (T9) and it is 2.5 units for the enzyme from *P. polycolor* (N2).

4.3.2. *Absorbance*

Coefficients of absorbance at 280 nm for cholinesterase are given in Table 8. Apparently, none of these values has been corrected for light scattering, although corrections would appear to be significant for concentrated solutions of the enzyme (1 to 5 mg/ml). Teng *et al.* (T3) applied the procedures described by Leach and Scheraga (L16) and by Winder and Gent (W31) to their figures, as shown in Table 8, and obtained a dry weight coefficient, corrected for light scattering, of 14.6 units. A slightly higher value was obtained (T3) when protein concentrations were determined by differential refractometry (B1). Considering the diverse purities of these enzyme preparations, there is fair agreement between the values.

4.3.3. *Partial Specific Volume*

A partial specific volume ($\bar{v}$) for purified horse serum cholinesterase of 0.78 ml/g was derived (L19) from measurements by the D_2O method (E4). Workers in the same laboratory (T3) reported a value for $\bar{v}$ of

TABLE 7
Sedimentation Coefficients of Butyrylcholinesterase

Source	S_{20W}	Reference
Horse serum	9.9	J3
Horse plasma	10.7	H18
Horse plasma	11.5	L19
Horse plasma	11.1	M6
Horse serum	11.5	T3
Porcine parotid	9.7	T9
Human plasma	12.4	D7
Human plasma	11.1	M20
P. polycolor	2.5	N2

TABLE 8
COEFFICIENTS OF ABSORBANCE AT 280 NM FOR CHOLINESTERASE FROM DIFFERENT SOURCES

Source	$E_{1cm}^{1\%}$	Reference
Horse serum	25	J3
Human serum	14.5	H18
Horse serum	20	M7
Horse serum	11.3	L19
Horse serum	13.6	M6
Horse serum	15.2	T3

0.723 ml/g from accurate density measurements, and 0.710 ml/g when calculated from the amino acid and carbohydrate compositions. The latter pair of estimates agree within experimental error and are similar to other values, including one for eel acetylcholinesterase reported by Main *et al.* (M6), as presented in Table 9.

4.3.4. *Isoelectric Point*

Estimates of the isoelectric point of different plasma cholinesterases are shown in Table 10. There is a wide variation within the range pH 3 to 5, which is probably due to artifacts associated with the different methods which were used. Sources of error include evaporation, electroosmosis, and variation in texture of the supporting medium, with consequent variable adsorption of protein. Correction may be made for these factors by the electrophoresis, under identical conditions, of an uncharged reference substance such as dextran (S5, W1). When subjected to preparative isoelectric focusing, human plasma cholinesterase showed a ma-

TABLE 9
PARTIAL SPECIFIC VOLUME, $\bar{v}$, FOR HORSE SERUM CHOLINESTERASE AND EEL ACETYLCHOLINESTERASE

Enzyme	$\bar{v}$ (ml/g)	Method	Reference
Horse serum	0.78	D_2O	L20
Horse serum	0.732	Calculation	L20[a]
Horse serum	0.723	Density measurements	T3
Horse serum	0.710	Calculation	T3
Horse serum	0.688	Density meter	M6
Horse serum	0.712	Calculation	M6
Eel acetylcholinesterase	0.731	Calculation	L29

[a] Reported by Main *et al.* (M6).

TABLE 10

ISOELECTRIC POINTS OF HORSE AND HUMAN SERUM CHOLINESTERASES

Enzyme	Method	p*I*	Reference
Horse serum	Segmented U-tube electrophoresis	4.36	A20–25
Horse serum	Segmented U-tube electrophoresis	5.2	K34
Horse serum	Cellulose electrophoresis	4	H24
Horse serum	Paper electrophoresis	3	S49
Human serum	Paper electrophoresis	2.9–3.0	S50
Human serum	Isoelectric focusing—acrylamide	4.2	G1
Human serum	Isoelectric focusing—preparative	3.99 (4.28)	D7
Human serum	Isoelectric focusing—preparative	3.96, 4.03, 4.14 4.35, 4.48	W14
Human serum	Isoelectric focusing—acrylamide	3.85, 4.05, 4.40	P10

jor peak at pH 3.99 and a minor peak at pH 4.28 (D7). Using a similar technique, Whittaker (W14) determined the isoelectric points of several of the human plasma cholinesterase variants with the results given in Table 11. All the variants showed several components.

4.3.5. *Electrophoretic Properties*

It is well established that human plasma cholinesterase is an α_2-globulin, with slightly lower mobility at pH 8.6 than the main α_2-globulin fraction of human plasma. By changing the pH, the electrophoretic prop-

TABLE 11

ELECTROFOCUSING OF HUMAN PLASMA CHOLINESTERASE VARIANTS

Genotype	Number investigated	Values of p*I* for resolved enzymically active peaks						Reference
$E_1^uE_1^u$	4	3.96	4.03	4.14	—	4.35	4.48	W14
$E_1^uE_1^a$	4	3.93	4.05	4.15	—	4.30	4.41	W14
$E_1^aE_1^a$	4	3.97	—	4.14	—	4.32	4.51	W14
$E_1^uE_1^f$	3	3.94	4.06	—	4.21	4.30	—	W14
$E_1^aE_1^f$	1	3.93	—	4.11	4.21	4.33	—	W14
$E_1^fE_1^f$	1	3.87	4.04	—	4.24	4.37	—	W14
C_5 + $E_1^uE_1^u$	3	—	—	4.14	—	4.33	4.48	W14
$E_1^uE_1^u$	1	3.85	4.05	—	—	—	4.40	P10
$E_1^uE_1^u$	1	3.99	—	—	—	—	—	D5
$E_1^aE_1^a$	1	—	—	—	4.20	—	—	D5
$E_1^fE_1^f$	1	3.70	—	—	—	—	—	D5

erties of the enzyme can be shown to differ from those of the bulk of the α_2-globulins (S49). At pH 11, the enzyme migrates as a β-globulin, while at pH 5, it migrates like albumin, and at pH 4.2, like prealbumin (Fig. 4).

Removal of the sialic acid residues of cholinesterase by treatment with bacterial neuraminidase (*N*-acetylneuraminate glycohydrolase, EC 3.2.1.8) changes the electrophoretic mobility of the enzyme significantly, without loss of cholinesterase activity (S47). Thus, at pH 8.6, the native enzyme migrates between α_2- and β-globulin, while the neuraminidase-treated enzyme migrates with the γ-globulins (Fig. 4). Augustinsson (A29) suggested that a large number of sialic acid residues are in a terminal position in the enzyme. Removal of sialic acid from human cholinesterase changes the isoelectric point of the enzyme from *ca.* 3 to 7 (S51); similar findings have been reported for horse serum cholinesterase (S49). Ecobichon and Kalow (E3) have shown that neuraminidase-treated human plasma cholinesterase variants retain both their enzymic activities and their inhibition characteristics with respect to dibucaine and fluoride. The relative electrophoretic migration of cholinesterases from the plasmas of different vertebrates has been determined by column electrophoresis at about pH 8.6 (A22–A25) as well as by paper electrophoresis (G29, P11). Other comparative studies, using starch gel or agar plate electrophoresis, have been reported (e.g., L14, P4, O4, S42).

Saeed *et al.* (S1) studied the action of exopeptidases on the major isoenzyme of human plasma cholinesterase. Using polyacrylamide gel electrophoresis, they found that purified preparations of the major isoen-

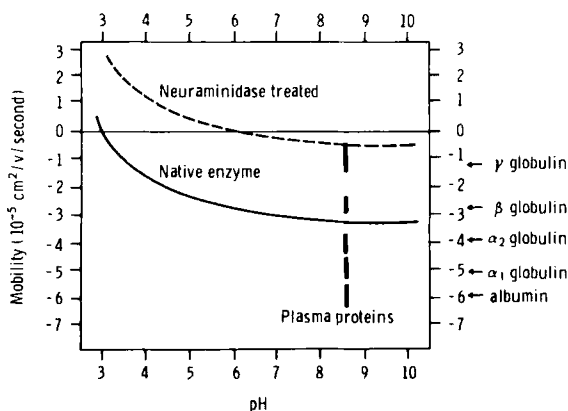


FIG. 4. Electrophoretic mobilities at 1°C as a function of pH for human serum cholinesterase before and after treatment with neuraminidase. The five vertical bars refer to the respective plasma proteins. (After Svensmark, S47.)

zyme, when treated briefly with trypsin, papain, ficin, or plasmin, produced cholinesterase-active bands which had the mobilities of other isoenzymes; the action of α-chymotrypsin was less marked. Somewhat conflicting results were reported by Chiu *et al.* (C7) for the proteolytic digestion of horse serum cholinesterase by trypsin. Polyacrylamide gel electrophoresis of the digest after 2 hours incubation showed a 20% loss of activity of the major isoenzyme, complete disappearance of the second slowest isoenzyme, and 80% loss of activity of each of the other two isoenzymes. Increasing the incubation time with trypsin produced no significant increase in other isoenzymes as the activity of the major band decreased. Such observations are consistent with those of Whittaker and Charlier (W17), who used starch gel electrophoresis in studying the effects of lipases and proteases on the "usual" and "atypical" plasma cholinesterase variants, with longer periods of incubation than those adopted by other workers. It is unfortunate that trypsin was not among the proteases studied, but α-chymotrypsin did cause the complete disappearance of the second slowest isoenzyme and some loss in activity of the major isoenzyme. Without exception, the atypical enzyme was the more vulnerable to degradation. Exopeptidases apparently had no effect on the usual enzyme: This could be explained by the presence of a terminal proline residue—a basic amino acid which is resistant to the action of carboxypeptidase A. More likely, the conformation of the molecule renders the terminus inaccessible to enzymic attack.

Other treatments *in vitro* that have been found to alter the electrophoretic migration of cholinesterase include ultrasonic vibration and the addition of urea. By such means, the major component of the enzyme produces enzymically active bands which have the respective mobilities of each of the other isoenzymes (A29, D25). Dubbs (D25) has suggested that sonication might release enzyme which was previously bound to β-lipoprotein, rather than convert one isoenzyme into another.

The administration of drugs *in vivo* can affect both enzymic activity and isoenzymic mobility. Changes in plasma cholinesterase isoenzymes have been observed in women taking oral contraceptives containing estrogens (W18). Moreover, the observed changes are promptly reversed when the therapy ceases, indicating that hepatic insufficiency is not an underlying mechanism.

4.3.6. *Relative Molecular Mass*

Several authors have measured the relative molecular mass of purified butyrylcholinesterase from horse or human plasma. The earlier findings (Table 12) differ considerably from the more recent results. Inspection of

TABLE 12
ESTIMATES OF THE RELATIVE MOLECULAR MASS OF PURIFIED CHOLINESTERASE FROM DIFFERENT SOURCES

Source	Value (daltons)	Method	Reference
Horse serum	750,000	Kinetics	H24
Pig parotid gland	370,000	Ultracentrifugation: sedimentation equilibrium	T9
Human serum	348,000	Ultracentrifugation: sedimentation equilibrium	H18
Human serum	366,000	Ultracentrifugation: density gradient	D7
Human serum	260,000	Ultracentrifugation: density gradient	L11
Human serum	280,000	Sephadex gel filtration	L4
Horse serum	315,000	Sepharose 6B gel filtration	M7
Horse serum	440,000	Ultracentrifugation: sedimentation equilibrium	L19
Human serum	345,000	Ultracentrifugation: sedimentation equilibrium	M20
Horse serum	317,000	Ultracentrifugation: sedimentation equilibrium	T3
Bacteria, *P. polycolor*	59,000	Sephadex G200 gel filtration	N2
Human serum	371,000	Sepharose 6B gel filtration: acrylamide electrophoresis	P10
Horse serum	326,000	Analytical ultracentrifugation	T6

the data for the horse serum enzyme shows that there is remarkable agreement among different workers. If the high value of 440,000 daltons reported by Lee and Harpst (L20) is replaced by 317,000 daltons—obtained in the same laboratory by Teng *et al.* (T3)—there is very good agreement between the more recent determinations. The same is true for later measurements of the human enzyme which, excluding those of LaMotta *et al.* (L11), lie between 345,000 and 371,000 daltons. Overall, it would appear that horse serum cholinesterase has a slightly smaller relative molecular mass than does the human enzyme. Such a difference is not unexpected for enzyme proteins prepared from different species (N2, T9).

High values for relative molecular masses are indicative of complex protein molecules with multiple subunit structures. Low values, such as 59,000 obtained for the *P. polycolor* enzyme, could be due to disruption of the complex structure during purification, although such an effect would appear to be improbable in this instance.

4.3.7. *Multiple Molecular Forms*

Modern techniques for protein separation have been used to demonstrate the existence of multiple molecular forms of cholinesterase. Such entities, which are groups of isoenzymes, can be separated either by electrophoresis or by chromatography (H8); moreover, they can be distinguished by kinetic means (e.g., R3).

Starch gel electrophoresis (S33) with a discontinuous buffer system (P13) was widely used in the 1960s for investigating the multiple forms of cholinesterase, not only in human plasma but also in the plasmas of many other species (Table 13). Criticism can be directed toward some of this work, in which nonspecific esterase stains were used to locate the enzymes, but such criticism does not apply to the starch gel electrophoresis method of Bernsonn *et al.* (B21) or to the polyacrylamide disc electrophoresis of Juhl (J7), who used butyrylthiocholine as the substrate for staining. The latter is slow-acting, but it has been found that it is unnecessary to stain the white precipitate of copper thiocholine with dithiooxamide. Although polyacrylamide forms a tougher gel and gives superior resolution to starch, staining by any method is slower. Electrophoresis of human plasma cholinesterase on starch usually gives four or five bands, the least mobile having about 80% of the total enzymic activity. The minor bands are probably cleavage products of the major band. When the enzyme is subjected to frequent freezing and thawing, "storage" bands appear. Such bands are not only less mobile than the major component but they are also less significant; little work has been done on them. Two-dimensional electrophoresis, coupling filter paper with starch gel, has been used to resolve the four areas of activity of cholinesterase (H6). Mascall and Evans (M9) obtained good resolution of the enzyme by two-dimensional electrophoresis in density gradient polyacrylamide, and were able to detect electrophoretic differences among plasmas from individuals with rare genetic variants of cholinesterase. This technique, together with one-dimensional electrophoresis in density gradient polyacrylamide disc- (N3) or slab-gels (M9), has real potential for the isolation and subsequent study of the unusual components which apparently characterize the rare genetic variants.

Most workers have adopted the international convention on nomenclature of multiple forms of enzymes (I2), so that the major isoenzymes of cholinesterase are numbered consecutively, with the form having the highest mobility towards the anode being designated one. Unfortunately, there are two sets of symbols for the electrophoretic bands of the enzyme, namely C_1–C_4 (H6) and ChE_1–$ChE_{5,6,7}$ (L8). It turns out that C_1–C_3 are identical to ChE_1–ChE_3, respectively, but C_4 is identified with ChE_4 +

TABLE 13

MULTIPLE MOLECULAR FORMS OF HUMAN PLASMA CHOLINESTERASE

Electrophoresis			Chromatography			Kinetics		Other techniques		
Medium	Component	Reference	Type	Component	Reference	Component	Reference	Type	Component	Reference
Starch	2	D26	Calcium phosphate and Dowex 2	2	M8	2	H37	Ultracentrifugation	5	L11
						2	W28			
Starch	6	B21				2	K12			
Starch	4	H6	Sephadex G200	4	H8	2	H22	Hybridization	4	S14
Starch	6	H28	Sephadex G200	4	G25	3	B22	Isoelectric focusing	3	L10
Starch	5	L8				2	H48	Isoelectric focusing	4	W14
Starch	4	H36	DEAE Sephadex	5	S48					
Starch	5	B37				2	C9			
Polyacrylamide	12	J7				4	M3			
Starch	6	V2				4	T6			
Starch	7	L7								
Polyacrylamide	7	G1								

ChE_5 since ChE_4 was not resolved by the earlier workers. Minor additional bands, ChE_6 and ChE_7, are sometimes found in human plasma (L7). The isoenzymes of human plasma cholinesterase have been shown to be interconvertible during concentration procedures such as ammonium sulfate precipitation and resolubilization, or dialysis against polyvinylpyrollidone (L8), and it was suggested that the various forms of the enzyme were aggregates of a common polypeptide subunit. Subsequent determinations of the relative molecular masses of the isoenzymes (L11) have indicated that these multiple forms are indeed molecular aggregates. This work, together with supporting data from other laboratories, is summarized in Table 14 and provides convincing evidence that the major component of human plasma cholinesterase is a tetrameric aggregate. The smallest functional subunit structure of the enzyme, C_1 (or ChE_1), appears to have a relative molecular mass of *ca.* 83,000 daltons. The other isoenzymes have different values which, although not simple multiples of this smallest subunit, are nevertheless compatible with the concept that the native isoenzymes differ from one another in their states of aggregation. At present, the evidence of LaMotta and Woronick (L10) is the most convincing that interconversions of the isoenzymes of human plasma cholinesterase occur by processes of molecular aggregation and disaggregation. There is, as yet, no compelling evidence that the components of the tetrameric molecule are identical. The relative molecular mass of the subunit of purified horse serum cholinesterase has been determined (Table 15). It can be deduced that the aggregated and dissociated forms of the purified enzyme from horse serum have relative molecular masses of *ca.* 316,000 and *ca.* 80,000 daltons, respectively, and moreover, that the aggregated molecule consists of four subunits with similar masses. It may be tentatively suggested that the subunits are identical, in the light of the additional evidence of Teng *et al.* (T3) who found a single N-terminal glutamate residue.

Main *et al.* (M5) found high concentrations of a butyrylcholine esterase, of relative molecular mass 83,000 daltons, in pooled rabbit serum. This was unexpected, since rabbit serum is classed with those mammalian sera that have low cholinesterase activities (A25, E2). Substrate specificity confirmed that the enzyme was a butyrylcholine esterase. Moreover, the active site concentration of the enzyme was five times that found for pooled horse serum, which is a rich source of the enzyme. The known fact that some rabbits can metabolize atropine, whereas others are unable to do so, can be explained by the presence or absence of serum atropinase, which is a genetic trait. Perhaps the subunit-sized butyrylcholine esterase is characteristic of some rabbits, but absent in others? This seems probable according to Ellis (E9) and Koelle

TABLE 14
ESTIMATES OF THE RELATIVE MOLECULAR MASSES OF HUMAN PLASMA CHOLINESTERASE ISOENZYMES

Electrophoretic band	Value (daltons)	Methods	Reference
C_4	300,000	Ultracentrifugation; density gradient	S46
C_1	81,000	Sephadex gel filtration	L3
C_2C_3	150,000–250,000		
C_4	280,000		
ChE_1	82,000	Ultracentrifugation; density gradient centrifugation	L12
ChE_2	110,000		
ChE_3	170,000		
ChE_4	200,000		
ChE_5	260,000		
C_1	86,000	Sephadex G200 gel filtration	B30
C_4	> 300,000		
C_1	85,000	8 *M* urea electrophoresis and ultracentrifugation: sedimentation equilibrium	M20
C_4	345,000		

(K31), who found that benzoylcholinesterase activity varies widely among the sera of various rabbits. Unfortunately, the relevant genetics were not investigated. The presence of butyrylcholine esterase may also be questioned; it acts—apparently—as a monomer in rabbit sera, but as a tetramer in human and horse sera. Perhaps the apparent absence of the tetramer and other polymers in some rabbits is the result of the inhibition or complete absence of an aggregating enzyme? Does such an enzyme control the interconvertibility of the isoenzymes which are present in other plasma? It is relevant that Main (private communication) isolated from rabbit liver cholinesterase two monomer forms of butyrylcholine esterase, one of which spontaneously formed a tetramer after purification.

TABLE 15
ESTIMATES OF MOLECULAR MASS OF HORSE CHOLINESTERASE SUBUNIT

Value (daltons)	Method	Reference
81,000	Sedimentation equilibrium at high pH	T3
79,000	Sedimentation equilibrium in guanidine HCl	T3
88,500	SDS–polyacrylamide electrophoresis	T3
77,300	SDS–polyacrylamide electrophoresis	M7
80,000	SDS–polyacrylamide electrophoresis	T6

Gaffney (G1) suggested that *N*-acetylneuraminic acid plays an important role in maintaining the stability of the multicomponent system of the human enzyme. Such an hypothesis could be readily verified with the subunit butyrylcholinesterase found in pooled rabbit sera. LaMotta *et al.* (L12) found that the relative molecular masses of neuraminidase-treated human isoenzymes are essentially the same as those of the untreated isoenzymes (see Table 16). This implies that the sialic acid residues which have been split off do not play a significant role in determining the state of molecular aggregation of human isoenzymes.

4.4. Structural and Catalytic Aspects

4.4.1. *Amino Acid Composition*

The amino acid composition of cholinesterase has been determined for the purified enzyme from porcine parotid glands (T9), for equine serum by Main *et al.* (M6) and by Teng *et al.* (T3), and for human serum by Muensch *et al.* (M20). The analytical results are given in Table 17, together with those of Leuzinger and Baker (L28) for electric eel organ acetylcholinesterase. For ease of comparison, the available data have been recalculated, where necessary, and expressed as moles of each amino acid per 1000 amino acid residues. In all cases, the values for threonine and serine were extrapolated to zero-time hydrolysis, and half-cystine was calculated from the cysteic acid content. No sulfydryl group was detected (T3) when cholinesterase in guanidine hydrochloride solution was titrated with 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman's reagent). Thus, all six cysteic acids appear to be derived from disulfide linkages, confirming earlier conclusions of Lee and Harpst (L20), from viscosity

TABLE 16
Estimates of the Relative Molecular Mass of Human Serum Isoenzymes before and after Treatment with Neuraminidase[a]

Isoenzyme	Untreated enzyme: mean value (daltons)	Neuraminidase-treated enzyme: mean value (daltons)
C-1	82,000	81,000
C-2	110,000	130,000
C-3	170,000	160,000
C-4	200,000	220,000
C-5	260,000	260,000

[a] From LaMotta and Woronick (L10).

TABLE 17
AMINO ACID COMPOSITIONS OF CHOLINESTERASES FROM DIFFERENT SOURCES

	Number of residues (moles/1000 amino acid residues) in purified enzyme				
Amino acid	Human serum[a]	Horse serum[b]	Horse serum[c]	Porcine parotid gland[d]	Electric eel organ[e]
Alanine	76	56	59	101	55
Arginine	54	53	51	44	54
Aspartic acid	96	112	107	115	108
Glutamic acid	96	114	107	114	94
Glycine	101	75	75	105	78
Histidine	18	9	15	19	23
Isoleucine	39	37	37	23	37
Leucine	74	92	85	76	90
Lysine	78	60	64	53	40
Methionine	11	17	18	10	30
Phenylalanine	48	64	55	51	53
Proline	45	64	53	76	81
Serine	67	62	71	89	69
Threonine	51	56	67	53	56
Tryptophan	34	26	27	Not stated	21
Tyrosine	51	36	34	21	39
Valine	48	55	58	40	71
Half-cystine	14	10	16	9	11

[a] Data of Muensch *et al.* (M20).
[b] Data of Teng *et al.* (T3).
[c] Data of Main *et al.* (M6).
[d] Data of Tucci and Seifter (T9).
[e] Data of Leuzinger and Baker (L28).

measurements, that the protein in guanidine hydrochloride solution contains disulfide bridges.

As expected, there are species variations in amino acid composition. For horse serum cholinesterase, the three sets of results (from two independent laboratories) are in very satisfactory agreement, with estimates for only four of the amino acids differing substantially. In all five sets, aspartic and glutamic acids occur in nearly equal amounts, and together account for about 20% of the amino acid content of the molecule. In a subunit of molecular mass 80,000 daltons, there would be about 540 amino acid residues, which would indicate that 100 to 110 of these would be either aspartic acid or glutamic acid. At the other extreme, histidine, methionine, and half-cystine together represent only 6% of the amino acids.

The data given in Table 17 do not permit a dogmatic statement to be

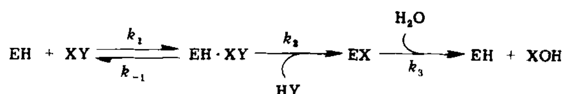
made about differences between acetyl- and butyrylcholinesterase. Acetylcholinesterase is rich in valine and methionine, and low in lysine compared with butyrylcholinesterase. Porcine parotid butyrylcholinesterase is rich in alanine, but poor in isoleucine compared with the other cholinesterases. Human serum cholinesterase is rich in tyrosine, which is low in the porcine parotid enzyme. There are other differences, e.g., in respect to glycine and proline, but additional evidence is required before these indications can be interpreted fully. Further studies of variants in a given species are likely to add to present confusion.

The carbohydrate content of the molecule appears to be *ca.* 20%–17.4% from the data of Main *et al.* (M6); 20% from Teng *et al.* (T3), and 24.5% from Muensch *et al.* (M20).

4.4.2. *Mechanism of Catalytic Action*

The three-dimensional structure of cholinesterase is not known. Until this information becomes available, it is necessary to deduce the mechanism of catalytic action from kinetic studies of substrate catalysis, by chemical probes of the active site, and by analogies drawn from chymotrypsin, which has become a model for serine hydrolases. The usefulness of this model has been demonstrated by X-ray crystallographic studies which have shown striking similarities in the three-dimensional structures of the three serine hydrolases, chymotrypsin, trypsin, and elastase. A dominant feature of these enzymes is the "charge-relay" system of hydrogen bonds formed by serine hydroxyl at the esteratic active site, together with a histidine imidazole ring and a carboxylate side chain in linear array (B25). It is significant that all of the experimental evidence indicates that both a serine hydroxyl and an imidazole ring of histidine are active components in the catalytic mechanism of cholinesterase at the esteratic site, but so far, evidence for a carboxylate side chain is not convincing.

Cholinesterase is now considered to react with substrates and competitive inhibitors by the initial formation of an enzyme affinity complex which enables the serine hydroxyl group at the esteratic site to become acylated. The acylated group can then react with any nucleophilic reagent to regenerate the free enzyme. The overall scheme is as follows:



where EH represents cholinesterase, with the H belonging to the hydroxyl group of the serine residue present at the esteratic active site, and XY is the substrate or competitive inhibitor. The rate constants are k_1,

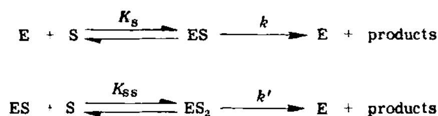
k_{-1}, k_2, and k_3; it is generally assumed that $k_{-1} \gg k_2$. The rate constant k_2 controls acylation, and k_3 controls deacylation. When XY is a substrate, the dissociation constant governing the formation of EH-XY is conventionally K_s, whereas when XY is an inhibitor, it is usually termed K_I or K_a. The rate-determining step is considered to be the formation of the acylated enzyme; deacylation is very fast, making it difficult to detect the acyl enzyme by direct observation. Experimental evidence in favor of this hypothesis will have to await technical developments in the detection of very short-lived intermediates.

It has been shown that both the substrate acetylcholine and the inhibitor tetramethylammonium chloride serve as activators of horse plasma cholinesterase (B35), but the tetramethylammonium ion does not have any activating effect on the phosphorylation of the enzyme. Brestkin and Brick (B35) concluded that the activation process is associated with the deacylation step and not with the acylation stage.

4.4.3. *Velocity and Substrate Concentration Relationships*

The kinetics of cholinesterase catalysis, as with other enzymes, is usually studied under such conditions that the initial substrate concentration, S_0, is much greater than the initial enzyme concentration, E_0, so that S remains effectively constant over the measured reaction (H37). Conditions are also adjusted so that the progress curves are linear over the early stages of the reaction. The slope of the linear progress curve so obtained gives the initial velocity, v, for a given value of S.

The plot of v against S should give a rectangular hyperbola according to conventional Michaelis–Menton kinetics. In fact, such curves are not obtained when the substrates are acetylcholine or other choline esters (B22, H17, H22, W28). A typical plot of v against v/S for butyrylcholine is shown in Fig. 5, from which it can be seen that there is an apparent activation in the presence of excess substrate. Hastings (H17) considered that the increasing velocity at higher substrate concentrations could be due to an excess of substrate binding with the enzyme–substrate complex as follows:



$$E + S \underset{}{\overset{K_S}{\rightleftharpoons}} ES \xrightarrow{k} E + \text{products}$$

$$ES + S \underset{}{\overset{K_{SS}}{\rightleftharpoons}} ES_2 \xrightarrow{k'} E + \text{products}$$

From this scheme, the rate equation can be deduced by assuming that the rates of the product-forming reactions are very much less than the rates of dissociation of the enzyme–substrate complexes, ES and ES_2, which reform the substrate.

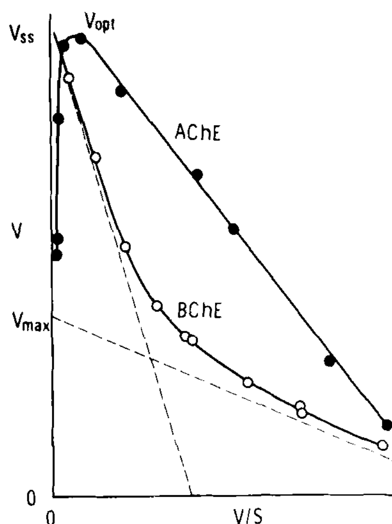


FIG. 5. Michaelis-Menton plot of v against v/S (V against V/S in figure) for acetylcholinesterase (AChE) and cholinesterase (BChE) using acetylcholine as substrate. (After Main, M3.)

Hence:

$$v = \frac{VK_sS}{1 + K_sS + K_sK_{ss}S^2} + \frac{V_{ss}K_sK_{ss}S^2}{1 + K_sS + K_sK_{ss}S^2}$$

$$= \frac{(V/K_{ss}S) + V_{ss}}{1 + (K_{ss}S)^{-1} + (K_sK_{ss}S^2)^{-1}}$$

where $V = kE_0$ and $V_{ss} = k'E_0$. By this analysis, the values of the Michaelis constant, K_m, for butyrylcholinesterase that were previously determined using high substrate concentrations are really K_{ss} values. Hastings (H17) determined K_m, K_{ss}, V, and V_{ss} by best-fit methods and found $K_m = 5 \times 10^{-4}\ M$ and $K_{ss} = 1.1 \times 10^{-3}\ M$ for horse serum cholinesterase with acetylcholine as substrate. He found that the K_m for butyrylcholine as substrate was $5 \times 10^{-4}\ M$ and $K_{ss} = 7.7 \times 10^{-4}\ M$. A similar analysis has been made by Augustinsson *et al.* (A30).

Activation at high substrate concentrations not only explains the failure of butyrylcholinesterase to follow simple Michaelis-Menton kinetics, but also explains the enigma of substrate inhibition of the enzyme using either benzoylcholine (A21, T7) or acetyl- or butyryl-salicylcholine as substrates. The proposal made by Hastings is analogous to that of Myers (M24, M25) for the inhibition of acetylcholinesterase by excess substrate, in this case acetylcholine.

4.4.4. *Effect of pH on Enzymic Activity*

Hase (H16) studied the effect of pH on the hydrolysis of acetylcholine by horse serum cholinesterase, and his results have been reanalyzed by Laidler (L5) and extensively discussed by Dixon and Webb (D21). The relationship between pH and the rate of hydrolysis of acetylcholine has been used to obtain information on the structure of the active site of the enzyme (B19, W28). Acetylcholine is a particularly suitable substrate for these studies since it does not change its charge in the pH range studied. Similar pH–activity curves have been obtained using other substrates for cholinesterase (H23, S20, P19). Moreover the pH dependence of enzymic activity varies with the buffer system (K3). By investigating the effect of pH and sodium chloride concentration on the rate of hydrolysis of benzoylcholine by human plasma cholinesterase, Kalow (K6) deduced that for this substrate, each enzyme molecule contains at least two binding sites which differ in their dependence on pH. Michaelis constants and maximum hydrolysis velocities were measured for each of the two binding sites, and p*K* values of the enzyme–substrate complexes were found to be 5.2, 6.7, and 9.2 for one site, and 5.2, 7.0, 8.4, and 8.8 for the other.

4.4.5. *The Active Sites*

The basis of our current understanding of the two types of active site which are present in cholinesterase has been provided by Wilson and Bergmann (W30), who substantiated the concept of anionic and esteratic sites introduced by Zeller and Bisegger (Z2). Although it is now generally accepted that the hydrolysis of the ester bond of the substrate occurs at the esteratic site, there is still some uncertainty regarding the existence of the anionic site in butyrylcholinesterase; an alternative site has been proposed by Augustinsson (A28).

The discovery that organophosphates such as diisopropyl fluorophosphate (DFP) inhibit cholinesterase by irreversible phosphorylation of a basic group at the esteratic site led to the use of [^{32}P]DFP to ascertain the chemical nature of the DFP-binding site. Jansz *et al.* (J2) found that the structure of the ^{32}P peptide of horse serum cholinesterase was Phe-Glu-Ser-Ala-Gly-Ala-Ala-Ser: This indicated the serine hydroxyl as the

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     |
     P
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designated basic group at the esteratic site. However, data on the pH-dependence of the enzymic hydrolysis indicated that the basic group showed a p*K* of about 7 (Table 18) which was inconsistent with serine, but approximated to the p*K* of the imidazole ring of a histidine residue (C11). Although the evidence for the presence of histidine at the esteratic

TABLE 18

DISSOCIATION CONSTANTS OF THE ACTIVE SITES OF CHOLINESTERASE

Cholinesterase	p*K*	Substrate or Inhibitor	Reference
Horse plasma	6.2, 7.7	Acetylcholine	H16
Human plasma, fraction IV–6–3	6–7, 9–10	Acetylcholine	W28
Human serum	6.2, 9.0	*n*-Propyl chloroacetate	B20
Human serum	6.4	Tetraethylammonium bromide	B20
Human serum	5.9, > 9	Acetylcholine	S20
Human plasma	6.6	Diisopropyl fluorophosphate	M19
Human plasma, fraction IV–6–3	6.7	β-Methylacetylcholine and β-methylacetylthiocholine	H23
Cholase	5.2, 6.7, 9.2	Benzoylcholine	K6
Horse serum	5.6	*o*-Nitrophenyl benzoate	W2
Worthington (CHE9KD6)	6.2, 10.1	Methanesulfonyl fluoride	P6
Horse serum	7.5, 9.5	Diethyl 4-nitrophenyl phosphate (Paraoxon) and 1-chloro-1-ethylcarbamoyl-1-propen-2-yl-dimethyl phosphate (Phosphamidon)	W4

site is indirect, it is accepted that serine and histidine are the basic groups at this site in all cholinesterases.

Pavlic (P7) measured the dissociation constants of the esteratic center of butyrylcholinesterase by sulfonylation of the enzyme with methanesulfonyl fluoride. This reaction has been shown to be a simple, irreversible, first-order reaction with respect to the substrate; furthermore, sulfonylation occurs at the esteratic site of the enzyme (P6). The plot of the pH-dependence of the rate of sulfonylation was found to be bell shaped, and Pavlic interpreted this in terms of the dissociation of two ionizable groups at the esteratic center. The dissociation constants of these two groups ($pK_1 = 6.2$ and $pK_2 = 10.1$) are indicative of histidine and tyrosine at the esteratic site of the enzyme. These results, together with the evidence of earlier studies, are summarized in Table 18. It is apparent that the pK_a of the basic group in all investigations is about 6 to 7. The only group known to exist in proteins with a pK_a in this range is the imidazole ring in histidine (C13). Moreover, the heat of ionization of 6.5 kcal/mol (S20) is in good agreement with that for the imidazole ring (7 kcal/mol). But discrepancies do arise; the analysis of pH–activity curves depends upon the mathematical approximations made, and the dependence of the enzymic activity on pH varies with the substrate even when the substrate is uncharged and therefore, should not affect the observed ionization constants in the enzyme (B20). These discrepancies may be explained by the presence of polar groups in the substrate which affect the dissociation constants of the enzyme. It is therefore essential that kinetic analyses should not be regarded as conclusive, but merely indicative of possible ionizable groups. The electron spin-resonance labels developed by Hsia *et al.* (H46), which parallel the action of organophosphate inhibitors, offer profitable approaches for studying the nature of the active site under catalytic conditions. The labels differ from DFP in that one or more of the ester linkages is an organic nitroxide molecule. Striking differences have been obtained between the spin-label spectrum of horse serum cholinesterase and that of α-chymotrypsin, the conventional model for this type of hydrolase. It is suggested that the active site of cholinesterase is less confined and, in contrast to α-chymotrypsin, probably on or near the surface of the enzyme.

It has already been mentioned that there are some doubts (A26) about the existence of an anionic site in human or horse cholinesterase. Comparative kinetic studies using a series of pyridylcarbinol acetates as substrates have shown that acetylcholinesterase from *T. marmorata* electric organ and the plasma cholinesterases from horse and man have similar esteratic sites. It was also shown that the electric eel organ enzyme has an anionic site, whereas the second site of butyrylcholine

esterase is believed to be nonionic and to use van der Waals rather than coulombic forces to hold the substrate (A28). Preliminary nuclear magnetic resonance studies of the interaction between acetylcholine and horse serum cholinesterase have not yet yielded clear-cut results, but there is some evidence that the quaternary ammonium group acts as an initial anchor of the molecule at the anionic site, followed by a more pronounced interaction between the acetate group of acetylcholine and the esteratic site of the enzyme (K17). Augustinsson's hypothesis gained support from binding energy studies of Purcell and Beasley (P18) which suggested the presence in horse serum cholinesterase of a specific hydrophobic binding site with definite size limitations.

Millner *et al.* (M14) prepared pyridostigmine analogs to probe the active sites of acetylcholinesterase and butyrylcholinesterase. They found that an increase in the negative character of the pyridine nitrogen affects both enzyme systems to the same extent, which is contrary to the hypothesis of Augustinsson (A28). However, Millner *et al.* did find some differences in the active sites of the two enzymes. Dimethylcarbamylated butyrylcholinesterase did not reactivate in aqueous solution as readily as did dimethylcarbamylated acetylcholinesterase; furthermore the esteratic site of butyrylcholinesterase could accommodate larger groups than could acetylcholinesterase.

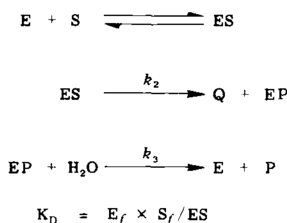
Comprehensive reviews (K1, U1) of the active sites of cholinesterase both postulated the presence not only of an esteratic site for butyrylcholinesterase but also of an anionic site. Additionally, in the region of the anionic site, there are two hydrophobic areas, one directly surrounding the anionic group and the second located at some distance from it (K1). The presence of hydrophobic areas has been established (B32, C3, H29, H45, M10) by the use of fluorescent probes with spectral responses which reflect the environment of the probe. Such probes can be used to monitor changes in the conformations of enzymes and can be designed to be active-site-directed, competitive inhibitors (H30). Aspects of the spectroscopy of intrinsic and extrinsic fluorescent probes have been reported (C3).

The hydrophobic area surrounding the anionic site plays a more important role for butyrylcholinesterase than for acetylcholinesterase. The greater importance of this hydrophobic area for butyrylcholinesterase could help to explain and resolve some of the opposing views of earlier workers (A26). Kabachnik *et al.* (K1) also proposed that in the vicinity of the esteratic site of butyrylcholinesterase there are two hydrophobic areas separated by a hydrophilic group. Differences in length and structure of the hydrophobic areas of the active surfaces of butyryl- and

acetylcholinesterase may be responsible for the different properties of the two groups of enzymes.

Thus, it is probable that the active site of cholinesterase comprises anionic and esteratic subsites and also, as with most proteins, hydrophobic areas. The anionic site determines specificity with respect to the choline moiety, while the actual catalytic process takes place at the esteratic site (F8).

DFP titrations of purified serum cholinesterase have indicated two active sites for the horse enzyme (M7) and two active sites for the C_4 component of the human enzyme (M20). In other studies using this kind of technique, human serum cholinesterase has been found to have two or three active sites (Y3), and purified horse serum, three or four (M6, T6). A slightly different approach (R9) was adopted by La Du (L34), who titrated the number of active sites of purified "usual" and "atypical" human serum enzymes using a carbamyl ester [*N*-methyl-(7-dimethylcarbamoxy)- quinolinium iodide], which is not fluorescent itself but gives a highly fluorescent hydrolysis product. The reaction of the probe (S) with cholinesterase (E) is as follows:



$$E + S \rightleftharpoons ES$$

$$ES \xrightarrow{k_2} Q + EP$$

$$EP + H_2O \xrightarrow{k_3} E + P$$

$$K_D = E_f \times S_f / ES$$

The subscript *f* refers to the concentrations of the free reactants. Q represents the fluorescent *N*-methyl-7-hydroxyquinolinium ion which is released when the enzyme is carbamylated, forming EP. The values for K_D, k_2, and k_3 were determined for the usual and atypical cholinesterases (L34). It was found that the kinetic constant k_2 was the same for both enzymes (k_2 = 5.0 minute^{-1}). This was also true for the kinetic constant k_3 (k_3 = 0.15 ± 0.05 hour^{-1}). On the other hand, the value of the dissociation constant K_D was found to be 0.16 ± 0.01 mmol/liter for usual cholinesterase, and 5.4 ± 0.1 mmol/liter for atypical cholinesterase.

Assuming that both enzymes had a relative molecular mass of 340,000, and a V_{max} of 188 μmol of benzoylcholine hydrolyzed per minute per milligram of enzyme protein (at pH 7.4 and 25°C), it was found that there were four active sites per enzyme molecule for both the usual and atypical forms of the enzyme. Similar results were obtained when protein

concentrations were determined from the absorbance at 280 nm. However, in the latter calculation, a value for the molar absorbance at 280 nm of 18 cm^{-1} was used instead of the reported value of 14.5 (H21) for human serum cholinesterase at a concentration of 10 g of protein per liter.

Many studies have shown that the atypical enzyme has properties which are distinct from those of the usual enzyme, and that these differences must be based upon differences in molecular structure. Direct physical evidence that atypical cholinesterase has a composition different from that of usual cholinesterase was obtained by Altland *et al.*, (A14) and by Muensch *et al.* (M21). Cholinesterases which had been purified from an $E_1^uE_1^u$ individual and from an $E_1^aE_1^s$ individual were labeled (A14) with [^{32}P]diisopropylfluorophosphate ([^{32}P]DFP), hydrolyzed with trypsin, and subjected to high voltage electrophoresis on silica gel at pH 3.5. The radioactivity in the hydrolysate from the usual enzyme migrated in several bands toward the anode, whereas the radioactive peptides from the $E_1^aE_1^s$ individual migrated in several bands toward the cathode. Muensch *et al.* (M21), using tryptic digests of [^{14}C]DFP-labeled cholinesterases prepared from the plasmas of several different genotypes, found that the labeled peptides obtained from $E_1^uE_1^u$-$E_2^-E_2^-$ enzyme and from $E_1^uE_1^u$-$E_2^+E_2^+$ enzyme migrated only toward the anode, whereas the labeled peptides from $E_1^aE_1^a$-$E_1^aE_1^s$ enzymes migrated only toward the cathode (Fig. 6). As expected, labeled peptides from $E_1^uE_1^a$ enzyme migrated in both directions, because the plasma of these heterozygotes contains both the usual and atypical molecular forms of cholinesterase. These findings are consistent with the hypothesis that the active sites of the two enzyme forms have different amino acid sequences or compositions, and that the atypical enzyme is less negatively charged in the vicinity of the active site. This is also consistent with the report of Das (D3) that the atypical enzyme has an isoelectric point (Section 4.3.4) of 4.20, while the usual enzyme has an isoelectric point of 3.99. The latter value agrees with the reports of LaMotta *et al.* (L11) that the isoelectric points of five of the multiple molecular forms of usual cholinesterase lie between pH 3.9 and 4.1.

4.4.6. *Turnover Number*

The turnover number reflects the number of moles of substrate hydrolyzed per minute per mole of occupied active site: The available data are given in Table 19. Detailed information on turnover numbers was derived from the fluorescent probe studies of Lockridge and La Du (L34) outlined in the previous section.

The turnover numbers for benzoylcholine at pH 7.4 (0.067 mol/liter

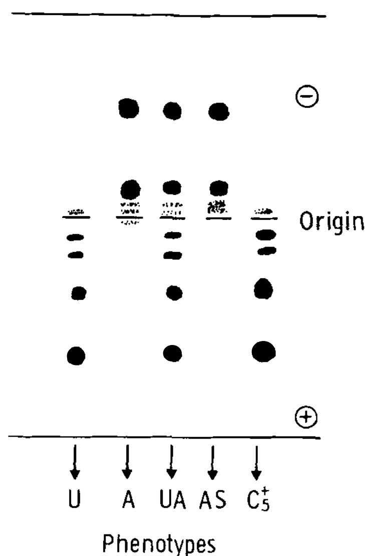


FIG. 6. Autoradiogram of diisopropyl fluorophosphate-labeled tryptic peptides of normal and variant plasma cholinesterases: U, enzyme from plasma of genotype $E_1^uE_1^u$; A, enzyme from plasma of genotype $E_1^aE_1^a$; UA, enzyme from plasma of genotype $E_1^uE_1^a$; AS, enzyme from plasma of genotype $E_1^aE_1^s$; and C_5^+, enzyme from C_5^+ positive plasma with usual kinetic properties. Electrophoresis was carried out in silica gel sheets with pyridine–acetate buffer (10% acetic acid adjusted to pH 3.5 with pyridine) at 40 V/cm for 3 hours. (After Muensch *et al.*, M21.)

TABLE 19
ESTIMATES OF THE TURNOVER NUMBERS, FOR DIFFERENT SUBSTRATES, OF HORSE AND HUMAN SERUM CHOLINESTERASES

Cholinesterase	Substrate	Turnover number (minute^{-1})	Reference
Horse serum	Sarin ^{32}P (inhibition)	84,000	J3
		89,400	E1
Horse serum	Butyrylcholine	171,000	M3
	Acetylthiocholine	61,000	M3
Human serum (usual)	*N*-methyl-(7-dimethylcarbamoxy) quinolinium iodide	0.0025	L34
	Benzoylcholine chloride	15,000	L34
	o-Nitrophenyl butyrate	48,000	L34
Human serum (atypical)	*N*-methyl-(7-dimethylcarbamoxy) quinolinium iodide	0.0025	L34
	Benzoylcholine chloride	15,000	L34
	o-Nitrophenyl butyrate	48,000	L34

phosphate buffer) and 25°C were found to be the same for the usual and atypical cholinesterases. The mean value was 14,700 ± 300 (±SE) mol benzoylcholine hydrolyzed per minute per mole of *N*-methyl-(7-dimethylcarbamoxy)-quinolinium ion consumed in the active site titration. The key assumptions in the calculations were that only cholinesterase active sites react with the quinolinium compound and that each active site reacts with only one molecule. It was determined that both enzymes had turnover numbers of 0.0025 minute^{-1} for *N*-methyl-(7-dimethylcarbamoxy)-quinolinium iodide, and 48,000 minute^{-1} for *o*-nitrophenylbutyrate at 25°C and pH 7.4.

When Michaelis constants for several substrates were estimated, it was found that the two substrates with positively charged quaternary nitrogen groups had different K_m values for usual and atypical cholinesterases. The K_m for *N*-methyl-(7-dimethylcarbamoxy)-quinolinium iodide was 0.08×10^{-6} mol/liter for the usual enzyme, and 2×10^{-6} mol/liter for the atypical enzyme—a 25-fold difference.

The K_m values for benzoylcholine were found to be 5×10^{-6} mol/liter for the usual enzyme, and 24×10^{-6} mol/liter for the homozygous atypical enzyme—a 4.8-fold difference. The K_m value for the uncharged ester *o*-nitrophenylbutyrate was found to be 1.4×10^{-4} mol/liter for both enzymes. This is not in agreement with the report of McComb *et al.* (M2), who found that the K_m for this substrate was about twice as great in the case of the atypical enzyme.

