IMMUNOCHEMICAL AND BIOCHEMICAL INVESTIGATION OF SERUM CHOLINESTERASE IN HYPERLIPIDAEMIA

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BY

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Abstract

Serum cholinesterase is an enzyme similar to acetylcholinesterase but of unknown function. Activity varies widely between individuals and numerous variants are known. Numerous studies have related the *activity* of serum cholinesterase to serum lipid concentrations but it is not known whether the enzyme plays a part in lipoprotein metabolism nor, if it does, whether this is related to its catalytic activity or to some other feature of the protein. It was therefore decided to compare the relationships of its *concentration and activity* to the concentrations of serum lipids and lipoproteins.

Cholinesterase was purified from human serum, and antiserum was raised in a rabbit. The antiserum was used to measure the concentration of the enzyme by radial immunodiffusion in sera from 117 blood donors and 282 patients for whom serum lipid profiles had been requested.

In the donor group the correlation between activity and concentration of the enzyme was 0.95 and there was little difference between the correlations of the two measures of cholinesterase with lipids. In the patient group the correlation between serum cholinesterase activity and concentration was only 0.88 and all the lipid indices correlated better with the concentration of cholinesterase than with activity. This was probably because of variable loss of activity during transport and storage, and because of enzyme inhibition by drugs.

For further analysis the patients were preferred because they had fasted before blood sampling and had a greater range of lipid concentrations. Correlations with cholinesterase *concentration* were in the order 'total LDL' (LDL + VLDL) > VLDL > triacylglycerols > cholesterol. The correlation coefficients were similar, 0.51 with 'total LDL' as against 0.41 with cholesterol, but the shapes of the plots were markedly different. The plot of cholinesterase against cholesterol showed very weak relationship. The plot against triacylglycerols showed a well-defined triangular shape. At lower triacylglycerol concentrations a wide range of

cholinesterase concentrations was found but with high triacylglycerol concentrations the enzyme concentration was always high. The possible nature of the relationship is discussed.

In the preparatory stages, impurities occurring in affinity-purified cholinesterase were investigated by western blotting; and in concentration measurement, reproducible variation in precipitin ring patterns between individuals was observed.

Key words: Serum cholinesterase concentration; serum lipids; immunological variation of serum cholinesterase.

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$A_{4, 8, 12}$	'asymetrical' forms of serum or acetyl-cholinesterase with a collagen-like tail and 1, 2, or 3 tetramers of the active subunit respectively
ApoA-I, ApoB, etc.	apolipoprotein A-I, B, etc.
bis	N,N'-methylene-bis-acrylamide
C_1, C_2, etc.	serum cholinesterase electrophoretic bands (see 1.5)
CRF	'complimentary risk factor' for ischaemic heart disease = serum cholinesterase activity/HDL-cholesterol
CV	coefficient of variation
DEAE	diethylaminoethyl; DEAE-Sephacel is a DEAE-cellulose anion-exchanger
DFP	diisopropylfluorophosphate
E_1, E_2,	the most commonly used symbols for two genetic loci affecting serum cholinesterase, introduced by Motulsky (1964). E_1 codes for the active subunit and the symbol has superscripts, u = usual, a = atypical, s = silent, etc. for variants. E_2 controls the expression of the electrophoretic variant C_5 which has about 30% higher activity but seems to be the result of association of serum cholinesterase with a non-enzyme protein coded by the E_2 locus. The loci were officially named CHE1 and CHE2 (Shows *et al.*, 1979) but the Motulsky nomenclature continued in use. Since it was shown that there is apparently only one gene coding for the

	active peptide CHE1 has been renamed BCHE (Masson *et al.*, 1990) while the other locus retains the official name CHE2.
EDTA	ethylenediaminetetraacetate
ELISA	enzyme-linked immunosorbent assay
ERF	'established risk factor' for ischaemic heart disease = serum total cholesterol/HDL-cholesterol
$G_{1, 2, 4}$	'globular' forms of serum or acetyl-cholinesterase, monomer, dimer and tetramer of the active subunit respectively
γGT	γ-glutamyl transferase, also called γ-glutamyl transpeptidase
GOT	glutamate oxaloacetate transaminase, also known as Glutamic-oxaloacetic transaminase, and as AST, aspartate transaminase, and as aspartate aminotransferase
GPT	glutamate pyruvate transaminase, also known as glutamic-pyruvic transaminase and as ALT, alanine transaminase, and as alanine aminotransferase
HDL	high density lipoprotein
HMG-CoA	hydroxymethylglutyryl coenzyme A
IDL	intermediate density lipoprotein
iso-OMPA	tetraisopropyl pyrophosphoramide
LCAT	lecithin:cholesterol acyltransferase
LDL	low density lipoprotein

PAGE	polyacrylamide gel electrophoresis (T in this context is the percentage of acrylamide in the gel, and C is the percentage *of the acrylamide* that is bis.)
PBS	phosphate-buffered saline. See section 2.3.2.
PNPB	p-nitrophenyl butyrate
2pq	proportion of homozygotes of two alleles of frequencies p and q predicted by the Hardy-Weinberg formula, which requires random mating and absence of selection
RID	radial immunodiffusion
RO 2-0683	dimethylcarbamate of (2-hydroxy-2-phenyl-benzyl)- trimethyl ammonium bromide
SDS	sodium dodecylsulphate
TEMED	N,N,N′,N′-tetramethylethylenediamine
'total LDL'	low density lipoproteins precipitated by calcium and heparin: LDL + VLDL
tris	tris(hydroxymethyl)aminomethane
Triton X-100	a non-ionic detergent. 'Triton' is a registered trade name of Rhom & Haas Co.
Type I, IIa, etc.	Fredrickson classification of hyperlipoproteinaemia. See Section 4.1.8 for reference.
U	unit of serum cholinesterase activity which, as used in this thesis, = 1 μmol of thiocholine released from butyrylthiocholine iodide per minute (Section 4.1.9)

VLDL	very low density lipoprotein

Chapter 1

INTRODUCTION

1.1 Objectives

There have been many reports of raised serum cholinesterase activity in hyperlipidaemia. Serum cholinesterase activity is routinely measured on all samples sent to The Charles A. Janeway Hospital for serum lipid profiles and these measurements had confirmed this association. The function or functions of cholinesterase in the serum are unknown. There are many variants of the enzyme, some of these having different substrate affinities. The basis of the relationship between serum cholinesterase activity and serum lipids or lipoproteins is not known, whether primary or secondary, or whether this is dependent on the enzymic activity or some other feature of the cholinesterase protein.

The first objective of this project was to purify cholinesterase from human serum, to use the purified enzyme to raise polyclonal antibodies in a rabbit, and to develop competence in a method of measuring cholinesterase concentration in human serum samples using the rabbit antiserum. The second objective was to establish normal ranges for concentration and specific activity of the enzyme and then to estimate these parameters in patients' serum samples in which the cholinesterase activities had already been measured. The first purpose of this was to investigate the nature of the relationship between serum cholinesterase and serum lipid levels, particularly to see whether the raised activity in hyperlipidaemia was indeed due to raised concentration of the enzyme, as was presumed by most authors, and to see whether the concentration of the enzyme correlated better or worse with lipid levels than did activity. The second purpose was to look out for samples with unusual specific activity, such as might be the result of inhibition, known to be caused by various drugs and toxins, or due to genetic variants.

These objectives were largely met, though the estimation of specific activity, based on two measurements (concentation and activity) and therefore having compounded measurement error, was not considered accurate enough for quantitative purposes, but served for qualitative comparison of specific activity and its variation in patients and controls. In addition, an exploration was made of impurities in the purified cholinesterase and immunological variation in serum cholinesterase between individuals was observed.

1.2 History and nomenclature

In 1914, the physiologist Henry Dale, having found that the physiological effects of acetylcholine are very short-lived and that acetylcholine is readily hydrolysed to choline and acetic acid, suggested the presence of an esterase in the blood. He did not appear at that time to have considered that the enzyme might be present in the tissues. In 1926, Loewi and Navratil established the existence of an acetylcholine-splitting enzyme by showing that the breakdown of acetylcholine by aqueous extracts of frog heart could be prevented by heating the extract or exposing it to ultraviolet light, or by the addition of physostigmine. Several esterases in animal tissues were found to be incapable of hydrolysing acetylcholine, but in 1932 Stedman *et al.* found an enzyme in horse serum that specifically hydrolysed choline esters, though it hydrolysed butyrylcholine faster than acetylcholine. They proposed the term cholinesterase for this enzyme.

Stedman and Stedman (1935) went on to show that blood cells also contain a cholinesterase, and in 1940 Alles and Hawes showed that the cholinesterase in human red cells is different from that in human serum. The activity of the serum enzyme rises steadily with increasing concentrations of acetylcholine whereas the red cell enzyme shows substrate inhibition with high concentrations of acetylcholine. They also showed that acetyl-β-methylcholine is hydrolysed at about the same rate as acetylcholine by the enzyme in red cells, but hardly at all by the serum enzyme. Mendel *et al.* (1943) found in contrast that the cholinesterases in horse serum and dog pancreas would hydrolyse

benzoylcholine whereas those in human red cells and dog brain would not. Nachmansohn and Rothenberg (1945) went on to show that in human blood, relative to the rate of acetylcholine hydrolysis, benzoylcholine hydrolysis is about 4% in red cells but about 60% in serum, and that for butyrylcholine the figures are about 8% and 270% respectively. Benzoylcholine and butyrylthiocholine are now probably the most commonly used substrates in specific assays of serum cholinesterase (Whittaker, 1986).