In summary, Lockridge and La Du (L34) have determined by direct methods that the usual and atypical cholinesterases have the same turnover numbers for a given substrate. This holds true whether a substrate has a positive charge or is uncharged. With *o*-nitrophenylbutyrate, an uncharged substrate, both purified enzymes have the same Michaelis constants. With positively charged substrates, the usual enzyme has greater affinity for the charged substrates, as compared with the atypical enzyme. [The latter observation has previously been made by others (H27, K5, K10), using serum from usual and atypical individuals.] With certain assumptions, the conclusion was reached that both cholinesterases have four active sites per molecule. It was also concluded that the esteratic site in the usual and atypical cholinesterases is the same, but that the anionic sites differ (K10, L34) and that a negatively charged amino acid at the anionic site is replaced by a neutral amino acid in the case of atypical cholinesterase (K10, L34).

4.5. Inhibition and Activation

For mechanistic purposes, inhibitors (I) of cholinesterase can be regarded as poor substrates (S) which react with the enzyme (E), forming

enzyme–inhibitor complexes (EI), which influence the reaction velocity by modification of the enzyme. The point of attachment of the inhibitor can be at the catalytic active site or at an allosteric site (distinct, and often remote, from either the anionic or esteratic active site), or at a hydrophobic area. Inhibitors which compete with the substrate for attachment at the catalytic active site are competitive; such inhibition is recognized by Lineweaver-Burk linear plots of reciprocal velocity ($1/v$) against reciprocal substrate concentration ($1/s$) for each specified concentration of inhibitor. If the lines given by each inhibitor concentration intersect at a same point on the $1/v$ axis, then the inhibition is competitive; intersection of the lines elsewhere indicates noncompetitive inhibition. Noncompetitive inhibitors are regulators of cholinesterase activity. Detailed presentations of the basic features and kinetics of the different types of enzyme inhibition can be found in standard textbooks.

Inhibitors of cholinesterase are traditionally considered as acting reversibly or irreversibly, and the two classes are distinguished by the type of bond which they form with the enzyme. Reversible inhibitors form only noncovalent bonds. Irreversible inhibitors are held by covalent as well as by noncovalent binding at the active site, and it is the covalent bonding which causes irreversibility. Noncovalent binding can be due to hydrogen bonds, to ionic forces resulting from ionic attraction, to ion–dipole couplets formed in hydration processes, or to van der Waals forces.

4.5.1. *Reversible Inhibition and Its Kinetics*

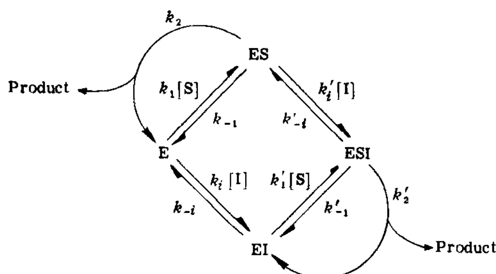
The activity of cholinesterase which has been inhibited by reversible inhibitors can be fully restored by dialysis. Many reversible inhibitors contain a quaternary ammonium group, and the positively charged nitrogen atom of such compounds is attracted to the negatively charged anionic site of the enzyme. There, they are bound by coulombic forces which may be augmented by van der Waals attraction of the alkyl groups to hydrophobic areas near the anionic site. This kind of inhibition, presumably, is due in part to occupation of the anionic site. While such a concept is appropriate to quaternary ammonium compounds, it cannot be extended to other types of reversible inhibitors. Thus, reversible inhibition by fluoride ion (H12, H15, H26) follows complex kinetics whose mechanism has yet to be resolved. Furthermore, there is some limitation on the chemical nature of the groups attached to the positively charged nitrogen atom. Primary and secondary amines are not good inhibitors of plasma cholinesterase, and the positive nitrogen atom must be either a protonated tertiary amine or a fully substituted quaternary ammonium group. Being strong bases, quaternary ammonium groups are charged at

all values of pH, in contrast to tertiary nitrogen groups, whose charge is pH-dependent.

The quaternary ammonium compound Tris [tris-(hydroxymethyl)-aminomethane] has been found to be a competitive inhibitor of horse plasma cholinesterase using butyrylcholine as substrate (P5), with inhibition occurring at concentrations commonly used in buffer solutions. Tris is believed to compete for the esteratic site but, in the absence of Mg^{2+} and Ca^{2+}, the enzyme was activated. The exact role of the cations was not investigated, but a complex of the cation with an amino group could be the effective inhibitor. Similar results were obtained using acetylcholinesterase from *T. marmorata*.

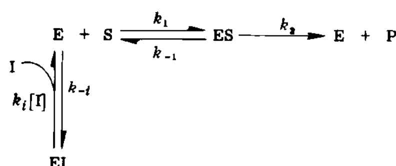
Many drugs of therapeutic importance, e.g., hexafluorenium, pancuronium (S9, S10), and succinylcholine itself, are bis quaternary ammonium complexes which are reversible inhibitors of cholinesterase. One of the charged groups occupies the anionic site, but there is uncertainty regarding the attachment of the second quaternary nitrogen group. Experimental evidence shows that the bis quaternary compounds are more than twice as potent (mole for mole) as inhibitors of cholinesterase than are simple quaternary ammonium compounds (M4).

A general inhibition mechanism that describes the interaction of substrate (S), and a reversible inhibitor (I), with the enzyme (E), is as follows:



In the absence of inhibitor, only the enzyme and enzyme substrate complex are present, whereas with inhibitor, two new complexes, EI and ESI, are formed. If all of the reactions operate in the above scheme, it leads to second-order kinetics (W33).

Main (M4), in an excellent review article on cholinesterase inhibitors, discussed the additional complication of substrate inhibition in the above general mechanism. However, in many inhibition studies Michaelis–Menton kinetics are obeyed quite closely. One simple reaction scheme which leads to such kinetics is as follows:



The rate equation deduced for this, as well as for other similar mechanisms (W33), has the form of a modified Michaelis–Menton equation; for the example given above, the rate equation is

$$v = \frac{SV_s}{S + K_m\,(1 + I/K_i)}$$

where the Michaelis constant $K_m = (k_{-1} + k_2)/k_1$; I is concentration of inhibitor; S is the concentration of substrate; E_0 is initial concentration of enzyme; and E is concentration of enzyme actually present; $V_s = k_2(E_0)$ and $K_I = k_i/k_{-i}$.

4.5.2. *Irreversible Inhibition and Its Kinetics*

Irreversible inhibitors are effectively esteratic site inhibitors which, like true substrates, react with the hydroxyl group of serine at the catalytic active site. Such inhibitors, sometimes referred to as acid-transferring inhibitors, include the organophosphates, the organosulfonates, and the carbamates. All form acyl-enzyme complexes which, unlike substrate–enzyme intermediates, are relatively stable to hydrolysis. Indeed, the phosphorylated enzyme intermediates have half-lives from a few hours to several days (A12), whereas the sulfonated or carbamylated enzyme complexes have much shorter half-lives—several minutes to a few hours. Several strong lines of direct evidence point to the formation of an acyl complex—the isolation of phosphorylated serine from hydrolysates of horse cholinesterase (J2), complex formation and carbamylation (O2), and the sulfonation of butyrylcholinesterase by methanesulfonyl fluoride in the presence of tubocurarine and eserine (P6).

The commercial use of some organophosphates and carbamates as systemic insecticides has resulted in the synthesis of a large number of these anticholinesterases. Structural formulae and some data for these inhibitors are given in the reviews of Usdin (U1), Aldridge and Reiner (A12), and Main (M4). Compounds which structurally resemble substrates are usually very good inhibitors of plasma cholinesterase. Thus, organophosphorus compounds modeled on acetylcholine are powerful inhibitors of the enzyme (Table 20). Quaternary aminophenylphosphates

TABLE 20

Inhibition Characteristics of Acetylcholinesterase and Cholinesterase for Various Organophosphates[a]

Inhibitor (PX)	Bovine erythrocyte acetylcholinesterase (AChE) k_2(minute^{-1})	$K_a(M)$	$k_i(M^{-1}$ minute$^{-1})$	°C	Horse serum cholinesterase (BuChE) k_2(minute^{-1})	$K_a(M)$	$k_i(M^{-1}$ minute$^{-1})$	°C	k_i BuChE / k_i AChE	Reference
Sarin	—	—	6.9×10^7	25	—	—	1.3×10^7	25	0.19	A20
Tabun	—	—	1.7×10^7	25	—	—	1.3×10^7	25	0.76	A20
O-Ethyl-S-ethyl methyl thiophosphonate	—	—	2.2×10^2	—	—	—	6.4×10^2	—	0.29	R10
O-Hexyl-S-ethyl methyl thiophosphonate	—	—	1.6×10^4	—	—	—	3.8×10^4	—	2.4	R10
Diethylphosphorochloridate	—	—	1.2×10^6	25	—	—	7.0×10^6	25	5.8	A18
Diethylphosphorofluoridate	—	—	1.0×10^5	25	—	—	6.6×10^6	25	6.6	A18
O-Isopropyl S-(diisopropylamino-ethyl)methyl thiophosphate	2.8	1.36×10^{-6}	2.1×10^6	20	0.22	4.0×10^{-8}	5.6×10^6	20	2.7	P3
Diisopropyl fluorophosphate	41	1.2×10^{-3}	3.4×10^4	25	145	2.6×10^{-5}	5.5×10^6	25	160	M4
Diisopropyl fluorophosphate	12	1.6×10^{-3}	7.4×10^3	5	11	9.9×10^{-6}	1.1×10^6	5	150	M4
5-(2-Diethylaminoethyl) diethyl thiophosphonate	160	2.8×10^{-4}	5.6×10^5	5	49	7.6×10^{-6}	6.5×10^6	5	12	M4
Maloxan	67	2.7×10^{-4}	2.4×10^5	5	6.6	6.2×10^{-4}	1.1×10^4	25	—	M4

[a] K_a, affinity constant; k_2, phosphorylation constant; k_i, bimolecular constant for the reaction $E + PX \overset{K_a}{\rightleftharpoons} EP$; $k_i = k_2/K_a$.

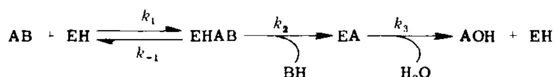
and quaternary quinolylphosphates are more potent inhibitors of cholinesterase than are the corresponding tertiary compounds (H31, H32, W28), and it has also been shown (H20) that the replacement of a quaternary nitrogen by a sulfonium ion results in a powerful inhibitor. The major requirement for potent inhibition appears to lie with the structural orientation of the substituent groups rather than with the positive charge itself. Some inhibition characteristics of various organophosphates for bovine erythrocyte acetylcholinesterase and for horse plasma cholinesterase are given in Table 21.

The effectiveness of a given inhibitor of the enzyme, as with substrate specificity, varies with the source of cholinesterase. I_{50} values, defined as the molar concentration of inhibitor giving 50% enzymic activity, are often used to compare the potency of inhibitors. By no means, however, do all investigators specify the experimental conditions under which their measurements were made. It is therefore advisable to use I_{50} values only as crude indices of inhibitor potency. Differences between the reactivities of horse serum isoenzymes with organophosphates have been reported (C6, R3). Inhibition of horse serum cholinesterase is stereospecific (B28), and thus the enzyme reacts with optically pure (−)-sarin at least 4000 times faster than with (+)-sarin.

Several organophosphates and carbamates do not inhibit cholinesterase. A classical example is the insecticide parathion—a thionophosphate—which will inhibit cholinesterase, *in vivo* or *in vitro*, only after oxidation to the corresponding oxo derivative, paraoxon. The thionophosphates in general do not inhibit the enzyme.

There are a number of reports of impurities being detected in organophosphorus compounds which are more powerful inhibitors of plasma cholinesterase than are the parent compounds (Table 22). Many of these impurities occur as a result of hydrolysis, transmethylation (H20), or other transformation during storage.

The reaction scheme for irreversible inhibition is



$$AB + EH \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EHAB \xrightarrow[BH]{k_2} EA \xrightarrow[H_2O]{k_3} AOH + EH$$

where AB is the inhibitor, EH the enzyme, EHAB the enzyme-inhibitor complex, and EA is the acyl enzyme. Kinetically, the difference between substrate and inhibitor lies only in the rates of individual reactions; the rate-limiting step for inhibition is k_3. It is believed that the rate constant k_1 for the Michaelis complex is one of the fastest reactions (G12) and that the constant k_2 for decomposition of the Michaelis complex is also fast. Detailed kinetic analysis of irreversible inhibition has been discussed by Aldridge and Reiner (A12) and by Main (M4).

TABLE 21

COMPARISON OF SUBSTRATE RATIOS AND INHIBITION RATIOS FOR HORSE ERYTHROCYTE ACETYLCHOLINESTERASE (ACHE) AND HORSE PLASMA CHOLINESTERASE (BUCHE) USING CHOLINE ESTERS AS SUBSTRATES[a,b]

Acyl group of choline ester	Relative activity			Alkyl group of inhibitor	Inhibitor ratio			
	BuChE	AChE	Ratio BuChE/AChE		*p*-Nitrophenyl phosphates	Fluoro-phosphonates	Pyro-phosphates	Fluoro-phosphoro-amidate
$-CO \cdot CH_3$	100	100	1.0	$:POCH_3$	—	—	—	—
$-CO \cdot CH_2 \cdot CH_3$	145	78	1.9	$:PO \cdot OCH_3$	0.68	—	—	—
$-CO \cdot CH_2 \cdot CH_2 \cdot CH_3$	216	2.3	94	$:PO \cdot O \cdot CH_2CH_3$	2.9	51	75	—
$-CO \cdot CH: (CH_3)_2$	91	46	2.0	$:PO \cdot N: (CH_3)_2$	—	—	—	22
$-CO \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot CH_3$	145	3.3	44	$:PO \cdot OCH_2CH_2CH_3$	—	—	—	—
$-CO \cdot CH_2 \cdot CH: (CH_3)_2$	50	0.19	263	$:PO \cdot OCH: (CH_3)_2$	10	270	500	3950

[a] Activities are based on acetylcholine as 100, and inhibitor ratios are calculated from the inhibitor concentrations that effect 50% inhibition of the enzyme in 30 minutes.

[b] After Aldridge (A12).

TABLE 22

CHOLINESTERASE INHIBITORS DETECTED AS ARTIFACTS IN ORGANOPHOSPHOROUS COMPOUNDS[a]

Organophosphate	Artifact	Reference
Parathion	S-Ethyl isomers	D18
Parathion	Paroxan	A11
Diisopropyl fluorophosphate	Not known	G28
Bisdimethylaminofluorophosphine oxide	Not known	A9
Diethyl (substituted phenyl) phosphates	Tetraethyl pyrophosphate	A10
Diethyl-8-quinolyl phosphorothionate	Not known	A11
Ethyl-4-nitrophenylphenylphosphonothionate	Not known	D20
Diethyl-7-(4-methylcoumarinyl) phosphorothionate	Not known	A9
Tri-2-tolylphosphate	Not known	A8
Trimethylphosphate	Not known	A12
O,O-Dimethyl-S-ethylthioethyl phosphorothiolate	Sulfonium derivative, sulfoxide, and sulfone	H20

[a] After Aldridge and Reiner (A12).

4.5.3. *Salt Effects*

The effects of inorganic salts on plasma cholinesterase (E16) are largely contradictory. Fruentova (F9) reported that divalent cations are more effective inhibitors of horse serum cholinesterase than are monovalent ions, whereas divalent ions are frequently reported to have a marked activating effect (H38, T8, V1). Lithium and sodium nitrates have been shown by *in vitro* studies of the reaction of human plasma cholinesterase with benzoylcholine to have identical inhibition profiles (W21), while sodium and potassium chlorides had very similar inhibitory actions on the hydrolysis of acetylcholine by human plasma (H47). Silver nitrate, copper sulfate, and mercuric chloride are powerful inhibitors of *P. polycolor* butyrylcholinesterase (N2). Cohen and Oosterbaum (C12) concluded that activation by cations occurring at the usual substrate concentration is highly dependent on the experimental conditions. This supposition is very relevant to the somewhat random choice of buffers and substrates in the work reported above.

Among the anions, fluoride and sulfate had the strongest inhibitory effect, with bromide being the weakest (F9). The exceptional inhibitory power of fluoride ion (in a concentration of about 10^{-5} mol/liter) has been much studied, without real insight to the mode of action (C12, H26). It has been used for the differential inhibition of the human genetic variants (H12). The inhibition of human cholinesterase by fluoride differs from that of chloride in many respects (H15), not least in the effective concentration. Sodium chloride operates in the mole-per-liter concentration range, and its inhibition appears to be very similar in character to that of the human enzyme by sodium bromide (D12).

4.5.4. *Solvent Effects*

Todrick *et al.* (T7) studied the effect of *n*-butanol on the activity of horse serum cholinesterase. With benzoylcholine as substrate, the enzyme was activated to a maximum at a butanol concentration of 0.25 mol/liter. The activation decreased with increasing butanol concentration, and inhibition set in at 0.42 mol/liter. In contrast, with acetylcholine as substrate, the enzyme was inhibited and the inhibition increased progressively with increasing butanol concentration.

Acetylcholinesterase from human erythrocytes or rat brain homogenates reacts with acetylcholine in the presence of *n*-butanol in a manner similar to the benzoylcholine–horse enzyme system. The unusual behavior of the acetylcholine–horse enzyme system—in which inhibition of the enzyme by excess substrate does not occur (A21)—indicates that inhibition by *n*-butanol is probably competitive. Todrick *et al.* (T7) con-

firmed this and suggested that the activation of the benzoylcholine system by *n*-butanol may reflect interference with the inhibition of the enzyme by excess substrate. Activation sets in immediately and is reversible, whereas inhibition sets in gradually and is irreversible.

Benzoylcholine has also been used as substrate in studying the effects of alkanols on human serum cholinesterase variants (W8, W9). Increasing concentrations of alkanols were found to activate the usual enzyme up to a maximum, which increased in magnitude as the chain length of the alkyl group increased. At higher concentrations, irreversible inactivation, presumably due to denaturation, occurred. The atypical enzyme was much more readily inactivated with all the alkanols, and only slight activation was observed at low alkanol concentrations.

The role of the apolar amino acid side chains in cholinesterase is still a neglected field of study, in spite of the development of solvent variation (B34, Y1), which provides new opportunities for experimentation. The resultant alteration in the configuration of the enzyme produced by hydrophobic solvents because of solvation of apolar side chains may expose changes in the configuration or stability of the steric structure of some of the enzyme variants, changes which could be of real value in their recognition and identification.

4.5.5. *Miscellaneous Inhibitors*

4.5.5.1. Plant products. Extracts of certain plant species inhibit the hydrolysis of choline esters *in vitro* by human plasma cholinesterase (B12, H19, O6–8). Thus, physostigmine is an anticholinesterase which can be extracted from the calabar bean. To identify naturally occurring substances which could inhibit human plasma cholinesterase, Orgell *et al.* (O8) studied a large number of alkaloids, glycosides, and phenolic compounds. Of various plant tissues, potato has been most extensively studied (H11, O8) and it has been demonstrated that the glycoside solanine and the alkaloid solanidine could be used to characterize some of the genetic variants of cholinesterase (H14).

4.5.5.2. Steroids. Although steroid hormones (F3) and the administration of oral contraceptives (R6) are known to reduce plasma cholinesterase activity *in vivo* (Section 5.2.3), it has been shown that a large number of steroids had no effect *in vitro* on the enzymic activity (R2). A notable exception was progesterone, inhibition by which was insufficient to account for the decreased enzymic activity associated with pregnancy.

Pancuronium bromide is a bis quaternary amino steroid which is used in clinical medicine as a muscle relaxant of the nondepolarizing type. It is a powerful inhibitor of plasma cholinesterase, but is about 1000-fold less

effective as an inhibitor of erythrocyte acetylcholinesterase (S43). The inhibition is reversible and competitive, with an I_{50} value of 2.3×10^{-7} mol/liter for human plasma cholinesterase with acetylcholine as substrate. Schuh (S10) found I_{50} values of 2.7×10^{-7} mol/liter for plasma cholinesterase using butyrylthiocholine, and 2.4×10^{-4} mol/liter for acetylcholinesterase using acetylthiocholine.

4.5.5.3. Surfactants. Several anionic surfactants, at low concentrations, strongly inhibit horse serum cholinesterase, whereas nonionic surfactants are without effect with acetyl- or propionylthiocholine as substrate (T8). Low concentrations of the hemolysin, alkylphenoxy polyethoxyethanol (Triton X-100) strongly inhibit human plasma cholinesterase activity with acetylcholine as substrate (S37).

4.5.5.4. Antibodies. Vodenicharova *et al.* (V9) showed that antibodies which almost completely inhibited horse plasma cholinesterase were synthesized when rabbits were immunized with purified horse serum cholinesterase. Preincubation of γ-globulin with horse serum cholinesterase was found to completely inhibit the hydrolysis of butyrylcholine, whereas simultaneous incubation of the enzyme, substrate, and antibody was not accompanied by any change in cholinesterase activity. Similar treatment with mipafox effected complete inhibition in both cases (E17). It is suggested that the antibody exerts its inhibitory effect at a site remote from the catalytic active site, and that the effect is probably due to steric hindrance.

4.5.6. *Activation and Reactivation*

The hydrolysis of benzoylcholine and procaine by human plasma cholinesterase has been shown to be enhanced by tryptamine (E12, E13), alkylamines (E14), and quaternary ammonium salts (E15). The activating effects of 12 narcotic analgesics on the hydrolysis of the same choline analogs by human plasma cholinesterase was reported by Erdos *et al.* (E14): Allyl substitution decreased the accelerating effect. Although morphine enhanced the hydrolysis of benzoylcholine by either human or horse cholinesterase, levorphan did not accelerate the activity of the horse enzyme, in contrast to the human enzyme. In general the activating effect of the narcotic analgesics on the hydrolysis of procaine varies inversely with their inhibitory effect on the hydrolysis of acetylcholine. The study of such activators is helpful in enlarging our knowledge of the nature of the active site of the enzyme and the mechanism of the catalytic action.

The activity of cholinesterase that has been newly inhibited by organophosphates can be restored, at least partially, by the prompt addi-

tion of nucleophilic agents which compete for the oxygen of the serine residue and thereby displace the phosphoryl group. Such nucleophilic agents are hydroxylamine, hydroxamic acids, and oximes. Pyridine-2-aldoxime methiodide (2-PAM), for example, rapidly reverses phosphorylation at quite low concentrations; only the *syn* configuration of the oxime is active in this respect (C2).

In storage, however, the phosphorylated enzyme gradually loses its ability to be reactivated by nucleophiles; the enzyme is then said to have "aged" (H33, J1). The chemical basis of this aging process has been shown (B18, J2) to be the spontaneous loss of one of the alkyl groups from the dialkylphosphonyl group of the phosphorylated enzyme, thereby producing a negative charge on the phosphonate bound to the cholinesterase. The induced negative charge resists nucleophilic reactivation by the oximate anion, which is the functionally active group of the oximes. The close correlation between the rates of aging *in vitro* and *in vivo* demonstrates that, for a given species, *in vitro* studies can be a useful guide to the elapsed time, after organophosphate intoxication, during which oxime therapy would be effective. Phosphorylated human plasma cholinesterase "ages" much more rapidly than the phosphorylated horse plasma enzyme (H25). Many oximes which are antagonistic to inhibitors of cholinesterase have been evaluated for clinical use (C6, D19, N5, W26), and those in most common use are the pyridinium aldoximines pralidoxime (2-PAM) and the bis compound Obidoxime (C8). A tris-quaternary trioxime was synthesized (L35), but it proved to be too toxic for therapeutic use.

A particular oxime is not equally effective either against all organophosphates or in all species.

Geldmacher von Mallinckrodt (G11) has shown that the choice of substrate is of paramount importance when measuring the activation by oximes of plasma cholinesterase which has been inhibited by alkyl phosphates. This is because oximes have been found to cause spontaneous hydrolysis of *o*-nitrophenyl butyrate and the thiocholines, but not of butyrylcholine or benzoylcholine.

Kinetic studies of the nature of reactivation of phosphorylated enzymes by nucleophilic agents are based on the mechanism:

$$EI + R \underset{}{\overset{K_D}{\rightleftharpoons}} EIR \xrightarrow{k_R} E + P$$

where EI is the inhibited enzyme, R the reactivator, E the regenerated enzyme, P the product formed, K_D the dissociation constant of the EIR complex and k_R the rate constant of breakdown of the complex. Activa-

tion energies for the breakdown of the complexes have been estimated (D9, P2, W3, W27), but it is difficult to assess the significance of the results, either in terms of variation of the phosphoryl groups or the structures of the activators. Wang and Braid (W4) measured thermodynamic and kinetic parameters for spontaneous reactivation as well as the oxime-mediated reactivation of two phosphorylated serum cholinesterases. The enthalpy and entropy changes in both the binding and dephosphorylation steps were found to be coupled, and resulted in a minor variation in free energies. Neither enthalpies nor entropies alone bore any relationship to the kinetic parameters K_D and k_R, but the expected linear correlation was found for the changes in free energy with log K_D and log k_R/K_D. Investigations of the spontaneous reactivation of dimethyl phosphoryl serum cholinesterase implicated two catalytic sites with pK values of 9.5 and 7.5 and heats of ionization of 5.3 and 9.6 kcal per mole, respectively. Keijer *et al.* (K19) reported that a protonated group with pK 7.35 was involved in the aging of phosphorylated serum cholinesterase. The influence of temperature and ionic strength on the pH–rate profile for these investigations suggests a more complicated mechanism than the participation of an undissociated carboxyl group of the enzyme catalyzing the formation of a carbonium ion as the rate-determining step.

The sterospecificity of aging of phosphorylated cholinesterase has been investigated (K18) with special reference to the influences of the configurations around the phosphorus atom and the α-carbon atom of the alkyl group. The former appeared to have a much greater influence on the aging rate than did the latter.

4.5.7. *Histochemical Applications*

Histochemical techniques for demonstrating cholinesterase are nonspecific, so that it is convenient to use appropriate inhibitors in order to distinguish between acetylcholinesterase and cholinesterase. Diegenbach (D13) studied the usefulness of several inhibitors and found that BW284c51 and BW62c47 (Wellcome Research Laboratories, Beckenham, Kent, England) showed comparable inhibition of acetylcholinesterase; for BW284c51, they confirmed the optimal concentration of 5 $\times$ 10^{-6} mol/liter proposed by Holmstedt (H39). Some organophosphorus compounds are powerful inhibitors of plasma cholinesterase but show marked differences in inhibitory powers for different animal species (A7, P9). The choice of inhibitor for histochemical studies therefore depends on the species under examination, and an empirical approach may be necessary. Discrepancies in inhibition characteristics may reflect the degree of purity of the enzyme in some preparations (M15).

It may be noted here that the neurotoxic effects of tetrazolium salts could be due to interference with cholinesterase in nerve tissue, since tetrazolium salts have been found to be powerful inhibitors *in vitro* of human plasma cholinesterase (F6).

4.6. Immobilization

Immobilization of cholinesterase, like that of other enzymes, has a number of potentially valuable applications, including active-site studies, economical use of the enzyme as an analytical reagent, and enhancement of the storage life of the enzyme.

Immobilization can be accomplished by entrapment of the enzyme in a gel, such as starch or agar (A6, B9, G30), or in cross-linked polyacrylamide (D11). It can also be covalently bound to paper (S36). Chromatography on gel filtration media such as Sepharose is essentially immobilization by covalent fixation. Guilbault and Das (G30) considered that the physical entrapment of cholinesterase in an acrylamide gel was preferable to entrapment in a starch gel: The enzyme appeared to be more stable on the former medium and to have a longer life on wet or dry storage. Starch gel entrapment is recommended only when long-term use of the gel is not required. It would be wrong to be biased toward a specific method of immobilization, and each situation should be reviewed before deciding on a particular supporting medium. Axen *et al.* (A31) found that the binding of horse serum cholinesterase to Sepharose produced no change in substrate specificity of the enzyme. However, Stasiw *et al.* (S36) observed a decrease in the apparent K_m of the bound compared with the free enzyme, using either acetylcholine or butyrylcholine as substrate. The changes in K_m may be caused by steric hindrance and other limiting changes imposed by the matrix. However, it was proposed (H43) that the observed decrease in K_m may be a result of enrichment of substrate, consequent upon attraction between the positively charged substrate and negatively charged sites in the matrix.

Enzyme electrodes are essentially immobilized enzyme systems. Crochet and Montalvo (C15) developed a technique for cholinesterase based on coupling a small pH electrode to a thin polymer membrane. At the electrode surface, cholinesterase interacted with acetylcholine to produce acetic acid, which was detected by the pH electrode. Excellent sensitivity was achieved by the use of a very thin film of enzyme solution, with extremely low strength of buffer containing the enzyme and almost complete suppression of spontaneous hydrolysis of the substrate.

A choline ester-selective electrode has been developed by Baum and his

co-workers (B6–8), using either acetylcholine, butyrylcholine, or acetyl-β-methylcholine as substrate, in order to monitor the enzymic activity of serum cholinesterase or acetylcholinesterase in erythrocytes. It was claimed that reproducibility was comparable with conventional analytical methods.

Such studies illustrate the rapid advances being made in the field of immobilized enzymes and point to the future importance of enzyme electrodes as analytical tools.

4.7. Half-life of the Enzyme

The half-life of usual serum cholinesterase has been calculated by measuring the rate of change of cholinesterase activity following transfusion of plasma or blood (G8, J4) or after injection of purified serum cholinesterase (P12, S10) into patients who have the silent phenotype. Jenkins *et al.* (J4) reported the half-life of serum cholinesterase in a Bantu girl to be about 10 days. Although two other investigators (P12, S10) used purified cholinesterase from the same commercial source, the half-life reported by Ploier (P12) was between 8 and 9 days, whereas that reported by Schuh (S10) was 1.86 days. It may be that the cholinesterase used by the latter investigator was partially denatured either during or after its preparation, and that it was therefore more susceptible to degradation after injection (S10). A careful half-life determination was performed by Garry *et al.* (G8) who reported that the first order elimination constant was 0.20 per day, from which a half-life of 3.4 days was calculated. In this particular case, the patient ordinarily had a serum cholinesterase activity that was about 5% of the usual value. If this had not been taken into account, an erroneous half-life of 6.7 days would have been calculated. Two of the investigators (J4, P12) were unable to detect residual plasma cholinesterase in their patients with the methods they used.

Reasons for the discrepancies in the reported half-lives are not apparent. In some cases, differences in the source of the enzyme or in the experimental technique may be responsible. In others, racial or idiosyncratic differences among the patients may be responsible.

The effect *in vivo* of diisopropyl fluorophosphate upon the plasma of several adults has been measured (M22). A distinct fall in enzymic activity was observed, but no clinical signs of intoxication appeared in any individual. The half-life of the cholinesterase was found to be 16 days, thus confirming that alterations in the plasma enzyme activity are of limited value in assessing the toxic effects of diisopropyl fluorophosphate.

5. Practical Applications of Cholinesterase Measurements

Three items of information are necessary to characterize the state of cholinesterase in human plasma—the genetic type of the enzyme, an estimate of the activity, and the molar concentration. Usually not all of these data are available, and if cholinesterase measurements are to be used for practical purposes, it is of crucial importance to know in what circumstances partial information suffices, and when incomplete information calls for caution in interpreting results. For instance, the isolated observation of a low level of enzyme activity can have three entirely different meanings. First, the low activity could be a consequence of reduced protein synthesis due to a pathological cause such as liver disease, cancer, or malnutrition; second, there may be enzyme inhibition, indicative of organophosphate poisoning; or third, the low activity could indicate the presence of a genetic variant in a perfectly healthy subject. In the past, these sources of ambiguity were not always recognized, and their presence must have tended to discredit the use of cholinesterase testing for clinical purposes.

Information on the molar concentration is least often available since it requires immunoassays (G18) or other specialized procedures. As will be explained below, information on enzyme activity, also, is not needed when the problem is to find an explanation for a prolonged effect of succinylcholine as used in anesthesia; in this situation, the important test is for the presence of genetic enzyme variants.

In all cases, it is best to take the stand that one isolated determination of the activity of cholinesterase in a human serum specimen provides information that cannot be interpreted with any degree of certainty. However, what can be useful is information on changes in enzyme activity over periods of time or after different manipulations, since cholinesterase activity is normally a stable trait of the individual (G32, H44).

5.1. In Anesthesia

5.1.1. *Succinylcholine Apnea*

As stated in Section 3.1, biochemical considerations make it necessary to use tests that are capable of indicating the presence of unusual esterase types rather than merely the levels of enzyme activity. However, different methods of esterase typing for use in anesthesia may yield different depths of information. Most often the purpose of the test is to document for future reference the cause of a prolonged effect of succinylcholine after recovery of the patient. This is usually a fairly simple task. By con-

trast, if the purpose of the test is to provide a basis for genetic counseling, at least the established variants in homozygous as well as in heterozygous conditions should be identified. There are undoubtedly numerous cholinesterase mutants (G18, R12) that are difficult or impossible to identify by inhibition tests alone (W13) but which may some day become recognizable by other means. Some comments on techniques and policies of testing are therefore appropriate.

Most clear-cut and usually without ambiguity is the identification of subjects who have only the atypical, dibucaine-resistant variant in their plasma—that is, subjects who are either homozygous for atypical esterase ($E_1^aE_1^a$) or heterozygous with one silent gene and one for atypical esterase ($E_1^aE_1^s$).

For many years, in about 800 cases with prolonged apnea, one of the present authors (W.K.) has faithfully determined both dibucaine and fluoride inhibition. Many of the fluoride inhibition data were inconsistent with the concept of there being a single, special allele conveying resistance to fluoride inhibition. Fluoride tests may be worth pursuing when the purpose is to conduct research into the genetics or biochemistry of cholinesterase. If the purpose is to explain prolonged apnea after succinylcholine, the fluoride test scarcely repays the effort, because any abnormality of fluoride inhibition also shows up as an abnormality of cholinesterase inhibition by dibucaine (see Table 23).

A special problem is the certain identification of "silent gene" cases ($E_1^sE_1^s$). There are obviously several different point mutations that may render cholinesterase virtually inactive, but the distinction between these types at the present time would be entirely a matter of research. The immediate problem is always to establish whether a given person's lack of

TABLE 23
CHOLINESTERASE VARIANTS IN PLASMA SPECIMENS SUBMITTED FOR TESTING BY ANESTHETISTS: SUMMARY FINDINGS[a]

Dibucaine number	Phenotype	Genotypes	Number found	Number expected	Ratio—found/expected
> 72	U	$E_1^uE_1^u$, $E_1^uE_1^s$	330	782	0.4
< 35	A	$E_1^aE_1^a$, $E_1^aE_1^s$	251	0.4	600
—	S	$E_1^sE_1^s$	10	0.008	1200
≥ 35 and ≤ 72	F	$E_1^fE_1^f$, $E_1^fE_1^s$	5	0.05	100
	I	$E_1^uE_1^a$, $E_1^uE_1^f$, $E_1^aE_1^f$	220	40	6
—	Not known	Not known	7	—	—

[a] Unpublished observations by W. Kalow.

cholinesterase activity has a genetic basis or whether it is produced by organophosphate poisoning or by some other artifact. An artifact has been, for instance, the undeclared use of oxalate in anticoagulant mixtures, causing complete inhibition of enzyme activity. It is therefore wise to subject a small portion of any serum without esterase activity to dialysis (e.g., against running tap water; see further comments below), and diagnose silent esterase only if there is no trend towards restitution of enzyme activity.

If the purpose of the test is to find preoperatively whether prolonged apnea is likely to occur, the type of test to be used depends on the assessment of cost–benefit relationship. If a thousand tests have to be done in order to detect one patient who will spend an hour longer in the recovery room than most others, the cost of testing would have to be very low to be justified (S4). If there is reason to suspect an esterase abnormality, however, because there is an affected close relative, the type of test to be made should yield data which allow genetic counseling.

The prescreening of surgical patients for low levels of cholinesterase activity and for phenotypes known to be most susceptible to prolonged apnea has indeed been advocated from time to time (see K21). At some hospitals, all surgical patients are tested (see comment by F. Madera-Orsini in D15 and D16). However, this approach is not universally accepted because it is inefficient from a cost–effectiveness point of view. Many anesthetists have the opinion that it is more efficient to observe the patient for spontaneous restoration of respiration, and provide assisted ventilation for those few patients who have prolonged apnea (B37, L30, M23). However, there appears to be general agreement that patients who have had unusual reactions after anesthesia should have their cholinesterase phenotype determined. It is also recommended that family members of susceptible individuals should be tested (B5, C1, D15, D16, F7, P20, W15, W23). It has been stressed that even for minor surgical procedures on ambulatory patients, the anesthetic procedure cannot be considered to be minor (P20). Succinylcholine should never be administered to anyone unless the physician has the facilities to mechanically ventilate the patient for long periods of time, if necessary. Periods of apnea lasting several hours are not uncommon among susceptible patients, and the longest period of apnea of which the reviewers are aware is 9 hours (D22).

It may be noted that systemic reactions to ester-type local anesthetics have also been reported to occur among some individuals who are homozygous for atypical serum cholinesterase (Z3, Z4).

The E_2 locus specifying electrophoretic variants is of research interest rather than of significance for clinical service, at least for the time being.

For most clinical chemists, experience with genetic typing of plasma cholinesterases is gathered at a very slow rate simply because the number of patients in trouble with succinylcholine is quite small in absolute terms. On the other hand, the tests particularly designed for genetic counseling require a fair number of fine distinctions between rates of enzyme activity under various conditions. As a rule, therefore, it may be best to have at least the more refined measurements for cholinesterase typing and the occasionally difficult interpretation of the measurements made in a central laboratory where the desirable experience can be gained within a relatively short time. Models of such specialized laboratories are the Cholinesterase Research Units in England (W23) and in Denmark (V6). The establishment of similar centers modeled after these can only be recommended.

5.1.2. *Malignant Hyperthermia*

Very rarely, succinylcholine may cause muscle rigidity or contractions as a consequence of abnormality or pathology of skeletal muscle, with no relationship to plasma cholinesterase (H42, O9). Contractions of short duration are typical for the myotonic diseases and for denervated muscle during a period of a few weeks after nerve injury. A special case is malignant hyperthermia (A5, B36, K9), a syndrome which is often fatal but which does not represent a uniform disease. The best defined and most frequent episodes occur after exposure to an anesthetic vapor such as halothane, and are characterized clinically by tachycardia (or other arrhythmias), rise of body temperature, and more or less extensive rigidity which may last for hours. Succinylcholine contributes to the development of this rigidity. It therefore draws attention to the developing attack and it does aggravate it—but it is usually not responsible for the attack. Nevertheless, there are often requests for cholinesterase tests in cases of malignant hyperthermia. In most of these cases, the cholinesterase is found to be normal in regard to type and activity. Recently, however, a statistical association between malignant hyperthermia and the fluoride-resistant variant of the enzyme has been described (E7, W22). There is at this time no rational explanation for such an association, so that cholinesterase investigations in this context should be regarded, at present, as a matter of research rather than clinical service.

5.2. In Various Disease States

Plasma cholinesterase activity varies with physiological factors such as age and sex (S19, S21) and pregnancy (W6), or with parameters such as

body fat (B23, C16), plasma lipids (C16) or lipoprotein fractions (C10, K36). There is also a rich literature pertaining to levels of human plasma cholinesterase activity in various diseases. In a general way, the data can be divided into two classes. One class comes from studies which were guided by the hope that some association between enzyme activity level and disease state would reveal information either about enzyme function or about the nature of the disease. To this category belong, for instance, the old attempts to relate plasma cholinesterase values statistically with thyroid function (see G14). This kind of investigational use of plasma cholinesterase determinations is speculative and is of no further concern in the present context.

There is another class of studies relating disease to plasma cholinesterase measurements, in which the assay of cholinesterase activity has come to be regarded as a sensitive indicator of protein level in plasma. Today, this seems to be the common interest if cholinesterase is measured in cases of hepatic or renal disease, cancer, or kwashiorkor. In all of these cases, the weakness of the test rests in the fact that esterase activity may be subnormal not because there is pathology, but because there is a different genetic type in which a low esterase activity is normal and does not reflect a lowered concentration of the enzyme protein. The ambiguities created by this situation are probably the main reason for cholinesterase tests not being more widely used. The difficulties are confounded by the occurrence of the silent gene, which cannot be detected by means of inhibition tests with dibucaine, fluoride, or other commonly used agents (Section 6.3.2).

Therefore, a single determination of plasma cholinesterase activity can never reveal a reduction of protein formation with certainty, although it may indicate with some probability whether protein production is normal. Because of this limitation, the greatest value lies in serial determinations of cholinesterase activities over a period of time (H44). In other words, the best use of the assay is for monitoring the course of serious disease. Indeed, the present reviewers' unpublished experiences as well as several published comments suggest strongly that a reduction of cholinesterase activity to about one quarter of a given person's normal activity usually heralds that person's death (H49, L9, T1). There is no reason for believing that death would be due to a lack of cholinesterase function. Most likely, it is simply an indication of breakdown of protein synthesis. It must, however, be stressed that discovery of a low level of cholinesterase activity in a given person does not necessarily have this foreboding meaning unless it is known whether the low value is normal for that subject on genetic grounds or whether it is due to the presence of an irreversible inhibitor.

5.2.1. *Liver Disease*

There are many reports on cholinesterase activity in hepatic diseases (see E18, F1, H49, K20, L9, T1, V7, V10), and there is some rationale for such studies. Plasma cholinesterase is formed in the liver, but its level in the liver is low, apparently because it is released into plasma immediately following its synthesis (V7). Fluctuations of cholinesterase activities in plasma can therefore be very sensitive indicators of changes in the rate of protein synthesis, i.e., in the functional state of the cell. Thus, it is well established that on the statistical average, activities are reduced when there is hepatitis and evidence of cellular impairment.

In the early stages of acute hepatitis, the plasma enzyme activity should be no more than slightly reduced; a substantial reduction is a danger signal and indicates the development of complications. In chronic hepatitis, cholinesterase activities are in the low-to-normal range, but quite low values are frequently found in liver cirrhosis. The cholinesterase activity cannot be used to differentiate between the various forms of cirrhosis. Generally, there is a good correlation between the functional state of the liver and the activity. A sudden drop in activity may indicate a developing hepatic coma.

Plasma cholinesterase determinations have been helpful in assessing the suitability of a patient for shunt operations in portal hypertension or for liver transplantation (E18). Obstructive jaundice is not accompanied by a reduction of cholinesterase activity unless there is also damage to the hepatocytes. In cases of fatty liver, the plasma cholinesterase activity is generally normal or high. As indicated above, there is a positive correlation between the enzyme activity and plasma lipids or lipoproteins (C10, C16, K36).

The greatest value of determinations of cholinesterase activity is as a prognostic tool. If esterase tests are performed together with other liver function tests at the time when the patient first presents, and if the tests are then repeated every few days afterwards, it is noticed that cholinesterase levels often do not change in parallel with other biochemical indicators of liver disease (E18, V7). However, it has been remarked several times that any change in cholinesterase activity is the most reliable indicator of damage to the hepatocytes. A marked fall in these circumstances indicates a deteriorating condition of the patient, and any rise, an improvement.

5.2.2. *Cancer*

While malignancy progresses, and particularly when metastases develop, levels of plasma cholinesterase activity always tend to drop (F1,

K35, M1, M16, W5, W2, W25) (cf. Section 2.6.3). This reflects, at least in part, a decreasing rate of protein formation, and in this sense, changes are to be assessed as in liver disease. However, specific reductions of cholinesterase levels—reductions beyond those seen with other plasma esterases, albumin, or proteins in general (W5)—have raised the suspicion that there is more selective humoral effect of cancer cells suppressing cholinesterase formation or activity (M1), just as bronchogenic carcinoma must release something to cause the myasthenic syndrome. The present reviewers have seen some very low plasma cholinesterase activities in cancer patients, but the accumulation of further data would be desirable before generalizations should be made.

In spite of the drastic fall of cholinesterase activity in many cases of cancer, the reduction does not come early enough or with sufficient regularity to serve for cancer detection. One possible exception can be the diagnostic distinction between tumor- or gallstone-produced biliary obstruction (F1, K35, W25). The monitoring of cholinesterase activity in cancer patients, however can serve as a sensitive index of a patient's state and prognosis.

5.2.3. *Other Pathological Conditions*

Because of the dependence of cholinesterase concentration in plasma on the rate of protein synthesis, there may be a lowering of the activity on a nonspecific basis in innumerable pathological conditions. There is no point in trying to enumerate all the published reports which attest to this concept, but a few items of information deserve attention.

As indicated above, cholinesterase levels rise with the amount of body fat. On the other hand, there is always a reduction of esterase levels under conditions of starvation (E5, S34), as for instance in kwashiorkor (B4, S35). Since in starvation there is a general tendency for the blood volume to shrink before there are major changes in the concentrations of blood constituents, the possibility must be considered that the reduction in the actual amount of cholinesterase protein is much greater than may be suggested by the reduction of cholinesterase activity per unit volume of plasma.

There is generally a fall in plasma cholinesterase activity after burn injury (P15, V6, V8). There is a clear-cut relationship between the severity of the injury, as expressed by the burn index, and the lowest measured cholinesterase activity if that is expressed in terms of percentage of the patient's normal values (V6). Particularly in severely burned patients, there may be a very sharp fall of esterase activity within one day; that is, in a shorter time than can be accounted for by the normal catabolism of the enzyme. The initial drop therefore seems to indicate a dilution effect,

the enzyme being lost through the capillaries and diluted further by intensive fluid therapy. It is known that burns of the skin may cause disturbances in liver function. Because of the persisting reduction of cholinesterase levels, particularly after severe burns, there is also likely to be reduced cholinesterase synthesis. Sequential determinations of plasma cholinesterase activity have served as indicators of a patient's prognosis, as they have in other cases of liver disease.

It is worth mentioning in this context that the use of oral contraceptives regularly leads to some modest diminution of plasma cholinesterase activity (R6, S21). The same may be true after administration of glucocorticoids in anti-inflammatory therapy or during immune suppression (Z1).

5.3. In Toxicology

Various classes of biologically active agents are inhibitors of cholinesterases (Section 4.5) (G21, K32, S23). The "nerve gases" of military arsenals (e.g., Tabun, Sarin) are potent inhibitors, as are insecticides (e.g., parathion, malathion) and cholinergic drugs such as those used in ophthalmology to treat glaucoma (e.g., physostigmine, echothiopate), in anesthesia to overcome the effects of curare (e.g., edrophonium, neostigmine), and in internal medicine to treat certain diseases of muscle or intestine (e.g., neostigmine, pyridostigmine). In addition, numerous drugs are capable of inhibiting plasma cholinesterase *in vitro*, and some of these (e.g., local anesthetics) are likely to be capable of exerting some inhibitory effect *in vivo*. Many of the potent inhibitors belong to one of two chemical classes—the organophosphates or the carbamates. The pharmacological effects of all these agents are solely or overwhelmingly due to inhibition of acetylcholinesterase in certain membrane structures of the nervous system (J6, K30, N4). However, the enzyme most readily inhibited by most of these substances, except the nerve gases, is plasma cholinesterase. This inhibition does not seem to be pharmacologically important, but it serves as an excellent biochemical indicator for the presence of some types of inhibitors in the human being (G31, H40). It can easily happen that plasma cholinesterase is almost completely inactivated, while acetylcholinesterase is inhibited only to 50 or 60%. However, this is not a fixed relationship; the proportion varies grossly among compounds and also with the route of absorption.

The organophosphates and the carbamates differ typically in speed or ease of reversibility of cholinesterase inhibition (O1). As a rule, reversibility is much greater with the carbamates, which include such drugs as neostigmine, pyridostigmine, and the alkaloid physostigmine or such in-

secticides as aminocarb. Because of this reversibility, cholinesterase tests in carbamate poisoning are useful only if false negatives are avoided; this may be achieved by diluting the inhibitor out, so that its effects are not noticeable *in vitro* even if they were prominent in the living organism. If it is assumed, for instance, that plasma contains sufficient neostigmine to cause 90% inhibition of the plasma cholinesterase in the living subject, then a sample of plasma taken from the patient with the purpose of determining cholinesterase activity needs to be diluted for almost all methods of testing. If the plasma is diluted 1 to 100, this both dilutes the enzyme and diminishes the inhibitor concentration by a factor of 100, so that it will cause only 10% inhibition, which will not easily be recognized as a reduction of activity. To demonstrate inhibition, the enzyme activity has to be tested with various degrees of plasma dilution. There should be at least a 10-fold difference, and preferably a 100-fold difference, between the highest and lowest dilution. The presence of reversible enzyme inhibition in plasma can then be deduced from the fact that the esterase activity is relatively greater in the dilute than in the concentrated samples of plasma. Since the interpretation of such measurements requires a substantial extrapolation to estimate activity in the undiluted serum, the measurements of enzyme activity have to be rather precise if the data are to be meaningful.

By contrast, organophosphates are sufficiently irreversible inhibitors that enzyme inhibition observed *in vitro* will tend to reflect the enzyme inhibition in the living subject. Therefore, the finding of normal cholinesterase activity in plasma excludes systemic poisoning by organophosphates (N4). The greatest difficulty here is to decide whether a lowered esterase activity is indeed due to inhibition by organophosphates. Modern analytical techniques may allow the toxicological diagnosis to be made by demonstrating the presence of the inhibitor or its metabolites in biological fluids (R5). Alternatively, a good measure is to determine the enzyme activity of acetylcholinesterase in erythrocytes. If both plasma cholinesterase and acetylcholinesterase are low, the likelihood of organophosphate poisoning is great. This is because the rules governing the fate of plasma cholinesterase (N4, W34) and that of acetylcholinesterase in erythrocytes are entirely different. This can be fairly stated even though the mechanisms controlling the rate of plasma cholinesterase formation and its release from the liver are not known in any detail. On the other hand, acetylcholinesterase activity per erythrocyte depends essentially on the rate of erythropoiesis (B24). Newly formed erythrocytes have a high level of this enzyme, which gradually diminishes with time; in old erythrocytes, there is barely one-third of the original activity. Hence, the level of acetylcholinesterase activity in blood is largely determined by the

number and average age of the red cell population. Because of the different physiological conditions which determine the levels of these two esterases, a substantial drop in the level of both enzymes below normal values is almost certainly an indicator of influence by an environmental factor. For practical purposes, this points to inhibition by an irreversible inhibitor like an organophosphate.

It is of some importance for the analyst to know that there are distinct phases of cholinesterase inhibition by organophosphates. In the first phase, the inhibitor competes relatively weakly with any natural or artificial substrate for binding sites at the enzyme. The initial rate of enzyme binding by the organophosphate therefore varies greatly under different circumstances. It may be very fast, if a person is exposed to a high concentration of a nerve gas. If the organophosphate is of the insecticide type, the insecticide molecule usually requires metabolic activation in the liver before it becomes an effective cholinesterase inhibitor. In acute poisoning with organophosphate insecticides, the onset of action is normally 5 to 20 minutes, and death occurs after 30 to 45 minutes (T1). However, the development of cholinesterase inhibition may sometimes take a few hours because of delay due to absorption, chemical activation, and slow combination with the enzyme. This initial phase is followed by a second one, which again may take many hours; this phase is characterized by a binding to the enzyme that is fairly firm. The inhibition will not be abolished by means of plasma dilution as with carbamates. However, the inhibition is reversible by the use of a reactivator such as PAM (Section 4.5.6), although this drug is more suitable for reactivating acetylcholinesterase than for plasma cholinesterase. In practice, at this stage, unmistakable—and sometimes substantial—reactivation of the enzyme can be achieved by dialysis. If serum is suspected to contain an organophosphate inhibitor, an aliquot should be taken to measure the enzyme activity in the usual way, and a second portion of the serum is dialyzed for a few hours against running tap water before redetermining the esterase activity. For comparison of the two measurements, esterase activity is expressed in relation to the protein concentrations before and after dialysis. In the presence of organophosphates, there may be a substantial gain of enzyme activity on dialysis. However, an estimate of the degree of inhibition is obtained only afterwards by making weekly determinations of the enzyme activity until a constant value is restored, and by assuming that this was also the level of enzyme activity prior to the organophosphate exposure.

The procedures described above are most useful to diagnose esterase inhibition in individual subjects. For reasons which are not clear, however, there are substantial person-to-person variations in response to cholin-

esterase inhibitors. For instance, in many glaucoma patients receiving on a regular basis echothiopate iodide in the form of eye drops, cholinesterase activity is not much depressed (E8). In other subjects on the same drug regimen, plasma cholinesterase may be completely inhibited (L27), and acetylcholinesterase may be sufficiently inhibited to endanger the patient's life through augmentation of bradycardia-producing reflexes. It is possible that such responses can be explained by the genetic variability of paraoxinase (G10), an enzyme which occurs in human plasma and is capable of inactivating some organophosphates.

A biological problem of concern to industrial and environmental toxicologists is the question of chronic toxicity as a consequence of continual exposure to low concentrations of organophosphates (B16, V3, V4). If it is necessary to compare exposed and nonexposed populations in order to assess relative organophosphate uptake, the determination of population averages of cholinesterase activities appears to be all that is needed.

5.4. In Forensic Medicine

In human biochemical genetics, a large number of biochemically or immunologically defined traits is known, so that almost every person can be demonstrated to be biochemically unique. In human blood, it is not only the blood group substances which are used for a biochemical identification but a fairly large number of enzymes as well, especially the erythrocyte enzymes.

In principle, the use of plasma cholinesterase for forensic purposes is no different from the use of other enzymes that are genetically variable. However, there are two advantages which have proved to be very helpful for the purpose of forensic investigations. In the first place, plasma cholinesterase is very rugged; it deteriorates only slowly (A16), and even old malodorous blood samples can often still be used for cholinesterase typing. As long as some cholinesterase activity can be measured with reasonable confidence, it has been always possible to determine the presence of the atypical variant (E_1^a). Also, scrapings of dry blood stains, when redissolved in buffer, may retain enough activity to allow cholinesterase typing. There is, however, insufficient experience to allow statements to be made on any other genetic type but the atypical variant.

It must be realized, of course, that the low activity usually present in old samples may call for prolonged testing; for instance, if—as in one of the standard procedures—a test run for 3 minutes suffices for precise rate determinations in normal plasma, with partially deteriorated plasma, the time of testing may have to be increased to 30 or 60 minutes.

A second advantage of the use of cholinesterase typing over that of

many other enzymes is the fact that the atypical variant (E_1^a) can be recognized in the heterozygous state; this renders pedigree determinations fairly efficient. The frequency of this genetic variant in different populations can be derived from published data (Section 2.7), but of course, the interpretation of data for forensic purposes requires a full understanding of the genetics of cholinesterase variation.

6. Methodological Aspects

6.1. Specimen Collection and Storage

Much of the work involving cholinesterase variants has involved collecting serum specimens at one location and shipping them to another location for analysis. In some cases the specimens have been shipped in the frozen state (W19) but in others they have been shipped at ambient temperature (O5), even though they were in transit from one continent to another. The effects of time and temperature of storage on the stability of cholinesterase variants have received comparatively little attention. There are conflicting reports concerning the stability of cholinesterase activity in blood. This subject has been included in the review of Witter (W32), who stated that the enzyme in plasma is stable for several weeks at 0 to 5°C, and for several months in the frozen state. The use of blood dried on filter paper was also discussed. as were reports that the enzyme is unstable when the plasma is stored for several days. However, other investigators have drawn opposite conclusions. Johnston and Huff (J5) reported that an average of 31% of the cholinesterase activity was lost within 3–4 months when plasma was stored frozen under their conditions.

This stability of cholinesterase in stored plasma was systematically studied by Braid and Nix (B33). They found that one reason for apparent loss of activity at room temperature is that the specimen slowly coagulates, and as it does, cholinesterase activity accumulates in the coagulum. Full activity could be restored by very vigorous mixing; 5-fold dilution of the plasma before storage prevented coagulation. Another way in which the activity was reduced was through bacterial contamination. Apparent increases in activity were produced through evaporation from inadequately stoppered specimens. It was also noted that the activity increased by about 10% during the first 24 hours after the specimen was collected, but returned to the original value within 48 hours. These studies demonstrated that plasma cholinesterase is very stable. At −20°C, specimens have retained more than 95% of their original activ-

ity after 3 years of storage, and more than 85% after 7 years. Eight freeze-thaw cycles over a period of 8 days resulted in less than 5% deviation from the original value. At 5°C, 80% of original activity remained after 4 months of storage. At 23°C, the enzyme appeared to be completely stable for up to about 80 days, and retained 85% of its original activity at 240 days. Storage at 37°C resulted in a loss of about 1% of the original activity per day. At 45 to 55°C, the loss of activity occurred in two phases—an initial rapid phase, and a slower second phase. The higher the temperature, the less activity remained at the time of switchover from phase 1 to phase 2. The rate of the phase 1 reaction increased with increasing temperature, whereas the rate of the phase 2 reaction *decreased* with increasing temperature. The latter fact, which can be deduced from the published data, was not commented on by the authors (B33).

In regard to the stability of the phenotype determined on fresh *vs* stored serum or plasma, there are numerous observations that the chemical tests lead to the same phenotype after specimen storage in the frozen state. This is true of the enzymes produced by the E_1^u, E_1^a, E_1^f, E_1^s, E_1^t, E_2^t, and E_2^- genes. It is possible that some undiscovered variants may be missed because of changes occurring in the serum or plasma before it has been analyzed. Most investigators seem to disregard this possibility.

The recommended method of collecting, transporting, and storing the specimen for serum cholinesterase measurement and phenotyping is as follows: The site to be punctured should be washed free of possible cholinesterase inhibitors by cleansing with alcohol, which is allowed to evaporate before skin puncture. In those cases where only the plasma cholinesterase is to be studied, the blood is collected in the absence of anticoagulant and allowed to clot for 1 hour at room temperature. The tube is then centrifuged at 1000 g, and the serum separated from the clot. It is important to avoid hemolysis, especially if acetylcholine or acetylthiocholine is used as substrate. If the analysis is not to be performed immediately, the serum may be stored for several days at 4°C. Otherwise it should be stored at −20°C, or lower. If the serum is to be shipped, it is safest to freeze the separated serum, place it in a precooled, insulated container along with solid carbon dioxide, and ship by an appropriate means of transportation such that it arrives at the laboratory while still frozen. This is recommended in order to avoid unwanted extreme increases in temperature during transportation. If it is not possible to follow this latter recommendation, the specimens should be kept sterile. In this case, the transported specimens may still be analyzed for phenotyping purposes, but it should be recognized that a reduction of enzyme activity may have occurred during shipment. If the erythrocyte

acetylcholinesterase is also to be studied, plasma instead of serum may be collected for the cholinesterase studies. In this case, the anticoagulant of choice is heparin, because other anticoagulants are reported to be associated with greater losses of enzyme activity (L13).

6.2. Screening Tests for Cholinesterase Variants

Several investigators have described the use of various screening procedures for detecting cholinesterase variants. In order to be useful, any screening procedure for abnormals should be capable of detecting *all* abnormal cases. It is acceptable to have a small percentage of the normals misclassified as abnormals on the basis of the screening procedure, because the suspected abnormals should always be confirmed by a specific test. Most of the proposed screening methods for cholinesterase variants fail to meet these criteria reliably. None of the procedures can separate all the non-$E_1^uE_1^u$ genotypes from the $E_1^uE_1^u$ genotype. As far as being able to separate the individuals or phenotypes that are hypersensitive to the effects of succinylcholine from those that are not, the reports are conflicting. Two groups (H9, M17) have developed screening procedures in which α-naphthylacetate is used as the substrate, and the α-naphthol produced by hydrolysis is coupled with the diazonium reagent from 5-chloro-*o*-toluidine to produce a purple-colored azo derivative. The tests are performed with and without Ro2-0683 (Roche) as the discriminating inhibitor. The genotypes $E_1^uE_1^u$, $E_1^uE_1^s$, $E_1^uE_1^f$ $E_1^fE_1^f$, $E_1^fE_1^s$, and E_1^s E_1^s usually produce very little color in the presence of the inhibitor, whereas the genotypes $E_1^aE_1^a$, $E_1^aE_1^u$, $E_1^aE_1^f$, and $E_1^aE_1^s$ usually produce a definite amount of color, even in the presence of the inhibitor (H9). However, Morrow and Motulsky (M17) report that with their procedure, the $E_1^fE_1^f$ genotype will also produce color.

In the procedure of Harris and Robson (H9), two sets of agar gels are set up, each containing wells. One gel contains the inhibitor and the other does not. Diluted serum is placed in wells in each type of agar, and is allowed to diffuse overnight. The agar is then reacted with substrate and diazo reagent, and examined for zones of activity. Negative sera produce a colored zone of over 1 cm in diameter in the non-inhibitor-containing gel, and almost no color in the inhibitor-containing gel. Positive sera produce about the same sized colored zone in each gel. Homozygous "silent" sera produce no color in either gel. It is claimed that only sera from carriers of the atypical gene will produce color. Simpson and Kalow (S28), using this agar diffusion screening method of Harris and Robson (H9), found that 3% of all $E_1^uE_1^a$ individuals tested were misclassified as belonging to the Ro2-0683-sensitive group, and 4% were

in an "undecided" category. It seems probable that a variation of this screening procedure as applied by Szeinberg *et al.* (S53) has produced results that are in error to a similar degree. However, the way in which Simpson and Kalow (S28) used the screening procedure resulted in much higher reliability in detecting $E_1^uE_1^a$ individuals. Their experimental procedure called for screening parents *and* all their children. If any family member was positive by the screening procedure, all family members were tested by the dibucaine procedure of Kalow and Genest (K11). The probability of the screening procedure missing two or more positive individuals in the same family is most likely small.

On the other hand, Lee and Robinson (L18) reported that the agar diffusion procedure produced reliable results in discriminating the group containing the Ro2-0683-sensitive phenotypes from nonsensitive phenotypes. However, their group of subjects contained only the $E_1^uE_1^u$, the $E_1^uE_1^a$, and possibly the $E_1^uE_1^f$ genotypes.

Morrow and Motulsky's (M17) screening procedure consists of a tube method in which the color development in tubes with or without the inhibitor are compared. In contrast to the previously described procedure, it is claimed here that $E_1^fE_1^f$ sera will produce color. Das *et al.* (D6), using a modification of this procedure, failed to obtain reliable discrimination between the sensitive and insensitive phenotypes.

Karahasanoğlu and Özand (K16) proposed a screening procedure based upon the yellow color produced when butyrylthiocholine is hydrolyzed in the presence of 5,5′-dithiobis-(2-nitrobenzoic acid). This procedure is reported to distinguish between the $E_1^uE_1^u$ variant; the variants $E_1^aE_1^a$, $E_1^aE_1^f$, and $E_1^uE_1^f$ as a group; and the "silent" homozygotes. Because the procedure is based strictly upon the enzyme activity present, it is not capable of distinguishing between the presence of an enzyme variant and a low enzyme activity produced by other causes. The test consists of measuring the amount of time required for the yellow color of the test solution to match the color intensity of a suitable standard.

Swift and La Du (S52) proposed a screening method based on the color change of the indicator phenol red by the acid produced upon hydrolysis of benzoylcholine. This method does not reliably distinguish between the $E_1^uE_1^u$ and $E_1^uE_1^a$ genotypes.

Evans and Wroe (E22) conducted a study of the hydrolysis rates of four commonly used substrates as related to cholinesterase genotypes and succinylcholine hypersensitivity. They concluded that the propionylthiocholine hydrolysis rate is capable of detecting over 90% of hypersensitive individuals, with no false positives. The cutoff value was the mean for the usual phenotype minus 2.5 SD. Acetylcholine and butyrylthiocholine were slightly inferior, and benzoylcholine was almost useless. If

the cutoff point were reduced to the mean minus 2.1 SD, almost 4% of the hypersensitive individuals would still be missed, and the false positive incidence would increase to 12%. However, a screening method that is incapable of detecting all susceptible individuals is generally considered not acceptable.

Dietz *et al.* (D14) have demonstrated that a commercially produced cholinesterase test paper is not suitable for use in screening.

Dietz *et al.* (D15, D16) have proposed that the assay system used in their procedure for detecting genetic variants could be used as a screening procedure (Section 6.4). Although Price and Brown (P14) concur with this proposal, very few data concerning the efficiency of this screening procedure have been published.

6.3. Detection of Cholinesterase Variants

A plethora of methods has been proposed for detecting cholinesterase genetic variants. In most cases, the proposed methods have not been widely adopted, and therefore, there are insufficient data on which to judge the effectiveness of the new methods when used in other laboratories. Also, there are two quite different reasons for determining whether a genetic variant is present and for identifying the variant. One way in which this type of determination is commonly used is in a clinical setting, where the physician decides it is necessary to determine whether a patient might be subject to prolonged apnea following a standard dose of succinylcholine. The other common reason for this type of determination is in the field of population genetics, in which it is desired to determine which genes controlling the cholinesterase variants may be present, and the frequencies with which these genes occur in the population under study. The requirements of these two uses of cholinesterase variant detection are quite different. In the clinical setting a "yes" or "no" answer to the question concerning increased sensitivity to succinylcholine is necessary, along with an identification of the variant. In the case of population genetic studies, the investigator usually strives to identify accurately the genes controlling cholinesterase variants in the population, and the frequency with which each gene occurs.

In determining the type of cholinesterase variant present in a given specimen, the ideal situation would be one in which a single, simple test would detect and identify all variant forms of the enzyme. No such test currently exists for the serum cholinesterase variants. In fact, it is not even known how many different kinds of cholinesterase variants exist in the human population. It has often been noted that the range of serum

cholinesterase activities measured in populations considered to be of the usual phenotype is very broad. While part of the variability is undoubtedly environmental in origin, it still appears likely that a substantial portion of the variability is caused by genetic factors, such as genetic control over the quantity of enzyme produced or the type of enzyme molecule produced. Although little is known about the amino acid compositions or sequences of the cholinesterase variant molecules, the situation may be analogous to the case of human hemoglobin, where the genetic variants are usually formed by the substitution or deletion of a single amino acid. Potentially, the number of cholinesterase variants may be very large, and indeed, there is evidence that a number of previously unrecognized variants do exist, as discussed in Section 2.

6.3.1. *Methods Based upon Substrate Hydrolysis Rates*

One of the earliest attempts to detect and identify cholinesterase variants was based on the observation that individuals who had experienced prolonged apnea tended to have low levels of cholinesterase activity as measured with benzoylcholine. Methods for detecting susceptible individuals by activity measurements have been proposed by Evans and Wroe (E22) and by Dietz *et al.* (D15, D16) (Section 6.2). However, it is generally agreed that determinations based on the amount of enzyme activity present, using a single substrate, are not very efficient for detecting overly sensitive individuals and are of very limited value for determining phenotypes. Fishtal *et al.* (F2) have proposed measuring hydrolysis rates of succinylcholine as a more direct way to detect patients who may be overly sensitive to the effects of this agent. Although there was little overlap with this method among the usual, intermediate, and atypical phenotypes as compared with a more standard method, there was one conspicuous expection out of the 58 individuals studied. Furthermore, only limited data were presented concerning the relationship between sensitivity to succinylcholine and the hydrolysis rate of the drug.

Methods based upon measuring substrate hydrolysis rates of more than one substrate are, in principle, better able to detect differences among various kinds of phenotypes. Investigations of this type have been performed by Smith and Foldes (S32) and by Becker (B11). Each laboratory measured the hydrolysis rates of acetylcholine, benzoylcholine, procaine, and tetracaine, and measured dibucaine numbers. These authors concluded that the ratio of the hydrolysis rates of procaine/tetracaine was useful for differentiating the $E_1^uE_1^u$, $E_1^uE_1^a$, and $E_1^aE_1^a$ genotypes. However, Becker (B11) also noted that this was truely useful only in the absence of the fluoride variant gene—an absence he recommended should be determined separately by means of inhibition measurements.

6.3.2. *Methods Based upon Use of Inhibitors*

Several compounds of different classes have been found to be inhibitors and/or activators of serum cholinesterase, and to be useful for distinguishing among some of the cholinesterase variants. These include inorganic anions, tertiary amines, quaternary ammonium compounds, alkyl alcohols, organophosphorus compounds, and carbamates. However, only a few compounds have been extensively used in many laboratories to distinguish variants.

Some of the compounds that have been reported to be capable of distinguishing some of the variants, but which have either been tested on an insufficient number of variants or have been used in an insufficient number of laboratories include sodium chloride (G6, W10), *n*-butanol (W9), formaldehyde (W12), solanine and solanidine (H14), urea (H1, H2), sernylan (B10, B11), Ro2-0683 (B11), and others (K5, K10). Double testing with Ro2-0683 and butanol (D8), and with urea and dibucaine (H1) have each been reported from one laboratory.

6.3.2.1. Succinylcholine as a competitive substrate. Because succinylcholine is responsible for producing prolonged apnea in some patients, several groups have thought that better differentiation of hypersensitive phenotypes might be possible if the drug itself is used as a competitive substrate of a second substrate. This operation is not difficult to perform if the hydrolysis of succinylcholine does not interfere with the assay of the second substrate. McComb *et al.* (M2) devised a spectrophotometric procedure capable of differentiating the usual, intermediate, and atypical phenotypes by determining the inhibition of the hydrolysis of *o*-nitrophenylbutyrate by succinylcholine. The percentage of inhibition of the formation of the yellow *o*-nitrophenylate anion was called the succinylcholine number (SN).

A somewhat different method of determining an SN value was reported by King and Griffin (K25). The inhibition by succinylcholine of the rate of hydrolysis of benzoylcholine was determined spectrophotometrically using sera from six phenotypes. When the SN values so determined were compared with dibucaine, fluoride, and chloride number values, it was found that the best differentiation among the six phenotypes occurred when the SN values were plotted against the fluoride numbers. There was good correlation between the succinylcholine and dibucaine numbers. A method involving the use of a programmable calculator to assign phenotypes using the four inhibitor numbers has been reported (B39).

A similar method of determining SN values was reported by Raj *et al.* (R1). These authors also noted a good correlation between suc-

cinylcholine and dibucaine numbers, except for three patients out of the 50 studied. These three had dibucaine numbers typical of the usual phenotype, but had SN values typical of usual–atypical heterozygotes and had experienced prolonged apnea after the administration of succinylcholine.

Although the SN may be of value for genetic studies, it has not been widely used under standardized conditions in clinical situations.

6.3.2.2. *Differentiation based on dibucaine inhibition.* The most commonly used agent for differentiating serum cholinesterase variants is dibucaine, which acts as an inhibitor of the enzyme (Sections 2.2 and 6.2). Its use for the differentiation of cholinesterase variants was first introduced by Kalow and Genest (K11) in 1957, and it has been and continues to be extensively used for this purpose. The assay is performed in two cuvettes, one with substrate alone and one with substrate plus dibucaine. Both cuvettes contain M/15 phosphate buffer (Sorensen) at pH 7.4 and 5×10^{-5} mol/liter benzoylcholine chloride. One of the cuvettes contains 1×10^{-5} mol/liter dibucaine. The initial velocity at 26°C is measured in both cuvettes by determining the rate of change of absorbance at 240 nm. The dibucaine number, DN, is the percentage of inhibition by dibucaine, and is calculated as $DN = 100(v - v')/v$, where v is the uninhibited velocity and v' is the velocity measured in the presence of the inhibitor. DN values obtained by this method for the most widely studied cholinesterase variants are presented in Table 1 (see Section 2.1).

Since the introduction of this assay procedure, a host of variations of the procedure have been reported. Many of these variations are purported to be improvements, but this is not always obvious. The most frequent variations involve using a different substrate in place of benzoylcholine. In general, with the assay conditions reported (K11), the results obtained with other substrates tend to parallel those obtained with benzoylcholine, although it has been claimed that the efficiency of differentiation varies with the substrate and in certain cases, with the genotype (B27). Some of the other substrates used for DN determinations include acetylcholine (B2, B27, G3), butyrylcholine (B27, G4), propionylthiocholine (D15–17, G3), and succinylcholine (A3). However, most laboratories have used the procedure of Kalow and Genest as briefly described above (K11). A procedure using propionylthiocholine is described in Section 6.4.

6.3.2.3. *Differentiation based on fluoride inhibition.* None of the reported methods utilizing dibucaine as an inhibitor is capable of differentiating all the known cholinesterase variants. Therefore, fluoride ion is also commonly used to help distinguish the genetic variants. When

used alone, neither dibucaine nor fluoride ion is capable of distinguishing all the reported variants. However, when the dibucaine number is used in conjunction with the fluoride number, it then becomes possible to differentiate additional variants (Section 5.1.1).

The method of determining fluoride numbers, as originally described by Harris and Whittaker (H12, H13), is identical to the method for determining the dibucaine number, except that in the inhibited reaction vessel, the dibucaine is replaced by 5×10^{-5} mol/liter sodium fluoride. The fluoride number, the percentage of inhibition by fluoride ion, is calculated as described for the dibucaine number (Section 6.3.2.2). A plot of the dibucaine number *vs* the fluoride number made it possible for the earlier investigators to readily detect new genotypes involving the E_1^f gene. However, the various E_1^f heterozygotes are frequently difficult to distinguish from certain other genotypes. Part of the problem probably stems from the fact that, in the case of fluoride inhibition, temperature control during measurement of the reaction is very important (Section 6.5). Partly because of these difficulties, other procedures for determining fluoride inhibition have been proposed.

Garry *et al.* (G7) reported a procedure in which the percentage of inhibition by 5×10^{-5} mol/liter NaF when the assay was performed in 0.05 mol/liter phosphate buffer is plotted against the percentage of inhibition by 5.5×10^{-4} mol/liter NaF when the assay was performed in 0.05 mol/liter *tris*(hydroxymethyl)aminomethane, both at 25–26°C and pH 7.4. The enzymes from six genotypes were tested, and it was found that five could be distinguished from each other. Values for several other genotypes were published in a later report (G6). Several genotypes have yet to be tested using this procedure.

6.4. Comments on a Selected Method for Identification of Serum Cholinesterase Variants

A procedure for identifying certain cholinesterase variants was proposed by Dietz *et al.* (D15). After a period during which comments and discussion were offered by others working in the field, the method was published in "Selected Methods of Clinical Chemistry" (D16). This method is based upon the Ellman reaction (E10), which was used by Ellman *et al.* (E11) for the assay of acetylcholinesterase, and by Garry and Routh (G9) for the assay of serum cholinesterase. In these assay procedures, a thiocholine ester is used as the substrate. The thiocholine produced upon hydrolysis reacts with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) to yield 5-thio-2-nitrobenzoate anion and other products. The rate of the reaction may be determined by measuring the rate at which

the absorbance at 410 nm changes owing to the formation of the 5-thio-2-nitrobenzoate anion.

The assay of Dietz *et al.* (D15, D16) is performed at pH 7.6 and 37°C with 0.1 ionic strength sodium–potassium phosphate buffer. The final concentrations in the reaction mixtures are as follows: DTNB, 0.253 mmol/liter; phosphate, 25 mmol/liter; and propionylthiocholine iodide, 2 mmol/liter. To one of a pair of reaction tubes is added 1 ml of a 1:100 dilution (water) of serum. After incubation for exactly 3 minutes, 1 ml of quinidine sulfate solution (5.0 g/liter) is added to each tube to inhibit the serum cholinesterase. To the tube serving as the blank is then added 1 ml of the 1:100 serum dilution. The difference in absorbance at 410 nm between the blank and test solutions is read within 30 minutes.

Two other pairs of tubes are prepared to determine the percentage of inhibition by dibucaine (concentration in reaction mixture: 0.03 mmol/liter) and by sodium fluoride (concentration in reaction mixture: 4 mmol/liter). In each case one out of the pair of tubes serves as the blank. The inhibitor is added to all tubes with the substrate. The remainder of the assay procedure is identical to that of the uninhibited reaction. The percentages of inhibition by dibucaine (dibucaine number) and by fluoride (fluoride number) are calculated as described in Section 6.3.2.2.

For preanesthesia screening, the authors (D15, D16) recommend that any patient with a serum cholinesterase activity of less than 4 International Units per milliliter be considered likely to develop prolonged apnea if succinylcholine is administered. They also recommend that DN and FN values be determined for these patients and, where appropriate, for their family members. Others have agreed that the 4 IU/ml activity level is suitable as a cut-off point for detecting susceptible patients (P14). However, out of the 20 patients with prolonged apnea who had been tested (P14), one had a cholinesterase activity of 4.9 IU/ml. This value is about 2 standard deviations below the mean activity measured for the usual phenotype, whereas the 4 IU/ml value is 2.5 standard deviations below the mean.

The method as originally proposed (D15) was evaluated in several laboratories, and some of the comments and corrections have been included in the final version (D16). A rather extensive evaluation of the proposed method (D15) was published by Brown and Price (B40) and Price and Brown (P14). One criticism presented by these authors is that propionylthiocholine, as compared with butyrylthiocholine, has been used in too few laboratories to be made the basis of a Selected Method. In their hands (P14), the method produced coefficients of variation that were unacceptably large. The method was modified to make it more reproducible by incubating the reaction tubes in a shaking water bath

and measuring the change in absorbance within 5 minutes after adding quinidine inhibitor. The percentage of inhibition by dibucaine and by fluoride determined for five of the phenotypes varied in some instances (P14) from those reported by Dietz *et al.* (D15, D16). Also, for some of the phenotypes, the 95% confidence limits reported by Price and Brown (P14) were considerably larger than those calculated from the data of Dietz *et al.* (D15, D16). It was suggested that the use of the shaking water bath gave more rapid temperature equilibration, and that this could account for some of the discrepancies between reports of the two laboratories.

6.5. Importance of Temperature Control

One potential source of error in determining activities of serum cholinesterase in the presence and in the absence of inhibitors is failure to properly control the temperature of the reaction, or failure to reproduce the temperature used in other laboratories or on another occasion. King and his co-workers have reported the results of studies on the effect of temperature on activities of eight serum cholinesterase genotypes using benzoylcholine as substrate. It was found that the temperature of the activity maxima varied from 32 to 35°C for the $E_1^aE_1^a$ genotype to 50–51°C for the $E_1^uE_1^u$ genotype (K23, K26–28). There were considerable differences for the activity at 25°C *vs* the activity at 37°C for the various genotypes. There was also variation with temperature of the dibucaine numbers, fluoride numbers, and chloride numbers (K24). The fluoride numbers decreased considerably as the temperature was increased from 15 to 50°C. It is for this reason that precise temperature control while determining fluoride numbers is crucial. The dibucaine numbers tended to vary slightly between 15 and 25°, and then decrease as the temperature was increased. The chloride numbers changed only slightly with changing temperature. King and Dixon (K23) and King and Morgan (K26) have recommended that, for purposes of determining susceptibility to succinylcholine, all serum cholinesterase activity measurements and inhibitor studies be performed at 37°C, the temperature to which the enzyme is subjected under physiological conditions. Some laboratories follow this recommendation, but many do not. King (K22), in a published abstract, has reported activation energies for the usual, atypical, and fluoride variants of serum cholinesterase.

6.6. Recommendations

At the present time, the only reliable method for determining the genotypes of the various heterozygotes involving products of the "silent"

genes with other genes is by family studies. Although the mean activity of the "silent"-nonsilent heterozygote is usually lower than the mean activity of the nonsilent homozygote, the ranges of activity of the two genotypes usually overlap. Furthermore, the percentage of inhibition by various inhibitors is statistically nondistinguishable for the "silent"-nonsilent heterozygote as compared with the nonsilent homozygote. Thus, the genotypes of "silent" gene heterozygotes must usually be inferred from family studies.

Given the limited amount of experience accumulated with the method of Dietz *et al.* (D15, D16), as compared with the large amount of experience gained with the method of Kalow and Genest (K11) with the inhibitor dibucaine, and the method of Harris and Whittaker (H12, H13) with fluoride ion, it would seem prudent to proceed with caution in assigning genotypes based upon the results obtained with the new method. At the time of this writing, no one method or combination of methods can be recommended as being clearly superior to the methods that have been commonly used. All methods have problems with overlapping to some extent between the various genotypes. Regardless of the method used, the results reported in one laboratory are frequently found to be nonreplicable in all particulars in another laboratory.

Because of the various problems, the search for better methods of differentiating the cholinesterase genotypes will undoubtedly continue. Ideally, what is required is a molecular probe that is capable of interacting in a distinctly different manner with each genotypic variant of the enzyme, and which will produce an unequivocal "signal" characteristic of each genotype. At the present time, such a universal probe is not in sight.

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BIOCHEMICAL EVENTS RELATED TO PHAGOCYTOSING CELLS

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1. Introduction

1.1 Enzymatic Equipment and Metabolic Pathways of Leukocytes

The enzymatic equipment of the polymorphonuclear cell is very complete and the main metabolic pathways are present in white cells. Biochemical disorders in leukocytes may present a picture of metabolic impairment of the white cells themselves or that of general pathological manifestations in the human organism (F1). Therefore, since leukocytes can be isolated and purified with relative ease, these cells have become a valuable tool for the study or even the diagnosis of inborn errors and acquired deficiencies and disorders of ubiquitous enzymes and metabolic systems. Thirty five inborn errors of metabolism have been diagnosed with the aid of leukocytes (see H2 for a review). If the abnormality can usually be detected in the white blood cells of homozygotes, by continuously adapting the techniques, it is generally possible to detect the carrier state in heterozygotes (M6).

A decrease or increase in a number of leukocyte enzyme activities can be demonstrated in pathological situations, but the specificity of such changes and the way in which they are brought about have not been elucidated. Frei *et al.* (F2) reported a significantly diminished activity of three glycolytic enzymes in three groups of diabetics. Other aspects of the metabolism of diabetic leukocytes have been reviewed by Esman (E2). Alkaline phosphatase (EC 3.1.3.1) has been shown to increase in Hodgkin leukocytes (F2), and to be significantly different between men and women, but the function and localization of this enzyme in leukocytes remains unclear (B7).

Cellular elements of human blood contain RNases and DNases. An attempt has been made to use ribonuclease activity in the diagnosis of protein-calorie malnutrition. Indeed, in subjects suffering from kwashiorkor alkaline RNase (EC 3.1.4.22) levels in plasma and leukocytes are markedly raised and they return to normal after therapy (P1), but this activity remains unchanged in moderate form of malnourished children. Another possible role of these nucleases in the defense of the organism against infections by viral nucleic acid has been suggested (H1). More information on the specificity and mode of action of these ribonucleases is brought by Keddi (K1), who partially characterized a RNase, which specifically catalyzes the cleavage of the secondary phosphate esters of uridine 3′-phosphates in human granulocytes.

Works devoted to the study of a large variety of enzymatic activities in

leukocytes are very numerous. Some other, more specific questions of the enzymology of these cells will be mentioned in subsequent sections.

The metabolic pathways have been studied as multienzyme systems or by characterizing their individual enzymes. It has long been known that granulocytes have a high aerobic and anaerobic glycolysis compared with a low respiration (B6). Glycolysis has been shown to be decreased in newborns (J2) and in diabetic cells (E2); this observation could provide an explanation for the decreasing ability of these cells to resist infection. A Crabtree and a Pasteur effect can both be demonstrated, indicating that ATP is formed in the respiratory chain (E1). The existence of the Krebs cycle has been proved by measuring the effect of its intermediates on respiration either in intact or broken cells (M4), or by isolation of some of the cycle intermediates (malate, fumarate, and succinate) (S2). Jemelin and Frei (J1) have demonstrated that ATP production in a leukocyte mitochondrial preparation was stimulated by many substrates, succinate, glutamate, and 1-glycerol phosphate being the most active. The existence of a phosphorylation coupled to the oxygen consumption has been clearly demonstrated in the work of Nessi *et al.* (N1). The oxidation of glucose via the hexose-monophosphate shunt pathway has been studied by Beck (B5). The absence of four of the enzymes necessary for gluconeogenesis has been reported (S3). Thus, glucose cannot be synthesized from pyruvate or from amino acids.

Even if the incorporation of [^{14}C]acetate into lipids has been observed (M3), a *de novo* synthesis of fatty acid is not possible due to the lack of acetyl-CoA carboxylase (EC 6.4.1.2) (M1). The incorporation of acetate into fatty acids represents chain elongation of preformed fatty acids.

Malec *et al.* (M2) discovered the synthesis of antibacterial proteins and nuclear RNA in isolated leukocyte nuclei. In granulocytes, more than one half of mRNA is unstable, quickly and continuously renewing itself and ensuring protein synthesis (C1).

1.2 Phagocytosing Cells

Phagocytosing cells include the polymorphonuclear leukocytes or neutrophils, the mononuclear leukocytes that can give rise to macrophages in tissues, and the eosinophils. Neutrophils are the most numerous phagocytes and ensure the early tissue response to infection, phagocytosing the invading microorganisms. Bacterial killing is a complex process which includes the migration of the cells to the site of microbial invasion, recognition and ingestion of the foreign particles, and biochemical changes within the cells (S4).

Because there are some striking differences between phagocytosing

cells of different species (B3) and between cells obtained from blood and from peritoneal exudate (A1), as well as between different types of white cells (F3, B4), we shall focus our study mainly on the phagocytosing human polymorphonuclear neutrophil (PMN). Reference to other white cells will be made specifically.

The cytoplasm of mature human neutrophils contains a bactericidal battery of enzymes in roughly two populations of granules. The azurophil or primary granule is considered a special kind of primary lysosome and contains the microbicidal agents, peroxidase (EC 1.11.1.7) and lysozyme (EC 3.2.1.17), in addition to the characteristic digestive enzymes (hydrolases). The specific granules contain the remaining lysozyme, but are devoid of other components of the azurophil granules (B7).

Superoxide anion (O_2^-), which has been shown to be produced by phagocytosing PMN (B2), undergoes spontaneous dismutation as well as enzymatic breakdown by superoxide dismutase (SOD; EC 1.15.1.1), generating H_2O_2 and O_2. This enzyme has been shown to protect the organisms from the reactive superoxide radical (M5). It has been found that PMN contains the same types of enzymes as do other eukaryotic tissues. A copper- and zinc-containing SOD which is cyanide sensitive is located in the cytoplasm, whereas a mangano-enzyme, also cyanide resistant, is contained in mitochondria (D1, S1, R1). A recent study (R2) on the total activity of SOD demonstrates an increased activity in PMN of children with bacterial infections and infectious hepatitis; this effect could explain the diminished recovery of O_2^- by stimulated PMN from patients suffering from bacterial infections (see Table 3). In addition to its protective function, an alternative role has been proposed for the enzyme, involving the generation of H_2O_2, one of the oxidizing agents used for bacterial killing by phagocytes (D1, K2). However, this role is less clear, since this enzyme does not enter the phagosomes (R3). Another protective system against hydrogen peroxide injury is present in the PMN cytosol. The glutathione peroxidase (EC 1.11.1.9)-glutathione reductase (EC 1.6.4.2) system maintains low levels of H_2O_2. The glutathione peroxidase catalyzes the reoxidation of reduced glutathione by H_2O_2 (B1).

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2. Isolation and Purification of Leukocytes

Several procedures are available for the isolation of leukocytes from blood; the choice must be made according to the investigations in question. However, it must be realized that it is difficult to obtain pure populations of leukocytes without altering their integrity and viability; indeed, in any isolation procedure, removing white cells from their natural environment and bumping them around in an artificial medium always represents an aggression against their morphological, biochemical, and functional state. In addition, excessive handling of the isolated cells can also lead to changes in leukocyte metabolism. Crowley *et al.* (C1) demonstrated that centrifugation and cell washings caused a reduction in the stimulation of oxygen consumption in human leukocytes ingesting polystyrene latex. Others (W2, H1) have observed that enzymes leak out of the resting cells during incubation in isotonic medium, and the leakage is influenced by the energy level of the cell; by adding adenosine triphosphate to media used for isolation, Frei *et al.* (F1) could demonstrate a possible protective effect on the activity of some enzymes. Despite these facts, the elimination of contaminating elements is primor-

dial for quantitative biochemical studies, because significant differences between ubiquitous metabolic systems have even been shown in different white blood cell populations (W1, S1, B1).

Most of the methods for separation (reviewed in C2) are based on the different properties of the blood cells: phagocytosis of iron particles by granulocytes, adhesion of monocytes and granulocytes to a variety of surfaces, and differences in the specific gravity of the various cell types.

Most of the works devoted to phagocytosing cells use a one-unit (1 g) gravity sedimentation step with a commonly used agent, such as fibrinogen, gelatin, or dextran, for enhancing red blood cell sedimentation (S2). The plasma enriched in leukocytes is usually freed from the remaining erythrocytes by lysing them with ammonium chloride (155 mmol/liter). When the erythrocytes have been eliminated, most of the thrombocytes still remain, and this can be a source of error (F2). Since the work of Böyum (B2), many investigators rely on centrifugation on Isopaque-Ficoll discontinuous gradients, and, for the further separation of granulocytes and erythrocytes, they undertake a dextran sedimentation. Slight modifications and improvements have been brought to the basic method (W1, N1, B3), which has been shown to yield more than 95% of granulocytes (N1). Following application of the Ficoll-gradient technique, the lymphocyte population is still contaminated by monocytes and granulocytes. To avoid this, Goldrosen *et al.* (G1) preincubate whole blood with carbonyl iron and adenosine diphosphate (ADP), prior to centrifugation on the gradient. The carbonyl iron is phagocytosed by the monocytes, which can then be removed in a magnetic field, and thrombocytes agglutinate in the presence of ADP. Monocytes can also be further purified after their centrifugation on Ficoll gradient by using their adherent properties. The problem of their recovery for subsequent manipulations has been circumvented (R1) by treatment of the cells adhering to foreign surfaces with lidocaine, which releases them without alteration; scraping them from the surface causes irreversible damage.

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3. Isolation of Subcellular Particles of Leukocytes

The mature PMN contains abundant cytoplasmic granules, only a few mitochondria, a multilobed nucleus, and glycogen particles. In order to elucidate the biochemical behavior of these organized structures, homogenized cells have been submitted to fractionation.

3.1 Granules

Since the work of Cohn and Hirsch (C4), who first characterized the granules of rabbit PMN by differential centrifugation, many investigations have been devoted to human PMN. By zonal sedimentation and zonal isopycnic equilibration, Bretz and Baggiolini (B4) characterized two main populations of granules. The azurophils or primary granules, containing acid hydrolases, myeloperoxidase, part of the lysozyme, and

neutral proteases, have been identified as lysosomes; the specific granules contain the remaining lysozyme, vitamin B_{12}-binding proteins, lactoferrin, and a proteolytic enzyme, collagenase (EC 3.4.24.3) (B2). The data concerning the location of this latter enzyme are conflicting: Its presence has been reported in azurophil (K3) as well as in specific granules (M2). Biochemical investigations have shown cationic proteins to be present predominantly in the azurophil granules (W1, D3). A recent cytochemical study to detect arginine-rich cationic proteins mentions that the cationic proteins of human PMN are located only in the large "crystalline" eosinophilic granules (B5). With the aid of differential centrifugation (S2) or isopycnic sucrose gradients (W2), other authors separated human PMN granules into three main fractions.

When exposed to phagocytic and nonphagocytic stimuli, PMNs selectively release the content of their granules into the phagocytic vacuoles, where the destruction of ingested microorganisms occurs (Z2, G1). Myeloperoxidase, H_2O_2, and halide ions have been shown to be potent antibacterial (K2) and cytotoxic systems (C2). Other oxygen-dependent mechanisms using superoxide anion, hydroxyl radicals, or singlet oxygen have been described (see B1 for a review). Lysozyme, lactoferrin, and cationic proteins are bactericidic compounds which can kill microorganisms *in vitro* in the absence of oxygen (B6, O2, O1). Though the mechanism by which degranulation occurs is not known, it has been demonstrated that some regulatory agents are involved in this process, principally cyclic nucleotides, microfilaments, microtubules, and calcium (Z1, B3). In a recent paper (D2), the importance of microtubules and cyclic AMP in the control of enzyme release is questioned.

These PMN enzymes originally sequestered within their granules also appear in the extracellular fluid and have been shown to be responsible for tissue damage in inflammatory processes (B2).

Neutral proteinases, elastase (EC 3.4.21.11), cathepsin G, and a third serine proteinase seem to be the most important mediators of these effects. In addition to their degradative properties, it was found that PMN elastase and cathepsin G can stimulate lymphocyte B antibody formation *in vitro* (V1). The granules have been intensively studied for their role in the oxidative burst of the PMN; this aspect is discussed in Section 4.4.

3.2 Mitochondria

Compared with the extensive investigations that have been performed on many aspects of the metabolic processes in leukocytes, few studies have been devoted to the biochemical analysis of isolated mitochondria (reviewed in K1). This situation can be attributed to four main factors: (*a*) The difficulty in obtaining pure populations of leukocytes gives rise to

erroneous results because of contamination (J1); (*b*) the mature PMN possess fewer mitochondria than do other cells, such as these of liver or heart; (*c*) no satisfactory homogenization procedure seems to have been available, since the PMN membrane is not easy to destroy, but more drastic techniques are too traumatic for the mitochondria; and (*d*) an important contamination by granules cannot be avoided.

Nessi *et al.* (N1) succeeded in rupturing the PMN membrane by adding heparin to the cell suspension. The oxygen consumption, which was measured by a photometric method, could be stimulated by the addition of ADP. An oxygen curve in stage 3, an acceptor control index, and an oxidative phosphorylation quotient with different substrates were obtained according to the scheme of Chance and Williams (C1).

The activities of mitochondrial respiratory chain enzymes, NADH (quinone) dehydrogenase (EC 1.6.99.5), NADH (cytochrome c) dehydrogenase (EC 1.6.99.3), succinate dehydrogenase (EC 1.3.99.1), and cytochrome c oxydase (EC 1.9.3.1) in five leukocyte populations were estimated (N1).

3.3 Nucleus

As the PMN matures, the nucleus changes from the round into the characteristic lobular form. In addition, the cells released into the blood from bone marrow are not capable of further division.

In smears, the nuclear chromatin appears to be clumped and the nucleus exhibits two, three, or four segments. A higher number of segments is considered to be a sign of cellular aging or of a congenital anomaly. Nucleoli are absent (D4).

If the DNA content of white cells remains stable around 0.7×10^{-12} g of DNA phosphorus per cell in different white cell populations within a given species, the RNA content is very variable. In lymphocytes, the RNA content is twice that of granulocytes (C3).

Most investigations on DNA and RNA content, synthesis, or catabolism in leukocytes have been performed to evaluate differences between leukemic and normal cells (T1, S1, M1).

Very few studies concerning the isolation of nuclei have been reported in the literature. The main reason for this is the lack of a method for disrupting the membrane without alteration of the intracellular organelles. Nuclei from guinea pigs have been isolated by the use of a combined osmotic-mechanical technique (D1). In a recent work, the nuclei from bone marrow and peritoneal exudate cells of guinea pig were extracted with citric acid 0.05 mol/liter and homogenized by hand in a Potter-Elvehjem homogenizer (R1). It was found that the synthesis of chromatin

proteins and the RNA content of the nucleus decrease markedly during maturation. However, the amount of histone, nonhistone protein, and phosphoprotein was not different in chromatin of immature and mature granulocytes, which suggests that the rate of turnover of these proteins must decrease significantly as maturation proceeds.