Mendel and Rudney (1943) found that the enzymes that they purified from horse serum and dog pancreas would catalyse the hydrolysis of some non-choline esters as well as choline esters, whereas the enzyme from brain and red cells from several species was specific for choline esters. They also noted that the former have only slight activity at low concentrations of acetylcholine whereas the latter exhibit maximum activity at low acetylcholine concentrations. Because of these findings, they proposed the name 'pseudocholinesterase' for the former, reserving the name 'cholinesterase' or 'true cholinesterase' for the latter.

Augustinsson (1948), reviewed the already voluminous literature, and reported extensive work of his own on the cholinesterases in a range of tissues in a range of species, including invertebrates, using a range of substrates. He was against the use of the term 'pseudocholinesterase' and cited several other authors who had called for its abandonment. He pointed out that both enzymes hydrolyse choline esters at higher rates than non-choline esters, and generally split acetylcholine at a higher rate than other choline esters, whereas unspecified esterases split all sorts of esters at the same rate or even at higher rates than acetylcholine or other choline esters. He also mentioned that both enzymes are inhibited by very small amounts of physostigmine whereas other esterases are not. He therefore considered them both to be choline esterases, and started by referring to them as 'specific cholinesterase' for, the one most active on acetylcholine at low concentrations, and 'non-specific cholinesterase' for the other. He found that both enzymes are present in a variety of tissues, and that their specificities vary from tissue to tissue within a given species, as well as in a

given tissue from species to species. He therefore came to regard the cholinesterases as a family of enzymes with widely divergent properties, and divided them into two groups, Group I corresponding to the 'specific' and Group II to the 'non-specific' cholinesterases. His data convinced him of the identity of the enzymes in erythrocytes and brain, and he noted that in the erythrocyte it had been demonstrated to be membrane-bound and presumed that this would also be true in the nervous system.

In only the next year (1949), Augustinsson and Nachmansohn proposed that those in Group I, which had been shown to have well-defined bell-shaped acetylcholine concentration optima, should be renamed 'acetyl-cholinesterase', and that those in Group II might be called 'cholinesterase'. They pointed out that the name of the former would associate this type of enzyme with its physiological substrate, at least in nerve and muscle tissue, where a function of acetylcholine appeared fairly well established, and that as the physiological function of the latter was unknown, calling them cholinesterases was at least temporarily appropriate.

Subsequent work on structure and tissue localisation of these enzymes, reviewed by Massoulié and Bon (1982), Brimijoin and Rakonczay (1986) and Chatonnet and Lockridge (1989) has shown that they occur in two homologous sets of molecular forms. Each enzyme may exist as a monomer, dimer or tetramer. In addition, one, two or three tetramers may be attached by disulphide bonds to the same end of a 'collagen-like' tail, consisting of three peptides in a triple helical arrangement. Each monomer has a single active serine residue. Bon *et al.* (1979) proposed that these six molecular forms should be designated as G_1, G_2 and G_4 for the non-tailed or 'globular' forms and A_4, A_8 and A_{12} for the tailed or 'asymmetrical' forms, where the subscript in each case represents the number of catalytic subunits. These forms have been found throughout vertebrates and even in Drosophila acetylcholinesterase. The picture is however more complicated than this. The globular forms of acetylcholinesterase may each exist in hydrophilic and amphiphilic forms. The amphiphilic nature can be

conferred by either of two hydrophobic anchors which attach the protein to cell membranes. One is a small glycolipid containing *myo*-inositol, glucosamine and ethanolamine in amide linkage to the C-terminus of the catalytic subunit (Roberts *et al.*, 1987) The other is a 20 kDa component which contains fatty acids but no inositol and no ethanolamine or glucosamine with free amino-acid groups. It is asymmetrically linked to two catalytic subunits by disulphide bonds. Similar membrane-bound forms of the 'non-specific cholinesterase' are thought to exist because detergent-soluble globular forms of the enzyme have been found in vertebrate tissues. Additional complication is added by the fact that the proteins are glycosylated. There is evidence that the glycosylation of acetylcholine varies from tissue to tissue and in the course of development and differentiation, but it is not known whether the same applies to the 'non-specific cholinesterase'. Another complication is that in some species (the electric ray, *Torpedo* and the chicken, and therefore probably also other species) there appear to be other associated peptides (see Massoulié and Bon, 1982).

The various forms of the enzymes are found in different locations. The tail proteins attach the enzymes, probably by ionic interactions, to extracellular basement lamina whereas the hydrophobic components enable the globular forms to be attached to cell membranes. For example, the bulk of asymmetric acetylcholinesterase is found in the synaptic cleft, attached to the basement lamina, the G_4 form of acetylcholinesterase is also found in brain and is attached to cell membranes by the 20 kDa anchor, while the red cell acetylcholinesterase, G_2 in humans and cattle, but G_1 in the rat, is also amphiphilic but has the small glycolipid anchor. The principal cholinesterase found in human serum is the 'non-specific cholinesterase'. About 94% is the G_4 form (Atack *et al.*, 1987). The multiple forms of the remainder will be discussed later. Evidence for the presence of acetylcholinesterase in human serum was found by Rubinstein *et al.* (1970) and confirmed by Sorensen *et al.* (1986), but its concentration is lower by a factor of more than 400 (Brimijoin and Hammond, 1988).

The name 'acetylcholinesterase' rapidly became generally adopted after its

proposal in 1949, but to this day the other enzyme is referred to by different authors as 'cholinesterase', 'pseudocholinesterase', 'serum cholinesterase', 'plasma cholinesterase', 'non-specific cholinesterase' and, in those species (including our own) in which it hydrolyses butyrylcholine tho most quickly, 'butyrylcholinesterase' and in other species, in which propionylcholine is hydrolysed more quickly, 'propionylcholinesterase'. The Enzyme Commission (Bielka *et al.*, 1979) has given the latter enzyme the systematic name 'acylcholine acylhydrolase' (EC 3.1.1.8) and adopted 'cholinesterase' as the trivial name. However, as the term cholinesterase has not only been used in the past for acetylcholinesterase but is commonly used as a general term to cover both enzymes, this seems a little unfortunate. Though the 'non-specific' enzyme occurs in the plasma *in vivo*, the term 'serum cholinesterase' is used much more than 'plasma cholinesterase'. In this work the term 'serum cholinesterase' will be used except when referring to the enzyme in other tissues, when 'butyrylcholinesterase' will be used.

1.3 Human serum cholinesterase

Lockridge *et al.* (1979) showed that tetrameric human serum cholinesterase exists as a dimer of dimers of apparently identical subunits. The members of each pair are joined by a single disulphide bond. After reduction and alkylation of the inter-chain disulphide bonds the enzyme was found still to be a tetramer and still fully active. It was therefore concluded that the disulphide bonds were not necessary for either the tetrameric structure or for activity, and that the four subunits were held together by non-covalent bonds. Heat-inactivation studies showed, however, that the inter-chain disulphide bonds increase stability. The easy reduction and alkylation of these bonds suggested that they are near the surface of the molecule. This was reinforced when Lockridge and La Du (1982) showed that limited proteolysis could remove a small peptide containing the disulphide bond, and the subunits still stayed together.

The complete amino-acid sequence has since been determined (Lockridge

et al., 1987a) and confirmed from the sequences of overlapping cDNA clones from fetal brain and liver (Prody *et al.*, 1987) and cDNA clones from neonatal brain (McTiernan *et al.*, 1987). The protein contains 574 amino-acids per subunit. The vast majority of the polyclonal antisera and monoclonal antibodies raised against acetylcholinesterase had no measurable affinity for the other cholinesterase and none of the antibodies against the latter reacted with the former (Weitz *et al.*, 1984; Brimijoin and Rakonczay, 1986; Sorensen et al., 1986). This led to the suspicion that, despite the homology of the quaternary structures of the two kinds of cholinesterases, the amino-acid sequences and protein folding might be significantly different. It was therefore a surprise to find that the amino-acid sequence of human serum cholinesterase is 53.8% identical to that of the acetylcholinesterase of the electric ray (fish) *Torpedo californica* (Schaumacher *et al.*, 1986). *Torpedo* acetylcholinesterase has almost the same number of amino-acids too, 575, and distances of the active site serines from the amino-termini are 198 in human serum cholinesterase and 200 in *Torpedo* acetylcholinesterase. Human serum cholinesterase is 38% identical to *Drosophila* acetylcholinesterase. It shows no significant homology with the serine proteases but shows similarities to a rabbit liver microsomal esterase, to esterase-6 of *Drosophila*, to bovine thyroglobulin (Chatonnet and Lockridge, 1989) and to lipase of the fungus *Geotrichum candidum* (Slabas *et al.*, 1990).

From the established amino-acid composition and the percentages of protein and carbohydrate in the enzyme reported by Haupt *et al.* (1966), Lockridge *et al.* (1987a) calculated the subunit molecular weight of human serum cholinesterase as about 85.5 kD, giving about 342 kD for the tetramer. This is in good agreement with the figure of 348 kD determined by Haupt *et al.*.

During sequencing, glycosylated asparagine showed up as a blank, since the carbohydrate chain prevented extraction of the phenylthiohydantoin derivative into the sequencing solvents. Out of the 40 asparagine residues in the peptide, 9 were distinguished in this way. Every one of these was in the sequence Asn-X-(Ser or Thr), where X can be any amino-acid except proline.

This sequence is known to be common to all N-glycosidically-linked carbohydrate chains (Bause, E., 1983). A tenth asparagine residue which also fitted into this sub-sequence was eliminated as a possible carbohydrate-bearer because it is next to one of the asparagines that does have a carbohydrate chain (Asn-Asn-Ser-Thr - the first has no chain) and carbohydrate chains have never been found to occur on adjacent asparagines. Lockridge *et al.* (1987a) cite the data of Haupt *et al.* (1966) as supporting their conclusion of nine carbohydrate chains and as suggesting that the chains are of the complex type, ending in sialic acid. The possibility that other carbohydrate chains might be present as O-linked oligosaccharides was not examined.

Lockridge *et al.* (1987b) determined the positions of the disulphide bonds. Each subunit has eight 'half-cystines'. Six of these form three intra-chain disulphide bridges, one - four residues from the carboxyl terminus - forms the inter-chain bond, and one could not be alkylated. Comparison with *Torpedo* acetylcholinesterase showed that they have the same number of disulphide bonds, with the same number of amino-acids in each loop and very similar 'hydropathy indices' (*sic*, devised by Kyte and Doolittle, 1982) suggesting that their folding is very similar. However, the explanation of the difference in antigenicity of the cholinesterases of a single species could well lie in the carbohydrate chains. *Torpedo* acetylcholinesterase has only four asparagine residues that have the possibility of being glycosylated and only two of them are in the same places as glycosylated residues in human serum cholinesterase.

1.4 Clinical significance

In the very paper in which the existence of serum cholinesterase was first demonstrated (Stedman *et al.*, 1932) it was noted that the activity in the serum from different horses varied considerably. By 1948 Augustinsson was able to report that investigations of human serum cholinesterase activity had been carried out with a great number of patients with various diseases, some showing an elevation and some a depression of mean activity, but that the data did not

seem to have any diagnostic value because, in humans also, the enzyme's activity varies markedly between normal healthy individuals. This is, with notable exceptions, still true today; Whittaker, 1986, gives tables of conditions associated with increased and (more) with decreased serum cholinesterase activity.

Much evidence has been collected that indicates that serum cholinesterase is synthesized in the liver (Kaufman, 1954). Recently, this has been proved by the finding that in a person who was a heterozygote for a genetic variant ('atypical', see next section) serum cholinesterase, the variant was eliminated and replaced with the 'normal' variant when the person had a liver transplant (Khoury *et al.*, 1987). Serial measurements of the enzyme in an individual can therefore be used to monitor liver function in liver disease and after porto-caval shunting or liver-transplantation (Whittaker, 1986), but there are other indices of liver function. The two principal areas of clinical interest in serum cholinesterase are in succinylcholine sensitivity and organophosphate poisoning.

Succinylcholine (suxamethonium) is a usually-short-acting muscle- relaxant used in general anaesthesia for surgery and electro- convulsive therapy. Its introduction into clinical practice led to the realization that normal healthy individuals differ in their inherited abilities to metabolize different drugs - opening a field which has since become known as 'pharmacogenetics' - and to the discovery of the first of many genetic variants of serum cholinesterase. Its physiological effects were first investigated in 1906 (Hunt and Taveau), and in 1941 Glick showed that it was hydrolysed (slowly) by horse serum cholinesterase, but it was not until 1949 that its potent neuromuscular blocking activity was described by Bovet *et al.*. These authors pointed out that it might be valuable in clinical applications because it was hydrolysed rapidly by serum cholinesterase and therefore gave only transient effects.

By 1952 it had been commercially available for long enough for Bourne *et al.* and Evans *et al.* to be able to report on its effects in hundreds of patients. The former group found that in the majority of patients the paralysis lasted from 2-4

minutes, the latter 2-6. Both groups noted that in a few of their patients the duration of action of the drug was prolonged, quoted several other reports of the same finding that year (the longest being three hours), investigated the serum cholinesterase activity, and found that it was much lower in those with prolonged paralysis. The latter group demonstrated an inverse relationship between the serum cholinesterase activity and the period of apnoea. They also suggested that the explanation for the action of succinylcholine lies in a difference between acetylcholinesterase and serum cholinesterase such that the former is subject to competitive inhibition by it, but hardly hydrolyses it, while the latter usually rapidly removes it. Both groups suggested the need for caution in the use of the drug in patients likely to have low cholinesterase activity such as those with liver disease or malnutrition, or poisoning with anticholinesterase compounds such as certain insecticides and war gases. They counselled avoiding it, or giving a reduced dose, and only where facilities existed for prolonged artificial respiration.

The following year it occurred to Forbat *et al.* (1953) to investigate the relatives of a patient who had exhibited prolonged apnoea with the drug. The only available one was a healthy brother and he too had a very low serum cholinesterase. Subsequent studies (see next section) led to the establishment of inherited variation in serum cholinesterase as the explanation for this clinical phenomenon and to the finding that the posession of a variant enzyme is more important than the cholinesterase activity, as usually measured, in determining duration of apnoea.

The other main area of clinical interest in serum cholinesterase is in poisoning by anticholinesterases. This is reviewed by Whittaker (1986). Many compounds inhibit cholinesterases. These include drugs used in the treatment of such disorders as myaesthenia gravis, glaucoma, urinary retention and paralytic ileus, and to reverse the effects of non-depolarising (competitive) muscle relaxant drugs such as tubocurarine (George *et al.*, 1990). However, the inhibitors which most frequently cause toxicity are the systemic insecticides (and nerve gases). Most of these are either carbamates - which cause reversible inhibition and only

short-lasting effects (Vandekar *et al.*, 1971) - or organophosphates, which cause irreversible inhibition and therefore much longer-lasting effects. The pharmacological effects of these compounds are due to the inhibition of acetylcholinesterase but most of them inhibit serum cholinesterase to a greater extent. The measurement of serum cholinesterase can therefore be useful, in these cases, not only for diagnosing intoxication, but for monitoring workers at risk of exposure to such compounds for changes which occur before symptoms develop, as well as in determining when an individual might return to work. As has already been stated, serum cholinesterase activity is very variable between individuals. It is therefore often not possible to diagnose toxic exposure with a single post-exposure measurement if a baseline pre-exposure measurement has not been made. Coye *et al.* (1987) cite numbers of cases of agricultural workers exposed to the organophosphates mevinphos, phosphamidon and diazinon who complained of symptoms but were sent back to work because of serum cholinesterase levels within the normal range. They showed that diagnosis can still be made by removing the workers from exposure and making serial measurements. Serum cholinesterase activity is then seen to rise until eventually stabilising again.

1.5 Genetics and molecular biology

The first report of low serum cholinesterase in siblings, ascertained by the finding of prolonged apnoea with succinylcholine in one of them (Forbat et al., 1953), was followed by further reports of family studies. Lehman (the chemical pathologist author of Evans et al., 1952 and Forbat et al, 1953) and Ryan (1956) used a single dividing line between normal and low serum cholinesterase activity, which led them to believe that some patients had normal levels and that the inheritance of low levels was recessive. Allott and Thompson (1956) suggested that, in a family they reported, heterozygotes had intermediate levels. The data of Lehman and Ryan were also compatible with this idea.

In the meantime, another line of enquiry was being pursued, namely that

different individuals might have qualitatively different enzymes rather than just different amounts. Foldes et al. (1954) investigated the hydrolysis of acetylcholine, benzoylcholine, succinylcholine and procaine by the serum of 16 human subjects and found 'no parallelism' between the rates. From this they concluded that either there is more than one enzyme in the serum capable of hydrolysing acetylcholine or that marked qualitative differences exist between individuals. Following this up, Stovner (1955) pointed out that determination of the rate of hydrolysis of succinylcholine is complicated by the fact that it is a dicholine. He therefore decided to investigate the affinity of the serum cholinesterase of different individuals for succinylcholine by determining the amount of succinylcholine required to cause 50% inhibition (I_{50}) of the ability of the enzyme to hydrolyse acetylcholine. He found that the I_{50} was fairly uniform in 8 patients responding normally to succinylcholine but increased in 2 who had prolonged apnoea.

Around the same time, Kalow had a similar idea. Heralded by a number of abstracts and letters, e.g. Kalow and Lindsay (1956), Kalow, Genest and Staron (1956), Kalow (1956), he published in detail in 1957 the results of a large study in three papers describing his method of detecting the 'atypical' serum cholinesterase (Kalow and Genest), the relationship between dose of succinylcholine and duration of apnoea (Kalow and Gunn) and the distribution and inheritance of atypical forms of the enzyme (Kalow and Staron) and was apparently unaware of the work of Foldes *et al.* or Stovner. His method differed in that instead of determining the amount of inhibitor necessary to cause a fixed percentage of inhibition, he used a fixed concentration of inhibitor and determined the percentage inhibition. The substrate he chose was benzoylcholine and the inhibitor was dibucaine (also known as cinchocaine, Nupercaine and Percaine), each used at 10^{-5}M. The percentage inhibition was termed the 'dibucaine number'. This method was subsequently adopted as a standard test. Investigation of nearly 1700 sera indicated that dibucaine numbers could be divided into three groups. Those above 70 - usually around 79 - were

considered typical, between 70 and 40 intermediate, and below 20 atypical. None was found between 40 and 20. Kalow and Genest (1957) found, as Stovner had done, that the inhibition was independent of activity, except that activity was always low in those with very low dibucaine numbers. They also found that dibucaine number remained constant over time in individuals.

Kalow and Gunn (1957) gave different doses of succinylcholine to the same individuals on different occasions when they received a series of electroconvulsive treatments. They found a linear relationship between the logarithm of apnoea-time and the logarithm of dose with the same slope in all individuals but different intercepts. Individuals with intermediate dibucaine numbers were found to have serum cholinesterase activities within the normal range more often than to have normal responses to succinylcholine. Those with very low dibucaine numbers had much longer apnoea than those with normal or intermediate numbers, and their serum did not appear to hydrolyse succinylcholine, though it did hydrolyse other choline esters.