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4. Biochemical Mechanisms Involved in Phagocytosis

4.1 Recognition and Opsonization

How does a phagocyte recognize its "prey"? It is known that the process of recognition and ingestion are facilitated by humoral factors, immunoglobulin G, and heat labile opsonins, especially a fragment of the

third component of the complement (C_3) (G2, J3). The attachment of such opsonin-coated particles on the phagocytic surfaces has led to the concept of receptors for immunoglobulins and complement (H5, S2). However, some particles (e.g., latex) are already ingested in the absence of serum components, which means that the phagocyte should be covered with many specific receptors. Contrary to this point of view, another group (S7, S6) felt that opsonins were not essential for phagocytosis. Their theory is usually summarized in terms of surface phenomena as recently reviewed (O3): The more the surface of a particle is hydrophobic compared with that of the phagocyte, the better it is ingested.

Thus, although the basic biochemical mechanisms by which a particle activates serum opsonins are not yet established, it is known that complement deficiency is frequently associated with increased susceptibility to infections (A3). Measurement of serum activation is one way of detecting phagocytic dysfunction as a consequence of opsonic disorders (S10), such as C_3 or immunoglobulin deficiency.

Different methods can be used to evaluate the opsonic activity of a serum. Stossel (S10) measured the initial rate of ingestion of paraffin oil droplets coated with *Escherichia coli* lipopolysaccharides after their pretreatment with serum; in addition to the detection of opsonic deficiencies, he found that leukocytes of patients suffering from bacterial infections revealed an increased ingestion rate. Allen (A1) employed the initial chemiluminescent response from stimulated leukocytes. He used serum pretreated with different concentrations of *E. coli* to remove various amounts of opsonins by absorption; these treated sera were used to opsonize fresh bacteria for initiating phagocytosis, and the decreased chemiluminescent intensity observed was found to be proportional to the quantity of bacteria used for the absorption of opsonins. The time required for initiating the enhanced oxygen consumption by zymosan-stimulated neutrophils has been shown to reflect the opsonic activity of a serum (M5). Figure 1 shows how the latency period varies when oxygen consumption in the same preparation of neutrophils is stimulated with nonopsonized zymosan and serum in the medium (curve A) or with preopsonized zymosan and no serum (curve B). The delay in response varies for different conditions (Table 1). Preopsonization of zymosan with autologous serum reduces the delay from 7.9 to 0.9 minute, but does not abolish it. When pooled serum is used for opsonization, a slight increase in all delays is observed, possibly due to storage of the serum at $-20°C$, which might alter the activity of the complement pathway. The lag period is also modified when the composition of the medium is changed. Indeed, when a nonagitated medium which contains dextran replaces the buffer (Table 1C), the mean delay in response to stimulation is 4.5

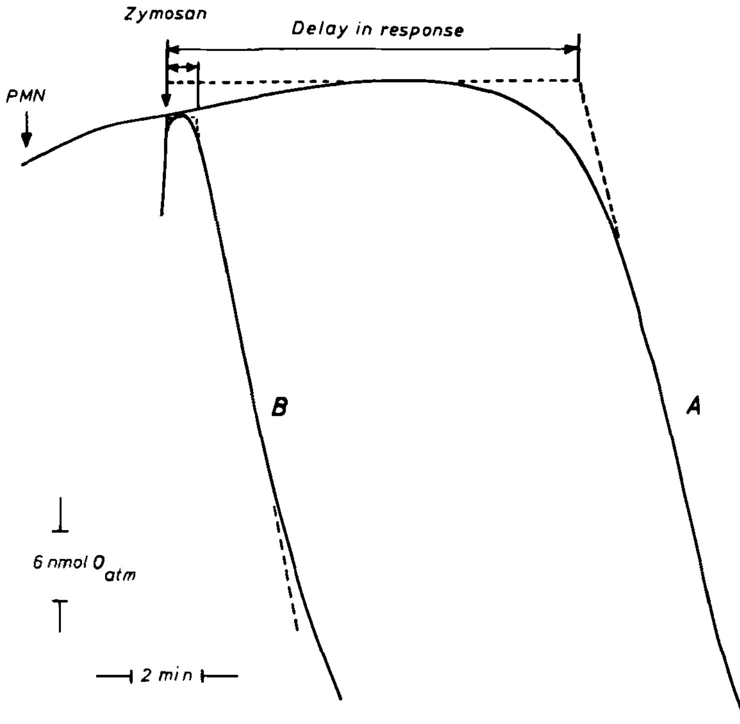

FIG. 1. Continuous monitoring of O_2 consumption. Conditions as in Table 1. (A) Nonopsonized zymosan; (B) opsonized zymosan. The rate is determined from the linear portion of the curve. Representation of original tracings. O_{at}, atomic oxygen.

minutes, as compared with the 7.9 minutes measured in a stirred glass cuvette. It has been demonstrated that dextran could mimic the opsonic effect of C_3 without directly stimulating the ingestion (E1). Its effect on opsonization could be the establishment of a better contact between particles and phagocytes. When calcium and magnesium are omitted from the medium, extra oxygen is consumed after a latency period of more than 20 minutes, when nonopsonized zymosan initiates phagocytosis in the presence of serum. The stimulation with preopsonized zymosan in a medium free of these divalent cations induces a greatly reduced oxygen uptake. The latency period and oxygen uptake are restored to their initial levels only after additions of both cations; these observations suggest that Ca^{2+} and Mg^{2+} are required not only for the activation of the alternative complement pathway, but also for the initiation of the respiratory burst. According to Stossel (S11), opsonins and divalent cations may activate ingestion rather than cell–particle affinity by acting on the contractile proteins of the leukocytes.

TABLE 1
RATE OF OXYGEN UPTAKE AND DELAY IN RESPONSE TO ZYMOSAN-STIMULATED PMN

Serum	Oxygen consumption (nmol O_{at}/minute/10^6 PMN)			Delay in response (minutes)		
	Mean	SD	*n*	Mean	SD	*n*
(A) Nonopsonized						
Autologous	18.16	6.69	10	7.9	2.0	10
Pooled	11.55	5.78	10	10.0	1.4	10
(B) Opsonized						
Autologous	10.19	2.42	9	0.9	0.4	9
Pooled	8.57	2.14	9	1.5	0.6	9
(C) Nonopsonized[a]						
Autologous	11.45	3.08	27	4.5	1.0	27

[a] Values from (M4).

A,B,C: O_2 consumption, PMN isolation, zymosan opsonization as in (M4).

A,B: Measurement in a stirred glass cuvette (B5) of 0.24-ml final volume. Medium for O_2 measurements: Phosphate-buffered saline (PBS), 110 mmol/liter NaCl; 2.2 mmol/liter KCl; 5.7 mmol/liter Na_2HPO_4; 1.2 mmol/liter KH_2PO_4; 0.7 mmol/liter $CaCl_2$; 0.9 mmol/liter $MgCl_2$; 13.4 mmol/liter glucose pH 7.4; 0.060 mmol/liter HbO_2; (considering the monomer); serum 10% (vol/vol) with nonopsonized zymosan.

A: PMN resuspended in the above PBS, paired experiments.

B: PMN resuspended in the above PBS free of Ca^{2+}, Mg^{2+}, and glucose.

A,B: PMN in the cuvette: 0.5–1.1 (10^6). Injected zymosan: 2 mg.

The latency period varies similarly if the production of the superoxide anion is measured by continuously monitoring cytochrome c reduction under the same conditions (Fig. 2). A comparative study of the latency period for oxygen consumption and superoxide production is illustrated in Table 2. In both cases, pooled serum is not as efficient as autologous serum for opsonization. In addition, the omission of calcium and magnesium has the same effect as that recorded with the measurement of oxygen uptake, and EDTA completely abolishes either the oxygen uptake or the superoxide production. These results suggest that a similar triggering mechanism could be postulated for these two events.

Differences in opsonic activity occur between normal serum and serum from infected patients (Table 3). The infected serum produces the same diminished latency period on normal cells as on cells from infected subjects. Normal serum induces the same response either with normal or "infected" cells. The rates of O_2 consumption and O_2^- production are slightly increased when the stimulation is initiated with serum from infected subjects; this observation suggests that the efficiency of the opsonic activity determines the metabolic response of the cells. The ability of PMN to produce a normal respiratory burst depends upon both humoral and

TABLE 2
RATE OF SUPEROXIDE ANION PRODUCTION AND DELAY IN RESPONSE TO ZYMOSAN-STIMULATED PMN COMPARED WITH THE RATE AND DELAY OF OXYGEN UPTAKE[a]

Opsonized serum	Rate of oxygen consumption (nmol O_{at}/minute/10^6 PMN)			Delay in response (minutes)			Rate of superoxide production (nmol O_2^-/minute/10^6 PMN)			Delay in response (minutes)		
	Mean	SD	*n*	Mean	SD	*n*	Mean	SD	*n*	Mean	SD	*n*
Autologous	10.56	2.38	9	0.9	0.3	9	4.74	2.13	9	0.6	0.2	9
Pooled	9.90	3.52	9	1.5	0.6	9	3.69	2.58	9	1.0	0.3	9

[a] Conditions as described in Table 1. Superoxide anion production was measured by following the reduction of cytochrome c at 546 nm using a $\Delta\epsilon$ = 8.1 $cm^2/\mu mol$ (M3). Cytochrome c concentration in the cuvette, 0.1 mmol/liter.

TABLE 3

EFFECT OF CROSSREACTING SERUM OF NORMAL CONTROLS AND INFECTED PATIENTS ON OPSONIC ACTIVITY, OXYGEN CONSUMPTION, AND SUPEROXIDE RADICAL PRODUCTION[a]

Serum	Normal cells				Infected cells			
	Rate of O_2[b] consumption	Delay in response (minutes)	Rate of O_2^-[c] production	Delay in response (minutes)	Rate of O_2 consumption	Delay in response (minutes)	Rate of O_2^- production	Delay in response (minutes)
Normal								
(1)	6.02	11.8	2.64	9.3	6.70	11.1	1.74	8.9
(2)	7.53	11.6	1.18	8.8	7.08	12.1	0.84	6.5
(3)	7.44	9.2	3.52	8.4	4.22	7.8	0	—
Infected								
(1)	4.98	6.7	2.82	3.9	7.19	6.9	1.74	3.3
(2)	10.68	4.8	4.50	3.8	8.42	6.4	2.31	2.2
(3)	13.68	1.1	5.88	1.9	11.07	1.3	2.41	3.5

[a] Conditions as described in Tables 1 and 2; individual values from paired experiments.

[b] In nmol O_{at}/minute/10^6 PMN.

[c] In nmol O_2^-/minute/10^6 PMN.

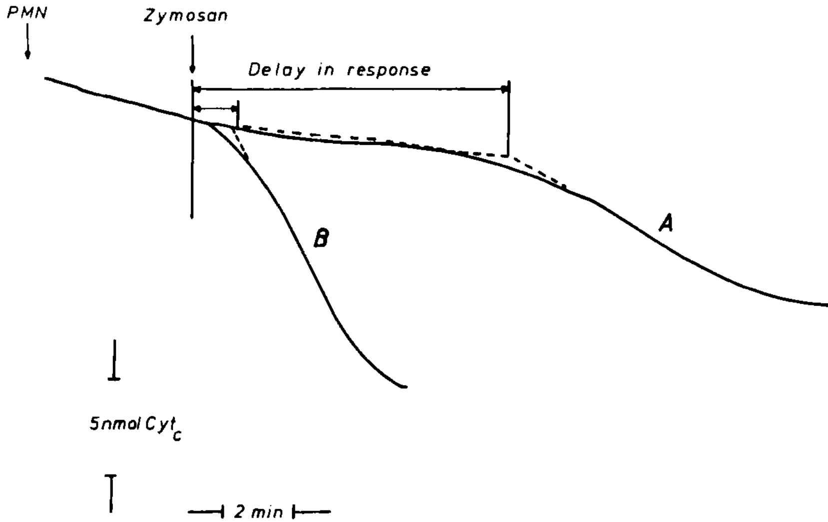

FIG. 2. Continuous monitoring of O_2^- production. Conditions as in Table 2. (A) Nonopsonized zymosan; (B) opsonized zymosan. The rate is determined from the linear portion of the curve. Representation of original tracings.

cellular factors. Our measurements allow the detection of opsonic as well as cellular defects.

The role of complement factors with respect to opsonization and phagocytic function has led to the development of a number of new tests which take into consideration criteria other than counting ingested particles under the microscope (Y1, W5, L1). However, even when a defective opsonization has been recognized, the question of the biochemical level of the opsonic defect remains to be elucidated.

4.2 ENERGY REQUIREMENT

The rate of lactate production provides data on glycolytic energy, whereas the amount of oxygen utilized by the mitochondria reflects the energy provided by oxidative phosphorylation. Under resting conditions, alveolar macrophages have relatively high level of O_2 consumption as compared with PMN (O2). Glycolysis was reported to be more intense in PMN than in monocytes (B6). It is now well accepted that phagocytosing PMN derive their energy mainly from glycolysis, whereas alveolar macrophages get their energy from mitochondrial phosphorylation (K2). These data were supported by results obtained with various metabolic inhibitors of these pathways.

By using simultaneously a glycolytic and a mitochondrial inhibitor,

Kvarstein (K6) obtained the greatest inhibition of latex uptake with a combination of 2-deoxyglucose (2DG) and antimycin or oligomycin, demonstrating that the energy may be required from both glycolysis and respiration, the main source being glycolytic energy.

Boxer *et al.* (B7) observed that ATP levels remained elevated in guinea pig PMN incubated with 2DG, potassium cyanide, and pyruvate, which is consistent with the idea that energy could be derived from sources other than glycolysis or oxidation in the Krebs cycle. Activation of the respiratory burst is an energy-requiring process. 2DG inhibits the enhancement of oxygen uptake only if present in the medium before the stimulation. If it is injected once the burst is initiated, no effect is observed (M5). In digitonin-stimulated guinea pig PMN (C4) 2DG inhibited the O_2^- generation only partially, whereas the addition of cyanide and dinitrophenol caused complete inhibition. Again, this effect occurred only when the inhibitors were added before complete activation of the oxidase system; such results suggest a possible role for mitochondrial ATP formation, and indicate that the activation process is energy dependent.

The contractile proteins, actin and myosin, have been discovered in phagocytes (B8). Their interaction could explain at least part of the energy dependence of phagocytosis (S12). Creatine kinase (EC 2.7.3.2) is present in human lymphocytes, PMN, and macrophages of guinea pig exudate, so that creatine phosphate could represent another source of energy (M4).

4.3 Metabolic Alterations

Upon phagocytosis of invading microorganisms, PMN undergo perturbations of most of the metabolic pathways which were described in Section 1.1 for resting conditions.

Anaerobic glycolysis is greatly enhanced, whereas the aerobic process is only slightly affected during ingestion of killed *Staphylococci albi* and *Salmonellae typhi* (F1). Glycogen breakdown was shown to be increased during phagocytosis (S9).

Another pathway of glucose utilization as measured by [1-^{14}C] glucose oxidation is the hexose monophosphate (HMP) shunt, which is stimulated by the addition of particles (S1). How this activation occurs is not yet settled, and a number of mechanisms involving different enzymes have been put forward (R10) to explain a possible link between the activity of the oxidase responsible for the increased oxygen consumption and the pentose cycle. The coupling between H_2O_2 and the HMP shunt via the glutathione redox system has recently been demonstrated in PMN deficient in glutathione reductase (R6).

During phagocytosis, new phospholipids synthesized from triglyceride may be utilized to form the membrane of the phagocytic vesicles (S8).

It could be shown that as much as 35% of the plasma membrane was involved in the formation of phagocytic vesicles (T5). It has been found (K3) that if PMN are able to ingest particles when RNA and/or protein syntheses are blocked (C2), a 18–26% decrease in phagocytosis occurs, which means that PMN continue to synthesize new proteins to maintain their phagocytic function at full capacity.

4.4 The Stimulated Oxygen Metabolism

Apart from the increases in metabolic rates occurring during phagocytosis of particles discussed in Section 4.2, the most striking change remains the marked stimulation of oxygen consumption which was first described by Baldrige and Gerard in 1933 (B4). Since this pioneer work, a great number of investigations has accumulated using different methods and stimuli (Table 4). The various conditions for PMN isolation and procedures used for the study of the phagocytic function are responsible for the variability of the results obtained by different authors (Table 4). The data in Table 4 reflect the ability of the phagocyte to enhance its oxygen uptake from 2 to more than 15 times the basal rate.

Iyer *et al.* (I3) demonstrated that part of this oxygen consumed was converted into hydrogen peroxide (H_2O_2), and several groups have confirmed that PMN release large quantities of H_2O_2 (H4, R8). The discovery by Babior *et al.* (B1) that a proportion of the oxygen consumed was converted to superoxide radical (O_2^-) initiated great interest in this phenomenon (C6, C7, B3, W2, S5, T1, G4). The relationship between the oxygen and its metabolites has been worked out from stoichiometric studies which revealed that the superoxide anion is an important product of oxygen reduction which was then further converted to H_2O_2 by dismutation in a reaction sequence O_2, O_2^-, H_2O_2 (R5). By treating the phagocytes with cytochalasin B, which prevents vacuolization of the membrane, it could be demonstrated that all the O_2 consumed was recovered as O_2^-, which then dismutates into H_2O_2 (R7). However it should be pointed out that the recovery of O_2^- and of H_2O_2 is not complete. Indeed, studies of phagocytosing leukocytes have revealed pathways for O_2^- utilization other than dismutation, such as oxidative attack of membranes containing polyunsaturated lipids (K4), or the participation of O_2^- in reactions with H_2O_2 to produce the hydroxyl radical and/or singlet oxygen (T2, G6, K5). From the measurements of maximal rates of both oxygen uptake and O_2^- production during continuous recordings (Fig. 3) it is possible to obtain good recovery of O_2^- (Table 2).

TABLE 4
SURVEY OF SOME DATA FROM HUMAN PMN OXYGEN CONSUMPTION[a]

Method	Stimulus	Resting O_2 consumption	Stimulated O_2 consumption	References
lanometric	*E. coli*	2.75	7.40	C5
lanometric	Zymosan	—	1.12	R9
lanometric	Latex	—	1.16	B1
lanometric	*Salmonellae typhi*	1.10	2.24	F1
:lark electrode	Zymosan	—	2.80	R1
:lark electrode	Zymosan	0.14	0.82	B3
:lark electrode	Zymosan	0.10	0.70	G6
:lark electrode	Latex (0.81 μm)	0.16	2.25	W1
:lark electrode	Latex (0.81 μm)	0.14	0.73	B3
:lark electrode	Latex (1.1 μm)	0.90	2.00	K7
:lark electrode	Latex (1.1 μm)	—	5.68	C5
:lark electrode	Heat-killed *E. coli*	—	3.35	N1
:lark electrode	Latex-coated IgG	0.33	3.50	S3
:lark electrode	Latex-coated IgG	0.10	1.90	G6
:lark electrode	Phorbol myristate acetate	0.10	1.20	G6
:lark electrode	Oil red O	3.70	12.40	O1
'hotometric	Zymosan	0.34	5.70	M5
'hotometric	Zymosan	—	5.10	This work

[a] The values are recalculated on the same basis unit: nmol O_2/min/10^6 PMN, but the linearity of the ction is not guaranteed in every case.

The maximal rates provide an estimate of the burst before the phagocytic vacuoles are sealed; it has been shown that the rate of PMN respiration is enhanced before microorganisms could be detected within completely fused vacuoles (Z1). Furthermore, when measuring these parameters after 5 or more minutes, dispersion of the oxygen metabolites and recycling of O_2 when O_2^- undergoes dismutation become more important. The production of activated oxygen, especially singlet oxygen, has been postulated to be responsible for the light generated in phagocytosing PMN (A2, J2). Later, it was demonstrated that light emission was a result of reactions between certain unspecified constituents of the ingested particles and some or all of the oxidizing agents, H_2O_2, O_2^-, $OH^\bullet$, and singlet oxygen, produced by the stimulated cells (C1).

From the flood of results obtained by measurements of the different manifestations of the respiratory burst—O_2 consumption; O_2^-, H_2O_2, and $OH^\bullet$ production; and chemiluminescence—some general features can be outlined which are common to all these events, suggesting that they could all depend on a single initial triggering mechanism.

All these events show a similar time course of activation, characterized by a lag period, a short linear maximal rate and a late decline (Fig. 3)

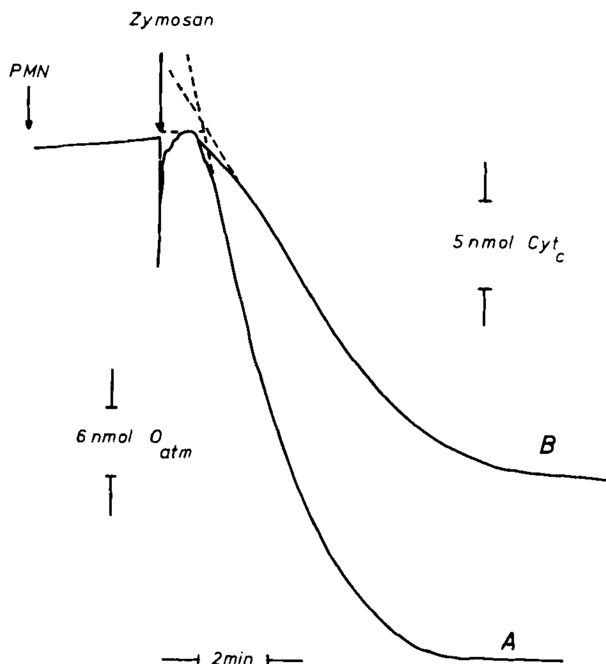

FIG. 3. Continuous monitoring of O_2 consumption (A) and O_2^- production (B) by opsonized zymosan-stimulated PMN. Conditions as in Tables 1 and 2. Original recordings from the same preparation of cells.

(S3, R8, T2, G5). The shortest lag periods, 1 minute or less, are observed with opsonized particles (see Figs. 1 and 2). Contrary to these results, it was found that if oxygen is consumed after a lag of 30 seconds, O_2^- production is almost complete by this time (S4). To our knowledge, only very few studies with continuous monitoring of more than one parameter of the metabolic burst have been performed. Most of the assays for O_2^- production are discontinuous, so that it is difficult to make accurate observations of the very early time course of activation. Cohen and Chovaniec (C3) have developed a continuous assay for O_2^- generation by guinea pig leukocytes stimulated by digitonin. They also continuously monitored the H_2O_2 production and found a similar lag time to that obtained for that of O_2^-. This lag time depends on the concentration of the stimulus and not on that of the cells (C3). Continuous spectrophotometric recording of O_2^- production by phorbol myristate acetate (PMA) and opsonized zymosan-stimulated neutrophils has been performed by Johnston and Lehmeyer (J1). The lag period is greater for the surface-active agent than for zymosan. Furthermore, it can be influenced by the PMA concentration. This same phenomenon has been observed by Curnutte *et al.* (C9) with fluo-

ride as the stimulus. The length of the delay was dependent on the fluoride concentration. It could be further shortened, though not abolished, by performing the incubation at much lower salt concentrations. The lag period of the stimulated O_2 consumption varies also with the particle concentration (Fig. 4). When serum is present in the medium and when non-opsonized particles stimulate the cells, the delay in response increases significantly (see Figs. 1 and 2) (C6, R9); under these conditions, this increased lag time is required first for particle opsonization (see Section 4.1) and then for the activation of the system.

Oxygen uptake, O_2^-, H_2O_2, $OH^{\bullet}$ production, as well as chemiluminescence, are stimulated by many agents, particulate or soluble, which include bacteria, latex, and zymosan (Table 4) (C6, B3), PMA (J1, G6, R2), ionophores (W4), complement peptide C_{5a} (G3), fluoride ion (C9, H2), and a chemotactic factor *N*-formylmethionyl peptide (S5). These events have been demonstrated to be independent of phagocytosis and even of degranulation; they only require contact of the stimulus with the cell membrane (M2, R4, G3, H2, C9).

The respiratory burst is insensitive to cyanide and azide or to inhibitors of the respiratory chain, whereas it is inhibited by sulfhydryl reagents and glycolytic inhibitors (C7, I3, S1, C4).

The dependence of the burst on the divalent ions, calcium and magnesium, has been demonstrated. Magnesium has been shown to have a better stimulatory effect on O_2 consumption than calcium (K7).

Stossel *et al.* (S13) found the same effect on the rate of ingestion of par-

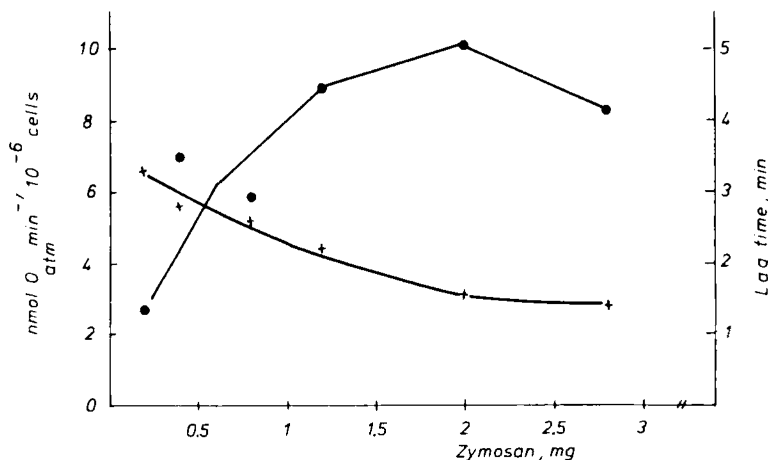

FIG. 4. The effect of the concentration of opsonized zymosan on the rate of O_2 consumption (circles) and the lag time (crosses). Conditions as in Table 1. One representative experiment.

ticles. Others have reported that calcium was more potent for stimulation of the superoxide production (C4, C9) or chemiluminescence (W4).

A decreased activity of the stimulated oxygen consumption 3 hours after cell isolation has been shown (M5). When superoxide production is measured on the same preparation of leukocytes, the decrease observed over the same period of time is comparable to that found when measuring oxygen consumption (Fig. 5). Takeshige *et al.* (T1) reported a 30% loss of activity 6 hours after blood sampling when they measured O_2^- production and a 50% loss of activity after 5 hours when they determined H_2O_2 production.

In addition to the similar behavior outlined above, the strongest evidence in favor of a unique mechanism initiating the burst remains an inherited disease, the chronic granulomatous disease, in which the increases in oxidative metabolism when exposed to particulate or soluble stimuli are completely lacking, though the cells appear to have normal ingestion (J4, K8, H4, H3). Even this convincing evidence has recently been questioned, since a disorder was described (H1) in which PMN have a defective initiation of O_2 consumption, superoxide production, and chemiluminescence during phagocytosis of particles, but respond normally to soluble stimuli.

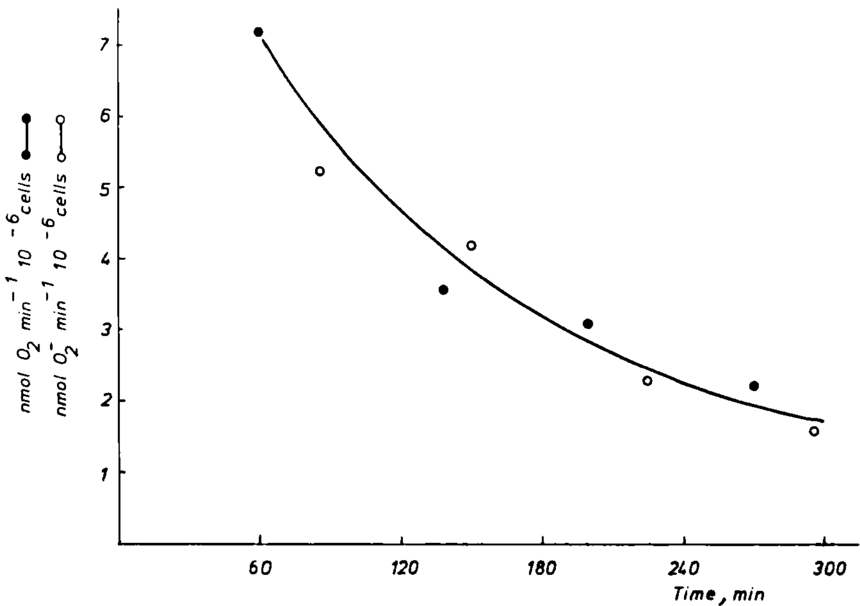

FIG. 5. Effect of storage of leukocytes at 0°C on O_2 consumption (filled circles) and O_2^- production (open circles).

In addition, two patients with recurrent bacterial infections ingested opsonized and nonopsonized particles, but only IgG-coated latex induced the stimulation of O_2 consumption, O_2^- formation, H_2O_2 production, and HMP shunt activity (W3). The turning on of the metabolic stimulation would then depend on the nature of the stimulus, which could modulate a variable membrane perturbation [the ingestion of very small particles (0.088 μm diameter) have been shown to be accompanied by no change in the resting oxygen uptake (R3)].

No situation has been reported in which the existence of stimulated oxygen metabolites was observed without increased oxygen consumption; on the contrary, it has been found (T3) that latex particles failed to stimulate superoxide production alone, although the responses of O_2 consumption, H_2O_2 production, and the HMP shunt were similar to these initiated by opsonized zymosan.

All these results would favor the existence of more than one mechanism for initiating the burst.

It is generally agreed that activation of an oxidative enzyme system catalyzing the reduction of O_2 to O_2^- is the initial step in the respiratory burst. Among the various candidates that have been proposed (reviewed in D1), the enzyme most likely to be involved is a NADPH oxidase whose activity is recovered in the particulate fraction of PMN homogenates (usually 27,000 *g*), but then only if cells have previously received a "pulse" with the same agents that trigger the oxidative burst in whole cells. This particulate fraction has been shown to consume O_2, to produce O_2^-, H_2O_2, OH^-, and chemiluminescence (P1, B2, A4, T4, K1, M1). The natural substrate of the system is still a source of controversy; NADH and NADPH can serve as electron donors, but it has been suggested that NADPH oxidation has more physiological significance than that of NADH (D1, B2, I1).

These activities are deficient in granular fractions from PMN of patients with chronic granulomatous diseases (C8). All these data provide evidence that this fraction contains the enzyme system responsible for the respiratory burst. However, the exact subcellular localization of the system is not yet settled. This fraction is heterogeneous and is composed of plasma membrane fragments, azurophil and specific granules, and occasional mitochondria (B2). By using sucrose density gradients, Iverson *et al.* (I2) found an oxidase activity in dense particulate material distinct from specific or azurophil granules and plasma membranes, whereas Dewald *et al.* (D2) using a similar sucrose density gradient centrifugation found the superoxide-forming enzyme associated with membrane fragments. In the latter study, the same distribution profiles of the oxidase activity was obtained when cells were activated by particulate or soluble

stimuli. In a recent work (G1), the extraction from the phagocyte membrane of an O_2^--forming system using NADPH as electron donor has been reported.

Purification and reconstitution of the enzyme system will be required before elucidation of the mechanism of its activation.

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5. Paraphysiological Changes and Exogenous Agents

The process of phagocytosis is affected by a large number of conditions and agents. Indeed, in many situations, there is a change of susceptibility to infections.

It has been reported that peripheral blood leukocytes isolated from pregnant women have phagocytic and bactericidal activities greater than those in leukocytes from non-pregnant women. An increased myeloperoxidase activity was also found in phagocytosing leukocytes from pregnant women (M1). Others found that the capacity to reduce nitroblue tetrazolium was depressed in PMN from pregnant women and that pregnancy serum inhibited phagocytosis of *E. coli* by control PMN, while neutrophils from pregnant women showed increased chemiluminescence during phagocytosis of opsonized zymosan. A normal chemiluminescence was obtained by control PMN stimulated with zymosan preopsonized in pregnancy sera (B1).

X-irradiation and impaired metabolism of leukocytes from guinea pig exudate have been demonstrated (S1).

A variety of pharmacological agents have been investigated. The administration of corticosteroids is usually associated with enhancement of infections. It was found that bactericidal activity and HMP shunt activity of leukocytes were depressed following ACTH administration, whereas the glycolytic activity was not altered (S2). As a concomitant, increased

level of circulating cortisol was measured; this latter compound could also have been responsible for the impaired bactericidal activity.

Hydrocortisone was demonstrated to depress the bactericidal capacity of PMN *in vitro*, while methylprednisone failed to diminish neutrophil function below that of controls (F1). It is possible that a diminished risk of infection could be expected with the use of methylprednisone. However, the precise mechanisms of all these observed effects have not been defined.

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6. Pathological Deviations

The leukocyte response is inadequate in a great number of clinical conditions. Most of the disorders described have been associated with an increased susceptibility to infections. However, the biochemical or molecular level of the various defects have not been clearly established, except for some genetically determined deficiencies. A few examples of neutrophil abnormalities will be given in the two subsequent sections, but for a more complete insight into PMN phagocytic disorders, general reviews and monographs are available (K2, B2, W1, W3, K3).

6.1 Acquired Diseases

Phagocytic dysfunction is known to occur during the course of a large number of acquired conditions, but the relationship between the altered function of the PMN and the clinical state of the patient is difficult to find out in many cases.

PMN from patients with alcoholic cirrhosis were shown to have defec-

tive chemotaxis, defective O_2^- production, and defective iodination of bacteria; defective ingestion was less frequently observed. Serum of patients with cirrhosis diminished PMN cytochrome c reduction. Serum-independent PMN abnormalities were also found, in addition to the serum-induced abnormalities in some patients (F1). Others could demonstrate that serum from patients with severe cirrhosis have diminished bactericidal activity against *E. coli* (F2). The abnormality was associated with decreased immunoglobulin M bactericidal activity and not with deficiencies of complement or lysozyme.

In the course of chronic granulocytic leukemia (CGL), abnormalities of granulocyte function exist. The phagocytic capacity has been reported to be decreased (P1, W2, B8) or normal (P2) in CGL. A significant decrease in the capacity of CGL neutrophils to reduce nitroblue tetrazolium (NBT) was found and this decrease was more obvious in the individuals with the Ph^1 chromosome (P2). It was observed that the initial rate of phagocytosis, O_2 consumption, and bactericidal capacity were decreased in a CGL patient group (O1). In addition, specific and primary granule protein deficiencies were found, and these deficiencies were more pronounced near blast cell transformation. The authors demonstrate that granulocyte function was progressively deteriorating during chronic phase CGL as an expression of an increasing disturbance of the differentiation process. In a study on 14 patients with untreated CGL, abnormalities observed were found to be different among the subjects (C3). Granulocytes of 4 patients were shown to be functionally in the control range with respect to particle ingestion, NBT-stimulated reduction, cyanide-insensitive O_2 consumption, superoxide anion production, hydrogen peroxide production, and iodination, while granulocytes of another group of 4 patients showed an ingestion defect associated with a decrease of all oxygen-dependent metabolic events. Granulocytes of the 6 remaining patients were in the normal range, except for iodination, which was strikingly decreased. The ultrastructural study of the PMN of one patient in the latter group showed a degranulation defect. Results concerning phagocytosis in acute granulocytic leukemia are conflicting. Defective microbicidal activity was measured in untreated patients (P2), while others failed to demonstrate a defect (B8). Although phagocytosis was reported to be normal in PMN of children suffering from protein-calorie malnutrition, bactericidal capacity was depressed (D1, S1); HMP shunt and degranulation were found to be normal, while opsonic activity was slightly increased (D1). NBT reduction was increased both in marasmus and kwashiorkor (W5, A1).

PMN of anemic subjects with iron deficiency were capable of producing normal quantities of H_2O_2 under resting and phagocytosing condi-

tions as measured by [^{14}C]formate oxidation, but in severe anemia with iron deficiency, the PMN have a reduced capacity of iodinating heat killed *E.coli* (P3).

PMN function was studied in children suffering from recurrent otitis media (G1): A depressed chemotactic response in 17.5% of 87 patients, depressed bactericidal activity in 23% of 30 patients, and depressed chemiluminescence in 15.8% of 19 patients were found. However, it is not known if PMN dysfunction is a consequence or a cause of the clinical problem.

PMN from patients with periodontis were reported to have a decreased phagocytosis of *Staphylococcus aureus*, and chemotaxis (C2). Frequent infections occur in patients with poorly controlled diabetes and it has been observed to be associated with altered granulocyte function (B3). PMN adherence, chemotaxis, phagocytosis, and microbicidal function were decreased in the poorly controlled diabetic, and these functions were corrected by treatment (B3, V1, B4). However, the exact biochemical impairment has not been elucidated.

6.2 Inherited and Congenital Diseases

A number of rare inherited conditions have been well studied and have allowed our better understanding of phagocytic basic processes, although they are far from being completely explained.

The chronic granulomatous disease (CGD) is a clinical syndrome characterized by susceptibility to pyogenic organisms without any determined defect in immunoglobulin (K1). CGD is a genetic disease inherited either as a X-linked (W4) or autosomal recessive trait (A2). The fundamental abnormality is the inability of the phagocyte to produce a normal respiratory burst (see Section 4.4) despite a normal ingestion. PMN are unable to destroy certain strains of bacteria, while those producing H_2O_2, such as streptococci and pneumococci, are readily ingested (Q1, K2). It is not known whether the impaired response of the PMN is due to a lack of the enzyme responsible for the burst, or whether the enzyme is inactive. It could be shown that lysates from the cells of CGD patients after freeze–thaw procedures were able to produce O_2^- in the presence of NADPH similar to O_2^- production by control lysates (B1). The same authors (C4) found that a cell-free system from CGD granulocytes prepared by another method failed to generate O_2^-. They concluded that the lesion lies outside the oxygen-metabolizing system, or that the granulocyte contains two separate O_2^--forming enzymes.

Hereditary deficiency of myeloperoxidase (MPO) is a very rare condition: About 13 cases from 10 families have been described, and this deficiency is an autosomal recessive disorder with varying degrees of gene

expressivity in the heterozygous state (C1). The stimulated oxygen consumption and O_2^- and H_2O_2 production have been shown to be increased in MPO-deficient cells (C1, R3). The phagocytes have a diminished fungicidal and bactericidal activity, but the defect is not as severe as in CGD (C1, L1). The accumulation of H_2O_2 or other reactive intermediates could accumulate to levels which are microbicidal and could compensate for the lack of MPO (K2). The Chediak-Higashi syndrome (CHS) is an autosomal recessive genetic disease characterized by partial albinism, the presence of giant granules in leukocytes as well as in other cells, and an increased susceptibility to bacterial infections (B5). Although the cells exhibit a normal ingestion of microorganisms, they have an impaired bactericidal activity which is linked to a defective discharge of their granule content into the phagocytic vacuole (R2). An abnormal elevated concentration of cyclic adenosine monophosphate was found in CHS PMN which could impair the motility of the granules by inhibiting microtubular assembly (B6). It was found that the addition of cyclic guanosine monophosphate to CHS PMN *in vitro* corrected the release of the lysozomal enzyme β-glucuronidase (EC 3.2.1.31; B7).

By increasing the cytoplasmic Ca^{2+} concentration with the ionophore A 23187, Zabucchi *et al.* (Z1) did not observe any enhancement in releasing the granule-associated enzymes of CHS PMN. In addition, the solubilization of MPO with Triton X-100 from the CHS granules was lower than that from the normal ones. The authors suggest that the CHS granules have a more resistant membrane and that the defective exocytosis does not likely depend on an impaired mobilization of Ca^{2+} from intracellular stores.

Loos *et al.* (L2) described a leukocytic glutathione reductase deficiency in three children of one family. The PMN glutathione-reductase activity was 10–15% of normal controls.

O_2 consumption, H_2O_2 generation and iodination were normal during the first 5–10 minutes of the burst; thereafter, these reactions stopped completely. O_2^- production as measured by cytochrome c reduction was normal. Respiration was no longer activated during phagocytosis after incubation of the cells with a H_2O_2-generating system; the addition of exogenous cytochrome c protected the cells against these oxidative damages (R1). These results suggested that the deficient cells were very sensitive to H_2O_2 toxicity either from outside or during phagocytosis.

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7. Conclusion

It is now recognized that the failure of the PMN to respond normally to invading microorganisms is associated with various defects. Indeed, the correct functioning of the defense systems against infection requires a

competent immune pathway for generation of chemotactic and opsonic factors and their recognition; chemotaxis, ingestion of foreign particles, oxidant generation, and lysozomal degranulation are interdependent components of the bactericidal process.

It can be concluded that the initiation of the metabolic burst depends on the activation of an "oxidase" by a mechanism as yet unknown. It is not known how molecular O_2 is converted to reactive products, but kinetic analyses provide observations which are consistent with a similar triggering mechanism for O_2 consumption and O_2^- production.

Further intensive studies by biochemists and clinicians are needed not only to clarify the molecular basis of the main functions of the PMN, but also to provide insight into the origin of the disorder and to allow therapeutic approaches to the dysfunctions of this cell.

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ADVANCES IN CLINICAL CHEMISTRY, VOL. 22

THE MEASUREMENT OF SERUM ALKALINE PHOSPHATASE IN CLINICAL MEDICINE

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1. Introduction

The measurement of alkaline phosphatase in serum or plasma constitutes one of the most widely employed tests in the clinical chemistry laboratory (Table 1). The subject has been repeatedly reviewed in recent years (F10, F11, K5, Mc3, P24, P29). An attempt will therefore be made in this article to list some of the more recent references and to indicate under what circumstances serum alkaline phosphatase measurements are of clinical value.

1.1. Serum or Plasma?

Ladenson *et al.* (L1), Breuer and Stucky (B43) and Fleisher *et al.* (F13) demonstrated that identical values are obtained with heparinized plasma and serum. Lum and Gambino (L20) found lower mean values in heparinized plasma than in serum but the differences were small. On the other hand, EDTA causes an irreversible inactivation of several human alkaline phosphatases (C27) and is not suitable as an anticoagulant for biological fluids if alkaline phosphatase measurements are to be made (Mc3). In this review, we shall refer to "serum alkaline phosphatase" values even if the actual measurements were made on plasma.

1.2. Patient Preparation and Collection of Specimen

It is generally considered unnecessary to keep patients fasting prior to blood collection (S59, S60, S61). A midday meal consisting of sandwiches

TABLE 1
Serum Enzyme Determinations[a]

	1964–1967	1967–1970	1970–1973	1973–1976	1976–1979
Acid phosphatase	685	860	694	621	781
Aldolase	151	221	261	262	280
Alkaline phosphatase	15,618	15,723	28,876	38,112	50,178
Amylase	538	1,061	1,755	2,411	3,308
Creatine phosphokinase	156	5,078	10,100	11,412	10,489
Glutamic-oxaloacetic transaminase	8,092	16,602	29,031	36,005	43,123
Glutamic-pyruvic transaminase	480	14,150	28,789	33,478	41,451
Lactic dehydrogenase	2,944	16,639	28,184	32,888	40,821

[a] Tests per triennium performed in the Biochemistry Department of Sydney Hospital over the period 964–1979. Sydney Hospital is a 360-bed general hospital which does not use these tests for screening rocedures of healthy individuals. Data by courtesy of F. C. Neale.

and a beverage caused no significant changes in mean serum alkaline phosphatase values if *p*-nitrophenylphosphate-based methods were used (S60, S61). Even a highly unphysiological dose of fat causes a mean rise of only 25% in the serum alkaline phosphatase values of normal subjects (K25).

Mild physical exertion (S60) and participation in normal sports activities (K21) are without effect on serum alkaline phosphatase. Prolonged bed rest causes no significant changes (H6, H22). Even a "marathon" run produced a mean increase of only 25% (R22), while the assumption of the erect posture for 30 minutes caused only minor rises (D15, S62). Complex protocols for patient preparation are therefore unnecessary from a clinical point of view.

Tourniquets do not appear to cause artefactual elevations of serum alkaline phosphatase activities provided the period of venous occlusion does not exceed 30 seconds (S62). Longer periods of occlusion may cause "tourniquet effects" similar to those seen with protein-bound calcium (R3) and other circulating proteins. This factor may well be responsible for some of the variability between specimens obtained from the same individual at different times (L10).

1.3. Separation of Cells and Storage of Specimens

A delay of 1–2 hours between venepuncture and blood centrifugation does not influence serum alkaline phosphatase values (B43). Prolonged standing "on the clot" causes minor rises (K4, N6).

Serum may be left at room temperature for relatively long periods of time. Aaron Bodansky (B29) stated that 24–48 hours at room temperature (as in mailed specimens) caused errors of ± 20%, but this has not been the experience of more recent workers. Tietz and Green (T7) found that freshly drawn human plasma showed 3–10% less activity than the same specimen left at room temperature for several hours. No further change in activity occurred after that time. Massion and Frankenfeld (M10) showed that fresh human serum may be stored at 25° for a period of 96 hours with only a 6% increase in alkaline phosphatase activity.

Bodansky (B28) found that human serum stored "in the refrigerator" for 24 hours showed a 10% increase in alkaline phosphatase activity. Kaplan and Narahara (K3) noted an increase of "5–30%" after overnight refrigeration. Massion and Frankenfeld (M10) found only a 4% increase in refrigerated serum after 96 hours. This was less than the increase noted in the same material at room temperature.

The effect of freezing and thawing must be distinguished from the effect of storage at freezing temperatures. Serum alkaline phosphatase

seems stable for long periods when frozen (M10). However, after thawing there is a time-dependent, temperature-dependent increase in activity (B46, M10), with rises of 6% over 6 hours at room temperature (M10). Some of the reported discrepancies concerning the effect of storage at -20° may be due to postthawing phenomena. After the reconstitution of lyophilized sera, the time-dependent increases in activity are even more marked and may account for discrepancies of up to 45% (B46).

1.4. Choice of Assay Methods

There are large numbers of assay methods differing from one another in types of substrates employed, substrate concentrations, buffer types, buffer strengths, pH values, and temperatures of incubation. Some methods have been officially recommended by national institutes (G11, S7) and the entire subject is reviewed in detail in a recent book (Mc3).

For historical reasons, results of alkaline phosphatase estimations have been expressed in a variety of eponymic units. Some of these are listed in Table 2, which also gives generally accepted normal ranges (D11, E3, G9, T8). Efforts are currently being made to standardize methods and to express results in units based on the "Système Internationale" (I7).

TABLE 2
Normal Ranges for Serum Alkaline Phosphatase[a]

Method	Unit	Normal ranges
Bodansky (B28)	Bodansky	1.5–4.0 units/deciliter
King and Armstrong (K20)	King-Armstrong	3.5–13 units/deciliter
Buch and Buch (B50)	Buch and Buch	2.2–6.6 units/deciliter
Shinowara *et al.* (S37)	Shinowara Jones-Reinhart	2.2–8.6 units/deciliter
Bessey *et al.* (B18)	Bessey-Lowry	0.7–2.7 units/milliliter
Babson *et al.* (B1)	Babson	11–44 units/liter
Sigma (S40)	Sigma	0.8–3.0 units/milliliter
Bowers and McComb (B36)	International[b]	6–110 units/liter
German Society for Clinical Chemistry (G11)	International[b]	30–170 units/liter
Scandinavian Society for Clinical Chemistry (S7)	International[b]	70–390 units/liter

[a] Values are approximate and were obtained in adults by different methods and are expressed in different units. If males and females are treated separately, different ranges are obtained (G4).

[b] These methods differ from one another in relation to reaction conditions (buffers, temperatures), so that different values are obtained even though all results are expressed in "International Units" (I7).

1.5. Reference Values

Serum alkaline phosphatase activities of apparently healthy individuals are not evenly distributed about a mean (Fig. 1) but are skewed toward the higher values (E10, G4, K13, R23). The biological reason for this skewness is not known. Posen (P25) suggested that it may be related to the laws governing the turnover rate of circulating proteins.

In spite of the skewed distribution of serum alkaline phosphatase values, many authors continue to speak in terms of "means and 2 standard deviations" even though such cut-off points do not identify the central 95% of observed values. Elveback *et al.* (E10) therefore suggested that the upper and lower reference limits be set at $2\frac{1}{2}$ and $97\frac{1}{2}$ centiles above and below the median value. This is standard practice in expressing other biological values, such as the height of growing children.

There are many publications concerning serum alkaline phosphatase in healthy individuals (B30, B37, D8, K13, O4, R23). In the following section, normal reference populations are divided according to age groups,

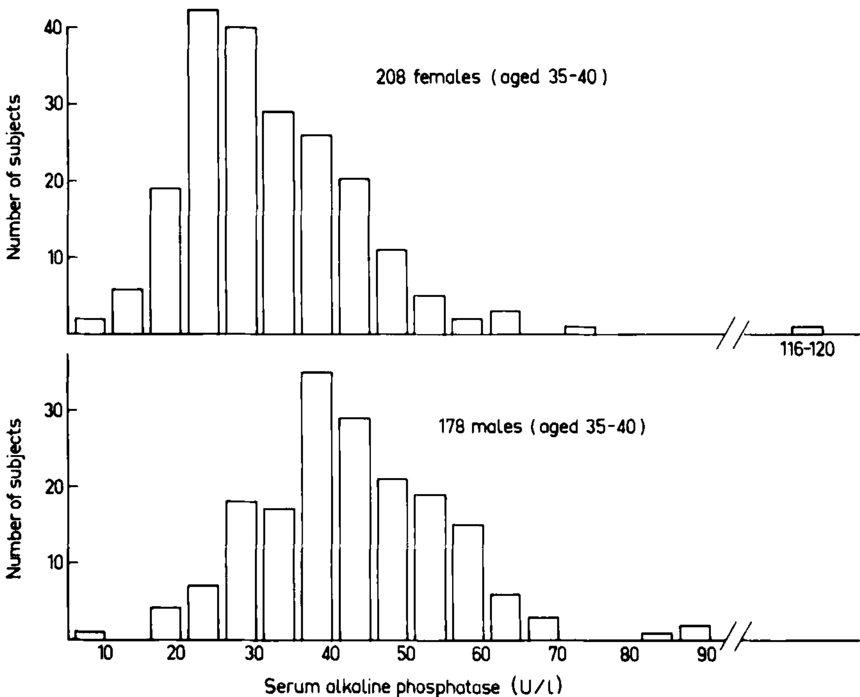

Fig. 1. The distribution of serum alkaline phosphatase values in 208 normal females and 178 normal males examined during the Busselton Survey (B37). Note the non-Gaussian distribution and the higher values of males. Data by courtesy of Dr. D. Curnow.

because serum alkaline phosphatase shows major age- and sex-related differences (C22, D8, R10). An extensive literature review may be found in McComb *et al.* (Mc3).

2. Effects of Age and Sex

2.1. Neonates (Aged up to 1 Week)

Table 3 shows the range of alkaline phosphatase activities in neonatal cord blood or peripheral blood as reported in a number of publications. In most of these reports, the mean values of neonates were higher than those of adult reference groups. However, some workers obtained mean values lower than those of adults. Kitchener *et al.* (K22) speculated that these relatively low activities may have been due to the use of citrated plasma.

Premature but otherwise healthy infants were said by Reddemann and Weinke (R8) to have higher and more varied alkaline phosphatase levels than full-term infants, but this finding was not confirmed by Hilderbrand *et al.* (H13) or Kulkarni *et al.* (K36). However, very low-birth-weight infants are liable to develop rickets within the first few weeks of life with spectacular serum alkaline phosphatase elevations (C19, K36) (see also Section 6.3).

It is not surprising that no correlation exists between the alkaline phosphatase activity of maternal blood drawn at term and that of the infant's cord blood (K22, L5), since most of the enzyme in the maternal circulation is of placental origin, while no placental enzyme is present in the serum of newborns (K22, P11, P27).

2.2. Infants and Children

Mean serum alkaline phosphatase values rise during the first months of extrauterine life (B6, S64, V3), reaching a plateau variously documented as 1.5 (F15), 1.9 (G7), and 2.6 (B6) times the upper reference limit for adults. It is generally agreed that after the initial rapid rise, no significant changes occur during childhood (Table 4), although mean serum alkaline phosphatase values remain considerably higher than those of adults (A16, C22, F13).

2.3. Young Adolescents

When boys and girls are treated as a homogeneous group, the combined mean serum alkaline phosphatase values show little alteration be-

TABLE 3

MEANS AND OBSERVED RANGES OF SERUM ALKALINE PHOSPHATASE ACTIVITIES IN NEONATES AS DETERMINED BY 17 GROUPS OF INVESTIGATORS

Reference	Authors' units (see Table 2)	Mean	Range	Ratio mean neonatal value and URL[a] for adults (approximate)
Stearns and Warweg (S64)	Kay	Not stated	0.1–0.25 (approximate)	Insufficient data
Meranze *et al.* (M14)	Bodansky (modified)	5.0	1.8–6.6	0.8[b]
Vermehren (V3)	Lundsteen and Vermehren	146	92–215	2.2
Von Sydow (V6)	Buch and Buch	7.5	2.4[c]	1.1
Barnes and Munks (B6)	Bodansky	7.1	2.7[c]	1.8
Speert *et al.* (S54)	Bodansky	5.4	2.9–7.9	1.3
Lapan and Friedman (L5)	Shinowara	7.0	3.8–11.3	0.9

Christiansson and Josephson (C17)	Bessey-Lowry and Brock	5.5	2.3[c]	1.8[b]
Gautier *et al.* (G7)	Bessey-Lowry and Brock	3.6	1.1[c]	1.2
Meade and Rosalki (M11)	International	134	60[c]	1.9
Zuckerman *et al.* (Z4)	Bodansky	8.3	Not stated	2.0
Kitchener *et al.* (K22)	King-Armstrong	16.6	7–28	1.3
Tandon *et al.* (T2)	King-Armstrong	7.6	3–14	0.6
Izquierdo *et al.* (I12)	King-Armstrong	8.2	1.97[c]	0.6
Watney *et al.* (W12)	King-Armstrong	17.8	8.7[c]	1.4
Hilderbrand *et al.* (H13)	King-Armstrong	12.3	Not stated	1.0
Petitclerc (P11)	International	130	Not stated	1.4

[a] URL, upper reference limits for adults are based on the method used by the authors.

[b] These investigators report adult values significantly different from those generally accepted by users of the relevant method.

[c] One standard deviation

TABLE 4
SERUM ALKALINE PHOSPHATASE IN HEALTHY BREAST-FED INFANTS AGED 28–112 DAYS[a]

Age (days)	Sex	N	Mean serum alkaline phosphatase[b]	Standard deviation
28	M	32	22[c]	6
28	F	31	19	5
56	M	40	21[c]	7
56	F	28	17	5
84	M	35	21	8
84	F	32	17	5
112	M	44	18	7
112	F	32	17	5

[a] Data from Fomon *et al.* (F15).
[b] King-Armstrong units/deciliter.
[c] Significant sex difference ($p < 0.05$)

tween prepubertal and pubertal years. However, when subjects are separated on the basis of age and sex, clear peripubertal rises are seen (Fig. 2). These rises parallel the adolescent growth spurt (F13, R13), and are less pronounced in girls than in boys. They occur several years earlier in girls than in boys (F13, R13), presumably because of the earlier onset of the growth spurt in females.

Peak serum alkaline phosphatase activities show a better correlation with sex-maturity ratings than with chronological age (B15). Variations in developmental age may account for some of the differences between the serum alkaline phosphatase values of individual adolescents of the same sex and age (F13).

Some dramatic rises and subsequent falls in serum alkaline phosphatase have been described between the ninth and fifteenth years of life (F13, S27). Pettifor *et al.* (P12), who studied a group of black children, found no such rises, but in other series, values up to 7 times the upper reference limit for adults were present during a rapid growth spurt (F13).

2.4. Older Adolescents and Young Adults

A steady decline in serum alkaline phosphatase activity toward adult values is seen in late adolescents of both sexes (Fig. 3). This process begins at approximately 11 years of age in females (F13), so that by the time they reach their twentieth year, their levels are almost indistinguishable from those of older females. In males, the decline toward adult values commences later and is more prolonged, so that levels do not merge into those of older adult males until well into the third decade (C22, F13, K23, K33).

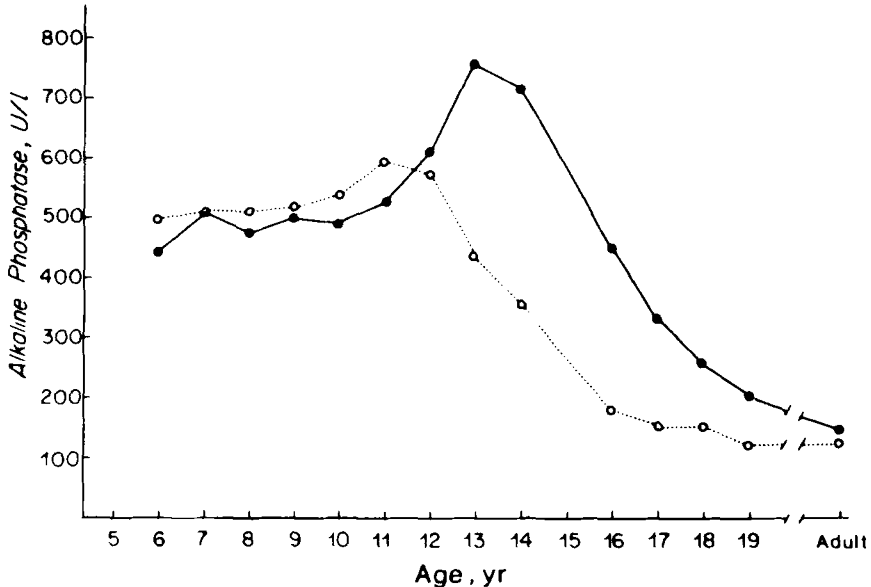

FIG. 2. Plasma alkaline phosphatase values in children and adolescents plotted as "moving" geometric means. Each mean is based on data from 80 children. The next mean is based on data from the 30 oldest of these 80, plus the next 50 in order of age. The solid circles denote males, the open circles females. The upper reference limit for adults obtained by the method employed was 256 U/liter. Note that the peripubertal rise occurs later in boys and is more pronounced than in girls. From Fleisher *et al.* (F13) with permission.

2.5. ADULTS

There are now many studies that demonstrate sex- and age-related differences in serum alkaline phosphatase activities in adults, although here, the age-related changes are much less marked than those observed in children. There is general agreement (B37, G4, K23, O5) that up to the age of about 50 years, serum alkaline phosphatase values are higher in males than in females (see Fig. 1).

An age-related increase in serum alkaline phosphatase values has been demonstrated in both sexes (K23, O5, W17). However, this increase is greater in females than in males, so that the sex-related difference disappears between 50 and 60 years of age (K13, K23, W17, W21).

2.6. PREGNANCY, CONFINEMENT, AND PUERPERIUM

Alkaline phosphatase rises significantly in maternal serum during normal pregnancies (K22, Mc9, P27), with mean values approximately 1.5 times the upper reference limit for non-pregnant females. Spectacular

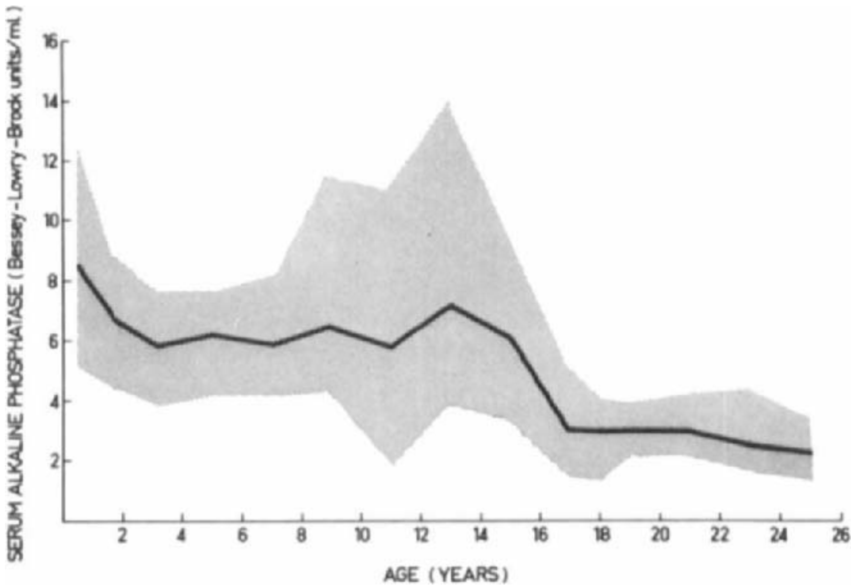

FIG. 3. Means and observed ranges of serum alkaline phosphatase values during childhood and adolescence in males. The upper reference limit for adults is 3 Bessey-Lowry-Brook units/milliliter. Note the wide observed range and the persistence of high values in some individuals. Data from Clark and Beck (C22) with permission.

elevations are uncommon, although values of 8 (B32) and 12 times (B10) the upper limit of the non-pregnant reference range have been reported.

The degree of hyperphosphatasemia in pregnancy is not correlated with maternal age or fetal sex (K22). Repeated pregnancies lead to serum alkaline phosphatase values similar to one another (B10), suggesting that interpatient variations may be due to different turnover rates (P25).

Cord blood alkaline phosphatase, which is predominantly of skeletal origin (K22), bears no relation to maternal serum alkaline phosphatase, which is predominantly of placental origin (L9, P27). The placental material, which is identifiable by its heat stability (G17, Mc9) and its immunological characteristics (B22), first appears in normal women some 5–6 weeks after conception (F12). The value then rises with advancing pregnancy, until a peak (Fig. 4) is reached at term (L9, S56). After confinement, placental alkaline phosphatase disappears from the maternal circulation with a half-life of 7 days (K22).

Women who breast-feed their infants have higher serum alkaline phosphatase than women who gave birth at the same time but whose infants had been weaned (C18, V4). Measurements of total serum alkaline phos-

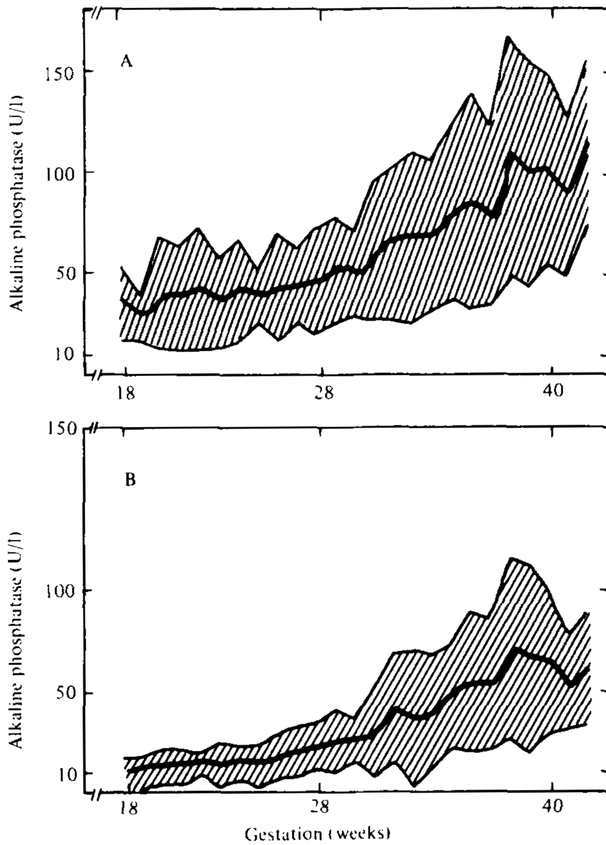

FIG. 4. Serum alkaline phosphatase in 31 healthy women during the second and third trimesters of pregnancy. The continuous line represents the mean and the shaded area, the 2.5–97.5 percentiles. (A) represents total alkaline phosphatase and (B) the placental fraction. Most of the increase is due to the placental enzyme. From Romslo *et al.* (R26) with permission.

phatase or the placental fraction are not useful in the diagnosis of fetal or placental emergencies (Mc3).

2.7. OLDER ADULTS

Mean serum alkaline phosphatase values have been variously reported to decrease (S28), to remain unchanged (H15), or to rise (K23, S30) in old age.

Adams *et al.* (A2) stated that the higher means which they found in a

group of elderly females were due to extremely high serum alkaline phosphatase values in a few subjects, at least two of whom had Paget's disease. Hodkinson and McPherson (H15) claim that many allegedly "normal" elderly females suffer from osteomalacia and that the high values of some geriatric age groups may be due to this disorder. Sharland (S30) and O'Carroll *et al.* (O1) attributed the higher total alkaline phosphatase activities of elderly subjects to an increase in the liver rather than the bone isoenzyme.

3. Constancy of Serum Alkaline Phosphatase Activity in Individual Subjects

The relatively wide variation in serum alkaline phosphatase activities observed in groups of apparently healthy adults (see Table 2) is in sharp contrast to the remarkable constancy of values found over long periods of time in any one subject (L11, S63, Y3).

One physician who measured serum alkaline phosphatase in himself repeatedly over a period of 8 years, found that despite the prolonged period of observation, the relative standard deviation of the values obtained was only 6.5% (Mc3). When this figure is compared with the relative standard deviation of 4–5% for the analytical variability of the method employed, it seems highly probable that the true intrasubject variation was smaller than indicated by the relative standard deviation and that the observed fluctuations were due, in part, to test imprecision.

Kanabrocki *et al.* (K1) claimed to have observed diurnal variations in serum alkaline phosphatase values. McComb *et al.* (Mc3) were unable to confirm this observation. De Merre and Litofsky (D5) found no changes in serum alkaline phosphatase values during the menstrual cycles of 41 healthy women.

4. Dietary and Other Environmental Influences

4.1. Fat, Carbohydrate, and Protein

Small but definite diet-induced changes in serum alkaline phosphatase activities have been reported in man. The most noticeable effects occur in association with fat ingestion, particularly in subjects of blood group O and in blood group secretors (K25, W4). Kleerekoper *et al.* (K25) found a mean rise of 25.6% in the serum alkaline phosphatase values of a group of volunteers 5 hours after a meal of high fat content. This rise was due, predominantly, to the appearance of intestinal alkaline phosphatase in

the circulation. Statland *et al.* (S61), who demonstrated smaller elevations in subjects taking smaller amounts of fat, pointed out that a significant rise in serum alkaline phosphatase in response to fat ingestion is obtained only if phenylphosphate rather than *p*-nitrophenylphosphate is used as the substrate. Statland *et al.* (S61) speculated that this phenomenon was due to the substrate specificity of intestinal alkaline phosphatase.

A significant elevation in serum alkaline phosphatase values has also been observed in response to chronic ingestion of relatively large amounts of refined carbohydrate (I11), although the acute ingestion of refined carbohydrate is without effect (L4).

Protein malnutrition appears to be associated with a reduction in total serum alkaline phosphatase activities (E7, W11). Schwartz (S19), in a study of the tissue sources of circulating alkaline phosphatase in kwashiorkor, claimed that there was an increase in the isoenzyme of hepatic origin, but a concomitant fall in total serum alkaline phosphatase activity due to a decrease in the bone isoenzyme. This finding was supported by clinical and radiological evidence of retarded bone growth.

4.2. Calcium and Phosphate

Variations in dietary calcium intake from 300 to 2000 mg/day do not influence serum alkaline phosphatase values (I8).

Phosphate depletion may result in nutritional osteomalacia and hyperphosphatasemia (see Section 6.3.2). In contrast, grossly elevated serum inorganic phosphate levels in nonuremic patients appear to have no detectable effect on serum alkaline phosphatase (M17).

4.3. Zinc

Zinc depletion, which may occur as a consequence of long-term parenteral nutrition, causes a decrease in serum alkaline phosphatase. The values become normal following zinc repletion (W14).

When oral zinc supplementation is used therapeutically in patients with rheumatoid arthritis, there is a rise in serum alkaline phosphatase values which correlates highly with the increase in serum zinc (S42). This positive correlation between serum zinc and serum alkaline phosphatase levels has also been demonstrated in patients with congenital acrodermatitis enteropathica (W15) and it has been suggested that serum alkaline phosphatase measurements may be employed as a useful index of serum zinc levels in these patients (W15).

4.4. Iron

Crosby *et al.* (C36) measured serum alkaline phosphatase and serum iron in a group of ostensibly normal subjects. High serum iron levels were found in 30 subjects, and 2 of these had serum alkaline phosphatase elevations. Other liver function tests were also abnormal in several subjects with serum iron elevation, and it is likely that at least some of the apparently normal subjects were suffering from subclinical hemochromatosis.

4.5. Vitamins

Vitamin A intoxication may result in hepatomegaly (S70) or hepatic cirrhosis (R34). While hyperphosphatasemia accompanies the more severe degrees of hepatic damage, cases with normal serum alkaline phosphatase values in the presence of hepatomegaly have been described (S70).

Vitamin B_{12} administration may cause a rise in serum alkaline phosphatase in patients with vitamin B_{12} deficiency (V2).

Vitamin C deficiency is said to be associated with low serum alkaline phosphatase activities (S48).

Vitamin D deficiency may give rise to rickets and osteomalacia, with marked hyperphosphatasemia (B30, S48, S76). This problem is further discussed in Section 6.3.1.

4.6. Obesity and Starvation

Obesity per se does not appear to lead to changes in serum alkaline phosphatase (M29) although venepuncture is more difficult in the obese so that artefacts associated with venous stasis are more likely to occur (see Section 1.3.).

Fasting for periods of 14–28 days does not lead to significant alterations in serum alkaline phosphatase values (B38). In patients with anorexia nervosa, serum alkaline phosphatase values are in the low normal range before treatment and increase significantly during the treatment period (K2).

4.7. Smoking, Drugs, and Ethanol

The effects of smoking on serum alkaline phosphatase do not appear to have been investigated in humans. The effects of ethanol and various other hepatotoxic agents are discussed in Section 7.5.1. An extensive list

of therapeutic and nontherapeutic agents that may alter serum alkaline phosphatase activity has recently been published (Mc3).

4.8. Altitude Changes

A 20-day stay at an altitude of 3800 m caused no change in the mean serum alkaline phosphatase activity of 25 volunteers (B20). However, the mean value increased sharply after the subjects returned to sea level and then fell to normal over the following 2 weeks.

5. Unexplained High and Low Serum Alkaline Phosphatase Activities

Physicians and laboratory scientists are occasionally confronted with isolated high or low serum alkaline phosphatase values which remain unexplained even after thorough investigation (B42). Such high or low activities may persist for prolonged periods of time (Mc3). Wilson (W28) described several members of a family with gross hyperphosphatasemia in the absence of demonstrable disease. The biochemical abnormality appeared to be inherited as an autosomal dominant trait.

Transient hyperphosphatasemia has been described in infants and children (A18, R31, S67). The features of this syndrome, which is currently unexplained, are summarized in Table 5. Posen *et al.* (P31) believed that the circulating alkaline phosphatase in this syndrome was of skeletal origin and suggested a sudden stimulus to osteoblastic activity as a possible etiological factor. Wieme (W20) concluded that the circulating enzyme derived from both the liver and the skeleton, suggesting that there may have been some interference with the mechanisms that normally control the removal of alkaline phosphatase from the circulation. Posen *et al.* (P31) suggested that an as-yet-unrecognized viral disease similar to that causing serum lactic acid dehydrogenase elevation in the mouse (M4) might block alkaline phosphatase degradation.

The clinical approach to the patient with unexplained hyperphosphatasemia is set out in Table 6.

6. Skeletal Disorders

The elevation of serum alkaline phosphatase in skeletal disorders such as Paget's disease and various types of rickets was described by Kay (K11,

TABLE 5
CLINICAL DATA OF FIVE PATIENTS WITH TRANSIENT HYPERPHOSPHATASEMIA OF INFANCY[a]

Initials	Age (months)	Sex	Clinical feature	Highest serum alkaline phosphatase	Tissue of origin as determined by isoenzymic studies[b]	Time until normal value recorded (weeks)
C.D.	18	F	Irritable behavior. Had received pipenzolate bromide, promezathine hydrochloride, and dicylcomine intermittently. Urinary catecholamines transiently increased.	3830 U/liter[c]	Bone	6
M.F.	14	M	Abdominal distension. Bilateral hydroceles. Distension disappeared spontaneously.	2800 U/liter[d]	Bone	5
J.H.	7	F	Feeding problem.	2410 U/liter[d]	Bone	7
N.L.	5	F	Fits. Mental retardation. No anticonvulsants.	2485 U/liter[c]	Bone	7
D.B.	9	M	Failure to thrive. Lowest third centile height and weight.	6300 U/liter[d]	Bone	5

[a] From Posen *et al.* (P31) with permission.
[b] Heat inactivation (P32) in Cases 3–5, and both heat inactivation and electrophoresis (P30) in Cases 1 and 2.
[c] Converted to U/liter from King-Armstrong units/deciliter by conversion factor of 7.1 (Mc3).
[d] Method of Morgenstern *et al.* (M22).

TABLE 6
APPROACH TO THE PATIENT WHO IS FOUND TO HAVE AN ELEVATED SERUM ALKALINE PHOSPHATASE VALUE AS AN ISOLATED ABNORMALITY

1. Is the value abnormally high?
   Children have higher activities than adults (F13). Pregnancy causes serum alkaline phosphatase to rise (P27).
2. Could this denote skeletal disease?
   Paget's disease may give rise to serum alkaline phosphatase elevation in the absence of clinical signs and in the absence of other biochemical anomalies.
3. Could this denote hepatobiliary disease?
   Space occupying lesions of the liver, partial biliary obstruction and hepatotoxic drugs, may raise serum alkaline phosphatase in the presence of normal serum bilirubin (see Fig. 11).
4. Has this patient recently received an albumin infusion?
   Values may take weeks to return to preinfusion levels (C23).
5. Transient or permanent elevation for which no detectable cause will be discovered. See Section 5.

K12) some 50 years ago. Since then, numerous references to the diagnostic significance of serum alkaline phosphatase elevations in disorders of bone have appeared in the literature and the subject has recently been reviewed in detail (Mc3, P29).

It is now generally agreed that the circulating enzyme in skeletal disorders is derived from osteoblasts, that osteoblastic proliferation results in an increase in circulating alkaline phosphatase, and that the degree of hyperphosphatasemia reflects the severity of skeletal disorders (see Table 7). A rough correlation is demonstrable between serum alkaline phosphatase values, skeletal roentgenograms (B5), urinary hydroxyproline excretion (C8), bone scintiscans (K17), and skeletal histology (B5).

However, there is only an indirect correlation between bone resorption and serum alkaline phosphatase activity. Patients with predominantly lytic lesions of the skeleton, as in multiple myeloma, have alkaline phosphatase values that are within reference limits or are only slightly elevated (S17). In addition, no correlation has been demonstrated between serum alkaline phosphatase and bone accretion rates as measured by bone-seeking isotopes (S36), which has prompted the suggestion that elevated serum alkaline phosphatase values in skeletal disorders might reflect the formation of bone matrix rather than bone (S36).

Serum alkaline phosphatase is a relatively insensitive indicator for the presence of metabolic bone disease (P29), and clinical decisions are rarely based on alterations of this parameter (P30).

TABLE 7

THE PREVALENCE OF SERUM ALKALINE PHOSPHATASE ELEVATION IN SOME SKELETAL DISORDERS

Diagnosis	How established	Number studied[a]	Number elevated[a]	Reference
Paget's disease	Radiology	61	53	Posen (unpublished) See also Fig. 5.
Osteomalacia—post-gastrectomy	Bone histology	15	1	Garrick *et al.* (G5)
Osteomalacia—renal failure	Bone histology	16	8[b]	Ingham *et al.* (I5)
Hyperparathyroidism—primary	Parathyroidectomy	60	11	Pratley *et al.* (P36) See also Table 8.
Hyperparathyroidism—secondary (renal failure)	Bone histology	29	2[b]	Ingham *et al.* (I6)
Osteoporosis	Radiology	31	0	Cooke (C28)
Fibrous dysplasia	Radiology and bone histology	74	37	Pritchard (P37)
Fractures	Clinical signs, radiology	17	1	Howard *et al.* (H18)

[a] Patients whose serum alkaline phosphatase was shown to be of nonskeletal origin are included in the "number studied" but not in the number with serum alkaline phosphatase elevation.

[b] Some patients had osteomalacia as well as histological hyperparathyroidism.

6.1. Paget's Disease of Bone

Patients with clinically evident Paget's disease usually have high serum alkaline phosphatase values (Fig. 5). Only 6% of Pagetic patients referred to Sydney Hospital had normal values, possibly because of the selection of patients with severe clinical or radiological involvement. Patients with asymptomatic Paget's disease may have significant serum alkaline phosphatase elevations, and we believe that this group accounts for a large number of patients with unexplained hyperphosphatasemia.

Serum alkaline phosphatase values in Paget's disease correlate well with urinary hydroxyproline (Fig. 6).

While serum alkaline phosphatase values constitute a reasonable index of the extent of the Pagetic process as determined by X-ray (S65) or bone scintiscan (K17), they are of no value in the diagnosis of complications such as osteogenic sarcoma (P22).

When serial estimations of serum alkaline phosphatase are performed in patients with Paget's disease there is little variation if initial levels are

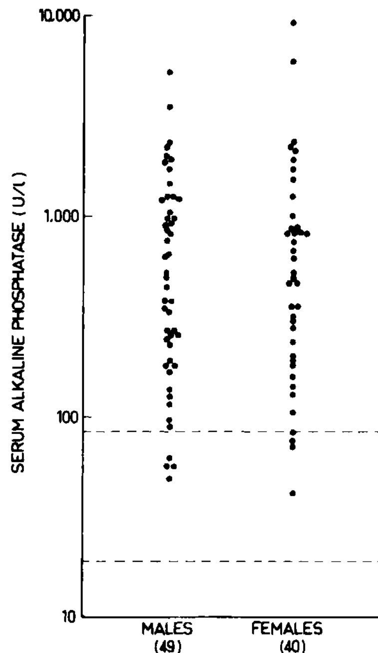

Fig. 5. Serum alkaline phosphatase values in 89 consecutive patients with Paget's disease during their first attendance at Sydney Hospital and prior to any anti-Pagetic therapy. The normal range is indicated by the horizontal lines. Spectacular elevations of this degree are seen only in Paget's disease. From McComb *et al.* (Mc3) with permission.

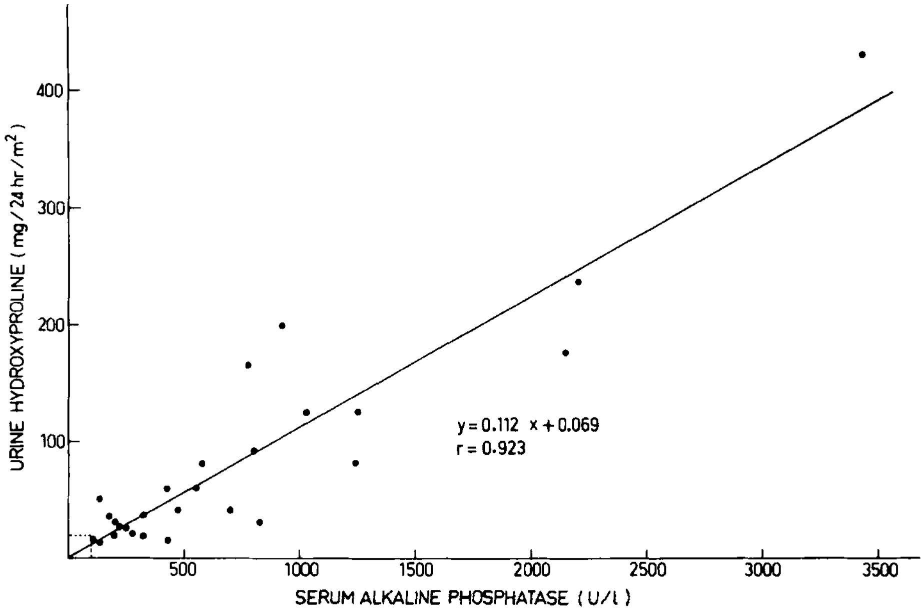

FIG. 6. The correlation between serum alkaline phosphatase and urine hydroxyproline in 27 patients with Paget's disease. The box in the bottom left hand corner denotes normal values obtained by the methods employed.

only slightly or moderately raised (G2). It has been claimed that if initial values are markedly elevated, they tend to undergo spontaneous fluctuations (G2). It is our opinion that such fluctuations are due to errors of dilution rather than to genuine alterations in activity (S. Posen, unpublished).

A number of agents are now available for the treatment of Paget's disease, including mithramycin (R36), a low phosphate and high calcium regime (E18), sodium etidronate (K18), and the various calcitonins (A21, H4, M9, P17, S44, S74). Each treatment schedule has been assessed by its effect on serum alkaline phosphatase values, but these do not correlate well with clinical improvement (P17).

After 6–14 months of therapy with adequate doses of porcine, salmon, or human calcitonin, a 50% reduction in serum alkaline phosphatase is observed (D12, H4, S74, W30). Large doses of calcitonin do not achieve a greater reduction in serum alkaline phosphatase. Plehwe *et al.* (P17) obtained a maximum fall in serum alkaline phosphatase values with a dose of 160 MRC units porcine calcitonin per day and were unable to achieve a greater fall by exceeding this dose.

Continuation of calcitonin therapy after 12–14 months does not lead to

further reductions in serum alkaline phosphatase values (D12, S74). Indeed, in some patients, the values rise in spite of continuing medication and in spite of persisting clinical remission, with the result that the mean after 12 months of therapy is higher than it was after 6 months (Fig. 7). The mechanism of this "escape" may be related to the formation of anticalcitonin antibodies (S45).

Discontinuation of therapy results in a return of serum alkaline phosphatase activities to pretreatment values. In the case of salmon calcitonin, this occurs within 6 months (A21, H4).

The biochemical response of patients with Paget's disease to other therapeutic agents is similar to that detailed for calcitonin except for the occurrence of the "escape" phenomenon, which has been observed only with calcitonin therapy. During mithramycin therapy, serum alkaline phosphatase declines more rapidly than during calcitonin therapy (L7)

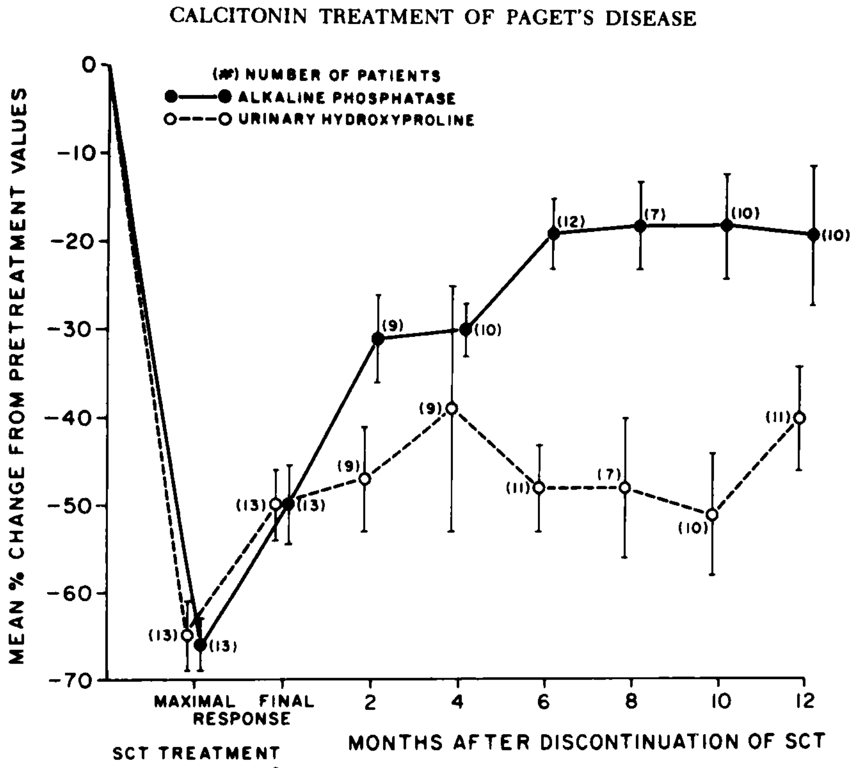

Fig. 7. The response of serum alkaline phosphatase and urine hydroxyproline values to treatment with salmon calcitonin. Note the biochemical "escape" during and after therapy. From Avramides *et al.* (A21) with permission.

and values may continue to fall for some months after withdrawal of the drug. After discontinuation of sodium etidronate therapy, serum alkaline phosphatase activities return to pretreatment values less rapidly than after discontinuation of calcitonin therapy (K18). Combination therapy with sodium etidronate and human synthetic calcitonin reduces mean serum alkaline phosphatase to 28% of the mean pretreatment value by the end of 6 months (B19).

6.2. Primary Hyperparathyroidism

The majority of patients with primary hyperparathyroidism have normal serum alkaline phosphatase values (Table 8). This test is therefore of little diagnostic significance. Serum alkaline phosphatase activities greater than twice the upper reference limit tend to be associated with radiological (D8, U1) and histological (P36) evidence of parathyroid osteopathy. The converse is not true: Histological examination of skeletal biopsy material may reveal bony lesions even when serum alkaline phosphatase values are within reference limits (P36).

Patients with gross hyperphosphatasemia in the presence of mild hyperparathyroidism should be suspected of suffering from Paget's disease as well as hyperparathyroidism (P26).

Bone *et al.* (B33) used preoperative serum alkaline phosphatase values as a predictive test to indicate which patients might show calcium hyperabsorption in the postoperative period. Persistent postparathyroidectomy hyperabsorption of calcium was associated with significantly higher preoperative serum alkaline phosphatase values even though postoperative values were the same as those of patients with normal calcium absorption (B33).

After successful parathyroidectomy, serum parathyroid hormone levels

TABLE 8
Serum Alkaline Phosphatase in 56 Patients with Primary Hyperparathyroidism[a]

Within normal limits:	43[b]
Elevated, but hepatic in origin:	2
Elevated, of skeletal origin:	11[c]
Total	56

[a] Data from Pratley *et al.* (P36).
[b] Less than 18 King-Armstrong units/deciliter.
[c] 10/11 patients with elevated skeletal alkaline phosphatase had evidence of bone disease on X ray or biopsy.

fall within minutes (K26), urine cyclic AMP within hours (S55), and serum calcium within 1–2 days (P36). In contrast, even in the absence of concurrent disorders associated with hyperphosphatasemia, serum alkaline phosphatase activities may not return to normal for weeks or months (K28, Mc3), and may even show a temporary increase (G29).

6.3. Osteomalacia and Rickets

The terms "rickets" and "osteomalacia" are not synonymous. Rickets refers to specific cartilaginous changes seen at the epiphyses in growing bones, whereas osteomalacia refers to lack of calcification of bone and the presence of abnormal amounts of uncalcified osteoid. The two coexist in children; in adults, only osteomalacia is found.

For reasons that are not entirely clear, rickets and osteomalacia, which may occur in a number of clinical situations, are characterized by increased osteoblastic activity with a resulting increase in serum alkaline phosphatase (B31). Vitamin D treatment may temporarily enhance this osteoblastic activity, with a further rise in serum alkaline phosphatase before a return to normal levels is observed (Fig. 8).

While severe osteomalacia is usually accompanied by hyperphosphatasemia, histological osteomalacia may be present in the absence of serum enzyme changes (A17, P29). Serum alkaline phosphatase measurement is therefore inadequate as a screening procedure for osteomalacia.

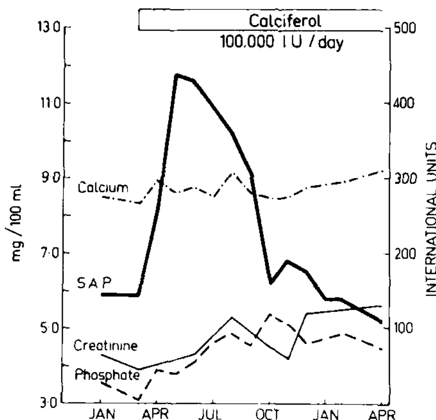

Fig. 8. Serum alkaline phosphatase (heavy line) in a patient with osteomalacia before and after treatment with ergocalciferol. Note the initial rise after the commencement of therapy and the subsequent fall to within the normal range. From Ingham *et al.* (I5) with permission.

6.3.1. *Osteomalacia due to Vitamin D Deficiency*

In recent years, several reports have appeared, particularly from Britain, concerning vitamin D-responsive osteomalacia due to nutritional causes (C29, H16, S76, W27). Most of the patients described were immigrants from India or Pakistan whose diet contained a high content of phytic acid (W27). Serum alkaline phosphatase activities in these patients ranged from normal to 20 times the upper reference limit for adults. After the commencement of therapy, activities fell to half the mean pretreatment values within approximately 4 months (C30, S76), although in some patients, this fall was difficult to distinguish from that normally seen in the peripubertal period (C29). In patients with very high serum alkaline phosphatase values, vitamin D therapy produced more spectacular reductions than in patients with mild elevations (H16, S76).

6.3.2. *Osteomalacia due to Phosphate Depletion*

Osteomalacia may be produced by phosphate depletion (L19), which may occur as the result of intravenous hyperalimentation (L12) or the prolonged consumption of aluminum hydroxide-containing antacids (B26, D10). Patients with hypophosphatemic osteomalacia may have normal serum alkaline phosphatase values (B26, D10). (See also Section 6.4.)

6.3.3. *Osteomalacia Following Gastrectomy and Other Malabsorptive States*

The prevalence of postgastrectomy osteomalacia (M20) depends largely on definition. Minor histological abnormalities are demonstrable in at least 12% of gastrectomized patients; clinical cases are relatively rare (E5, G5). Serum alkaline phosphatase activities in patients with severe forms of this disorder may be as high as 5 times the upper reference limit, whereas in patients with milder forms, the values are usually normal (G5).

Patients with gluten-induced enteropathy may develop osteomalacia with hyperphosphatasemia (H7). Normal serum alkaline phosphatase values in patients with gluten-induced enteropathy do not exclude the presence of osteomalacia (M13).

6.3.4. *Osteomalacia Associated with Anticonvulsants*

Serum alkaline phosphatase elevation and other indices, both biochemical and radiological, of rickets and osteomalacia are more common in patients receiving anticonvulsant drugs than in control subjects (B13, C16, C37, R16, T10). The severity of these abnormalities has been reported to relate directly to the duration of therapy (K35, T10), to the

number of anticonvulsants prescribed (L14, K35), and to drug dosage (K35).

Wark *et al.* (W7) measured serum 25–OH vitamin D in a group of cardiac patients receiving phenytoin and found values no different from those of a control group of patients not receiving this drug. However, serum alkaline phosphatase values were significantly higher in the phenytoin-treated patients.

Richens and Rowe (R16) found that the rise in serum alkaline phosphatase in patients receiving anticonvulsants was due to elevation of the hepatic as well as the bone isoenzyme. It was subsequently reported that significant elevations in hepatic enzymes other than alkaline phosphatase occur in patients on long-term anticonvulsant therapy (A11, R27) and that the rise in the hepatic isoenzyme of serum alkaline phosphatase precedes the rise in circulating skeletal enzyme (H3).

6.3.5. *Familial and Nonfamilial Vitamin D-Resistant Osteomalacia*

The combination of hypophosphatemia, vitamin D resistance, osteomalacia, and rickets is seen in a number of syndromes (W24). These include (*a*) familial hypophosphatemic vitamin D-resistant rickets, a sex-linked, dominant disorder (P3), (*b*) familial vitamin D dependency, an autosomal recessive disorder due to 1α-hydroxylase deficiency (F19), and (*c*) nonfamilial hypophosphatemic osteomalacia (D9), considered by some workers (P3) to be a separate disease entity because of its late onset, its severity, and its lack of response to therapy. In addition, there are many inherited and acquired disorders which are associated with impairment of renal tubular reabsorption of phosphate, and these may be accompanied by hypophosphatemia, rickets, and relative vitamin D resistance. Serum alkaline phosphatase values in these disorders correlate poorly with the severity of the disease (A14) and with the response to therapy (E4, Mc10, P7, S50).

Stickler et al. (S69) claimed that serum alkaline phosphatase is increased in all patients with vitamin D-resistant rickets and used serum alkaline phosphate measurements to diagnose familial hypophosphatemic rickets in the neonatal period (S16, S68). However, it is generally thought that vitamin D-resistant rickets is not always accompanied by serum alkaline phosphatase elevation (Mc3).

6.3.6. *Osteomalacia Associated with Connective Tissue Tumors*

A number of case reports have appeared concerning the association of osteomalacia with benign connective tissue tumors, such as sclerosing

hemangiomas (S1) and nonossifying fibromas (P20), or malignant tumors, such as bone sarcomas (W31). Biochemical abnormalities have included hypophosphatemia and serum alkaline phosphatase elevation. In some cases, these improved dramatically after removal of the tumor. It has been postulated that these tumors may cause hypophosphatemic osteomalacia by the elaboration of a humoral substance, the nature of which is as yet unknown (P20, W31).

6.4. Azotemic Osteodystrophy

The skeletal abnormalities associated with renal failure include osteomalacia, parathyroid osteopathy, osteoporosis, and osteosclerosis, in various combinations and degrees of severity (I6).

The prevalence of skeletal disease among patients with nonterminal renal failure is not known. The majority of such patients do not complain of skeletal symptoms and their serum alkaline phosphatase activities are not markedly elevated. The mean for all patients in one study (I5) was 1.5 times the upper reference limit for adults. In some patients with nonterminal renal failure, systemic acidosis is out of proportion to the degree of nitrogenous retention, and it currently seems that acidotic patients are more liable to develop skeletal abnormalities. Mean serum alkaline phosphatase in a group of such patients was 3.5 times the upper reference limit, with individual values of almost 10 times the upper reference limit (I5). The rise in total serum alkaline phosphatase, which is largely due to increases in the bone isoenzyme (I5, P15), shows a significant positive correlation with the severity of parathyroid osteopathy, irrespective of the presence or absence of concurrent osteomalacia (P15).

End-stage renal failure is associated with histological skeletal lesions in 75–80% of patients (I6). Fewer than 10% have serum alkaline phosphatase values in excess of 1.5 times the upper reference limit (I6).

Skeletal disease is common in patients with renal failure treated by chronic dialysis. The majority of these patients have histologically demonstrable bone disease, even in the absence of clinical symptoms (B21). However, serum alkaline phosphatase activities are either within reference limits or only moderately elevated (B21), with spectacular elevations occurring only rarely in both adults (B21, K10) and children (P34). High serum alkaline phosphatase values have been described in patients on chronic dialysis with severe hypophosphatemic osteomalacia (M3).

After successful renal transplantation, the various biochemical and histological abnormalities associated with azotemic osteodystrophy

regress (A6), although mild hyperparathyroidism may persist for a number of years (K26). Some transplant recipients have elevated serum alkaline phosphatase activities (I2) and in these instances, the circulating enzyme is largely of hepatobiliary origin. Ireland *et al.* (I9) claim that the immunosuppressant azathioprine is not responsible for the hepatic damage in these cases and implicate other factors, particularly infection.

Serum alkaline phosphatase values in transplant recipients with necrosis of bone are not significantly different from those of patients without this complication (I2). Woo *et al.* (W29) claimed that serum alkaline phosphatase values measured at the time of renal transplantation are higher in transplant recipients who later develop avascular necrosis of bone than in those who do not.