Kalow and Staron (1957) studied not only a large population, made up of students, labourers, unselected general hospital patients, and mental hopital patients, but the nuclear families of five students with intermediate dibucaine numbers and two extended pedigrees of patients found because of prolonged apnoea with succinylcholine. The dibucaine numbers were found to fall into three distinct groups explainable by the existence of two codominant alleles, inherited in Mendelian fashion. The frequency of the atypical allele in the mental hospital patients was higher than in the others, so in case there was some association with mental disorder, the population frequency was estimated from the healthy subjects only. This gave an incidence of atypical homozygotes of about 1 in 5,100 and of heterozygotes of about 1 in 36. Though the mean esterase activities were different in the different groups, there was very extensive overlap so that activity cannot be used to distinguish genotypes.

The investigators determined the experimental error of measuring dibucaine

numbers and found that the variation in results of people in the high, usual or typical group could just about be explained by experimental error. However, the numbers in the intermediate group varied too widely to be so explained. Furthermore their mean was not midway between those of the other two groups and their distribution was skewed, with a tail to the left. Examination of the family data led to the conclusion of the probable existence within the 'typical' group of several alleles, giving different intermediates with the atypical allele. Amongst these they were able to predict what later became known as 'The Silent Gene' (allele would be better now), which contributes nothing to the activity but does not affect the dibucaine number created by the other allele of its bearer either. Thus in combination with a typical allele the activity will be below average but the dibucaine number normal, but when passed on to a child receiving an atypical allele from the other parent the dibucaine number will be low rather than intermediate. Subsequent work has revealed that there is more than one 'silent' allele and that there are indeed other alleles, but before giving further information on these it is worth mentioning the discovery of a second locus affecting serum cholinesterase.

Investigating several reports that starch-gel electrophoresis of serum revealed a series of bands which displayed serum cholinesterase activity, Harris *et al.* (1962) examined human sera by two-dimensional electrophoresis with paper in the first dimension and starch gel in the second. They found four bands that were present in all individuals and named them C_1, C_2, C_3 and C_4, the last being nearest to the origin in the starch-gel dimension and much more intense than the others. A further (diagonal) band overlapping the C_4 band could often be seen in trace amounts in adult serum and was much more prominent in neonatal sera, and two other bands which they called 'storage bands', S_1 and S_2, migrating faster than C_4 in paper but more slowly than it in starch gel, appeared only after storage of the sera for ten or more days. Yet other bands have been identified by other workers who have used different nomenclature. Harris *et al.* found that some individuals had an extra band which they called C_5. Like S_1 and

S_2 this band moved slightly more slowly in starch gel than C_4 but unlike them it moved more slowly in paper. Family studies (Harris et al., 1962, 1963a) suggested that the extra band was inherited and that individuals carrying it were heterozygous for a gene determining it. Masson (1979) showed that bands C_1, C_3 and C_4 correspond to the serum cholinesterase monomer, dimer and tetramer, and Lockridge and La Du (1982) showed that another band, detected between C_3 and C_4 (CHE-4 of LaMotta *et al.*, 1965; possibly equivalent to band c of Bernsohn *et al.*, 1961), was the trimer. Masson (1989) has shown that the C_2 band represents the monomer linked to albumin by a disulphide bond, but the nature of C_5 has still not been established.

Harris *et al.* (1962, 1963a) designated individuals with and without the extra band C_5+ and C_5-. The band could not be detected in all individuals who would have to be heterozygotes but the expression of the band was found to be quite variable, leaving the possibility of its presence at concentrations below the level of detection in obligate heterozygotes who were apparently C_5-. The mean cholinesterase activity was found to be about 30% higher in C_5+ individuals than in those who were C_5-, suggesting that the C_5 component might be an extra component with no direct homologue in C_5- individuals (Harris et al., 1963a). Subsequent work (Harris et al., 1963b) showed independent assortment of C_5+/C_5- and typical/atypical serum cholinesterase in families in which both genes were segregating, indicating that the two are not allelic. The loci were designated E_1 and E_2 (in order of discovery) by Motulsky (1964). In his system, variants at the first locus are specified by superscripts - e.g. E_1^u and E_1^a for the usual and atypical alleles respectively - and the presence or absence of the C_5 band is specified as E_2+ or E_2-. Later, 'An International System for Gene Nomenclature' (Shows et al., 1979) proposed the naming of the loci as CHE1 and CHE2 with variants specified thus: CHE1*U, CHE1*A, CHE2*C5+, CHE2*C5-. The latter system was never universally adopted and has now been superceded (see below).

By exposing duplicate electrophoresis gels to substrate with and without the

inhibitor *RO* 2-0683 (dimethylcarbamate of (2-hydroxy-2-phenyl-benzyl)-trimethyl ammonium bromide), Harris *et al.* (1963b) demonstrated that in individuals with the atypical allele who were also C_5+, the C_5 component is inhibited by dibucaine just as much as is C4, indicating that the presence and properties of the C5 component are determined by genes at (at least) two loci. Scott and Powers (1974) purified the C_5 component and investigated its properties with various substrates and inhibitors, and managed to form a hybrid between it and neuraminidase-treated C_4. They came to the conclusion that the two loci produce similar enzymes that differ in charge rather than in size and differ slightly in nearly all of the properties they examined and that the C_5 component is a hybrid between polypeptides produced by the two loci. This seems rather unlikely for two reasons.

Firstly, since the gene at the second locus could not be expected to have the same mutation as that of the first locus when the latter happened to code for the atypical variant, this solution could not be expected to result in a hybrid enzyme that was inhibited to the same extent as the tetramer produced from alleles at the first locus only. Secondly, if two active enzyme proteins were being produced, and since the enzyme is a dimer of dimers, one would expect not one extra tetramer band, moving more slowly than the tetramer produced by the first locus alone, but at the very least, two, one corresponding to a tetramer composed of a dimer produced by each locus and one corresponding to a tetramer of the polypeptide from the second locus, and there should be an extra dimer band and an extra monomer band. If heterodimers could form and could lead to the formation of tetramers composed of three polypeptides from one locus and one from the other, then there should be two extra dimer bands and four extra tetramer bands in C_5+ individuals.

Scott and Powers (1974) explained the higher cholinesterase activity in C_5+ individuals by their second enzyme hypothesis. Altland *et al.* (1971) showed that the mean serum concentration of immunoprecipitable active cholinesterase protein of C_5+ individuals was very significantly greater than the population

mean. Simpson (1966) found that posession of the C_5+ heterozygote genotype also seemed to increase the activity of the atypical form of cholinesterase. She did not propose a mechanism either for this or for the appearance of the C_5 band but a possible alternative to the hypothesis of Scott and Powers would be that the second locus produces a non-enzyme protein that associates only with the tetrameric enzyme (or some other fixed number of subunits, monomer or dimer) and increases its plasma half-life, as well as reducing its electrophoretic mobility.

Tsim *et al.* (1988a) immunopurified an A_{12} form of cholinesterase from chicken muscle using a monoclonal antibody previously shown to react specifically with acetylcholinesterase and not with butyrylcholinesterase. They found that this enzyme showed the active site characteristics, substrate specificity and subunit properties of both acetylcholinesterase and butyrylcholinesterase and concluded that each catalytic tetramer consisted of two acetylcholinesterase and two butyrylcholinesterase subunits linked by disulphide bonds to the collagen-like tail. They also reported (Tsim *et al.*, 1988b) that while this is the dominant cholinesterase form in the muscle of one-day-old chickens, it and an asymmetrical form containing only butyrylcholinesterase as the catalytic form give way during development to homogeneous acetylcholinesterase. This work does show that hybrid cholinesterases can exist as well as promoting new speculation as to the rôle of butyrylcholinesterase. It may be that this hybrid form can appear in human serum, probably without the tail. Jones and Evans (1983) noted an extra cholinesterase band - running more slowly but close to the C_4 band - in human fetal serum on polyacrylamide gel electrophoresis. They said that it had not previously been described but did not refer to the fetal band of Harris *et al.* (1962). Jones and Evans found that their fetal band hydrolysed both acetyl and butyrylthiocholine but did not investigate whether it had the characteristics of both enzymes. However, evidence to follow suggests that C_5 is not a two-enzyme hybrid.

The CHE1 locus was shown to be linked to the transferrin locus (Robson *et al.*, 1966) and the transferrin gene was later shown to be on chromosome 3

(Lavareda de Sousa and Lucotte, 1985). Lovrien *et al.* (1987) found tentative linkage of the CHE2 locus to the α-haptoglobin gene, which was already known to be on chromosome 16. Marazita *et al.* (1989) combining their own and other published data obtained a lod score of 2.51 (not statistically significant) for this linkage at a recombination distance of 0.32 but this was increased to a score of 3.2 (just significant) when information from three other markers on chromosome 16 was included, and to 4.1 with information from a further marker. Soreq *et al.* (1987) carried out *in situ* hybridization of chromosome spreads with cloned butyrylcholinesterase cDNA and reported hybridization to chromosome 3 and the long arm of chromosome 16 and later (Zakut *et al.* (1989) that there were three sites of hybridization, two of them on chromosome 3. This work appeared to confirm the linkage results and the theory of Scott and Powers (1974) that CHE2 codes for a peptide of similar sequence to that coded by CHE1. However, Eiberg *et al.* (1989) found a higher lod score with a restriction-fragment length polymorphism in the γ-crystallin gene cluster, which is on chromosome 2. This was not based on a large amount of evidence. Information from 832 families and a large number of genetic markers was searched, but the families were not all tested for all markers. The linkage found was based on just one family in which there were 15 children and no crossovers. The authors noted an apparent very weak linkage to a chromosome 8 marker with information from 27 families. They pointed out that apparent deviations from Mendelian distribution had been encountered in previous studies as well as their own and put forward their finding of linkage to the γ-crystalin gene cluster as a useful tool to investigate whether these deviations were trivial or reflect some real deviation such as heterogeneity or distortion of segregation.