6.5. Osteoporosis

It is generally believed that, at the time of diagnosis, patients with osteoporosis have bone formation rates that are normal or only slightly elevated (K34). Serum alkaline phosphatase activities in osteoporotics are therefore, as one might expect, either normal or only slightly elevated (H23, J8, J9, T3). Indeed, the presence of significant hyperphosphatasemia should alert one to the fact that the disorder under investigation is something other than osteoporosis (C14).

A variety of therapeutic agents have been given to patients with osteoporosis. Riggs *et al.* (R19) treated nine women aged 43–67 with estrogens and noted a decrease in bone resorption after 12 weeks of therapy. Over the same period, serum alkaline phosphatase activities, which had all initially been within reference limits, decreased. Riggs *et al.* (R19) found this fall paradoxical, since their histological studies had shown no change in bone formation rates. Similar results have been obtained with a treatment regime consisting of a combination of androgens, estrogens, and a high calcium intake (L2). Patients given fluoride for osteoporosis have been variously reported to show a rise in serum alkaline phosphatase (M7) or to show no change (J9).

Patients with juvenile osteoporosis were found by Dent and Friedman (D6) to have normal serum alkaline phosphatase activities, which were uninfluenced by various treatment schedules.

6.6. Osteogenesis Imperfecta

The majority of patients with osteogenesis imperfecta have normal serum alkaline phosphatase values (C4). In some patients with this

disorder, hyperplastic calluses form at the site of fractures. In such cases, hyperphosphatasemia may be present (B4).

6.7. Fibrous Dysplasia

The lesions of fibrous dysplasia are characterized by the arrest of bone maturation at the woven bone stage. Patients with the monostotic form of fibrous dysplasia have normal serum biochemical parameters, including normal serum alkaline phosphatase activities (R4). A number of complications known to be associated with the polyostotic form of fibrous dysplasia may cause serum alkaline phosphatase elevation. These include various endocrinopathies (A13, B14, E8, F7), congestive cardiac failure resulting from a high cardiac output state (Mc6), the occurrence of sarcomatous change within the dysplastic lesions (H28), and the development of osteomalacia at sites other than those involved by the dysplastic process (D7). Even in the absence of such complications, polyostotic fibrous dysplasia may cause hyperphosphatasemia (F7, Mc6), although there is only a poor correlation between radiological appearances and the extent of this biochemical abnormality (Mc6).

6.8. Hyperostosis Frontalis Interna

Gegick *et al.* (G8) found serum alkaline phosphatase elevation in approximately one half of a group of 65 women with radiologically demonstrable hyperostosis frontalis interna. While the actual activities were not stated, the elevation does not appear to have been marked in any instance. Since the patients described were more obese than their controls, it is possible that the hyperphosphatasemia was due to difficulties associated with venepuncture.

6.9. Familial Hyperphosphatasemia

Patients with this disorder, which is probably inherited as an autosomal recessive trait, suffer from marked skeletal deformities due to rapid turnover of lamellar bone with failure to lay down compact cortical bone (E19, T4). Abnormal amino acids are excreted in the urine and serum alkaline phosphatase, which is indistinguishable from that circulating in patients with other skeletal disorders (E19), is spectacularly elevated.

The syndrome of familial hyperphosphatasemia in the absence of disease is described in Section 5.

6.10. Familial Hypophosphatasia

This rare disorder is one of the few conditions in which the diagnosis is made on the basis of low serum alkaline phosphatase activities (R6). It is characterized by radiological and histological features resembling rickets (F18, R6), but differs from the other varieties of rickets by the presence of craniostenosis, dental abnormalities (P16), and the excretion in the urine of abnormal quantities of phosphoethanolamine (F20) and inorganic pyrophosphate (R33). Alkaline phosphatase activities are low in a number of tissues, including the skeleton (Mc3), while the circulating enzyme has the characteristics of intestinal alkaline phosphatase (W10).

The full syndrome is probably inherited as an autosomal recessive trait (M28). Heterozygotes, it has been claimed, lack skeletal deformities but show low serum alkaline phosphatase activities as well as elevated urinary phosphoethanolamine levels (Mc1). Nevertheless, there are reports of so-called carriers with normal serum alkaline phosphatase values (F18, P16).

The question of whether low serum alkaline phosphatase activities are the cause or the effect of the skeletal deformities remains unanswered. However, a condition known as pseudohypophosphatasia has been described, in which all the clinical features of hypophosphatasia are present but the enzyme activity levels in serum are normal (S20).

6.11. Benign and Malignant Tumors Arising within Bone

Patients with benign bony tumors, such as chondromas and osteomas, have normal serum alkaline phosphatase activities (S43). Similarly, normal or only slightly elevated activities are observed in association with osteoclastomas (C5).

Early workers found serum alkaline phosphatase values among patients with osteogenic sarcoma to be very variable (C1), but observed considerably higher values in patients with blastic lesions than in those with lytic lesions (S43). Attempts have since been made to use serum alkaline phosphatase measurements for prognostic purposes. Studies such as those of McKenna *et al.* (Mc7) and Thorpe *et al.* (T5) agree that preoperative hyperphosphatasemia is associated with a poor prognosis. Thorpe *et al.* (T5) were unable to demonstrate any consistent relationship between postoperative serum alkaline phosphatase levels and prognosis,

whereas McKenna *et al.* (Mc7) concluded that elevated levels which persist after amputation have particularly grave prognostic implications.

6.12. Nonbony Malignancies with Bony Metastases

Serum alkaline phosphatase estimations are less reliable in the detection of skeletal metastases than are bone scintigrams (O3). More than 30% of patients with positive bone scans have normal serum alkaline phosphatase activities (C35). Patients with skeletal metastases from prostatic carcinoma have higher mean serum alkaline phosphatase values than do patients with skeletal metastases derived from other sites (S17), particularly when hypocalcemia is also present (R5). It has been suggested (L21) that prostatic metastases cause oncogenic osteomalacia but serum alkaline phosphatase measurements do not distinguish patients with this complication from those without (L21). The use of serum alkaline phosphatase measurements in carcinoma of the breast is discussed in Section 10.1.

6.13. Eosinophilic Granuloma

Eosinophilic granulomas of bone, whether isolated or multiple, are usually associated with normal serum alkaline phosphatase values (A20, Y1).

6.14. Extraskeletal Calcification and Ossification

Extraskeletal calcification occurs in several conditions unrelated to demonstrable abnormalities of calcium metabolism, such as scleroderma, myositis ossificans, calcinosis universalis, calcinosis cutis, and local tissue damage in the kidney. These disorders are not usually associated with changes in serum alkaline phosphatase (B30). Elevated values have, however, been reported in paraplegic patients with ectopic bone formation (F23), and a positive correlation has been demonstrated between the activity of serum alkaline phosphatase and the progression of this disorder. Nicholas (N4) claimed that hyperphosphatasemia in such a situation may help to differentiate thrombophlebitis, septic arthritis, and cellulitis from ectopic bone formation.

6.15. Fractures

In an early study, Hunsberger and Ferguson (H25) found major serum alkaline phosphatase elevations in patients with fractures. More recently,

it has been established that such elevations are not spectacular. Nilsson and Westlin (N5), in a study of patients admitted to hospital because of fractures of the femoral neck, found that mean serum alkaline phosphatase activities rose by approximately 50% after 1 week and then gradually returned to initial values over the next 2 months (Fig. 9). Sharland and Overstall (S31) noted, in a group of older patients with femoral neck fracture, that mean serum alkaline phosphatase values rose progressively until the fourth week to reach a peak which represented a doubling of baseline values. The limited isoenzyme studies performed by Sharland and Overstall (S31) showed that the increase in serum alkaline phosphatase was not always due to elevation of the bone isoenzyme. In some cases, it could be entirely accounted for by a rise in the circulating liver isoenzyme.

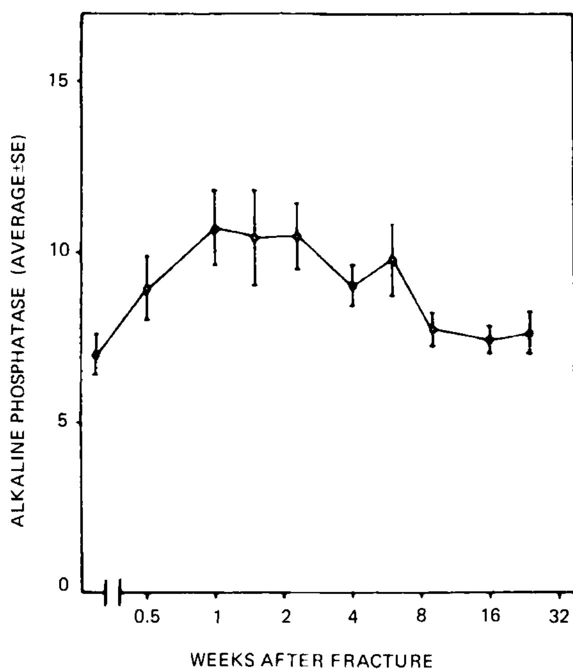

FIG. 9. Means and standard errors of serum alkaline phosphatase values (King Armstrong units/deciliter) in 63 patients with fractures of the femoral neck. Note the significant but relatively minor rise during the first week. The upper limit of normal by the method employed was 14 King-Armstrong units/deciliter. From Nilsson and Westlin (N5) with permission.

6.16. Osteomyelitis

Waldvogel *et al.* (W3) studied 62 adults and children with acute osteomyelitis. Serum alkaline phosphatase activities were normal in all cases with adequate data.

7. Disorders of the Liver and Biliary Tract

7.1. Source of the Circulating Alkaline Phosphatase in Patients with Hepatobiliary Disorders

The association between hyperphosphatasemia and hepatobiliary disorders was first reported in 1930 (R24) and until the late 1960s, this association was explained in terms of the "excretion" theory. According to this theory, skeletal alkaline phosphatase is normally excreted by the liver and failure of this excretory process, as in biliary obstruction, results in serum alkaline phosphatase elevation (G28).

The excretion theory is now thoroughly discounted and there is considerable evidence to suggest that in hepatobiliary disease, the circulating alkaline phosphatase comes from the liver and/or the bile passages (H15a, K7, P19, R21, S24). It now appears established that biliary obstruction leads to increased synthesis of alkaline phosphatase in the hepatobiliary system (K6) and that the newly synthesized enzyme then reaches the blood via canalicular-sinusoidal connections (B23).

The alkaline phosphatase(s) circulating in patients with hepatobiliary disorders can be shown to be distinct from skeletal, intestinal, and placental alkaline phosphatases by a variety of techniques (Mc3). Placental and intestinal alkaline phosphatases are believed to differ from human hepatobiliary alkaline phosphatase because they are synthesized in response to different structural genes (S23), whereas the differences between skeletal and hepatobiliary alkaline phosphatases may be due to post-translational events (S23).

7.2. General Principles in the Use of Serum Alkaline Phosphatase Estimations in the Diagnosis of Hepatobiliary Disorders

While unexplained hyperphosphatasemia is sometimes found in otherwise healthy patients (see Section 5), this is a relatively uncommon occurrence. Hence, serum alkaline phosphatase elevation, unless clearly due to a rise in nonhepatic enzyme, calls for thorough investigation of the hepatobiliary system.

Absence of serum alkaline phosphatase elevation, however, does not necessarily imply absence of disease (S17). The same general rule applies to other serum enzyme determinations, so that there is at present no single biochemical test which can be relied on to exclude hepatic disease.

Enzymatic estimations are only moderately helpful in distinguishing the various hepatic disorders from each other. The belief that high serum alkaline phosphatase activities are indicative of biliary obstruction while normal or only moderately elevated activities occur in association with hepatocellular damage is now known to be an oversimplification. There is considerable overlap between values in obstructive and nonobstructive hepatic disease (Fig. 10), so that serum alkaline phosphatase elevation or nonelevation in the differential diagnosis of hepatobiliary disease is useful only in a statistical sense (H14).

7.3. Inherited Disorders of Metabolism Affecting the Liver

Abnormalities of hepatic structure and/or function have been described in a number of inherited metabolic disorders. Serum alkaline phosphatase values in patients suffering from such disorders are presented in Table 9.

7.4. Infectious Diseases Affecting the Liver

7.4.1. *Viral Infections*

7.4.1.1. *Viral Hepatitis A.* During the acute icteric stage of infectious hepatitis, the mean serum alkaline phosphatase activity is approximately

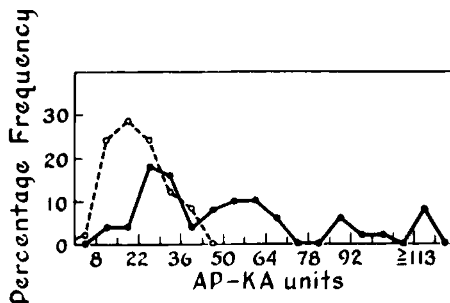

Fig. 10. Serum alkaline phosphatase values (King-Armstrong units/deciliter) in 49 patients with documented bile duct obstruction (solid circles) and in 49 patients with hepatic cirrhosis or hepatitis (open circles). Note the significant overlap between the groups. Note also that the highest value in biliary obstruction was less than ten times the upper limit of normal. From Hill and Zieve (H14) with permission.

TABLE 9
SERUM ALKALINE PHOSPHATASE IN A NUMBER OF INHERITED METABOLIC DISORDERS AFFECTING THE LIVER

Disorder	Serum alkaline phosphatase
Inherited disorders of bilirubin excretion	
Dubin-Johnson syndrome	Normal (B54, S29)
Rotor's syndrome	Reputedly "low" but probably normal (A15, S14)
Gilbert's syndrome	Normal (F17, S13)
Crigler-Najjar syndrome	Normal (E15, K8)
Familial cholestasis	Normal initially, but rising as disease progresses to cirrhosis (G20, W25)
Tyrosinemia	Elevated in presence of cirrhosis and/or rickets (L18, S5, S21)
Galactosemia	Elevated depending on extent of liver damage (D1, M19, S47)
Glycogenoses	
Type I (Von Gierke's disease)	Normal (B41)
Type II (Pompe's disease)	Normal (E11)
Type III (Cori's disease)	Normal even in presence of hepatic cirrhosis (M25, S56)
Type IV	Normal or intermittently elevated (R11, S39)
Type V (McArdle's syndrome)	May be elevated during episodes of severe rhabdomyolysis (G25)
Types VI–X	Normal (isolated case reports only) (H21, J10)
Fructose intolerance	Elevated in some cases with severe hyperbilirubinemia (L10)
Mucopolysaccharidoses	
Hurler's syndrome	Normal despite hepatomegaly and widespread skeletal abnormalities (Mc8)
Abnormalities of metal metabolism	
Hemochromatosis	Mildly elevated in approximately 20% of cases (P5, W26) but may return to normal after venesection therapy (W26)
Wilson's disease	Elevated in presence of hepatic damage (F6, M21, S11) or osteomalacia

twice the upper reference limit for adults (G13, R9). Higher values are seen in fewer than 10% of patients and the diagnosis of infectious hepatitis should be questioned if such high values are observed (G13, R9). In one thoroughly studied outbreak of infectious hepatitis (W1), hyperphosphatasemia reached its peak on the forty-second day after exposure, concurrently with the peak in mean serum bilirubin. Serum alkaline phosphatase values declined gradually over the next 40 days so that by the eightieth day, they were normal in all patients. In some patients with hepatitis, serum alkaline phosphatase remains within reference limits throughout the illness (S15).

Normal serum alkaline phosphatase activities have been found in association with anicteric hepatitis (C34), but this has not been a consistent finding (R9).

Occasionally, patients with acute infectious hepatitis may show a "cholestatic" clinical picture which is characterized by intense jaundice and marked hyperphosphatasemia (Mc11, O7). Serum alkaline phosphatase activities as high as 10 times the upper reference limit have been reported in such cases (Mc11).

7.4.1.2. *Viral Hepatitis B.* Serum alkaline phosphatase values in patients with serum hepatitis are similar to those seen in patients with acute infectious hepatitis (K19). In the majority of cases, they are less than 3 times the upper reference limit (K19).

Although serum bilirubin concentrations, serum aminotransferase, and serum alkaline phosphatase activities have been reported to return to normal more rapidly in hepatitis patients treated with steroids than in untreated patients (G23), these biochemical indices of apparent improvement are of no consequence in the long-term rate of recovery (G23).

7.4.1.3. *Other Viral Infections.* Hepatitis may occur in association with other viral infections. Patients with infectious mononucleosis hepatitis may have relatively high serum alkaline phosphatase activities in the presence of normal serum bilirubin concentrations (B7), and it has been suggested that such biochemical findings favor a diagnosis of infectious mononucleosis as against one of the other forms of infectious hepatitis (S38). Only minor increases in serum alkaline phosphatase occur in most patients with cytomegalovirus infection complicated by jaundice (J5), although in occasional instances, levels may be as high as 6 times the upper reference limit (S53).

Hyperphosphatesemia has been reported in infants with the congenital rubella syndrome (E17, P18). In this condition, however, there may be skeletal as well as hepatic involvement and no isoenzymatic studies appear to have been performed to determine the source of the circulating alkaline phosphatase.

7.4.2. *Rickettsial Infections*

7.4.2.1. *Q Fever.* Hepatic involvement is common in Q fever (P35), and it is now realized that a significant proportion of patients believed to be suffering from infectious hepatisis A or B actually have Q fever (A7). Powell (P35) found elevated serum alkaline phosphatase values in 36% of patients diagnosed as suffering from Q fever on serological grounds. Activities were greater than twice the upper reference limit in 7% of patients, while the highest level recorded was 5 times the upper reference

limit. In some patients, hyperphosphatasemia persisted for 6–8 weeks after the onset of symptoms (P35).

7.4.3. *Bacterial Infections*

7.4.3.1. ***Intrahepatic and Extrahepatic Sepsis.*** Bacterial infection of the liver, with microscopic or macroscopic abscess formation, results in hyperbilirubinemia and a rise in serum alkaline phosphatase (B53). Hyperphosphatasemia is greater in patients with microabscesses, particularly in cases of biliary tract obstruction, than in patients with macroscopic abscesses, who show only modest serum alkaline phosphatase elevation (R32).

Diagnostic difficulties may arise due to the fact that patients suffering from pyogenic infections may develop abnormalities of liver function tests, including serum alkaline phosphatase elevation, without obvious sepsis in the liver itself. This is a well-recognized phenomenon in the neonatal period and early childhood (B17), but it has also been reported in adults with systemic bacterial infections (E9). Hyperphosphatasemia is usually not marked in such patients (V5).

7.4.3.2. *Streptococcal Infections.* Jaundice may occur in association with scarlet fever. In the patients described by Fishbein (F9), serum alkaline phosphatase activities remained normal throughout the illness.

7.4.3.3. *Gonococcal Infection.* The majority of patients with gonococcal septicemia have serum alkaline phosphatase values appropriate to their age, although occasional patients may show significant elevations (H17).

7.4.3.4. ***Brucellosis.*** Hepatic granulomas are common in patients with brucellosis due to ***Brucella abortus*** (J6, Y4). Increases in serum alkaline phosphatase occur much less commonly. Joske and Finckh (J7) found minor elevations in three out of five patients, although one of these probably had unrelated chronic active hepatitis. Buchanan *et al.* (B51) found hyperphosphatasemia in only one of the nine patients with brucellosis on whom they performed liver function tests. Young (Y4), who studied five patients suffering from *Brucella melitensis* hepatitis, found that none of them had serum alkaline phosphatase values in excess of one and a half times the upper reference value.

7.4.3.5. *Tuberculosis.* Korn *et al.* (K30) reported serum alkaline phosphatase elevation in 41% of a group of patients with extrapulmonary tuberculosis and claimed that elevated values tended to be associated with granulomatous infiltration of the liver. Munt (M40) found hyperphosphatasemia in 34% of patients with miliary tuberculosis, but could demonstrate no correlation between serum alkaline phosphatase and liver histology. Many of the patients studied by Korn *et al.* (K30) had "fatty

metamorphosis" of the liver and portal fibrosis. The population studied by Munt (M30) contained a number of known alcoholics as well as three patients with neoplastic disorders and three pregnant women. Under these circumstances, it is impossible to say which condition was responsible for hyperphosphatasemia. Other granulomatous disorders involving the liver are discussed in Section 7.5.

7.4.3.6. *Syphilis.* Several reports have appeared in recent years concerning the development of hepatitis as a complication of secondary syphilis (B3, C3, L8). A clue to the diagnosis of syphilitic hepatitis is the disproportionate increase which occurs in the serum alkaline phosphatase level; values as high as 15 times the upper reference limit have been observed (L8). The abnormalities in liver function regress after standard therapy for syphilis, although serum alkaline phosphatase may take some weeks to return to normal (C3).

7.4.3.7. *Leptospirosis.* A rise in serum alkaline phosphatase occurs in approximately 60% of patients with leptospirosis at some stage during their illness (Mc5). Chinn *et al.* (C15), who studied six cases of Weil's disease, found mild hyperphosphatasemia (2–3 times the upper reference limit) in five of them. The peak was reached in the second or third week of the illness, in two instances, at a time when the serum bilirubin was on the decline. Normal serum alkaline phosphatase activities have been reported in *Leptospira pomona* infection associated with severe hemolysis and acute oliguric renal failure (B2).

7.4.3.8. *Pasteurella infections.* Butler (B52) studied 40 patients suffering from bubonic plague. None of them developed hyperbilirubinemia, although 9 showed serum alkaline phosphatase activities above the normal range. The values in these 9 patients varied from just above normal to six times the upper reference limit. Patients with clinical and hematological evidence of severe infection had higher serum alkaline phosphatase activities than did patients in whom the disease ran a milder course.

7.4.4. *Protozoan Infections*

7.4.4.1. *Malaria.* Transient hepatic dysfunction commonly occurs in childhood malaria (P6). Patwari *et al.* (P6), who studied a group of 80 children with *Plasmodium vivax* malaria, claimed that serum alkaline phosphatase values were elevated in 46% of patients during the acute attack, with a return to normal within the ensuing 6 weeks. While several abnormalities of liver function have been reported in adult patients with malaria, hyperphosphatasemia has only rarely been observed (G16). Among 81 patients with either falciparum or vivax malaria studied by

Goldstein (G16), there was not a single case with a serum alkaline phosphatase activity greater than 6.0 Bodansky units/dl.

7.4.4.2. *Toxoplasmosis.* Although it is well known that toxoplasmosis may be accompanied by disordered liver function (R12), there are remarkably few case reports that mention serum alkaline phosphatase. One patient has been described who died of overwhelming toxoplasmosis but had normal serum alkaline phosphatase values throughout his illness (K9).

7.4.4.3. *Amebiasis.* The majority of patients with hepatic amebiasis have serum alkaline phosphatase values within reference limits (L3).

7.4.5. *Metazoan Parasitic Infections*

7.4.5.1. *Schistosomiasis.* Patients who contract schistosomal infections show serum alkaline phosphatase elevations (B8, C21), which may persist despite subsequent remission of symptoms (C21). Portal hypertension associated with schistosomiasis does not, as a rule, lead to hyperphosphatasemia (G3).

7.4.5.2. *Echinococcosis.* Hydatid cysts of the liver may lead to hyperphosphatasemia (J6), but normal values may be found even in the presence of large cysts (J11). When cysts rupture into the biliary passages, hyperbilirubinemia and hyperphosphatasemia may follow (N7).

7.4.5.3. *Ascariasis.* Hyperphosphatasemia is not a consistent finding in ascariasis. When present, it tends to be associated with a greater immunological response, as judged by the severity of asthma and eosinophilia (P14). Adult forms of *Ascaris lumbricoides* may find their way into the biliary system and the rise in serum alkaline phosphatase associated with this invasion may be marked. Pfefferman *et al.* (P13) described a patient whose serum alkaline phosphatase was 10 times the upper reference limit in the presence of a normal serum bilirubin.

7.4.6. *Fungal Infections*

Fungal diseases of the liver that have been reported to be accompanied by serum alkaline phosphatase elevation include histoplasmosis (S49), cryptococcosis (S35), and actinomycosis (Y2). Successful chemotherapy usually leads to a return of serum alkaline phosphatase to normal levels (Y2), although antifungal agents may be hepatotoxic and may themselves lead to hyperphosphatasemia (M12).

7.5. Granulomatous and Infiltrative Disorders of the Liver

Sarcoidosis may lead to hyperphosphatasemia. Maddrey *et al.* (M2), who studied 20 patients with hepatic involvement, found that in one third, serum alkaline phosphatase values were normal; in another third,

there were slight elevations; and in the remaining third, more spectacular elevations were noted (up to ten times the upper reference limit).

Amyloidosis, whether primary or secondary, may similarly give rise to increases in serum alkaline phosphatase (B40). Values 30 times the upper reference limit have been described (B9).

7.6. Hepatotoxic Agents

7.6.1. *Alcoholic Liver Disease and Hepatic Cirrhosis*

The consumption of approximately 10 g of ethyl alcohol by a healthy person is without significant effect on serum alkaline phosphatase activities (S60). More substantial ethanol ingestion (approximately 0.75 g/kg body weight/day for 3 consecutive days) also produces no change in serum alkaline phosphatase values, although serum alanine aminotransferase and serum γ-glutamyl transferase activities rise significantly under these conditions (F21).

Even in patients with histologically proven alcoholic hepatitis, serum alkaline phosphatase may remain within reference limits. Green *et al.* (G21) found normal serum alkaline phosphatase values in 20 out of 46 patients with alcoholic hepatitis, while only 4 patients showed values greater than 2.5 times the upper reference limit. Bradus *et al.* (B39) described 83 patients whose liver biopsies showed fatty change but no evidence of cirrhosis. Mild increases in serum alkaline phosphatase (up to 2.5 times the upper reference limit) were found in about 50%.

Alcoholic cirrhosis of the liver, like cirrhosis from any cause, does not, in the majority of cases, lead to significant hyperphosphatasemia. Greene and Schiff (G22) showed that only 2% of patients with uncomplicated cirrhosis have serum alkaline phosphatase elevation greater than 7.2 Bodansky units/dl. Zieve and Hill (Z2), who studied a group of 30 patients with well-documented alcoholic cirrhosis, found a mean serum alkaline phosphatase of 16 King-Armstrong units/dl, with a standard deviation of 8 King-Armstrong units/dl.

Marked elevation of serum alkaline phosphatase in an alcoholic patient usually denotes the presence of biliary tract obstruction or space occupying lesions within the liver. However, in a few patients, alcoholic hepatitis and cirrhosis may be present, with marked hyperphosphatasemia due to intrahepatic cholestasis (P10).

7.6.2. *Hepatotoxic Agents Other than Ethyl Alcohol*

Numerous agents other than ethyl alcohol may have deleterious effects on hepatic structure and function. The nature of this hepatic damage determines the clinical manifestations, which may conform to a "hepatitic" or a "cholestatic" picture. The hepatitic syndrome is characterized

by elevations of unconjugated serum bilirubin and serum aminotransferase activities and, in severe cases, by hypoglycemia and disturbances in blood clotting factors. Serum alkaline phosphatase values are within reference limits or only slightly elevated. In the cholestatic syndrome, serum aminotransferase activities are relatively normal but there are marked elevations in conjugated serum bilirubin concentrations and in serum alkaline phosphatase, 5′-nucleotidase and γ-glutamyl transferase activities.

Although such purely hepatitic and cholestatic syndromes are recognizable, the clinical picture in individual patients may not be clear cut. Furthermore, there are many agents that tend to produce "mixed" effects. Representative hepatotoxins from each group are presented in Table 10.

7.6.2.1. *Narcotics.* Narcotics given to patients with proven biliary disease caused a significant rise in serum alkaline phosphatase within 6 hours of injection. Elevated activities persisted over the subsequent 18 hours (M26). No such elevations were noted in the absence of biliary disease (B16, S34).

7.6.2.2. *Antilipidemic Agents.* The administration of clofibrate leads to a significant decrease in serum alkaline phosphatase both in hyperlipidemic and in normolipidemic patients (F5, S8, W18). Isoenzyme analysis shows that this change is entirely attributable to a reduction in the circulating liver enzyme (W18).

Falls in serum alkaline phosphatase have also been reported with nafenopin (D17) and chlorophenoxyisobutyrate (H10). In contrast, colestipol therapy given over many months results in a significant rise in serum alkaline phosphatase (M15). It has been suggested that some of the effects of antilipidemic agents may be related to their action on vitamin D absorption (C25).

7.7. Biliary Obstruction

7.7.1. *Extrahepatic Obstruction*

Obstruction of the biliary passages, whether intrinsic or extrinsic, causes serum alkaline phosphatase elevation. The degree of elevation is very variable (B11, S12). In most instances, values are greater than 3 times the upper reference limit, but they rarely exceed 10 times the upper reference limit (see Fig. 10).

There is only a rough correlation between serum bilirubin and serum alkaline phosphatase, so that patients with similar serum bilirubin concentrations may have widely differing serum alkaline phosphatase values (H9). In addition, significant hyperphosphatasemia may be found in patients with little or no hyperbilirubinemia. This occurs when the common

TABLE 10
EFFECT ON SERUM ALKALINE PHOSPHATASE OF VARIOUS HEPATOTOXIC AGENTS

Agent	Type of syndrome produced	Serum alkaline phosphatase
Carbon tetrachloride	Hepatitic	Slightly elevated (A10, D4)
Chlorpromazine	Cholestatic	Elevations occur in approximately 20% of patients (S32). These may be marked and prolonged in some instances (R7), although, even in the presence of jaundice, fewer than half of the patients have activities greater than 4 times the upper reference limit (Z3).
Erythromycin esters	Mixed	Within reference limits or only slightly elevated (up to 1.5 times the upper reference limit) in patients with overt hepatic disease (C31, F2, F8)
Estrogen–progestogen combinations	Cholestatic	Elevations occur uncommonly when conventional doses are used (L6) and are minor, even in the presence of jaundice (T6)
Estrogens	Cholestatic	Elevations occur in most subjects on large doses of natural estrogens and may be greater than fourfold in some instances (M27)
Halothane	Hepatitic	Generally less than twice the upper reference limit in patients who develop jaundice (W6)
Iproniazid	Hepatitic	Mildly to moderately elevated (1–3 times the upper reference limit) in more than 80% of patients who develop jaundice (R29)
Isoniazid	Mixed	Elevated in 92% of patients with overt hepatic disease. Values greater than 4 times the upper reference limit are observed in 10% of cases (B25).
Methyldopa	Hepatitic	Ranges from within reference limits to as high as 7 times the upper reference limit in patients with overt hepatic disease (S25, T9)
Methyltestosterone	Cholestatic	Less than 4 times the upper reference limit in patients who develop jaundice (F16)
Nitrofurantoin	Mixed	Ranges from 3–5 times the upper reference limit in the presence of overt hepatic disease (E12, E14, J3)
Norethandrolone	Cholestatic	Slightly to spectacularly elevated (1–20 times the upper reference limit) in patients with histological evidence of cholestasis (S10)
Oxymetholone	Cholestatic	No abnormalities of liver function are observed in most series (A8, D3, Mc4). In one series, the hyperphosphatasemia attributed to the drug in some patients may have antedated its administration (S3).
Oxyphenisatin	Hepatitic	Less than 3 times the upper reference limit in patients who develop jaundice (R14)
Phosphorus	Hepatitic	Within reference limits or only slightly elevated (F14)
Progestogens	Cholestatic	Slight elevations may occur, but in most cases values remain within reference limits
Sulfonamides	Mixed	Elevated (2–7 times the upper reference limit) in patients who develop overt hepatic disease (D16, E16, F22)

bile duct is obstructed at a site proximal to its bifurcation or when areas of the liver are replaced by a tumor or abscess. Under these circumstances, bilirubin is cleared by the functioning liver cells of the uninvolved lobe and excreted by the nonobstructed hepatic ducts while increased alkaline phosphatase synthesis occurs in the involved lobe (Fig. 11).

Prolonged obstructive jaundice may be associated with osteomalacia, which can give rise to serum alkaline phosphatase elevation in its own right (A19).

7.7.2. *Intrahepatic Cholestasis*

Cholestasis in the absence of demonstrable mechanical biliary obstruction is usually referred to as "intrahepatic cholestasis," a term which implies that there is microscopic obstruction within the liver itself (P21). The condition occurs after the administration of various drugs, (see Table 10), in the presence of some infectious disorders (see Section 7.4), and during the last months of pregnancy. Mean serum alkaline phosphatase values in women with clinically overt cholestasis of pregnancy are higher than in matched controls (R13), although individual patients may have values appropriate to the last trimester of pregnancy (H2, R13). In some patients with intrahepatic cholestasis, no cause is identifiable (R35, S58). Serum alkaline phosphatase values in idiopathic cholestasis may be 7 times the upper reference limit (S75).

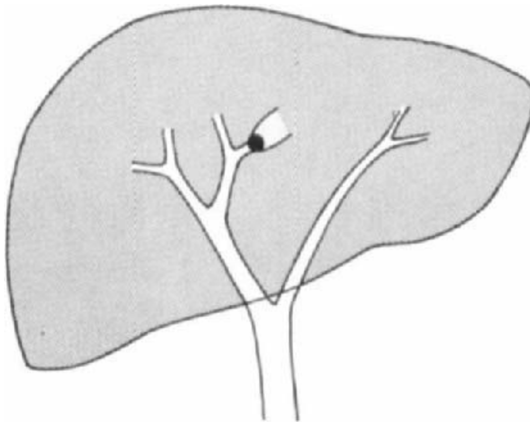

FIG. 11. Partial obstruction of the bile passages (as in patients with cholangiolar carcinoma or hepatic metastases) enhances alkaline phosphatase synthesis proximal to the site of obstruction. Such patients may have normal serum bilirubin values because the excretory processes in other parts of the liver are unimpaired.

7.7.3. *Primary Biliary Cirrhosis*

Patients with this disorder characteristically have high serum alkaline phosphatase activities. Only 2 of 100 patients with primary biliary cirrhosis studied by Sherlock and Scheuer (S33) had serum alkaline phosphatase values below 20 King-Armstrong units/dl, whereas 71 had values exceeding 50 King-Armstrong units/dl (Fig. 12). A reduction in serum alkaline phosphatase follows treatment with azathioprine (R30).

7.8. Neoplastic Diseases Involving the Liver

7.8.1. *Primary Hepatic Tumors*

High serum alkaline phosphatase values have been repeatedly described in patients with hepatoma (G22, S4, S6). In general, hyperphosphatasemia is observed twice as commonly as is hyperbilirubinemia (I4) and tends to be much greater in degree (E13). Although histochemical techniques fail to reveal alkaline phosphatase activity in the majority of hepatomas (S71), some such tumors contain unique isoenzymes (H11, W9). Atypical electrophoretic bands may be present in the serum of hepatoma patients, and it has been claimed that the presence of such bands is correlated with the presence of circulating α-fetoprotein (P23).

Angiosarcoma of the liver is a rare tumor associated with previous exposure to vinyl chloride. Patients with such a tumor may have serum alkaline phosphatase values within the reference interval or many times the upper reference limit (F1). Makk *et al.* (M5) screened over 1000 apparently well workers at a polyvinyl chloride factory and found hyperphosphatasemia in 32. In 15 of these cases, the circulating enzyme was predominantly of hepatic origin. Makk *et al.* (M5) stated that a "relative elevation of liver functions" could be detected in workers exposed to

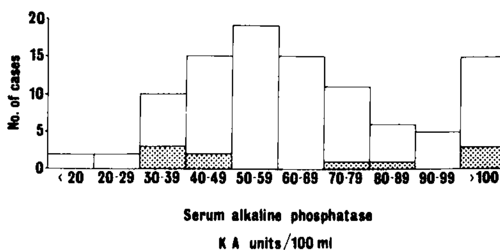

Fig. 12. Serum alkaline phosphatase values in 100 patients with primary biliary cirrhosis. Male patients are shown by stippled columns. Note the presence of hyperphosphatasemia in almost all the patients with this disorder. From Sherlock and Scheuer (S33) with permission.

polyvinyl chloride even when total serum alkaline phosphatase values were within reference limits.

7.8.2. *Hepatic Metastases*

Serum alkaline phosphatase elevation is of some value in screening for hepatic metastases (F4, G18, S9, W19). However, in one series (G18), 23% of patients with proven metastases had normal serum alkaline phosphatase values, while elevated values were seen in 6 of 23 patients who were subsequently shown to be free of hepatic metastases (G18). White *et al.* (W19), who studied 146 patients with metastatic breast cancer, found that only 2 of 39 patients with positive liver scans had normal serum alkaline phosphatase values (see also Section 10.1).

The degree of hyperphosphatasemia correlates poorly with the presence of jaundice in patients with hepatic metastases (F4, S9).

7.9. Surgical Procedures Involving the Liver: Vascular Disorders

7.9.1. *Partial Hepatectomy and Liver Transplantation*

Pack and Molander (P1) performed hepatic lobectomy in 23 patients suffering from "tumors and allied diseases." Following surgery, serum alkaline phosphatase increased in the majority of patients, although prompt decreases occurred in several patients with extremely high preoperative levels.[1] By the third week after surgery, 20 out of 33 patients had serum alkaline phosphatase values within reference limits. Decreases in serum alkaline phosphatase after hepatic resection were also observed by Almersjo *et al.* (A9). None of the 12 patients studied by Almersjo *et al.* (A9) showed a postoperative rise in serum alkaline phosphatase. Williams *et al.* (W23) reported massive falls in serum alkaline phosphatase values after liver transplantation.[1]

7.9.2. *Portocaval Anastomosis*

No significant differences were demonstrated between the mean serum alkaline phosphatase activities of patients who had portocaval shunts for the prevention of variceal bleeding and control patients who did not undergo such surgery (C26).

[1] An intraoperative fall in serum alkaline phosphatase is frequently due to the loss of phosphatase-rich blood followed by the transfusion of normal blood (G26).

7.9.3. *Ligation of the Hepatic Artery*

Brittain *et al.* (B44) described five patients in whom the right or common hepatic arteries were ligated accidentally during surgery. Although serum aminotransferase activities rose in several instances, serum alkaline phosphatase levels remained unchanged, or, if they had been elevated prior to surgery because of biliary obstruction, they fell in the postoperative period. Other case reports of hepatic artery ligation describe postoperative rises in serum alkaline phosphatase (A12, E6).

7.9.4. *Occlusion of Major Hepatic Veins (Budd-Chiari Syndrome)*

Serum alkaline phosphatase values in patients with this syndrome may lie within the reference interval or rise as high as 7 times the upper reference limit (C20, H19). Clain *et al.* (C20) were unable to demonstrate any correlation between serum alkaline phosphatase levels and serum bilirubin concentrations in this syndrome.

7.10. Gallbladder and Major Bile Ducts

7.10.1. *Cholelithiasis and Cholecystitis*

Patients with uncomplicated cholelithiasis generally have serum alkaline phosphatase values within reference limits (S2, S51). For this reason, serum alkaline phosphatase elevation in patients with gallstones should raise the suspicion of a stone in the common bile duct (S2, S57) or some other hepatobiliary disorder.

Normal serum alkaline phosphatase activities are found in patients with acute cholecystitis provided there is no associated bile duct obstruction (G19).

7.10.2. *Bile Peritonitis*

Bile peritonitis may be associated with varying degrees of hyperphosphatasemia. Serum alkaline phosphatase values ranging from 1 to approximately 15 times the upper reference limit have been reported (I3, Mc2, S57). The rise in serum alkaline phosphatase tends to lag behind the rise in serum bilirubin, the lag period varying from 2 to 14 days (Mc2).

7.10.3. *Bile Duct Obstruction*

Obstruction of the common bile duct by stones or tumors usually leads to hyperphosphatasemia (H14). (See also Section 7.6.1.) Values of between 3 and 10 times the upper reference limit are usually found (see Fig. 10).

8. Pancreas

8.1. Acute Pancreatitis

Acute hemorrhagic pancreatitis causes relatively minor serum alkaline phosphatase elevations, with values ranging from 4.0–13.5 Bodansky units/dl (K32) and a rough correlation between the severity of hyperbilirubinemia and the degree of hyperphosphatasemia (W16). Acute pancreatitis may be due to such disorders as alcoholism, gallstones, or hyperparathyroidism, which may themselves, by various mechanisms, give rise to serum alkaline phosphatase elevation (A4).

8.2. Chronic Relapsing Pancreatitis

Patients with this disorder frequently develop hyperbilirubinemia and hyperphosphatasemia during exacerbations. As each individual attack subsides, serum bilirubin concentrations return to normal at times when serum alkaline phosphatase values are still elevated. This sequence of events parallels that seen in most patients with extrahepatic biliary obstruction.

Snape *et al.* (S52) reported spectacular serum alkaline phosphatase elevations in six patients with calcific pancreatitis whose serum bilirubin concentrations were only moderately elevated. Littenberg *et al.* (L16) studied a group of patients with recurrent pancreatitis believed to be due to alcoholism. Persistent serum alkaline phosphatase elevation (more than twice the upper reference limit for 4 weeks or more) was associated with bile duct stenosis in 15 out of 16 patients.

8.3. Cystic Fibrosis of the Pancreas

Di Sant'Agnese and Blanc (D13) studied two groups of patients (children) with cystic fibrosis of the pancreas. In one group, who showed no clinical evidence of liver involvement, the mean serum alkaline phosphatase was 9.3 Bodansky units/dl, which was considered normal for this age group (D13). In a second group, whose disease was complicated by biliary cirrhosis, the highest serum alkaline phosphatase value was 20 Bodansky units/dl (D13).

Hsia *et al.* (H20) found that relatively few patients with cystic fibrosis of the pancreas had detectable intestinal alkaline phosphatase in their serum. They attributed this finding to the malabsorption of fat. However, the mean total values were considerably higher among the patients than among control subjects (H20).

8.4. Carcinoma of the Pancreas

Gullick (G27) studied 100 patients with carcinoma of the pancreas. Fifteen of 18 icteric patients had elevated serum alkaline phosphatase values, but there was no correlation between the severity of the hyperbilirubinemia and that of the hyperphosphatasemia. Among nonicteric patients, serum alkaline phosphatase elevation was usually associated with hepatic metastases (G27). The majority of patients with carcinoma of the pancreas without biliary obstruction have normal serum alkaline phosphatase activities (K32).

Warnes *et al.* (W8) described an alkaline phosphatase with distinctive electrophoretic and physical characteristics produced by a pancreatic tumor. Cha *et al.* (C9) suggested that pancreatic tumors constitute a source of circulating alkaline phosphatase.

8.5. Islet Cell Tumors

Mann *et al.* (M6) described an 11½-year-old child with an insulinoma. The preoperative serum alkaline phosphatase activity was 60 King-Armstrong units/deciliter and this value fell after removal of the tumor. This patient was taking anticonvulsants.

Ulcerogenic and diarrheogenic islet cell tumors of the pancreas may be associated with metastases and/or hyperparathyroidism (G24). Both may lead to hyperphosphatasemia.

Gjone *et al.* (G12) described a 72-year-old man with a pancreatic tumor which contained an inhibitor to gastric secretion. The patient, who suffered from watery diarrhea and hypokalemia but not from steatorrhea, had a serum alkaline phosphatase twice the upper reference limit. Autopsy showed no evidence of metastases.

Lightman and Bloom (L15) reported a patient with a glucagon-secreting tumor situated outside the pancreas but with the histology of a malignant islet cell tumor. Serum alkaline phosphatase was normal.

9. Endocrine Disorders

9.1. Pituitary

Total or partial hypopituitarism may result from disorders such as hemochromatosis (S72) or sarcoidosis (S26), which may raise serum alkaline phosphatase because of their effect on the liver. However, no correlation can be demonstrated in these conditions between serum alkaline

phosphatase activities and pituitary function. Patients with isolated deficiencies of growth hormone, follicle-stimulating hormone, thyroid-stimulating hormone, and ACTH have serum alkaline phosphatase values within reference limits (O2, R2), although significant rises may occur when growth hormone replacement therapy is given (Rabin, 1962).

Van Den Bosch *et al.* (V1) measured serum alkaline phosphatase in boys with delayed puberty who had been given very short courses of chorionic gonadotropin. The mean value almost doubled within 5 weeks of this medication.

Riggs *et al.* (R20) showed that patients with acromegaly had normal serum alkaline phosphatase values. Stepan *et al.* (S66), similarly, found that hyperphosphatasemia is often absent in acromegaly. However, significantly elevated levels of the bone isoenzyme were demonstrated in 84% of patients with elevated serum growth hormone values (S66).

9.2. Thyroid

Hyperphosphatasemia has been reported in 41% of patients with hyperthyroidism (C33). Huther and Scholz (H26) found the prevalence of hyperphosphatasemia to vary with the severity of thyrotoxicosis, although this finding could not be confirmed by Cooper *et al.* (C33). Serum alkaline phosphatase elevation in thyrotoxicosis appears unrelated to hypercalcemia (P4) or to the development of hypocalcemia following thyroidectomy (W22). Serum alkaline phosphatase elevation may become manifest or aggravated following therapy (M24) and abnormal values have been reported 24 months after ^{131}I therapy (C33). Richter and Ohlen (R18) stated that the circulating enzyme in thyrotoxicosis is of skeletal origin. Cooper *et al.* (C33) confirmed this finding only after ^{131}I therapy.

Talbot *et al.* (T1) stated that patients with juvenile hypothyroidism have lower serum alkaline phosphatase values than age- and sex-matched controls, and that the values become normal after replacement therapy. Chertow *et al.* (C13), who studied 15 hypothyroid patients, found normal serum alkaline phosphatase activities in each case.

Dalovisio *et al.* (D2) described three patients with subacute thyroiditis and serum alkaline phosphatase elevation. The values returned to within the reference range 8–12 weeks after the onset of symptoms.

9.3. Parathyroid

The relationship between hyperparathyroidism and serum alkaline phosphatase elevation is discussed in Section 6.2.

Patients with hypoparathyroidism, whether surgical (Posen, unpublished) or idiopathic (C12, K24), have normal serum alkaline phosphatase values. Steatorrhea (M23) and hepatic cirrhosis (K37) may be associated with idiopathic hypoparathyroidism and serum alkaline phosphatase elevation. Hyperphosphatasemia has been reported in some (C24) but not all (R25) patients with pseudohypoparathyroidism.

9.4. Adrenal Cortex

Hypersecretion of cortisol may be due to ectopic ACTH production by nonendocrine malignant tumors (L13, O6), which may raise serum alkaline phosphatase values by a variety of mechanisms (see Section 10). Hyperphosphatasemia has also been reported in association with adrenal carcinomas which may metastasize to the liver (H27).

Iannacone *et al.* (I1) studied 34 patients with Cushing's syndrome. Radiological osteoporosis was present in 22, of whom half had elevated serum alkaline phosphatase values. No such elevations were found in the 12 patients in whom osteoporosis was not detectable radiologically. Haas *et al.* (H1) suggested that hyperphosphatasemia in patients with Cushing's syndrome denotes unrelated pathology, such as osteomalacia. This suggestion does not seem in accord with the finding that hyperphosphatasemia disappears after appropriate therapy (I1). When corticosteroids are given for the therapeutic purposes, serum alkaline phosphatase values fall significantly (G7, M15).

Hypoadrenalism may result from a variety of disorders, some of which give rise to hyperphosphatasemia (I10). Addisonism not due to such causes is not associated with alterations in serum alkaline phosphatase.

Abnormalities of steroidogenesis known by the general term "adrenogenital syndrome" do not cause changes in serum alkaline phosphatase (B34, B48), nor are there any consistent changes associated with isolated hyper-or hypoaldosteronism (M8, P33).

9.5. Adrenal Medulla

In view of the known effects of adrenaline and noradrenaline on serum alkaline phosphatase activities of the dog (H12), it is surprising that reviews of pheochromocytomas barely mention this enzyme. High values may occur in this disorder because of such associated conditions as parathyroid adenomas (P2) or metastatic medullary carcinoma of the thyroid (S46) or, rarely, because pheochromocytomas may themselves metastasize to bone or liver (J1).

9.6. Diabetes Mellitus

It is difficult to ascertain from published papers whether hyperphosphatasemia in diabetes mellitus is due to the disorder itself, to its complications, or to unrelated pathological processes.

Belfiore *et al.* (B12) stated that mean serum alkaline phosphatase activities are 40% higher in patients with "uncomplicated" diabetes than in a control population. In the absence of careful documentation concerning the presence of hepatic disorders (such as hemochromatosis), renal disorders (such as glomerulosclerosis), and dehydration, such a statement is not very meaningful. Camerini-Davalos *et al.* (C2) measured serum alkaline phosphatase activities in 352 diabetic patients and found values to be higher than in a group of 30 nondiabetic patients. Although none of the diabetics had overt liver disease, many suffered from disorders other than diabetes. In a study of 101 diabetic inpatients and 200 outpatients, Goldberg *et al.* (G15) found 17% and 11%, respectively, to have serum alkaline phosphatase elevations. Patients with overt hepatobiliary activity were excluded from this study. It was concluded that hyperphosphatasemia is unrelated to duration, treatment, or complications of diabetes mellitus. Heath *et al.* (H8), who measured serum alkaline phosphatase in 82 diabetics, found a mean value 30% above that of a group of control subjects. There was no significant difference between untreated and treated diabetics and the degree of biochemical control made no difference to the magnitude of hyperphosphatasemia (H8).

Diabetic ketoacidosis, even when accompanied by marked hepatomegaly, does not lead to serum alkaline phosphatase elevations (B47).

9.7. Chromosomal Abnormalities

Patients with Klinefelter's syndrome have serum alkaline phosphatase levels appropriate to their age (H24).

In a study of juvenile patients with Turner's syndrome, five out of eight patients had serum alkaline phosphatase activities above the upper reference limit of age-matched controls (B49).

Patients with the XYY syndrome have serum alkaline phosphatase activities within reference limits (Z1).

9.8. Precocious Puberty

Precocious puberty may be associated with polyostotic fibrous dysplasia, which causes hyperphosphatasemia (A5). Some patients with precocious puberty suffer from convulsive disorders (L17), for which they receive anticonvulsant medication, resulting in osteomalacia and elevated serum alkaline phosphatase activities. Precocious puberty has also

been described as the presenting feature of a hepatoma (K31). It is therefore desirable to study isoenzymes of circulating alkaline phosphatase in children with precocious puberty, especially if there are any grounds to suspect associated hepatic dysfunction.

In the majority of girls with precocious puberty, no underlying cause is discernible. Such patients are likely to have a roentgenological bone age well in advance of their chronological age, while their serum alkaline phosphatase activities may reflect a period of rapid growth (S41).

10. Other Systems

It is our current belief that significant serum alkaline phosphatase elevations are not seen in association with human disorders unless these involve the skeletal or hepatobiliary systems. There are a few exceptions to this rule.

10.1. Carcinoma of the Breast

Hyperphosphatasemia in patients with breast carcinoma usually denotes the presence of hepatic or skeletal metastases (C32, C35, G1). Nathanson and Fishman (N1) claimed that, in at least some patients with breast carcinoma, hyperphosphatasemia was due to the ectopic production of alkaline phosphatase by the tumor. Measurements of serum alkaline phosphatase values are of little use in monitoring the effects of endocrine ablation and other therapeutic agents (D14, G1).

White *et al.* (W19) studied the correlation between hyperphosphatasemia and the presence of hepatic and skeletal metastases in patients with breast carcinoma. Elevated serum alkaline phosphatase values were present in almost every patient with a positive liver scan but in only 64% of patients with positive bone scans. Hyperphosphatasemia was associated with a significantly shortened median survival (W19).

10.2. Sickle Cell Anemia

Brody *et al.* (B45) found serum alkaline phosphatase elevations in patients with sickle cell anemia during symptomatic crises. Brody *et al.* (B45) concluded that the circulating enzyme was predominantly of skeletal origin.

10.3. Leukemias and Lymphomas

Hyperphosphatasemia has been reported in a variety of leukemias (J4, N3) and lymphomas (B35, J2, R28). As a rule, serum alkaline phospha-

tase elevation in these disorders is due to hepatic involvement (A3, B42). The claim by Neumann *et al.* (N3) concerning lymphoma-specific serum alkaline phosphatase has recently been refuted (D18, K14).

10.4. Rheumatoid Arthritis and Ankylosing Spondylitis

About 25% of patients suffering from these disorders have elevated serum alkaline phosphatase values (C10, K15, K16), probably because of hepatic involvement by the disease (K15, W13), by associated diseases (A1), or by salicylate therapy (R15, S22). The response to zinc therapy in patients with rheumatoid arthritis (S42) is discussed in Section 4.3.

10.5. Carcinoma of the Lung

The majority of patients with carcinoma of the lung have normal serum alkaline phosphatase values (G6). Hyperphosphatasemia in this disorder may be due to hepatic or skeletal metastases or, rarely, to the synthesis of alkaline phosphatase by the tumor (C11, S73). Pulmonary osteoarthropathy with hyperphosphatasemia has been described (C6a, H5).

10.6. Congestive Cardiac Failure

The prevalence of hyperphosphatasemia in congestive cardiac failure varies from 10% (R17) to 46% (F3). The values do not as a rule exceed twice the upper limit of normal (R17), although gross hyperphosphatasemia has been reported in association with severe congestive hepatomegaly (C6). Brensilver and Kaplan (B42) stated that the circulating alkaline phosphatase in patients with congestive heart failure was predominantly of hepatic origin.

10.7. Carcinoma of the Stomach

Cederqvist and Nielson (C7) used preoperative serum alkaline phosphatase values to predict the resectability of these tumors. Even in patients with normal values, only 50% of tumors were resectable. If hyperphosphatasemia was present, this proportion fell to 25%.

10.8. Crohn's Disease and Ulcerative Colitis

Both of these disorders may be associated with hepatic lesions, particularly pericholangitis (E1, E2, M18). Serum alkaline phosphatase values are elevated in 12% of patients with Crohn's disease (P8) and in 7% of patients with ulcerative colitis (P9). Spectacular hyperphosphatasemia is rare in both disorders.

10.9. Hypernephroma

Walsh and Kissane (W5) reported six patients with hypernephroma and hyperphosphatasemia. The biochemical abnormality disappeared after nephrectomy. It has been suggested (D19) that this syndrome is due to the synthesis of alkaline phosphatase by the tumor.

10.10. Carcinoma of the Prostate

The majority of patients with radiologically demonstrable skeletal metastases have elevated serum alkaline phosphatase values (G14, O3). Patients with skeletal metastases demonstrable only by bone scintiscans usually have normal serum alkaline phosphatase values (B24, O3).

There is no correlation between alkaline phosphatase and acid phosphatase in sera of patients with carcinoma of the prostate (G14). Estrogen administration causes no significant changes in serum alkaline phosphatase values in this disorder (K29). However, high pretreatment levels appear to be associated with a poor prognosis (W2).

10.11. Effect of Intravenous Therapy

In patients with hyperphosphatasemia, massive blood transfusions (from normal donors) lower serum alkaline phosphatase values by diluting the patient's plasma with normal plasma (G26) (Fig. 13).

Albumin solutions prepared from human placentas may contain alkaline phosphatase and cause hyperphosphatasemia in the recipient (M1, N2, P28).

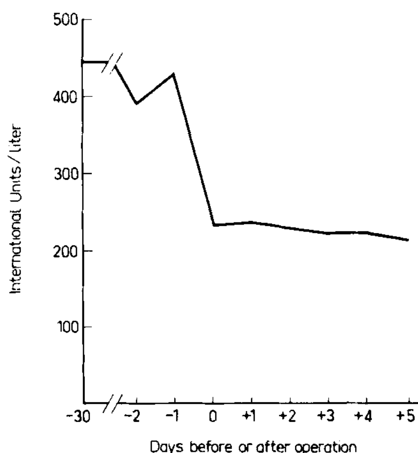

Fig. 13. Serum alkaline phosphatase in a patient with Paget's disease following total hip replacement. The rapid fall is attributed to the transfusion of 6 units of blood after massive blood loss during the operation. From Grunstein *et al.* (G26) with permission.

11. Summary

Serum alkaline phosphatase measurements continue to be widely used in clinical medicine. The test is relatively easy to perform, its cost is low, and a high degree of precision is readily achievable (Mc3). So long as the results (especially normal results) are not overinterpreted, serum alkaline phosphatase will continue to have a place in the diagnostic work-up of many clinical problems.

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ADVANCES IN CLINICAL CHEMISTRY, VOL. 22

HIGH-RESOLUTION ANALYTICAL TECHNIQUES FOR PROTEINS AND PEPTIDES AND THEIR APPLICATIONS IN CLINICAL CHEMISTRY

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1. Introduction

The human body is a complex and varied mixture of inorganic, organic, and organometallic substances, e.g., water, electrolytes, car-

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bohydrates, amino acids, steroids, vitamins, and proteins. During the past century, individual substances or groups of substances have been laboriously isolated from this mixture and characterized, and their functions have been studied. In addition, the variation of the levels of some of the substances in health and disease has been investigated. Subsequently, the measurement of individual or groups of substances has been demonstrated to be valuable in the routine detection, diagnosis, and/or management of disease.

Much remains to be learned of the composition of the mixture of molecules present in the human organism. With proteins, for example, it is not yet known how many different proteins and peptides are present or may be synthesized in man. It has been estimated that the human genome codes for between 30,000 and 50,000 proteins (A12). Only a few of the hundreds of possible gene products have been characterized or studied (A14).

In any particular cell type, fewer than 10% of the possible protein gene products appear, but in biological fluids, proteins derived from many different cell types may be present (Fig. 1).

Protein analyses have assumed an important role in diagnosis and it is to be anticipated that the range of proteins analyzed in the clinical chemistry laboratory will continue to expand. A wide variety of methods are used to determine protein concentrations, e.g., nephelometry, immu-

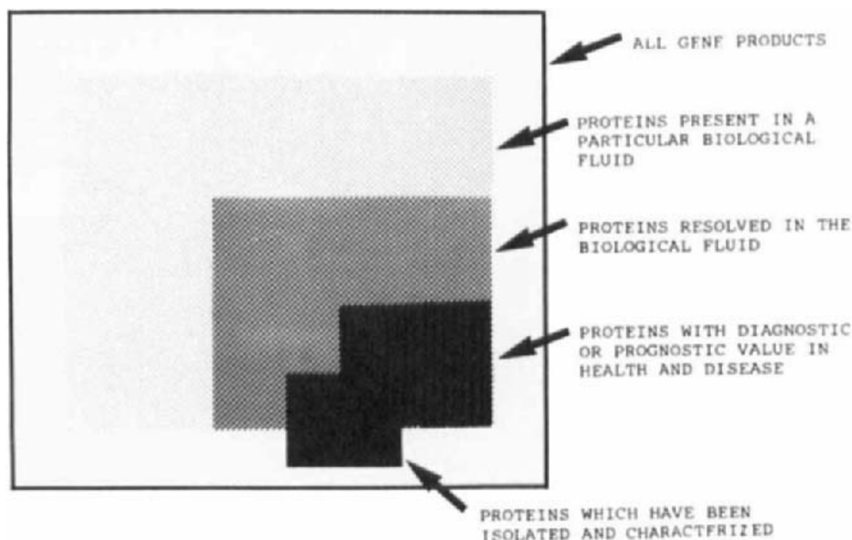


FIG. 1. Diagrammatic representation of our knowledge and ignorance of the proteins in a biological fluid.

nodiffusion, radioimmunoassay, and dye-binding. Such methods usually have good specificity and high sensitivity. In contrast, conventional techniques suitable for analyzing more than one protein in a mixture have a relatively low resolving power, e.g., gel-filtration chromatography. In order to facilitate the clinical chemist's search for new diagnostic and prognostic markers among the multitude of proteins known to be present in biological fluids, an analytical method is required with a resolving power matching the complexity of the sample to be analyzed (A12). This review examines three high-resolution analytical methods that are based on different analytical principles and are suitable for analyzing complex protein mixtures. Their principles and current applications in this field will be described. These methods, while not in routine use in clinical chemistry at present, exhibit great potential and may represent the next advances in clinical chemistry.

2. Isotachophoresis

2.1 Introduction

Charged particles migrate in solution when an electric field is applied; this phenomenon was described in the middle of the nineteenth century and from this early work, Kohlrausch (K12) developed a theory of ionic migration that can be used to describe all electrophoretic principles. However, it was not until 1923 that isotachophoresis was first used to separate rare earth metals and some simple acids (K3).

Development of the technique was slow until the early 1960s, when suitable apparatus became commercially available. Several names have been used for this separation technique: ion migration method, moving boundary method, displacement electrophoresis, and cons electrophoresis. The term used here, isotachophoresis (ITP), is based on the important associated phenomenon of the identical velocities of the sample zones in the steady state.

2.2 Principles

ITP is an electrophoretic technique for the separation of ionic species based on their differing mobilities in an electric field. In this method, a discontinuous electrolyte system is used, with a high-mobility ion as the leading ion and a low-mobility ion as the terminating ion. These electrolytes are chosen so that the mobility of the sample ions will lie between the mobilities of the leading and the terminating ions. There is no supporting electrolyte. In practice, ITP can be used to separate either

positively or negatively charged ions. The polarity of the applied electric field must be such that the ions in the leading electrolyte migrate in front of the sample ions (Fig. 2).

When the system, after a proper migration time, has reached equilibrium, all the ions will move with the same speed, individually separated into a number of consecutive zones, in immediate contact with each other, and arranged in the order of their mobilities.

A clear distinction must be made between (*a*) the first stage of the separation, where the ionic species have different migration velocities, and (*b*) the second stage, which is ITP proper, where a steady state has been achieved and all the ionic species are separated. At this second stage, the migration velocities of the zones are all the same, whence comes the name isotachophoresis, from the Greek *iso*, same, and *tacho*, speed.

The ITP system has several characteristics that are important in the interpretation of results:

(1) Because there is no supporting electrolyte and the current in the system is constant, the zones must follow each other with no gaps.

(2) With a constant current, the electric field strength is inversely proportional to mobility (Fig. 3). This means that each zone boundary will be characterized by a change in temperature and that a boundary-sharpening effect counteracts diffusion of the zones.

(3) The concentration of a species at any one point in the zone is defined by the mobilities of the participating ions. The concentrations are homogeneous and theoretically defined in all zones. Hence, an increase in the total amount of that species in the original sample will result in an increase in zone length. The height of the UV detector peak will be characteristic of the species and the zone length will be a measure of concentration of that species.

The high resolution of this technique is in part due to the zone-sharpening effects or "self-correction" of the zone boundaries. If an ion remains behind in a zone with a high electric field strength, it will acquire a higher migration velocity until it reaches its own zone. If it diffuses into a preceding zone, where the electric field strength is lower, its velocity will decrease and it will be overtaken by its proper zone.

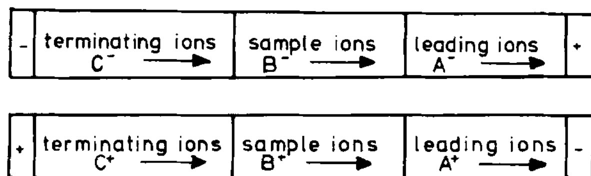


FIG. 2. Principles of separation in isotachophoresis.

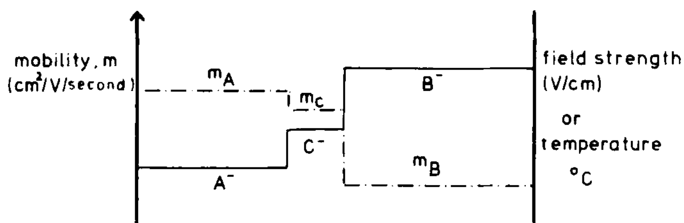


FIG. 3. Characteristics of isotachophoretic separations.

This section has presented a brief and simplified account of the principles of ITP. The interested reader is referred to the book by Everaerts and co-workers (E7) and other publications (B11, B12, E5–E9) for more rigorous accounts.

2.3 EQUIPMENT

Analytically, ITP may be carried out using either stabilizing agents, e.g., polyacrylamide and agar, or narrow-bore tubes, i.e., a capillary. The latter method is more usual. Currently, two instruments are available commercially and both utilize a capillary for separation. These are the LKB Tachophor 2127 (LKB, Bromma, Sweden), which has UV, thermal, and, more recently, conductivity detectors, and the Shimadzu IP-2A analyzer (Shimadzu, Tokyo, Japan), with a potential gradient detector (A3). The latter instrument has a two stage capillary: a first migration tube of 1 mm ID, which allows a high speed of analysis using a large current, and a second migration tube of 0.5 mm ID, where a lower current is used for high resolution. Recent developments in column coupling techniques (V4, V5) have allowed an increase in the maximal load capacity and the use of different electrolyte systems in the coupled columns. Column coupling can be used to improve the resolution in the analysis of complex samples.

ITP can be carried out preparatively using such supports as polyacrylamide or Sephadex. This method will be discussed further in Sections 2.4.4 and 2.4.5. On a microscale, both commercially available instruments can operate in a semipreparative mode. The LKB instrument, the Tachofrac, uses a cellulose acetate strip to collect the separated sample (A15, A16, M10), while the Shimadzu equipment relies on the manual removal of the zone of interest by means of a microsyringe. The use of the available equipment is described in Section 2.4. A detailed discussion of equipment and instrument design is to be found in the book on ITP by Everaerts and co-workers (E7).

2.4. Applications

This review is limited to high resolution techniques for the analysis of proteins, but the use of analytical ITP has no such limitations. Such widely differing substances as ionizable lipids, halogen ions, trace metals, drugs, organic acids, nucleotides, and proteins can be analyzed by ITP (A1, E7). However, it is perhaps in the field of protein analysis that both the greatest potential and the greatest problems lie, because of the complexity of most natural protein mixtures.

2.4.1. *Resolving Power*

It could be said that the resolving power of this technique depends on a difference in the effective mobilities of the constituents to be separated: If the mobilities do not differ sufficiently, the separation cannot be brought about. Much work has, therefore, centered on the selection of operating conditions that maximize the difference between the effective mobilities of the components to be analyzed. However, this approach has limited value for protein mixtures because, (*a*) the effective mobilities of many proteins are not known and (*b*) other factors will determine whether the separation can take place, e.g., since zones occupy a volume in the capillary between the injection point and detector, the sum of the zone volumes must be less than the volume of the column between these two points if separation is to take place. This restriction has led to the concept of the separation capacity of a column (B11), i.e., the maximum amount of the equimolar mixture of two selected components that can be separated.

In fact, for the separation of proteins, a more pragmatic view is taken and the operating conditions are usually optimized by experiment. Such factors as type, pH, and concentration of electrolytes, capillary length, temperature, column coupling, current, and use of spacers must all be considered.

2.4.2. *Detection Methods*

In the capillary ITP separation of proteins, the commonest detection method used is the UV-absorption (254 or 280 nm) of the proteins. The detection limits will therefore depend on the molar absorptivity of each individual protein.

The thermal trace (or differential thermal) is of limited use, because in complex mixtures of proteins, true "steps" are not obtained and the trace becomes difficult to interpret.

When ITP is carried out using solid supports, detection methods include the usual protein staining techniques (e.g., Coomassie Brilliant

Blue) and immunochemical reactions after separation in a semipreparative instrument (Section 2.3).

2.4.3. *Data Handling and Interpretation*

In simple systems, the interpretation and quantitation of isotachopherograms is relatively easy, compounds being separated in order of mobilities and zone length being a measure of concentration. However, in protein mixtures of biological origin, the UV traces obtained are complex and, for the reasons outlined in Section 2.2, data handling and comparison of isotachopherograms become difficult. In practice, data handling has been limited to the location of peaks and measurement of peak height or area, and few attempts have been made to analyze the sequences of peaks and the whole protein pattern (C1).

2.4.4. *Analysis of Body Fluids*

The protein analysis of body fluids [serum, urine, cerebrospinal fluid (csf), and sweat] by ITP has been limited because of analytical problems, namely, the effect on resolution of proteins coating detectors and the capillary wall, the purity of reagents, the need to use spacer solutions, and data handling and interpretation (D5, E1). These problems necessitate that operating conditions (capillary length, temperature, current, use of anticonvective agents) must be optimized and carefully controlled. The use of a spacer solution containing a mixture of varying mobility, non-UV-absorbing compounds, usually ampholytes and amino acids, may overcome some of the problems (C1, H8, H13, N2) in the separation of body fluid proteins.

One of the earliest applications of the method was in the analysis of serum and csf gammaglobulins. Several analytical systems and sample treatments have been developed (D4, H1, K5–K8); generally, short analysis times (30–40 minutes) and small sample volumes (microliters) are possible. Using ITP analysis of serum and csf immunoglobulins, it was possible to determine central nervous system (CNS) *de novo* synthesis of IgG, a hallmark of multiple sclerosis. The effects of various treatments on such synthesis were investigated (T6): The conclusion was that none of the therapies studied eradicated *de novo* CNS IgG synthesis.

Analytical ITP has been employed (C4) to characterize the protein matrix of quality control sera. Changes in the protein pattern were found after lyophilization, and interspecies and batch-to-batch variations in proteins were demonstrated. The analytical significance of such differences remains to be established.

Gallop and Hambleton (G1) have studied infection-related changes in serum components of rabbits by ITP. Their results suggested that the

onset of infection might be indicated by the appearance in the isotachopherogram of a low molecular weight (< 500) component (Fig. 4).

ITP has also been applied to the study of "middle molecules" in uremic toxemia (B4). It is known that there is a poor correlation between some toxic manifestations of uremia and the plasma levels of creatinine, urea, and uric acid. This is thought to be due to the presence of medium-molecular-weight compounds (possibly including proteins and peptides) in uremic serum. Adequate hemodialysis requires the retention of the right molecules and adequate removal of toxic molecules, including middle molecules. Hence, the measurement of these middle molecules may be valuable in diagnosis and the assessment of treatment. Several studies using ITP have found differences between normal and uremic serum and between pre- and postdialysis serum, the major differences occurring in the low-molecular-weight anionic solutes (M8, Z4).

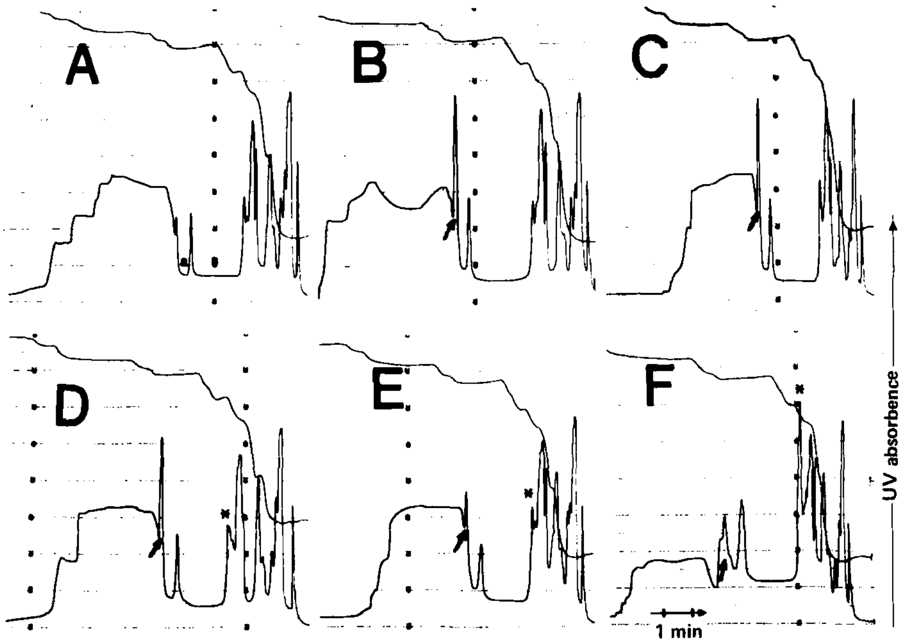


FIG. 4. Isotachophoretic profiles of rabbit sera obtained before and at intervals after experimental *Francisella tularensis* infection. For each analysis, 2.0 μl of appropriate serum, 1.0 μl SMGS, 0.5 μl asparagine (0.4 mg/ml), and 0.5 μl serine (0.4 mg/ml) were injected together. A–F are analytical UV-absorption profiles (lower curve) and thermosignals (upper curve) of sera obtained before and at 8, 24, 32, 48, and 72 hours after infection, respectively; a and s indicate the aspargine and serine zones, respectively; arrows indicate changes in proteins. From Gallop and Hambleton (G1), with permission of the authors and *Science Tools*.

Finally, there have been a few studies on the analysis of urinary proteins by ITP (T2, U1). Clark and co-workers (C5) studied the UV-absorbing constituents of urine from patients with rheumatoid arthritis as compared with osteoarthritis patients and healthy controls and were able to show significant differences (Fig. 5).

Serum proteins have been isolated by preparative ITP. Several stabilizing media have been used, e.g., polyacrylamide (B20, K15) and Sephadex (B7, B8). Preparative capillary ITP has been used for the separation of microgram amounts of some human serum proteins (M10). The separated protein zones were collected on the Tachofrac cellulose acetate strip and could then be further analyzed by immunoelectrophoresis.

2.4.5. *Other Proteins of Biochemical Interest*

Several applications of ITP have been in research fields of clinical biochemistry and these are discussed here briefly to give some indication of the potential of the technique.

The immunoglobulins have been extensively studied by ITP (in serum and csf; see Section 2.4.4) and in particular the subclasses of IgG have been studied (H8, H10, H11, Z3). An extension of this work has been the demonstration of soluble immune complex formation *in vitro* (H9), which has obvious implications, particularly for the assessment of immune complex diseases. Preparative work has involved the isolation of, for example, antibodies to pig lactate dehydrogenase (B21, P1) and IgD myeloma protein (J1). ITP has also been applied in the field of enzymology, not for the direct measurement of enzymes as proteins, but for the determination of enzyme reaction substrates and products, and hence has been of use in enzyme kinetics. This work is summarized in Table 1.

Protein interactions have been investigated by ITP (D6). It has, for example, been possible to determine the number of sodium dodecyl sulfate (SDS) molecules bound to bovine serum albumin (H14, H15) and to investigate the binding of drugs such as indomethacin to human serum albumin (H15). This method could be of value in pharmacokinetic studies.

Preparatively, ITP has had limited use; some of its applications are listed in Table 2. It is likely that with a development in the use of analytical ITP, the preparative applications will follow.

Finally, ITP has been applied to the investigation of proteins in tissues and membranes, e.g., brain tissue (L1, Z2), eye lens (B17–B19), and cell membranes (B14), and, although these uses are not relevant to clinical chemistry today, they do reflect the potential of the technique.

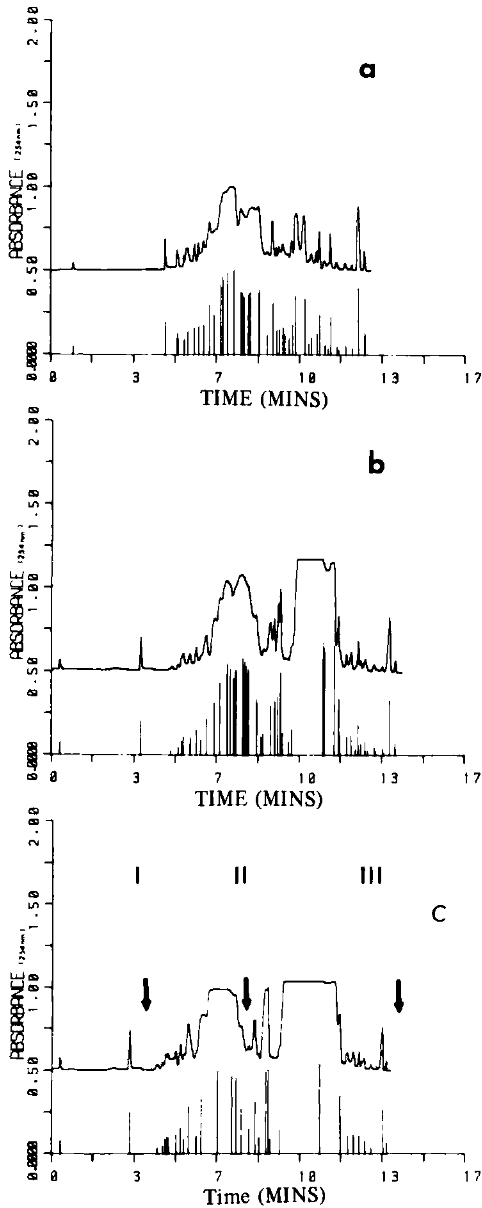


FIG. 5. UV traces and "stick" diagrams from the isotachophoretic analysis of urines from (a) a healthy control, (b) a patient with rheumatoid arthritis, and (c) a patient with osteoarthritis. Arrows indicate the limits of regions I, II, III. From Clark *et al.* (C5), with permission of the authors and Elsevier Scientific Publishing Company, Amsterdam.

TABLE 1
APPLICATIONS OF ITP IN ENZYMOLOGY

Enzyme	Reactants/products measured by ITP	Reference
Hexokinase/glucose-6-phosphate dehydrogenase	ATP, ADP, glucose 6-phosphate, 6-phosphogluconate, $NADP^+$, NADPH	K14
Calf heart mitochondria enzymes	Malate, phosphate, ADP, AMP	K13
Urokinase	*N*-α-acetyl-L-lysine	K2
LDH	Pyruvate, lactate, NAD, $NADH^+$	W6
UDP-glucuronyl transferase/nucleotide pyrophosphatase	UDPGA, UMP, GA-1-P,[a] inorganic phosphate, GA[b]	H16, H17
UDP-glucuronyl transferase	β-Glucuronides, UDP-glucuronate, UDP, phosphate glucuronate	H18, H19

[a] GA-1-P, glucuronic acid 1-phosphate.
[b] GA, glucuronic acid.

3. High-Performance Liquid Chromatography

3.1. INTRODUCTION

High-performance liquid chromatography (HPLC), also known as high-speed liquid chromatography and high-pressure liquid chromatography, has developed over recent years as a result of advances in instrumentation and column packings.

The characteristics of HPLC place certain demands on the design of

TABLE 2.
PREPARATIVE USES OF ITP

Source	Protein	Reference
Serum, rat	α-1-Macroglobulins	G4
Urine, human	Erythropoietin	P3
Plasma, human	Low-molecular-weight kininogen	A1
Bronchogenic tumors, human	Bronchogenic carcinoma antigen	F7
Pituitary tissue, canine	Prolactin	K9
Prostatic tissue, human	Acid phosphatases	V6
Legionella pneumophila	*Legionella pneumophila* toxin (causative agent of Legionnaires disease)	H8
Erythrocytes, human	Hypoxanthine guanine phosphoribosyltransferase	B1

the equipment. Short, high-performance columns are used, because with longer columns, elution times are excessively long and operating pressures excessively high, and there is no great gain in performance. Thus, small samples must be applied, usually into a pressurized system, sensitive detectors must be used, and the dead volume of the whole system minimized.

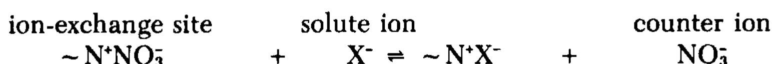
Modern liquid chromatography can be carried out in any of the classical modes, e.g., liquid–solid adsorption chromatography, liquid–liquid partition chromatography, reversed-phase chromatography, ion-exchange chromatography, and gel-permeation (size exclusion) chromatography.

These will now be considered briefly in relation to protein separation; the reader is referred to some of the numerous books and reviews on HPLC for further details of the theory and applications of HPLC (B22, C8, D9, H5, K10).

3.2. Principles

3.2.1. *Ion-exchange*

The principles of ion-exchange chromatography are well described and may be represented by the equation



$$\sim N^+NO_3^- \; + \; X^- \rightleftharpoons \; \sim N^+X^- \; + \; NO_3^-$$

Ion-exchange materials may contain either acidic groups, e.g., sulfonic acid or carboxylic acid for the separation of cations, or basic groups, e.g., amine or quaternary amine for the separation of anions.

Much of the early work on the ion-exchange separation of proteins by HPLC was carried out using the cation exchangers employed for amino acid analysis, usually porous polymers. However, the resolution obtained was not high and they had several other disadvantages, principally, that the supports were compressible and did not withstand high pressures. In addition, their volumes changed in different solvents, giving rise to variable bed sizes, and their mass transfer characteristics were poor. The next generation of HPLC ion-exchange supports were the coated bead resins, including the pellicular ion-exchange supports. More recently, the homogeneous bonded materials in which the ion-exchange groups are chemically bonded to microparticulate, fully porous silica gels have been developed.

3.2.2. *Gel-Permeation*

In conventional chromatography, gel-permeation is one of the commonest methods for separating proteins. The technique is based on the partial exclusion of large molecules from a porous matrix. In HPLC, gel-permeation has not been so widely used for the separation of proteins, which may be due to the lack of gel-permeation supports compatible with aqueous mobile phases and resistant to high operating pressures. However, suitable supports have recently been developed (e.g., TSK gels) and it is anticipated that this method for the high-resolution separation of proteins will expand in the foreseeable future (F8, H6).

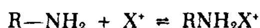
3.2.3. *Reversed-Phase*

The mechanism of separation in reversed-phase chromatography depends on the differential partitioning of the solutes into a bonded organic layer from the mobile phase (C8).

Reversed-phase chromatography has been widely applied in HPLC for numerous reasons: Polar and ionic molecules can be efficiently chromatographed, there is a wide choice of eluents and gradient elution techniques can be used, high pressures can be used, re-equilibration is fast and bleeding seldom occurs, results are reproducible, and the supports are more resistant to organic solvents and extremes of pH.

3.2.4. *Ion-Pair and Ion-Suppression/Ion-Enhancement*

These methods form an adjunct to reversed-phase chromatography and are based on the addition of compounds, e.g., sodium hexane sulfonate (ion-pair) or H^+ (ion-suppression/ion-enhancement) to the mobile phase, which will influence the equilibrium



$$R{-}NH_2 + X^+ \rightleftharpoons RNH_2X^+$$

and hence influence the solubility of the species to be separated and their partitioning between the mobile and stationary phases. Although not widely applied to protein separations, they may add a degree of flexibility to reversed-phase separations.

3.2.5. *Affinity*

Affinity chromatography is widely used in the biological sciences and involves the coupling of specific reacting groups to a column support, e.g., a column of immobilized antibodies can be used to separate a specific antigen from a mixture. This technique has not been widely used in the HPLC separation of proteins.

3.2.6. *Adsorption*

Adsorption chromatography, in which separation occurs due to the selective adsorption of the components of a mixture onto active sites on the surface of an adsorbent, has not been widely applied to the separation of proteins by HPLC. This may be because an organic mobile phase must be used and the presence of any water deactivates the column; hence, aqueous solutions of proteins cannot be analyzed.

3.3. Equipment

The equipment necessary for HPLC analysis includes column, pump, solvent reservoirs, a gradient former, and a suitable detector. It is beyond the scope of this review to cover the rapid, recent developments of HPLC instrumentation.

3.4. Applications

HPLC is widely used for the analysis of drugs, biogenic amines, steroids, and other low-molecular-weight substances in biological fluids. It has also been used with considerable success in the separation of peptides, both naturally occurring and synthetic, and some examples are collected in Table 3. Particular advantages of HPLC analyses are (*a*) ver-

TABLE 3.
Separation of Protein and Peptide Mixtures by Different Types of HPLC

Sample	Support	Detection	Reference
Ion-exchange chromatography: Cation-exchange			
Vasopressin Oxytocin Substance P Angiotensin II PTH[a]	Partisil SCX	Fluorescamine and amino acid analysis	R1
Basic proteins, 5,000–50,000 MW, *e.g.*, cytochrome c, collagen	Hydroxyapatite, and acrylic-based cation exchangers		V2
	Durram DC 4A	Ninhydrin	V1
Ion-exchange chromatography: Anion-exchange			
Human serum proteins	DEAE Glycophase/CPG, QAE Glycophase/CPG, SP Glycophase/CPG	254 nm	C2

TABLE 3. (*cont.*)

Sample	Support	Detection	Reference
Thyrotropin-releasing hormone analogs	Durram DC-1A	Ninhydrin	V7
Enzymes	Anion exchange CPG	Enzyme monitor	S2
Gel-permeation chromatography			
Thyroglobulin	Porous silica	254 nm	S9
Variety of proteins and peptides from fibrinogen (341,000 MW) to Gly-Gly (132 MW)	TSK-gel (type PW column)	220 nm	H6
Human serum proteins	Spheron P300	254 nm	S13
Glucagon, somatostatin	Glycophase G/CPG	280 nm	F5
Reversed-phase chromatography			
ACTH[b], somatostatin	ODS-Si	280 nm	M6
α and γ endorphins	μ Bondapak C_{18}	Biological activity	L6
ACTH, MSH,[c] α and β endorphins, leu- and met-enkephalins	Hypersil ODS	225 nm	N3
Di-decapeptides	Phenyl Corasil Poragel PN Poragel PS	220 nm	H3
ACTH, synacthen, α MSH, CLIP,[d] memory peptide β-lipotropin, enkephalins, endorphins	Hypersil-ODS	Fluorescence, ex 275 nm, em 270 nm[e]	N4
Insulins	LiChrosorb RP-8	215 nm	D8
Ion-pairing chromatography			
Insulin, glucagon, ACTH	μBondapak with phosphoric acid, with hexane sulfonic acid	215 nm 225 nm	H2

[a] Parathyroid hormone.
[b] Adrenocorticotrophic hormone.
[c] Melanocyte-stimulating hormone.
[d] Corticotrophin-like intermediate lobe peptide.
[e] Emission (em) and excitation (ex) wavelength (nm).

satility—separation may be based on the hydrophilic or hydrophobic properties of a molecule, on its size, or on its biological properties; (*b*) speed—separations may be achieved in minutes; (*c*) minimal loss of biological activity of resolved proteins; and (*d*) ability to be used in a preparative mode.

Despite the success of HPLC in peptide analyses, it has not been widely applied to the analysis of protein mixtures encountered in biological fluids or cell/tissue extracts (see Sections 3.4.4–3.4.8).

3.4.1. *Resolving Power*

Resolution of components by HPLC is governed by a range of factors, including the partition coefficient of a component between the mobile and stationary phase, column length, and particle size of the column packing. An account of the theoretical basis of resolution in HPLC has been published recently by Regnier and Gooding (R4), and the reader is directed to this for further information.

3.4.2. *Detection Methods*

Detection of resolved components in HPLC has several constraints. First, small samples are used because HPLC columns are easily overloaded; detectors must therefore be capable of detecting less than one part of solute in 10^6 parts of eluent. Second, to obviate peak broadening, the volume of the cell in which the measurement is to be made must be kept to a minimum, and the cell must be designed so that it is well swept by the flow of eluent.

In protein chromatography, two main types of detector have been used, the UV detector and the fluorometric detector.

UV Detection. The UV detector has been used most widely and usually without derivatization of the sample. Wavelengths in the range 200–340 nm are commonly employed. The wavelength 215 nm is the isobestic point at which random and helical peptide bond absorptions from the π–π^* transition are equal. Thus, at this wavelength, UV absorption is approximately independent of protein composition. This wavelength can therefore be used to compare concentrations of different proteins. It is not, however, specific for proteins.

Fluorometric detection. Greater specificity in detection may be obtained by derivatization and this has been used in conjunction with fluorometric detection. The two principal derivatization reagents employed have been fluorescamine (Fluram—trademark of Hoffmann-La Roche Inc.) (F4, F6) and *o*-phthalaldehyde (B3, C9). The latter reagent gives a higher quantum yield, is soluble in aqueous buffers, and is 5–10 times as sensitive as fluorescamine (B3, C9). Both fluorescamine

and *o*-phthalaldehyde react with primary amines; recently, a fluorescent derivatization agent (7-chloro-4-nitrobenzyl-2-oxa-1,3-oxadiazole), which reacts with prolyl secondary amine groups, has been described by Krol *et al.* (K17).

Miscellaneous. Reaction with ninhydrin and colorimetric detection of the reaction products (V1), and a moving wire in conjunction with a flame ionization detector (T7) have been used to detect proteins and peptides, but both methods have poor sensitivity. A recent innovation has been the development of reaction detectors for determining proteins with biological activity: For example, lactate dehydrogenase can be detected by adding NAD and lactate to the column effluent and monitoring the formation of NADH either fluorometrically or absorptiometrically (S4).

Radiochemical and immunochemical techniques of detection may offer improvements in sensitivity over conventional detection methods for proteins. These techniques have been applied in the HPLC of biogenic amines (M7) and of drugs and drug metabolites (T9), but not as yet in the HPLC of proteins. Similarly, there has been little study of the electrochemical detection of proteins.

3.4.3. *Data Handling and Interpretation*

Data handling in HPLC protein analysis has been limited to the determination of peak areas/heights, retention times, and, in the case of gel-permeation, molecular size. Commercial equipment is available for this.

In the analysis of complex mixtures, further information may be gathered by co-running pure samples in order to identify unknown peaks. Identity should be confirmed using a different column support.

3.4.4. *Ion-Exchange*

Ion-exchange HPLC separation of enzymes has been a particularly active area of research, mainly because of the clinical usefulness of isoenzyme measurements. Most separations employ a conventional colorimetric or fluorometric assay on the eluent (post-column) to detect resolved isoenzymes (F9).

A Glycophase DEAE-CPG column has been used to resolve arylsulfatase isoenzymes in serum and in concentrated urine samples from healthy controls, patients with colorectal cancer, and patients with malignant melanoma (B16). Creatine kinase MM and BB isoenzymes in brain and in muscle extracts have been resolved by anion exchange, but the MB isoenzyme was not detected either because of denaturation in the chromatographic process or because of adsorption to the column (K18). The MB isoenzyme has been successfully detected on a DEAE–Glycophase column in the serum of a patient who had suffered a myocardial

infarction (S3). This type of column is also effective in separating the three creatine kinase isoenzymes, and separation times of less than 5 minutes are possible (C2). Several groups have reported methods using DEAE anion exchange columns for resolving the isoenzymes of lactate dehydrogenase in both the serum (C2, D7, F9, K19, S3, S4) and in heart and spleen extracts (K19). Analysis of the serum isoenzymes could be completed within 0.5 hours (Fig. 6) and the method compared well with classical electrophoretic methods (F9, S3). An interesting observation by Kudirka *et al.* (K19) was the presence of up to three additional peaks of LDH activity in serum, but the identity of these as the extra isoenzymes of LDH described by other workers was not substantiated.

A popular use of ion-exchange chromatography has been in "metabolic profiling." The concept that individuals have a "metabolic fingerprint" that can be defined by the amount and variety of constituents in their biological fluids arose in the late 1940s (G3, S5). An extension of this idea allows a disease state to be identified by its "metabolic profile" (R7). Such a system relies on a high-resolution technique that allows the separation, detection, and quantification of many components in one sample in one analytical run. HPLC is therefore a suitable method for metabolic profiling. Early studies used ion-exchange columns with gradient elution and

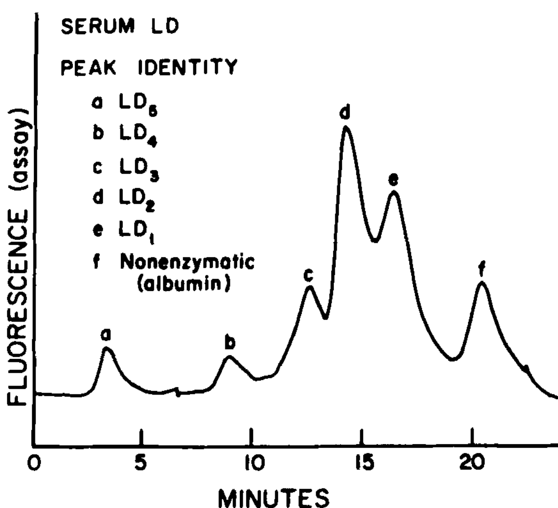


FIG. 6. Normal serum LD isoenzyme profile. The serum sample was directly chromatographed on an anion-exchange column with a 20-minute linear gradient at a flowrate of 1.25 ml/minute. The LD assay reagent was added at 0.8 ml/minute. The post-column temperature was 40°C and the resulting NADH was detected by its fluorescence. From Schlabach *et al.* (S3), with permission of the authors and *Clinical Chemistry*.

various detection systems, such as UV-absorbance and the ninhydrin reaction. Scott (S5, S6) and co-workers (S7, S8) have used such a system to detect over 140 components in urine, although analyses could take up to 40 hours per sample. Another group of workers (G5) has also used ion-exchange columns to separate urinary constituents with UV-absorbing and/or fluorescent properties, each analysis taking 20 hours. Using ammonium chloride/acetonitrile stepwise elution, Miyagi *et al.* (M9) were able to reduce the analysis time for urine to 2 hours. The number of peptides and proteins among the many components resolved by such profiling techniques is not known, since the detection methods used were nonspecific.

Analysis of human serum by anion exchange HPLC has demonstrated up to 16 components (A5, C2). Hemoglobins A1 and A2 from normal red blood cells have also been effectively resolved using a DEAE-Glycophase/CPG column and gradient elution (C2), and also by using a Synchropak AX 300 column (G10).

3.4.5. *Gel-Permeation*

Chromatographic media suitable for HPLC gel-permeation, such as the TSK–SW gels from the Toya Soda Company (Japan), have become available only recently and the number of applications described thus far has been very limited. Various animal proteins have been separated with very little loss of biological activity (T5), but preliminary results with human serum are not encouraging. Columns made using the TSK 3000 SW and TSK 2000 SW gels have resolved human serum into only 4–8 components (F8, W2). Controlled pore glass, modified by binding of glyceryl or glycerylpropyl groups to its surface, has also been utilized for gel-permeation HPLC of both native (C11) and denatured proteins (B9).

3.4.6. *Reversed-Phase*

The potential of reversed-phase HPLC in the chromatography of proteins and peptides has been amply demonstrated by results obtained with synthetic peptides (B10, F1) and mixtures of proteins (M11). Even diastereoisomers of short chain peptides can be separated by this method (K16, L7).

Reversed-phase chromatography has been used in metabolic profiling. Knudson *et al.* (K11) studied samples of serum, urine, and hemodialysate and, using gradient elution, were able to separate over 20 components in 80 minutes. The identification of components in the eluent was based on retention times, UV-absorption spectra, and mass spectra. Using this

technique, these authors concluded that most components originated from tissue fluids, and they were able to demonstrate that the concentration of most components of serum and hemodialysate fluid fluctuated regularly with time. Other workers (C6) have used reversed-phase chromatography for profiling urines from patients with rheumatoid arthritis (Fig. 7). Serum filtrates from a healthy control, a patient with lung cancer, and a patient with breast carcinoma and bone secondaries have been analyzed on a Bondapak/C18 column. A large number of constituents were resolved (UV detection 254 nm) but few were proteins or peptides (B22).

Reversed-phase (Hypersil ODS column) has also been applied in the analysis of proteins (25,000 MW) contained in extracts of medullary thyroid carcinoma cells and partially purified hypothalamus extracts (N5).

Analytical and preparative separation of proteins soluble in an organic phase (*n*-propanol) has been carried out on a LiChrosorb–Diol column. This type of chromatography has been termed "normal phase" (R8). It has been applied to the analysis of proteins in dialyzed fetal calf serum (R8) and in the separation of protected hydrophobic oligopeptides (N1), but as yet, it has not been used in a clinical chemical application.

3.4.7. *Ion Pair and Ion Suppression/Ion Enhancement*

Hearn and Hancock have surveyed the role of ion-pair HPLC in peptide and protein chemistry (H7). This mode of chromatography is effective in separating closely related peptides (R6) and has been used to analyze tryptic and CNBr digests of hemoglobin variants (H7). Detector response can be amplified by choosing a counter ion with a high molar absorptivity at the measurement wavelength. The resulting ion pairs have a greater absorptivity and hence, sensitivity of detection is increased dramatically. This strategy has been employed to resolve dipeptides using naphthalene-2-sulfonic acid as counter ion and a detector wavelength of 254 nm (C10).