Workers in the laboratory of La Du and Lockridge have subsequently isolated genomic clones for the human butyrylcholinesterase gene (Arpagaus *et al.*, 1990). There was very good agreement between the sizes of hybridizing fragments observed in Southern blots of total genomic DNA and a restriction map derived from the cloned DNA, strongly suggesting that there is only one gene.

This supported evidence from polymerase-chain-reaction amplification of a fragment of the gene containing the atypical mutation (McGuire *et al.*, 1989) prepared from genomic DNA of homozygous atypical individuals. Sequencing gels never showed any band heterogeneity, as would have been expected if there was more than one gene present, since the other gene could not be expected to have the same mutation. In the same laboratory, the DNA of three individuals expressing high-intensity C_5 bands was examined (Masson *et al.* (1990). Southern blots with probes for each of the four exons of the identified gene and seven restriction enzymes did not show any different bands from those of C_5- individuals.

It now seems fairly certain then that there is only one gene encoding the catalytic subunits of human butyrylcholinesterase and on this basis the Human Gene Nomenclature Committee has renamed the E_1 or CHE1 locus 'BCHE', while the other is still called CHE2 at present (Masson *et al.*, 1990). Scott and Powers (1974) explained the absence of their proposed second enzyme in about 90% of most populations by citing the precedent of intestinal lactase which is similarly absent in about 90% of the adults in most populations. Soreq *et al.* (1987) suggested that 'the CHE2 gene might have turned into a pseudogene in the majority of individuals'. The results from the laboratory of La Du and Lockridge are as much against the existence of a pseudogene as a functional one; and butyrylcholinesterase-like gene would have to be different enough that none of the probes would hybridize with it at the stringency used. The possibility of a non-enzyme protein would not require the postulation of a non-functional gene. It would only require that the majority of people produce a form of the protein (perhaps with a different charge, less hydrophobicity, or no free -SH group) that would not associate with serum cholinesterase. Masson *et al.* (1990) checked the C_5+ phenotype of their three subjects several times over six months to eliminate false positives and it is interesting that in one of them the extra band disappeared and reappeared again two months later. It would seem then that physiological changes may affect the association of the proteins. Masson *et al.*

suggested that another possibility for the function of the CHE2 gene was that it produces an enzyme that controls the assembly of butyrylcholinesterase subunits with another protein, but that possibility would, like the second butyrylcholinesterase enzyme hypothesis, require an explanation of non-function in the majority of people.

Returning to the atypical variant, Kalow and Davies (1958) investigated the difference between it and the usual variant with a series of inhibitors. They came to the conclusion that the esterasic sites of the two variants might be identical and that the difference was at 'the anionic site' (which accommodates the quarternary-ammonium choline part of choline ester molecules being hydrolysed) where one of two negative charges was missing in the atypical variant. (More details of this are given in the discussion.) This has subsequently been proved right by McGuire *et al.* (1989) who found that the only consistent difference of the atypical from the usual DNA sequence in 14 heterozygous and 6 homozygous atypical subjects was a single base change that results in the substitution of a neutral amino-acid (glycine) for an acidic one (aspartic acid).

The hypothesis of the existence of a silent allele was proved right when the first case of complete absence of serum cholinesterase activity was published (Liddell *et al.*, 1962; Hart and Mitchell, 1962). Serum from this individual did not affect the dibucaine numbers of sera from normal and atypical homozygotes when mixed with them *in vitro* and family data at this stage suggested that the silent gene was allelic to the usual and atypical alleles. Further work by Simpson and Kalow (1964) strengthened this impression though the possibility of a suppressor gene closely linked to the structural gene could not be ruled out. Absence of enzyme activity could be due to absence of production of the protein or to production of a truncated protein or a full length protein with no catalytic activity. In addition, very low activity could be due to an abnormal enzyme or very low production of the usual enzyme. There have been many reports of heterogeneity of the silent phenotype, based on activity measurements, immunological techniques and electrophoresis and more different types have been distinguished than named.

Goedde and Altland (1968) showed that in two cases there was no activity and no immunologically-detectable serum cholinesterase protein while in three others there was a little of each, but different amounts. Altland and Goedde (1970) found nine different electrophoresis patterns amongst cases with a little activity as well as relative differences between amount of enzyme protein and amount of activity, if any. They pointed out that since the the phenotype is rare individuals showing it are more likely to be heterozygotes for different 'silent' alleles than homozygotes. Scott (1973) proposed the names '*O* type' and '*T* type' for complete absence of activity and trace activity (usually 2-4% of normal) in different families of Eskimos and came to the conclusion that they were allelic. These subsequently became known as E_1^s and E_1^t, and Scott and Wright (1976) then proposed the name E_1^R (more correctly E_1^r) for the allele found in another family with activity under 10% in whom the C_4 band moved faster than the usual variant on electrophoresis. Whittaker (1990) has proposed the name E_1^x for the allele of another variant that has much more immunologically detectable enzyme protein than other types but no detectable activity with butyrylthiocholine iodide as substrate and very little with benzoylcholine, while showing that yet other types appear to exist.

Nogueira *et al.* (1990) have now published the DNA sequence of one silent allele. It has a change of sequence from GGT to GGAG at codon 117, an unusual combination of a transversion and an extra base. The frame shift changes the amino-acids coded from that point onwards, but more importantly results in the reading of a stop-codon after amino-acid 128 so that a very truncated protein with no esteratic site is produced. In the same paper the authors report partial sequencing of the DNA of another individual with no immunologically detectable protein. The abnormality had not been found but there was no abnormality at codon 117.

A series of other variants have been described which have the same inhibition characteristics as the usual variant but lower activity. Like the 'silent' variants the existence of these was first deduced from anomalous results in

families in which the usual and atypical alleles were segregating as their heterozygotes with the usual allele could not be distinguished from usual homozygotes. The first described was the J variant (Garry *et al.*, 1976). The authors calculated that the allele caused 66% reduction of usual enzyme molecules and (Rubinstein *et al.*, 1976) showed that heterozygotes for this variant and the usual or atypical one had less enzyme protein than individuals homozygous for the usual or atypical or their heterozygote, which all had about the same amount of protein. In the latter paper they concluded that there was indeed reduction in the number of circulating usual enzyme molecules. They allowed that they had not excluded the possibility that there could be decreased numbers of a variant molecule but considered this unlikely. Actually, inspection of their plots of concentration against activity clearly shows that the $E_1^aE_1^j$ genotype gives a specific activity between between that of $E_1^aE_1^a$ and $E_1^uE_1^a$, suggesting that E_1^j allele does indeed produce a variant enzyme.

In the same way, Rubinstein *et al.* (1978) identified the K variant and concluded that the E_1^k allele causes a 33% reduction in circulating usual enzyme molecules. From analysis of 795 caucasian serum samples, Evans and Wardell (1984) found the E_1^j allele to be rare but estimated the frequency of the E_1^k allele to be 0.115 giving the frequency of heterozygotes as about 20% of the population and homozygotes about 1 in 76. Mcguire *et al.* (1989) noted a DNA polymorphism present in usual and atypical alleles resulting in the substitution of threonine for alanine at amino-acid position 539 and after examining 40 individuals they estimated the frequency (Bartels *et al.*, 1989) of alleles coding threonine to be 0.125 and suggested that this could be the K variant. If this proves to be true it will show that a formerly-designated 'quantitative' variant is qualitatively different. Perhaps this change reduces the plasma half-life of the molecule as it is less easy to see how it could reduce synthesis.

Another allele detected by unusual results in families in which the usual and atypical alleles were segregating is E_1^h (Whittaker and Britten, 1987). The variant has less activity with benzoylcholine than does the atypical one. Immunological studies have not been carried out.

The known differential inhibitors of the usual and atypical variants were all tertiary-ammonium compounds until Harris and Whittaker (1961) found that sodium fluoride also differentially inhibited them, and (Harris and Whittaker, 1961, 1962) that in some individuals inhibition by dibucaine and fluoride was discrepant, revealing new phenotypes. Further work (Whittaker, 1967) strengthened the evidence for a fluoride resistant variant that was coded by another allele at the CHE1 locus.

Another variant was found (Simpson and Elliott, 1981) through a proband with prolonged apnoea with succinylcholine who had an unusually high dibucaine number for a usual-atypical heterozygote. Luckily the individual belonged to a large Newfoundland family available for testing. She proved to be a heterozygote for the atypical allele an allele coding a new variant, 'cholinesterase Newfoundland'. Though having a low activity with succinylcholine, like the atypical variant, the Newfoundland variant has a high percentage inhibition with dibucaine whereas the atypical variant has a low percentage inhibition. This difference in dibucaine numbers was found to have no overlap with the usual variant when succinylcholine was used as substrate.

Harris and Whittaker (1963) found that, at much higher concentrations than sodium fluoride, sodium chloride also differentially inhibits the usual and atypical variants but inhibits the atypical variant more, the opposite to inhibition with sodium fluoride and dibucaine. Further work (Whittaker, 1968c) revealed some individuals in whom chloride inhibition was discrepant with inhibition by dibucaine and fluoride, suggesting yet more phenotypes. The discrepant cases included individuals who were sensitive to succinylcholine but appeared to be of the usual phenotype when tested with dibucaine and fluoride (Whittaker, 1968d).

Various investigators observed that cholinesterases could be activated by alcohols at low concentrations but were inhibited by higher concentrations. Whittaker (1968a) found that the usual and atypical variants of serum cholinesterase were differentially affected, the atypical variant being activated

less and inactivated at lower alcohol concentrations. She found that the greatest differentiation between them was given by 1% n-butanol and proposed that alcohols could be used as another method of ditinguishing between the variants. She went on (Whittaker, 1968b) to define the alcohol number as the ratio of benzoylcholine activities in the presence and absence of 1% n-butanol under standard conditions multiplied by 100, and found that there were some individuals with whom the alcohol number was discrepant with the phenotype suggested by dibucaine and fluoride inhibition, revealing yet more new phenotypes.

Family data to confirm the phenotypes revealed by sodium chloride and n-butanol as due to inherited variants has yet to be published. There have been several other reports of different phenotypes without family studies. In the very near future it is likely that DNA sequencing will not only reveal the differences responsible for all the rest of the known inherited variants but show whether there is genetic basis for phenotypes for which family studies have not been done as well as discovering previously unrecognized polymorphism. The existence of so many variants allelic with the usual variant and no others shown to be allelic with the C_5+ and C_5- variants is suggestive independently of the evidence given earlier that the gene at the 'CHE2' or 'E_2' locus does not code for a second butyrylcholinesterase.

Goto *et al.* (1988) have reported a family in which a silent serum cholinesterase allele producing no immunologically detectable protein and the C_5+ gene are segregating. So far there is no family member who is both a homozygote for the silent allele and an obligate carrier of C_5+ but such a case could prove that the second locus does not produce an active enzyme.

C_5 is not the only inherited variant with increased activity or an extra band on electrophoresis. Neitlich (1966) reported an American family of unspecified ethnic origin in which the proband and three relatives had very high plasma cholinesterase activity (three of them 3-4 times the male mean) and an extra

band between the C_4 and storage bands. He investigated the enzyme *in vivo* by injecting the proband and controls with diisopropyl fluorophosphate (DFP) and concluded that the proband had a normal number of enzyme molecules but that some of them were much more active than usual. Yoshida and Motulsky (1969) reinvestigated the same family and named the variant E Cynthiana after its place of origin. They concluded from *in vitro* DFP inactivation and from reduction of activity by immunoprecipitation that the enzyme was of normal specific activity and present in increased numbers of molecules. They found that it migrated more slowly than the C_5 protein on electrophoresis and from varying starch gel concentration deduced that this was not due to difference in charge but higher molecular weight. Their explanation of this was that the extra band was due to a structurally diferent enzyme which associated in more than four subunits together and had an increased rate of synthesis.

Delbrück and Henkel (1979) reported two German families in which a total of 11 members had plasma cholinesterase activities up to four times normal and an extra band on electrophoresis but they did not compare the latter with a C_5+ serum. They found that the enzyme had the same inhibition with dibucaine, fluoride, succinylcholine and DFP, and the same pH optimum and heat inactivation as the usual enzyme, and rocket electrophoresis indicated that it was present in much increased concentration. This evidence suggested that these families also had the Cynthiana variant. Delbrück and Henkel also tried electrofocussing and found six bands in the region pH 4.4 to 4.9 and suggested that a difference in charge might be responsible for higher aggregation.

Yamamoto *et al.* (1986, 1987)reported a Japanese family with members serum cholinesterase activities about twice normal. Again the characteristics were the same as the usual enzyme except that the pH optimum seemed to be slightly lower. On electrophoresis two extra bands were found, one between the C_4 band and the origin and the other between the dimer and trimer bands. This latter band disappeared in the presence of heparin and the authors noted that the investigators of the Cynthiana variant had used heparinised plasma. Yamamoto

et al. noted that association between heparin and other serum proteins had been reported and speculated that the faster extra band disappeared because heparin bound to it and slowed it down to the same rate as another band. In the light of the knowledge that the C_2 band represents the association of the cholinesterase monomer with albumin and that the C_5 band is also almost certainly due to association of the enzyme with another protein it the possibility that the Cynthiana variant is due to association of the enzyme with yet another protein and that this protein prolongs the half-life of the enzyme seems more likely than the hypothesis of Yoshida and Motulsky of increased synthesis and increased aggregation of the enzyme. Though Yamamoto *et al.* did not realize it, their discovery of another extra band strengthens this possibility considerably since a band between the dimer and trimer bands could hardly be caused by increased aggregation.

Warran *et al.* (1987) reported a Saudi family with members with serum cholinesterase activities 3-5 times the reference mean. The proband also had a slow-moving extra band on electrophoresis and it showed the same inhibition as the C_4 band. Serum was used but staining was not enough to show the bands faster than C_4. There was no comparison with a known C_5+ serum and electrophoresis of the serum of the other family members was not done, but this could well be another case of Cynthiana as may a single case of high cholinesterase and an extra band slower than the C_5 band reported by Klein *et al.* (1967). All of the families were compatible with autosomal 'dominant' transmission; no homozygotes occurred. As none of the families appeared to be segregating for any of the known serum cholinesterase variants it was not possible to test whether the non-C_5 extra band variant(s) were allelic with them or not. This would have indicated whether the extra bands were indeed due to a protein coded by another locus or due to abnormality of cholinesterase (perhaps causing it to associate with a normal form of another protein).

There may be other variants with high serum cholinesterase activity. Ohkawa *et al.* (1989) reported a Japanese family in which six members tested

had an extra band of the same mobility as C_5 but four of whom had activities 3-5 times the reference mean, the other two having activities in the upper half of the normal range. The high results were much higher than expected for cases of C_5+. Raised serum cholinesterase activity is associated with hyperlipidaemia (see Section 1.7, p. 31) but the proband had normal serum cholesterol and triacylglycerol concentrations and ultrasound examinations for fatty liver were negative in all family members. Krause *et al.* (1988) claimed a new variant with increased specific activity in an Africaner mother and son on the basis that activity was raised but serum cholinesterase protein was not, as judged by rocket electrophoresis. However, the activities, said to be about twice normal, did not appear to be far above the upper limit of the normal range as judged from the activity of another family member said to be normal (the laboratory's range was not given) and the protein concentrations were a little higher than controls. The mother had a C_5 band and the son did not. The dibucaine and fluoride numbers were normal. The half-life at 52°C was increased. It may well be that this was not a new variant but C_5. Simpson (1972) showed that though polyacrylamide gel electrophoresis detected 33% more cases than the starch gel used by Harris *et al.* (1962) it still failed to detect the extra band in 50% of obligate carriers who were typed C_5– by starch gel electrophoresis.

There have been many other reports of other serum cholinesterase electrophoresis bands without increased activity, some present in all sera, some appearing in disease and some found in some individuals in population screens. The author detecting the most bands in normal serum was Juul (1968) who found twelve using a three-layer polyacrylamide gel and destaining electrophoretically. Of these the band with the most activity (80%) was the seventh, counting from the front. If true, and assuming that this represented the tetramer, this leaves two bands not accounted for by the monomer dimer trimer and C_2 compound. Bernsohn *et al.* (1961) did detect one such band using starch gel. These bands could represent two of the smaller molecules associated with some other serum protein, or one of them associated with two other proteins. On the same

assumptions, Juul found five bands moving more slowly than the tetramer. He pointed out that that bands could represent polymers, degradation products, or modification of the enzyme by the separation procedure as well as association with other proteins. LaMotta *et al.* (1968) detected two bands in purified cholinesterase moving more slowly than the band (their CHE5 of LaMotta *et al.*, 1965) which we now know to be the tetramer. They found that when they isolated and concentrated the material from these bands the mobility became the same as the tetramer band so they concluded that they could not be higher polymers but conformational isomers.

Gaffney (1970) suggested that the bands might represent hydrophobic association of the cholinesterase with other proteins after loss of some of the sialic acid residues from the cholinesterase. This was after finding that after treating serum cholinesterase with neuraminidase all the bands were replaced by a single band of low mobility. His preparation of the enzyme did not contain any C_2. Masson (1990) has shown that C_2 is not affected in this way and suggests that the albumin subunit protects the desialated cholinesterase subunit from self-aggregation by masking a hydrophobic area exposed by the removal of sialic acid residues. From this it is not hard to imagine that other blood proteins might well associate with serum cholinesterase to give rise to discrete electrophoretic bands. Loss of sialic acid residues could then be the explanation for the appearance of the storage bands. However, if this is so, the sialic acids involved must be lost from a different area from that which the putative protein responsible for the C_5 band abbuts because Simpson (1972) found that while there were two storage bands in C_5- individuals, C_5+ individuals showed four.

Ogita (1975) investigated the blood of a patient with a leiomyoma who had very little serum cholinesterase activity. He showed that when the blood was incubated with purified C_4, sialic acid was released and that a series of less mobile electrophoretic forms were produced. He also showed that the extent to which this occured varied with the patient's condition, and postulated - but did not demonstrate - that the band of C_5 mobility found in the serum of four of the

patient's relatives was formed by their production of lower levels of the same 'neuraminidase-like' enzyme. Augustinsson and Ekedahl (1962) had shown that stripping sialic acid residues from serum cholinesterase does not reduce its catalytic activity, so Ogita had to postulate that the patient was also producing a proteolytic enzyme active against cholinesterase.

Ogita called his idea of band production 'the epigenic modification hypothesis'. He suggested that the gene responsible for the neuraminidase-like enzyme has a 'normal' allele N^U and an allele N^R that may be activated by changed physiological conditions and thus raise the neuraminidase activity beyond the normal range. As a result, the 'C_4 component' would lose neuraminic acids and be transformed into C_5-like components of slower mobility. With further increases the C_6 or the $C_{7a,b}$ and additional slow components would be formed. The 'C_4 component' is of course the cholinesterase tetramer and the Ogita did not cover the expected effects upon the entities responsible for the faster bands. He did not propose that his hypothesis accounted for all cases of C_5+ but suggested that there might be heterogeneity, his hypothesis accounting for families in which some obligate carriers did not show the band.