3.4.8. *Affinity*

Ohlson *et al.* (O5) were the first to describe the combination of a bioaffinity support and HPLC. They used a silica support coated with aminohexyl AMP groups to separate horse liver alcohol dehydrogenase and porcine lactate dehydrogenase. Antibodies to human serum albumin bonded to silica separated bovine from human serum albumin within 5 minutes. The recovery of human serum albumin added to the column was quantitative ($>95\%$).

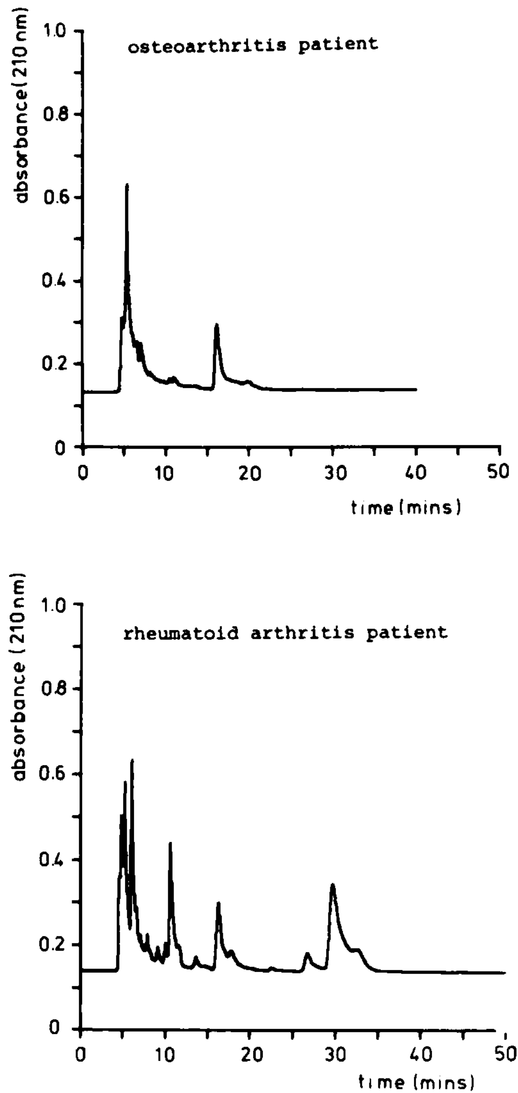


FIG. 7. HPLC trace of urine UV-absorbing constituents in the range 1,000–10,000 MW, from a patient with rheumatoid arthritis and a patient with osteoarthritis. From Clark *et al.* (C6), with permission of the authors and the *Journal of Chromatography*.

4. High-Resolution Two-Dimensional Electrophoretic Techniques

4.1. Introduction

Simple one-dimensional electrophoretic separation techniques can only partially resolve the complex mixtures of proteins and peptides found in biological fluids. Resolution may be improved by combining an electrophoretic separation with a second separation method at right angles. The second method will preferably be based on a different analytical principle. In theory, the resolution of such combinations is the product of the resolution of the component methods.

The two-dimensional approach to analysis was first described in 1956 by Smithies and Poulik (S12), who separated human serum proteins into more than 15 components using a combination of paper electrophoresis followed by starch gel electrophoresis. Since then, numerous two-dimensional systems have been described (Table 4). In many of these techniques, good separations are not achieved because the resolution in each dimension is not good, and some of these are only of historical interest (Table 5). In recent years, one of the most popular combinations has involved immunoelectrophoresis (IEP) in the second dimension. Combinations of IEP with electrophoresis in different media [e.g., agarose, starch, and agarose/polyacrylamide gel (PAG)] and with isoelectric focusing (IEF) have been reported (D3). Further variation can be introduced by including hydrophobic substances (e.g., Phenyl-Sepharose), biospecific molecules (e.g., lectins), or detergents in the first-dimension electrophoresis and detergents or lectins in the second-dimension IEP; or by including an intermediate gel containing proteins (e.g., antibodies),

TABLE 4
Varieties of Two-Dimensional Electrophoretic Techniques

First Dimension	Second Dimension										
	1	2	3	4	5	6	7	8	9	10	11
1 Paper electrophoresis	■	■									
2 Starch gel electrophoresis									■		
3 Cellulose acetate									■		
4 PAGE				■	■						
5 Gradient PAGE											
6 SDS–PAGE											
7 Gradient SDS–PAGE									■		
8 Agarose electrophoresis					■			■			
9 IEF				■	■	■	■			■	
10 NEPHGE							■				
11 ITP									■		

TABLE 5
Two-Dimensional Electrophoresis

	Application	Reference
Including IEF		
Cellulose acetate electrophoresis/IEF	50 serum proteins	A4
Starch gel electrophoresis/IEF	Alcohol dehydrogenase	H4
ITP/IEF	20 serum proteins	B20
IEF/IEP	α_1-Antitrypsin	D3, S10
Miscellaneous		
Paper electrophoresis/paper electrophoresis	Tryptic digests, e.g., immunoglobulins	W4
Paper electrophoresis/starch gel electrophoresis	Serum proteins	S11
PAGE/PAGE		R2, R3, K1
Agarose electrophoresis/agarose electrophoresis	Protein detergent binding	B5
PAGE/gradient PAGE	100 plasma proteins	M3, M4, W8
	Carried out in single slab	W3
Agarose/gradient PAGE	Serum proteins	F2, F3

between the first and second dimensions (B13, B15, V3). The use of immunochemical techniques can add a degree of specificity to two-dimensional techniques.

The most powerful two-dimensional systems are those employing combinations of isoelectric focusing and various forms of polyacrylamide gel electrophoresis (PAGE) and these are described in the following sections. In particular, this section of the review will be concerned with the principles and applications of IEF/PAGE.

4.2. Principles

4.2.1. *IEF*

Isoelectric focusing separates amphoteric molecules, e.g., proteins, in a gradient according to their isoelectric points (pI). A stable continuous pH gradient is established by means of carrier ampholytes (e.g., mixtures of low-molecular-weight polyaminopolycarboxylic acids) and an electrical field. Charged protein molecules migrate in the pH gradient under the influence of the electrical field until they reach the pH value equal to their pI. At this point, the net charge on the protein is zero and it remains stationary. If the protein migrates or diffuses away from its pI, it becomes charged (positive at pH $>$ pI or negative at pH $<$ pI) and is

repelled back to its p*I*. Separation of proteins with p*I*s differing by as little as 0.005 pH unit can be achieved (R5).

Focusing can be performed in liquids, but in two-dimensional systems, solid supports are used. Polyacrylamide gels have been perhaps the most widely used supports. The advantages of PAG as a medium are that it is chemically stable and has low electroosmotic flow, high optical clarity, and excellent anticonvection properties. Its disadvantages are that the acrylamide monomer is a neurotoxin and the polymer has molecular sieving properties that tend to retard the electrophoretic migration of molecules. The latter effect is minimized in low-concentration PAGs (i.e., $< 5\%$). More recently, agarose suitable for IEF (i.e., with very low electroendosmosis) has become available and it is to be expected that this will be widely used because it has none of the above problems of PAG.

Ampholytes spanning the pH range 2–11 or intervals within this range are available commercially. Ideally, the pH range of ampholytes used should be adjusted to suit the p*I*s of the proteins to be separated. However, the basic region of the pH gradient formed by ampholytes is unstable, particularly in the presence of urea, and consequently, basic proteins tend not to be resolved. This problem has been overcome using nonequilibrium forms of IEF, the so-called NEPHGE or BASO–DALT techniques, and these are described in Section 4.2.4.

Further details of the theory, practical aspects, and applications of IEF are contained in reviews by Allen (A4), Latner (L2), and Righetti and Drysdale (R5).

4.2.2. *PAGE*

PAG is a three-dimensional polymer network made from acrylamide and a cross-linker, e.g., *N*,*N*′-methylenebisacrylamide (Bis). Other cross-linkers include, *N*,*N*′-diallyltartardiamine (DATD), *N*,*N*′-acrylcystamine (BAC), and *N*,*N*′-dihydroxyethylenebisacrylamide (DHEB). Unlike Bis, these have the advantage of forming PAGs that can be solubilized under certain conditions.

Electrophoresis in PAG separates proteins according to their charge and molecular size, the latter because the gel also acts as a molecular sieve. To improve the separation of a given protein, the pore size of the PAG may be altered by varying the relative concentrations of acrylamide and cross-linker. In addition, the use of PAG with gradients of concentration will sharpen protein bands because the leading edge will be retarded more than the trailing edge. Separation of proteins in PAG depends upon both the charge on the protein and the relative sizes of the protein and the pores in the gel. Effects due to charge differences between proteins

can be eliminated by treating proteins with the detergent sodium dodecyl sulfate (SDS) (W1), which binds to proteins to form denatured SDS–protein complexes in which the charge per unit mass is constant.

Thus, the mobility of a protein in an SDS–PAG depends primarily on molecular size (weight). Note that the SDS must be present in the second-dimension gel, because it is removed from the first dimension during IEF.

4.2.3. *Sample Preparation*

Native or denatured proteins can be analyzed using the various types of IEF/PAGE outlined below. Prior treatment of samples with SDS-thioethanol or urea-thioethanol (dithiothreitol may be used in place of thioethanol) ensures both dissociation of subunits and cleavage of disulfide linkages. Dissociation in the first dimension is maintained by including urea (6–8 mmol/liter) and a detergent (Nonidet P–40) in the IEF gel. The presence of SDS in the sample does not interfere with isoelectric focusing because it is removed from the denatured proteins and forms mixed micelles with the Nonidet P–40. These then migrate to the acidic end of the gel (O2). If denatured proteins are being analyzed, it is important to maintain denaturing conditions throughout both stages of the two-dimensional separation. Thus, after the first dimension, the IEF gel is equilibrated with SDS-thioethanol before being sealed onto the second-dimension gel. Alternatively, SDS may be electrophoresed into the IEF gel *in situ* (O2).

4.2.4. *Varieties of Two-Dimensional IEF/PAGE Systems*

Dale and Latner (D1) were the first to describe the separation of human serum proteins using a combination of IEF followed by PAGE. Since then, the technique has been widely applied and a variety of modifications have been described. These involve (*a*) the presence or absence of urea in the first dimension, (*b*) the use of gradient or homogeneous PAG in the second dimension, (*c*) the inclusion of the detergent SDS in the second dimension, (*d*) reversal of the order in which the two separations are performed and (*e*) the use of microgels (0.12–0.36 mm thick) of PAG (Table 6).

The combination of IEF with gradient SDS–PAGE gives very high resolution. The technique was first described in 1975 (O2) and has been applied to denatured (C12, O3) and nondenatured protein mixtures (S1). A definitive account of the factors affecting reproducibility, quantitation of components, resolution, and sensitivity is detailed in the paper by O'Farrell (O2). Subsequently, Anderson and Anderson (A8, A9) have adapted O'Farrell's original method to allow the analysis of large

TABLE 6
VARIETIES OF IEF/PAGE

Type of electrophoresis	Details	Reference
IEF/gradient PAGE	4.5–26% concave gradient	K4
	—	W9
IEF/SDS-PAGE	Plant proteins, animal tissue, proteins (± 0.2 pH, units ± 3000 daltons)	B2, C12, M1, S1
IEF/gradient SDS–PAGE	Denatured proteins	S1
	Nondenatured proteins	S1
	ISO–DALT	A2, A3, A8, A9
	Gradient SDS-PAGE/IEF	T8
	NEPHGE	O4
	BASO–DALT	G7
	Microgels	G11, P2

numbers of denatured samples in parallel—this modified method has been named ISO–DALT (derived from ISOelectric and DALTons).

A variant of IEF/gradient SDS–PAGE in which the order of separation is reversed (i.e., gradient SDS–PAGE/IEF) has also been reported (T8). Its main disadvantage, apart from the expense of the ampholytes in the second dimension, was that proteins could not be electrophoresed directly into the second-dimension IEF gel because of the sieving properties of the gradient SDS–PAG. Instead, it was necessary to elute proteins electrophoretically from the gradient SDS–PAG into an intermediate stacking gel prior to IEF.

In order to resolve basic proteins using IEF/gradient SDS–PAGE, certain modifications are required because of the instability of the basic end of the pH gradient in the first-dimension gel (see Section 4.2.1). Normally, basic proteins that enter the IEF gel are not resolved well. This may be overcome by using very short electrophoresis times (so that equilibrium is not reached) for the first-dimension IEF and by reversing the direction of focusing, such that basic proteins lead in the separation (i.e., electrophoresis toward the anode as opposed to the cathode). O'Farrell (O4) has named this technique NEPHGE (nonequilibrium pH gradient electrophoresis), and has shown that NEPHGE/gradient SDS–PAGE has a similar resolving power to that of IEF/gradient SDS–PAGE. More recently, the NEPHGE technique has been utilized as the first dimension in the Anderson ISO–DALT method, and the modified method (BASO–DALT) has been used to analyze basic proteins from skeletal muscle (G7).

4.3. Equipment for IEF/PAGE

Isoelectric focusing of specimens is usually performed in PAG gels formed in narrow glass tubes (O2, O3). Alternatively, several samples may be focused on PAG slabs and the slabs cut into strips corresponding to the individual specimens (A6, I1, M3, S1). Anderson and Anderson (A9) have described an apparatus that allows both the simultaneous casting of 20 IEF gels and focusing of the gels—the so-called iso apparatus (Fig. 8).

Gel electrophoresis in the second dimension is performed in a slab of

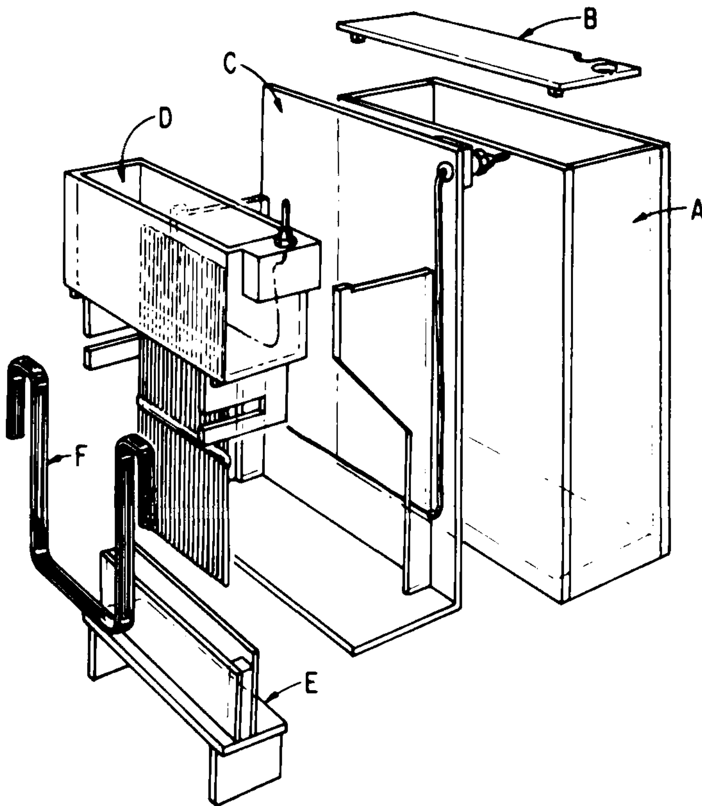


Fig. 8. Diagrammatic representation of the iso apparatus used for multiple-parallel isoelectric focusing in acryamide gels. Components are (A) lower buffer box, (B) top, (C) removable rack supporting one electrode and upper buffer reservoir, (D) upper reservoir with upper electrode and attached glass gel-holding tubes, (E) gel casting trough, and (F) a retainer. From Anderson and Anderson (A9), with permission of the authors and *Analytical Biochemistry*.

PAG gel formed between two glass or plastic plates. The size of the slab gel ranges from 80 × 80 × 3 mm (L4) to 180 × 180 × 3 mm (A8). Several authors have described the design and construction of apparatus suitable for casting and running slab gels, including gradient gels (D2, I1, M2, M3, W2, W7), but only the multiple slab gel electrophoresis system (the so-called DALT system) described by Anderson and Anderson (A8) is suitable for analyzing large numbers of specimens. An apparatus based on Anderson's ISO–DALT system is available from Electro-Nucleonics, Inc. (P.O. Box 451; Oak Ridge, TN 37830) while several other manufacturers (e.g., Bio-Rad Laboratories; 2200 Wright Avenue; Richmond, CA 94804; Pharmacia; Box 175, S–75104 Uppsala 1; Sweden) sell equipment suitable for simultaneously running a small number of second-dimension gels.

Recently, Jones *et al.* (J2) have described the design and operation of a low-volume electrophoresis tank suitable for running ten slab gels (size: 150 × 150 × 2 mm). The advantages of this tank are that its construction is relatively simple and its low volume makes it economical to dispense with the electrophoresis buffer after two or three runs.

4.4. Applications

The usefulness of IEF/PAGE methods depends on their resolving power, the sensitivity of detection of separated proteins/peptides, and also on the ease of interpretation and comparison of complex protein maps.

4.4.1. *Resolving Power*

In a two-dimensional system, resolution is a product of the resolution of the component methods. Both IEF and SDS–PAGE can separate approximately 100 protein/peptides, giving a combined resolution of 10,000 proteins/peptides (A12). However, an upper limit of approximately 30,000 has been proposed based on the number of 1-mm-diameter spots that would fit into a slab of PAG ≃ 17 × 17 cm (A9). In practice, fewer than 2,000 proteins/peptides have been resolved by such methods [e.g., 1,600 proteins from a rat liver hepatoma cell line (O3) and 1,400 components from monkey cells (O4)].

The effect of the size of the gels on resolution remains equivocal. Voris and Yound (V8) increased the size of the gels (32 cm vs 13 cm, first dimension; 40.64 × 40.64 × 0.8 cm vs 16.5 × 16.5 × 0.15 cm, second dimension) and were able to detect 1,750 proteins from rat thymus as compared with 500 on the smaller gels. This increased resolution was due to the increased sample size that could be analyzed and hence an increase

in the number of detectable proteins; in addition, autoradiographic spreading is minimized in large gels. In contrast, suitable resolution has been obtained with microgels (second dimension 3 × 3.5 cm × 0.25–1 mm) (P2).

4.4.2. *Detection Methods*

The most commonly used method for the detection of proteins and peptides in PAG is with dyes, such as Coomassie Brilliant Blue (R or G 250) or Kenacid Blue R. Such dyes can detect proteins in amounts down to 38 ng/mm^2 (S14), but for lower-molecular-weight proteins/peptides the limit may be 150 ng/mm^2 (O2).

More sensitive techniques for detection (by a factor of 100) include autoradiography (O2) and a silver stain (O1, S15). Although autoradiography is more sensitive than staining, it has several disadvantages: the need to radiolabel the proteins, dry the gels, and use long exposure times. Similarly, the silver stain, though sensitive, is expensive and involves a lengthy procedure.

4.4.3. *Data Handling and Interpretation*

There are two main aspects in analyzing the results from IEF/PAGE: quantitation of the separated proteins and comparison of protein patterns.

Individual protein spots can be quantitated by dye-staining, cutting out the spot on the gel, either eluting the dye or dissolving the gel (see Section 4.2.2), and spectrophotometric determination of dye concentration (A12, B23, M5). Such methods are generally lengthy, inaccurate, and imprecise. Alternatively, the wet gel can be cut into strips and scanned using a densitometer, or a negative photograph of the gel can be scanned (A12). The densitometers currently available for wet-gel-scanning are inappropriate for two-dimensional gels, and the scanning of photographs introduces another step into the procedure. Comparison of protein patterns generated by IEF/PAGE presents problems because the number of protein spots per gel makes comparison by eye difficult.

Effective comparison of patterns of proteins on IEF/PAGE gels requires some method of normalizing the protein patterns to compensate for distortions in the patterns due to gel-to-gel variation in the analytical conditions in either the first- and/or second-dimension separation. Internal standards are valuable in monitoring variation in analytical conditions: Isoelectric point standards for IEF based on carbamylated hemoglobin, fluorescein-labeled-hemoglobin, and creatine phosphokinase (A11, M5) can be prepared, as can molecular weight standards based on heart muscle proteins (G8).

Optical superimposition (comparator) allows comparison of two gels at one time and is not therefore suitable for a large number of gels (A12). Similarly, superimposition of negative photographs of the gels is not applicable to a large number of gels (C3).

Several prototype data-handling systems have been described (G2, L5, L8, T1). These generally scan negative photographs of the gel, digitize the data, subtract the background, and calculate/correct the position of the spot on the gel. However, no series of whole gels has been analyzed, many problems remain to be solved, and these systems are in no way routinely applied. When fully developed, such systems will allow the full potential of high-resolution IEF/PAGE methods to be realized.

4.4.4. *Analysis of Body Fluids*

The following section summarizes the applications of IEF/PAGE methods in the analysis of human body fluids.

The location of individual proteins or protein subunits has been established by analyzing pure proteins and immunoprecipitates prepared using specific antisera, or by co-electrophoresis. Proteins containing cysteine residues can be specifically labeled using [^{125}I]iodoacetamide and can be located autoradiographically. If analysis is performed under nondenaturing conditions, specific stains are available to locate certain types of protein, e.g., glycoproteins (A14, H12) and enzymes.

Serum and plasma. The pattern of plasma proteins following pretreatment with SDS and mercaptoethanol and then analyzed by IEF/gradient SDS-PAGE (ISO-DALT) is depicted in Fig. 9. About 300 proteins are visible in this Coomassie Blue-stained gel and the identity of some of these proteins is detailed in Fig. 10 (A7). The contrasting pattern of serum proteins analyzed under nondenaturing conditions by agarose electrophoresis/gradient PAGE is shown in Fig. 11. A notable feature of the ISO-DALT protein map is the microheterogeneity of many of the proteins—i.e., closely related groups of spots forming "runs" or "staircases." This microheterogeneity has been studied in several proteins, e.g., transferrin, haptoglobin *b* chain, α_1-antitrypsin, α_2-HS glycoprotein, arginine-rich lipoprotein, and the globulins (A7, A10). Heterogeneity of protein spots has been classified into three types. The first type is charge heterogeneity, which gives rise to spots in rows along the horizontal axis. This heterogeneity may be due either to the addition of charged residues, such as sialic acid, or to deamidation. The second type of heterogeneity is due to the addition of variable numbers of neutral carbohydrate side chains. These may be synthesized separately and transferred intact to plasma proteins, and could account for the vertical component of the "staircase" effect of many proteins in two-dimensional gels. Finally, it is

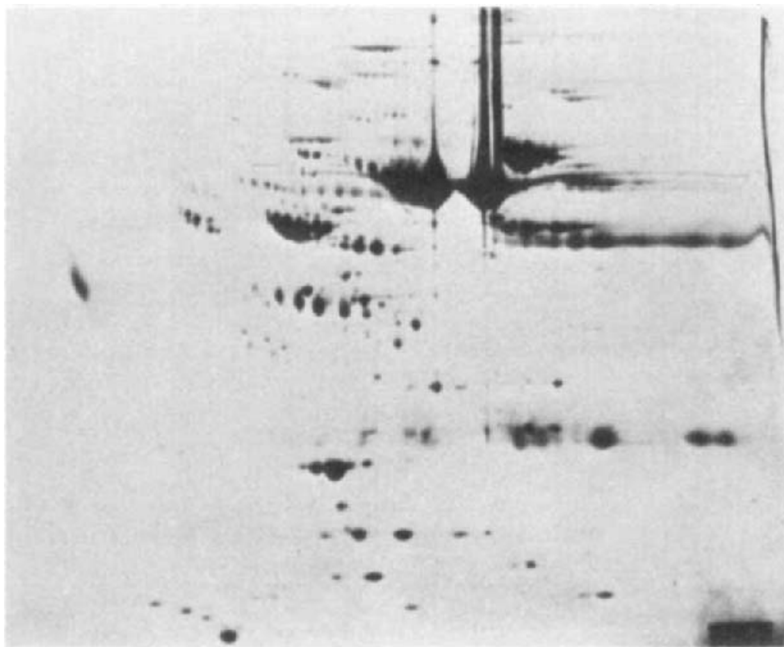


FIG. 9. Two-dimensional gel of human plasma proteins. The sample was 10 μl of fresh heparinized plasma, denatured in sodium dodecyl sulfate/mercaptoethanol. From Anderson and Anderson (A7), with permission of the authors and *Proceedings of the National Academy of Sciences (U.S.A.)*.

suggested that variations in the molecular weight of the transferred carbohydrate chains could account for the elongation of some of the spots in the vertical direction. These effects are thought to be due to an *in vivo* rather than an *in vitro* effect (A12, E1, E2).

The ISO-DALT technique has been used to investigate the pattern of proteins in sera from patients with, for example, Hodgkin's disease, Waldenström's macroglobulinemia, and myeloma (L4, T3). In addition, it has proved a valuable tool in the study of the protein matrix of animal- and human-based quality control sera (C7). The technique has also been successfully applied to the analysis of particular proteins, e.g., the apolipoproteins (Z1). Complementary studies, using nondenatured samples of sera from patients with, for example, myeloma, Waldenström's macroglobulinemia, hepatic cirrhosis and of the lipoproteins have also been carried out (E3, E4, L3) and are reviewed by Latner (L2).

Urine. Urine is known to contain a complex mixture of proteins and peptides in low concentrations, but early studies of this fluid using IEF/PAGE (nondissociating conditions) did not reveal many proteins (32)

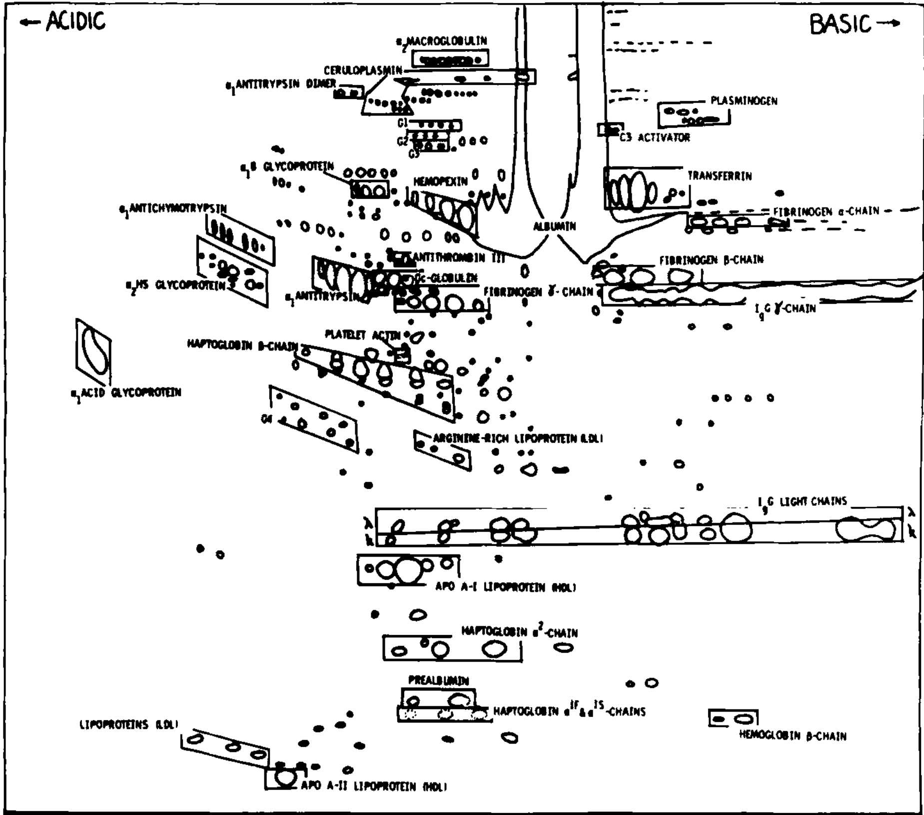


FIG. 10. Diagram drawn from the gel shown in Figure 9, and labeled to indicate positions of known plasma proteins. From Anderson and Anderson (A7), with permission of the authors and *Proceedings of the National Academy of Sciences (U.S.A.)*.

either in normal or pathological urine (nephrotic syndrome) (25). In order to improve the analysis, it is necessary to concentrate the urine at least 1000-fold (A13). When concentrated urine was analyzed under denaturing conditions, approximately 250 proteins were revealed, and some were identified (Fig. 12) (A12, T4). Differences in urinary proteins between the sexes were minimal, as were those in the low-molecular-weight range in several samples from the same individual. Using the ISO-DALT technique, changes in urinary proteins have been found in several diseases, e.g., bladder cancer (A12) (Fig. 13) and rheumatoid arthritis (C3).

Cerebrospinal fluid. Both PAGE/gradient PAGE and IEF/gradient PAGE have been used in the analysis of csf proteins (44–73 components

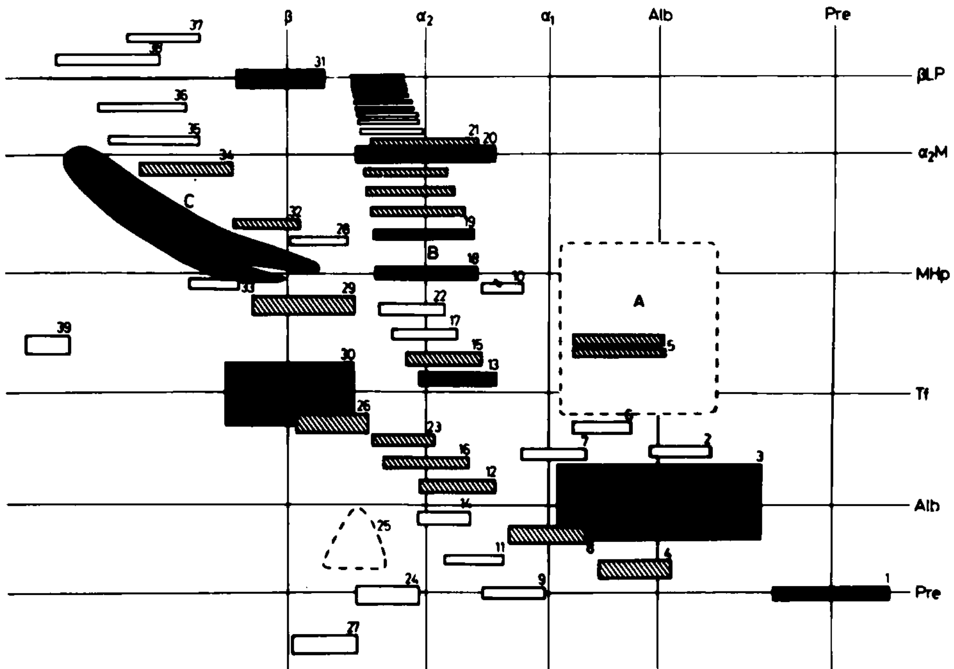


FIG. 11. Protein pattern of serum on two-dimensional electrophoresis, compiled from numerous experiments. The analytic grid is constructed from the final position of prealbumin (1, Pre), albumin (3, Alb), α_1-antitrypsin (8), α_2-macroglobulin (20, α_2-M), β-transferrin (30, Tf), and τ-globulin (30a), as well as monomeric haptoglobin (18, MHp 2–1) and β-lipoprotein (31, β-LP).

Localization of individual proteins (ordered according to charge): 1 = prealbumin, 3 = albumin, 4 = acid α_1-glycoprotein, 5 = albumin dimer, 7 = α_1-antichymotrypsin, 8 = α_1-antitrypsin, 12 = Gc-globulin, 13 = haptoglobin 1–1, 15 = ceruloplasmin, 16 = α_2-HS-glycoprotein, 18 = monomeric haptoglobin 2–1, 19 = monomeric haptoglobin 2–2, 20 = α_2-macroglobulin, 26 = hemopexin, 28 = C_4-complement, 29 = β_{1A}-globulin (C3c), 30 = transferrin, 31 = low-density lipoprotein, 32 = β_{1C}-globulin (C3), 34–36 = immunoglobulin oligomers, and 39 = γ trace-protein.

A = high density lipoproteins (3–5 subfractions), B = haptoglobin polymers (up to 15 bands), and C = immunoglobulins A and G.

Tentative identification: 6 = α_1T-glycoprotein, 10 = inter α_1-trypsininhibitor, 11 = 3.8 S-α_2-glycoprotein, 14 = Zn-α_2-glycoprotein, 17 = C_9-complement, 24 = β_2-glycoprotein I, 27 = β_2-glycoprotein III, 33 = plasminogen, 37 = C_{1q}-complex, and 38 = immunoglobulin M. The bands 2, 9, 21–23, and 25 have not been identified. From Felgenhauer and Hagedorn (F3), with permission of the authors and *Clinica Chimica Acta*.

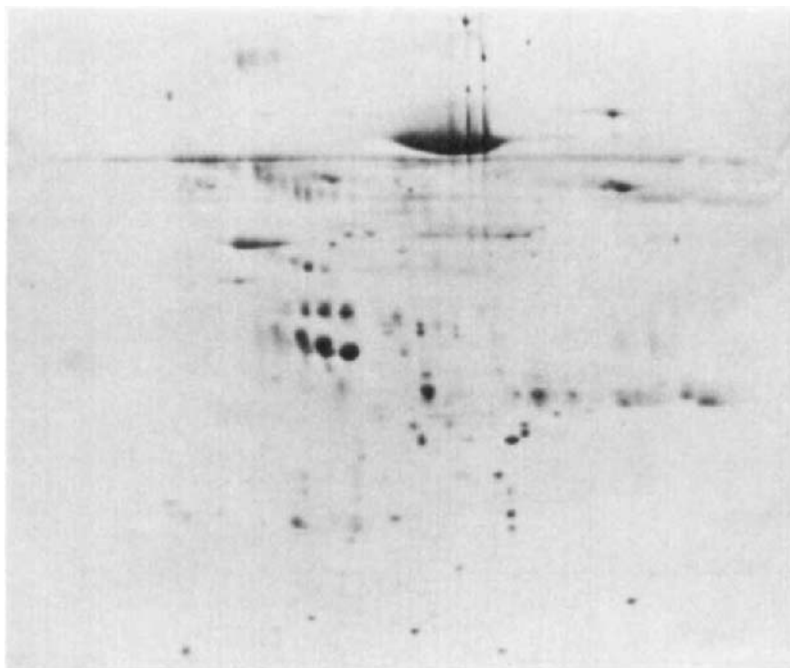


FIG. 12. Pattern of urinary proteins from a 27-year-old normal man. From Anderson *et al.* (A12), with permission of the authors and *Clinical Chemistry.*

resolved, of which albumin and transferrin have been identified) (L2, W9).

Using the more sensitive silver stain, however, Goldman and coworkers (G9) have been able to resolve more than 300 proteins and/or peptides (denatured sample) in csf using IEF/SDS-PAGE (Fig. 14). Twenty six of the proteins have been tentatively identified (Fig. 15), and comparison of the protein patterns with the corresponding plasma protein patterns has revealed several clusters of unidentified proteins that were more prominent in csf than in plasma and hence, may be of CNS origin.

4.4.5. *Other Proteins of Biochemical Interest*

The technique of IEF/SDS-PAGE has been applied to the study of several body fluids and tissues not usually analyzed routinely in the clinical laboratory (Table 7). Although the significance of many of the protein patterns and any differences demonstrated remains to be elucidated, this work does illustrate the wide applicability of the technique and its potential in such fields as genetic mapping.

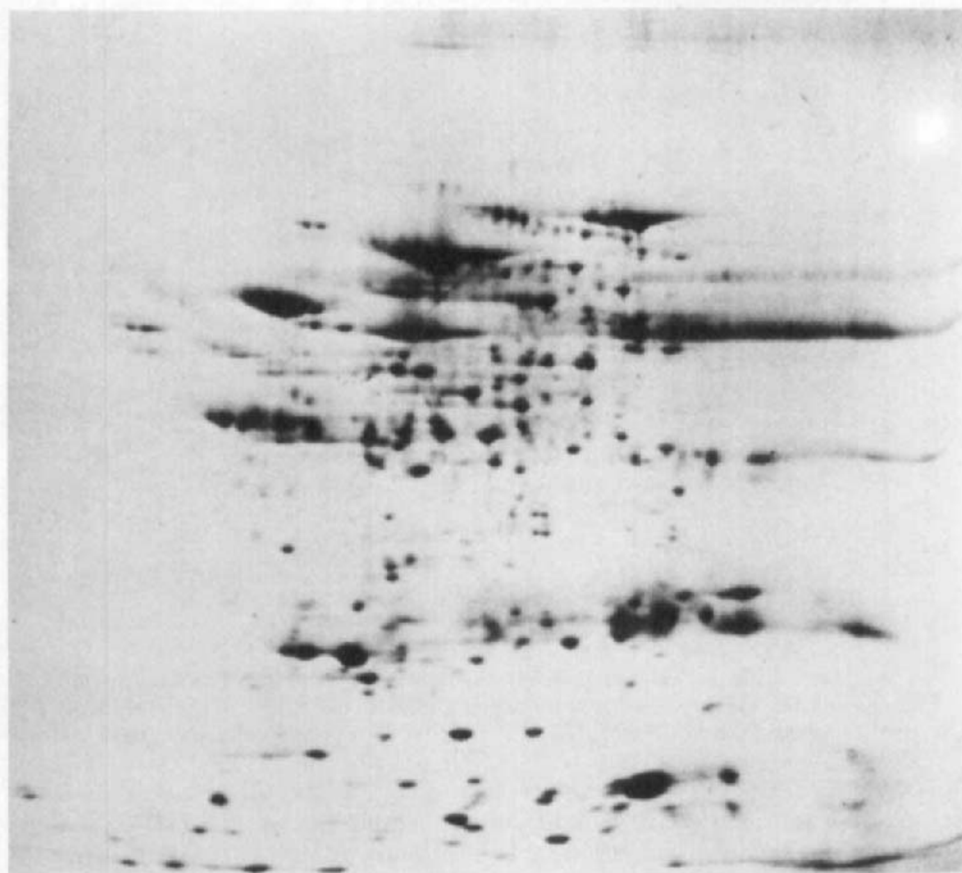


FIG. 13. Urinary protein pattern for a male patient with bladder cancer, illustrating very marked alterations in pattern and the presence of multiple additional spots in comparison with the usual pattern (e.g., Fig. 12). From Anderson *et al.* (A12), with permission of the authors and *Clinical Chemistry*.

5. Conclusions

The last decade has seen the development of several high-resolution protein separation techniques and three of these—isotachophoresis, high-performance liquid chromatography, and IEF/SDS gradient PAGE (ISO-DALT)—have been discussed in this review. The three techniques complement each other in both the method of separation and the method of detection.

The separation in both isotachophoresis and ISO-DALT is based partially on the charge of the sample species (isotachophoresis separates ac-

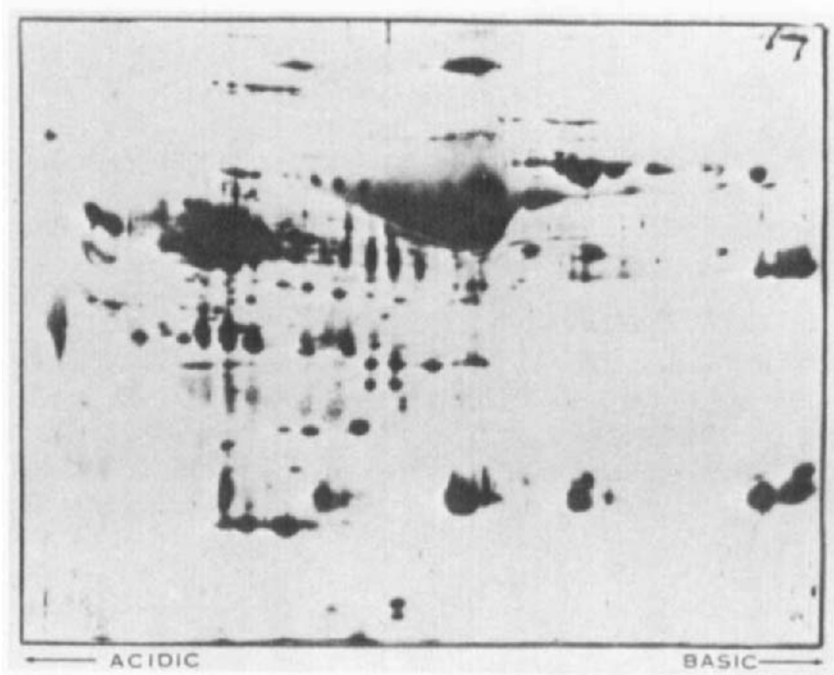


FIG. 14. Gel of csf, in which proteins were separated by IEF with 16 ml per liter 5/7 Biolyte and 4 ml per liter 3/10 Biolyte for 20 hours, followed by PAGE (100 g acrylamide per liter) for about 3 hours. From Goldman *et al.* (G9), with permission of the authors and *Clinical Chemistry*.

cording to the effective mobility of the sample species; the ISO–DALT technique involves separation by isoelectric focusing, i.e., p*I*, in the first dimension). In HPLC, the principle of separation depends on the support chosen, ion-exchange (charge) and gel-permeation (molecular size) being those most widely used in protein separations. Of the three methods, the ISO–DALT technique also differs in that the separation takes place in the presence of urea and SDS, and under reducing conditions. Therefore, the separation is of peptides and protein subunits. The specificities and sensitivities of the detection methods used in these three analytical techniques differ considerably.

ISO–DALT uses protein-specific detection methods such as autoradiography and dye-binding, e.g., with Coomassie Brilliant Blue. Isotachophoresis and HPLC both use UV-absorption for the detection of proteins, the equipment for the former being usually at a fixed wavelength and for the latter a variable wavelength. These detection methods are sensitive but not specific. With HPLC, there is the possibility of pre- or post-column derivatization, e.g., with fluorescamine, which may improve both sensitivity and specificity.

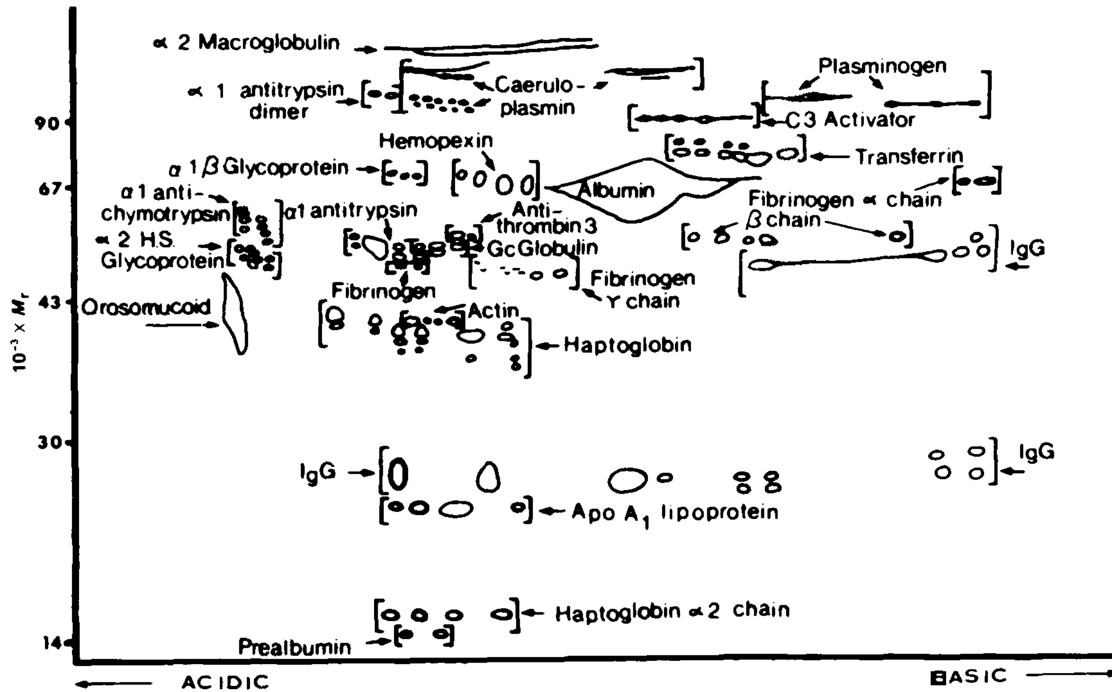


FIG. 15. Csf proteins identified by three different methods: (a) co-electrophoresis of purified proteins, (b) glycoprotein stain, and (c) position analysis, with the Anderson map of serum (cf., Fig. 10). The proteins identified, listed with the superscript letters corresponding to the method(s) of identification, are prealbumin[a,c], haptoglobin α 2 chain[c], apo-A_1-lipoprotein[a,c] (HDL), IgG heavy chains[a,b,c], orosomucoid[a,b,c], haptoglobin[b,c], actin[a,c], fibrinogen γ-chain[a,b,c], fibrinogen[a], α_1-antichymotrypsin[c], α_1-antitrypsin[a,b,c], Gc globulin[b,c], antithrombin III[a,c], IgG light chain[a,b,c], fibrinogen β-chain[a,b,c], α_1,β-glycoprotein[b,c], hemopexin[b,c], albumin[a,c], fibrinogen α-chain[a,c], transferrin[a,c], C3 activator[c], plasminogen[c], α_1-antitrypsin dimer[a,c], ceruloplasmin[a,b,c], and α_2-macroglobulin[c]. From Goldman *et al.* (G9), with permission of the authors and *Clinical Chemistry*.

TABLE 7
TWO-DIMENSIONAL ELECTROPHORETIC ANALYSIS OF TISSUES AND BODY FLUIDS

Method	Substance	Reference
	Erythrocytes	
	EDTA or Triton extracts 60 proteins	B6
	Lysates > 250 proteins	E1, E2
	Lymphocytes, lymphoblastoid cells, myeloma cells, HeLa cells	
ISO- and BASO-DALT	Lymphocytes before and after stimulation with concanavalin A protein, familial studies	W5
	Myeloma cells GM 1500	A14, H12
	Lymphoblastoid cells GM 607	A14, H12, W5
	HeLa cells	A14, H12, W5
	Virus proteins	D10
	Skeletal muscle	
ISO-DALT		G7
	Saliva	
ISO-DALT (Coomassie Brilliant Blue and silver stain)	Whole, parotid sublingual	G6
	Bile and gastric juice	
IEF/PAGE	Alkaline phosphatase	L2
	Vitamin B_{12}-binding protein	

Comparison of the convenience and cost of the three techniques may not be useful, in that commercial equipment for ISO-DALT has only recently become available, while that for HPLC and ITP has had a longer development time.

The important feature of the techniques is their high resolution. Many electrophoretic techniques currently used in clinical chemistry resolve a few bands and hence, their applications are limited diagnostically. The high-resolution techniques described in this review may prove to be suitable for mapping the products of the human genome to a greater extent, and this opens up possibilities in the fields of, for example, genetic counseling and carcinogenicity testing. For such projects to be realistic, the techniques will have to be developed (*a*) to allow rapid and easy analysis of a large number of samples and (*b*) to solve the problems of data-handling and interpretation.

Solutions to the foregoing problems (together with simplification in the instrumentation) may eventually lead to the routine application of high-resolution methods, perhaps coupled with pattern analysis/recognition, in the clinical chemistry laboratory. A more likely course of events is that they will be used as research tools to identify new proteins with

diagnostic value and that subsequently, conventional assays will be developed to allow measurement of these proteins in the routine laboratory.

The study of human gene products such as proteins should involve their characterization, determination of their biological function, and the elucidation of their role in health and disease. Such knowledge could lead to important developments in the role of clinical chemistry in the diagnosis and management of disease. High-resolution techniques, such as ITP, HPLC and ISO-DALT, may provide the analytical means of achieving this goal.

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