The C_6 band that Ogita referred to was described by Ashton and Simpson (1966) from starch gel electrophoresis in a single individual found in a large survey of families from Brazil. The man also had another fainter slower cholinesterase band that was not given a name. None of his four children showed either band. Bands C_{7a} and C_{7b} were described by Van Ros and Druet (1966) from two-dimensional paper/starch gel electrophoresis in two healthy African subjects out of 734 surveyed. These investigators also found a band which they called C_6 in four other healthy individuals in the same survey. All of these bands were distinct from the fetal and storage bands. Other slow-moving bands have been described in surveys of Cree 'Indians' and Eskimos (Simpson, 1972), in glaucoma patients (Juul, 1968; Juul and Leopold, 1968) and in myeloma patients (Gallango and Arends, 1969). Using three-layer polyacrylamide disc gel electrophoresis, Brock (1989) has surveyed plasma

samples of 193 healthy Danish volunteers and found the C_5 band at the remarkably high frequencies of 40.2% in men and 29.6% in women. He also reported three other extra bands, which he called C1′, C6 and C7, in 24, 53 and 9 of the volunteers respectively and concluded that all four extra bands occurred independently and that none of them (including C_5) had any influence on the total plasma cholinestease activity. Masson *et al.* (1990) say that so far Masson has eliminated albumin and degradation products of acetylcholinesterase, butyrylcholinesterase and collagenous tail as candidates for the non-enzyme component of C_5. When this protein has been identified and it is understood how it associates with the enzyme, understanding of the formation of the many other unexplained bands with cholinesterase activity may not be far behind.

Molecular biology techniques have been used to investigate the structure of the butyrylcholinesterase gene and its expression in development, in tumours and in organophosphate poisoning, but the work is not directly relevant to the findings presented here.

1.6 A note on function

Acetylcholinesterase has a very low substrate concentration optimum. Butyrylcholinesterase in contrast has a low activity towards acetylcholine at such a low concentration, but is not inhibited by much higher concentrations of acetylcholine (Augustinsson and Nachmansohn, 1949). It is not difficult to imagine therefore that the two enzymes might be complementary in the nervous system and muscles, butyrylcholinesterase taking over the hydrolysis of acetylcholine from acetylcholinesterase at higher substrate concentrations, and work on canine tracheal smooth muscle (Adler and Filbert, 1990) suggests that this may well be true. This however does not explain the presence of butyrylcholinesterase in the serum (or of acetylcholinesterase in the red cell membrane) where it presumably has some other function. This work investigates the relationship of the enzyme to serum lipids.

1.7 Association of serum cholinesterase with plasma lipoproteins

1.7.1 Clinical studies

Many observations have been made suggesting association between serum cholinesterase and food intake, levels of serum lipids or lipoproteins, or with rate of lipid turnover.

In 1950, Waterlow, noting that low serum cholinesterase had been found in undernourished subjects (McCance, Widdowson and Hutchison, 1948), measured cholinesterase in plasma and in liver biopsies before and after four or five weeks of feeding a high-milk diet to two malnourished African infants. In the liver, the cholinesterase increased more than other enzymes, and in the plasma it increased more than did the total protein. In 1954 Berry *et al.* noted that the same authors (Hutchison, McCance and Widdowson, 1951) had found, in their studies of undernutrition and feeding, that at the end of rehabilitation when men became 'fat', the average serum cholinesterase was higher than 'normal'. Acting upon this, Berry *et al.* measured the body fat (by skin-fold thickness) and plasma cholinesterase of 345 men and found a significant positive correlation.

Serum cholinesterase was found to be raised in hyperthyroidism, diabetes mellitus and hypertension by Antopol *et al.* in 1937 (though they thought that the enzyme was acetylcholinesterase), decreased in hypothyroidism (Thompson and Whittaker, 1965) and increased in nephrotic syndrome (Kunkel and Ward, 1947). These findings have subsequently been confirmed by other workers.

All of these findings have been linked by the association of butyrylcholinesterase with lipid metabolism. In 1963, Clitherow *et al.* pointed out that the penultimate product of fatty acid degeneration and the primary product of lipogenesis of fatty acids having even numbers of carbon atoms is butyryl-coenzyme A. They postulated that this, and to a lesser extent the acyl-coenzyme A derivatives of certain other higher fatty acids, might become involved in the choline ester synthetic pathway. The predicted products, particularly

butyrylcholine, have a powerful nicotinic action and if not rapidly destroyed would probably have undesirable and toxic effects. The authors suggested that the hydrolysis of these choline esters, almost at the site of formation, might be the principal biological function of butyrylcholinesterase. In support of this hypothesis they noted that a seasonal variation had been found in horse serum cholinesterase and pointed out that this closely parallels the well-known seasonal variation in fat metabolism. The first human case of homozygosis for silent serum cholinesterase had been reported the previous year (Liddell *et al.*, 1962; Hart and Mitchell, 1962) but Clitherow *et al.* did not mention this, and the fact that such individuals do not appear to suffer from the toxicity they predicted had obviously not come to their notice. Venkatakrishnan (1990) has now shown that inhibition of propionylcholinesterase activity with tetraisopropylpyrophosphoramide (iso-OMPA) in rats does not cause accumulation of choline esters in liver or serum, or increase their excretion in the urine. This does not however mean that butyrylcholinesterase could not have any rôle in lipid metabolism.

The most comprehensive series of clinical studies of the association between serum cholinesterase and lipid metabolism has been carried out by Cucuianu and colleagues (1968, 1973 (3 papers), 1975, 1976, 1978, 1985 (two papers)). In the first study (Cucuianu *et al.*, 1968) they looked at subjects divided into four categories defined by normal body weight *vs.* obesity and normolipidaemic *vs.* hyperlipidaemic. They found that both obesity and hyperlipidaemia were associated with increased serum cholinesterase, obese subjects having higher cholinesterase than normal weight subjects for a given level of lipid. The serum lipid indices used were cholesterol and triglycerides (now known as triacylglycerols). They found that both were positively correlated with serum cholinesterase and took this as support for the hypothesis proposed by Clitherow *et al.* (1963). They also investigated a few subjects with thyroid imbalance and noted that those with hyperthyroidism had high serum cholinesterase with low cholesterol and that those with hypothyroidism had low cholinesterase with high cholesterol. They noted that increased mobilization of

lipids was known to occur in hyperthyroidism but that high serum cholesterol is uncommon in this condition, and suggested that this is probably a result of increased elimination of cholesterol and oxidation of lipids. From this they concluded that serum cholinesterase should rather be correlated with increased influx of unesterified fatty acids and their uptake by the liver than with actual levels of lipoproteins. They mentioned that neonates have low serum cholinesterase and low levels of cholesterol, phospholipids and triacylglycerols but high concentrations of free fatty acids, and speculated that the mechanisms leading to maturation of lipogenesis and synthesis of serum cholinesterase are somehow linked. However the increase of serum cholinesterase as an expression of a nonspecific and rather general stimulation of protein synthesis in the liver of obese and hyperlipidaemic subjects could not be ruled out, and some of their later work was directed towards this question.

In 1973 Cucuianu and colleagues showed that treatment of 20 hyperlipidaemic patients with clofibrate lowered the mean serum cholesterol by 23% and triacylglycerols by 31% but did not reduce the raised serum cholinesterase significantly (Haragus *et al.*, 1973), whereas the treatment of leukaemic patients with L-asparaginase, which impairs hepatic protein synthesis, caused a decrease in both lipoprotein cholesterol and serum cholinesterase (Cucuianu *et al.*, 1973a). These papers were cited by Cucuiano (1988) who quoted a reference saying that clofibrate seems mainly to enhance the removal of VLDL-triacylglycerol and does not greatly depress the rate of VLDL synthesis. The inference from this would be that serum cholinesterase synthesis is related to VLDL synthesis rather than to the serum concentration of VLDL. Clofibrate is one of a class of drugs which induce proliferation of peroxisomes in hepatocytes and a marked increase in peroxisomal enzymes involved in lipid metabolism (for refs see Furukawa *et al.*, 1985). However, Brown and Goldstein (1990) say that the sites of action of the fibric acids (which include clofibrate) are only partially established and remain controversial. Though the primary effect of these drugs is to increase the activity of lipoprotein lipase, which in turn promotes catabolism

of the triacylglycerol-rich lipoproteins, VLDL and IDL, the drugs may also decrease hepatic synthesis and secretion of VLDL.

In 1975 Cucuianu *et al.* published the results of a more extensive study, including far more subjects than in 1968 and involving lipoprotein electrophoresis to investigate the relationship of serum cholinesterase to different types of hyperlipoproteinaemia, and the measurement of ceruloplasmin to see whether changes connected with hyperlipoproteinaemia involve non-specifically other enzymes secreted by the liver into the plasma. This added the information that serum cholinesterase was much higher in subjects with endogenous hypertriacylglycerolaemia - type IV and 'mixed hyperlipaemia' (roughly type IIb) - than in those with pure hypercholesterolaemia (type IIa). It also showed that many normolipidaemic overweight subjects had higher levels of serum triacylglycerol and prebeta fraction of lipoprotein than normal weight controls. Similarly, type IIa subjects who were overweight had higher serum triacylglycerols and cholinesterase than type IIa subjects who were of normal weight. Serum cholinesterase activity was found to correlate best with prebeta lipoprotein (i.e.VLDL), closely followed by triacylglycerols, with a poorer correlation with serum cholesterol and very weak one with 'relative body weight' (not defined), and a slight positive correlation with beta lipoprotein (i.e. LDL) was not significant. The interpretation was that serum cholinesterase activity is correlated with prebeta lipoprotein and that the lesser correlation with cholesterol occurs because a fairly large proportion of serum cholesterol is carried in the prebeta fraction. The serum cholinesterase was not significantly higher in the lean type IIa subjects than in the controls. The authors cited evidence that the main defect in this disorder is in catabolism of beta lipoproteins. They also noted from the literature that delayed production and turnover of lipoproteins had been found in hypothyroidism whereas in endogenous hypertriacylglycerolaemic and obese normolipidaemic subjects increased turnover of cholesterol and free fatty acids had been found as had increased turnover of serum lipoproteins in patients with the nephrotic syndrome. All of this pointed to the association of serum

cholinesterase with lipoprotein synthesis. They mentioned that the plot of serum cholinesterase activity against serum triacylglycerol concentration flattened with increasing triacylglycerol, a few patients with very high triacylglycerols having normal serum cholinesterase activities, and interpreted this as being due to defective or saturated mechanisms for removal of triacylglycerols. The serum ceruloplasmin differed very little between the groups studied, showing that the changes in cholinesterase could not be explained by entirely non-specific increase in protein synthesis by the liver, though they did note that serum levels of factor XIII and of lecithin:cholesterol acyltransferase had been found to be raised in obese and hypertriacylglycerolaemic subjects. The work on factor XIII was their own (Cucuianu *et al.*, 1973b). In that paper they also divided the hyperlipidaemic patients into different types and measured serum cholinesterase (though the numbers were smaller than reported in 1975). Inspection of their results shows that the pattern of factor XIII elevation in the different groups closely mirrored that of serum cholinesterase. They also measured plasma fibrinogen and though not discussed, the results show that it too was raised in all hyperlipidaemic groups but that the pattern was different, the highest level occuring in type IIa, followed by IIb and the lowest in type IV. Both factor XIII and fibrinogen were significantly lowered by treatment with clofibrate 1.5 g/day with a low-carbohydrate diet for 45-60 days in nine hypertriacylglycerolaemic patients whereas cholinesterase was not.

The paper by Haragus *et al.* (1973) - reporting reduction of VLDL by clofibrate treatment without influence on raised serum cholinesterase - is in Roumanian. The English summary does not mention the concomitant use of a diet and neither does Cucuiano (1988) in quoting it. Haragus *et al.* (1973) and Cucuianu *et al.*, 1973b (just discussed above) were written in the same year and have two authors in common but the number of patients and length of treatment are different. From this the assumption made here is that the patients of Haragus *et al.* (1973) were treated with clofibrate alone. However, returning to the patients of Cucuianu *et al.*, 1973b, one might have expected that VLDL synthesis

might be reduced by the diet and hence that serum cholinesterase might fall too, if its synthesis is related to that of VLDL, though not as much as it would if it were affected by increasing VLDL clearance. Myrtek *et al.* (1978) included serum cholinesterase amongst many other measurements on the blood of 190 patients with cardiovascular diseases before and after a 4-6-week rehabilitation programme of weight-reduction and improvement of physical fitness, without lipid-lowering drugs, and they did find a highly significant fall in serum cholinesterase along with falls in cholesterol, triacylglycerols and phospholipids.

Cucuianu *et al.* (1976) compared activities of serum γ-glutamyltranspeptidase (γGT) and cholinesterase in controls and different types of hyperlipoproteinaemia. Subjects with liver disease and other conditions in which γGT was known to be raised were excluded. Like the cholinesterase, γGT was found to be raised in hyperlipoproteinaemias, but again the pattern was different. Unlike serum cholinesterase, γGT activity did not correlate with serum cholesterol, and was not raised in type IIb hyperlipoproteinaemia. The γGT was much the highest in type V, in which the serum cholinesterase was lower than it was in type IV. Plotting both enzymes against triacylglycerol showed that while the cholinesterase activity reached a plateau, the γGT activity continued to rise with increasing triacylglycerol and correspondingly, a six-week course of clofibrate plus diet (type unspecified), which lowered the triacylglycerols of ten patients with hypertriacylglycerolaemia by 72%, brought about a 47% fall in γGT but, as before, no change in the serum cholinesterase activity.

Cucuianu *et al.* (1978) compared the activities of lecithin:cholesterol acyltransferase (LCAT) and serum cholinesterase in the types of hyperlipoproteinaemia, in obese subjects without hyperlipidaemia, in patients with hepatocellular diseases and in ones with cholestasis. The changes in the two enzymes between different groups were very similar and their activities were more strongly correlated with one another than with either cholesterol or triacylglycerols. Correlations with lipoproteins were not given. The authors put forward the possibility of a functional relationship between increased lipoprotein

turnover, induction of liver enzymes and thrombotic tendency and went on to publish (Cucuianu *et al.*, 1985a) a study including measurement of plasma fibronectin and factor VIII-related antigen (VIIIR:Ag) along with cholinesterase, fibrinogen and factor XIII in hyperlipoproteinaemias, cirrhosis, nephrotic syndrome and controls.

Fibronectin showed a similar pattern of elevation in the groups to cholinesterase except that it was lower in hyperlipoproteinaemia type IV than in type IIb. It was slightly better correlated with cholesterol than with triacylglycerols but was better correlated with serum cholinesterase than with either cholesterol or triacylglycerols, or with fibrinogen, VIIIR:Ag or factor XIII. Factor XIII was better correlated with triacylglycerols than with cholesterol, but only a little better. It too correlated better with cholinesterase than with the lipids or any other protein, and better than did fibronectin. The correlations of VIIIR:Ag with lipids and cholinesterase were not given but the results show that it was equally elevated in hyperlipoproteinaemias type IIa and IIb and slightly less so in type IV (and grossly elevated in cirrhosis and low in nephrotic syndrome, i.e. directly opposite to changes in cholinesterase).

The same year, Cucuianu *et al.* (1985b) published yet another study comparing the levels of clotting factors and serum cholinesterase. Not having the specific plasma (from beagle dogs genetically deficient in factor VII) required to measure human factor VII, they used a measurement roughly corresponding to the combined activities of factors VII and X in controls and hyperlipidaemic, cirrhotic, major surgery post-operative, and cholestatic patients. The clotting index was found to be raised in the types of hyperlipidaemia in a similar pattern to that of cholinesterase (except that in type IIb the clotting index was closer to its value in IIa than to that in type IV). However, unlike cholinesterase, it did not go down significantly in post-operative or cholestatic patients. The correlation of the clotting index with cholinesterase improved when these groups of patients were excluded (0.578 to 0.716) and 'remained significant' when the cirrhotic patients were excluded (actually fell to 0.475). What the authors failed to point out was

that these exclusions made virtually no difference to the correlation of the clotting index to serum triacylglycerol (0.637, 0.661 and 0.645 respectively, all 6 correlations $p < 0.001$), i.e. that the clotting index is much more closely related to the absolute level of triacylglycerol than is cholinesterase.

These studies show that though many proteins secreted by the liver are elevated in hyperlipidaemias the patterns are different. Thus the particular relationship of cholinesterase cannot be taken merely to be part of a general trend. In particular, serum cholinesterase activity seems to be associated with an increased rate of synthesis of VLDL or its release into the bloodstream and not at all with its catabolism. In this regard it is significant that the protein studied that most closely approximated the relationship of cholinesterase to serum lipids and lipoproteins was LCAT, since the cholesteryl ester in VLDL is derived mainly, if not entirely, from the action of LCAT in the plasma. Several papers important to the establishment of the latter theory were published in 1978 (reviewed by Frohlich *et al.*, 1982), the same year that Cucuianu *et al.* published their study on comparison of LCAT and serum cholinesterase levels, so Cucuianu *et al.* could not have been aware of the full implication of their findings at the time. It is surprising, however, that when Schouten *et al.* (1987) challenged association of serum cholinesterase activity and lipoprotein metabolism as fortuitous, Cucuiano (1988) in defence of his theory that 'raised serum cholinesterase activity and high levels of lipoproteins (mainly VLDL) represent different effects of a common cause acting upon the hepatocyte and leading to an accelerated rate of synthesis and turnover of lipoproteins' did not mention this point. He said that the association of increased serum cholinesterase activity and accelerated lipoprotein turnover could be due to specific induction, unspecific stimulation or hyperreactive hepatocytes. His evidence discussed above suggests that it may be fairly specific.

Schouten *et al.* (1987) it might be noted, had based their challenge on their finding that reduction of LDL-cholesterol with 'MK-733' in 11 heterozygotes for LDL-receptor deficiency did not cause a fall in serum cholinesterase activity.

'MK-733' blocks cholesterol synthesis by competitive inhibition of 3-hydroxy-3-methylglutaryl-CoA reductase. The work of Cucuianu and colleagues had shown not only that cholinesterase seemed to correlate with VLDL synthesis rather than absolute level, but that its apparent correlation with cholesterol was only through the association of raised cholesterol with raised triacylglycerols and that cholinesterase is not raised in lean subjects with pure hypercholesterolaemia (type IIa). Schouten *et al.* (1988) admitted that their data on patients with types IIa, IIb and IV hyperlipoproteinaemia agreed with those of Cucuianu *et al.* (1975).

The group publishing the next-largest number of papers on this topic from clinical studies have been Kutty and colleagues. They have also reported *in vitro* studies and much animal work which will be discussed in the following sections. Way, Hutton and Kutty (1975) reported a study of 16 children with nephrotic syndrome. Serum cholinesterase, dextran-precipitable lipoproteins (LDL + VLDL), triacylglycerols and cholesterol were all found to be raised compared with values in controls. In 7 of the patients further measurements were made after recovery, as judged by serum albumin concentration as well as 'the clinical picture'. In all cases the serum cholinesterase returned to within normal limits along with falls in the beta-lipoprotein to normal or below-normal levels. These findings were interpreted as evidence for the theory, developed from *in vitro* studies (Section 1.7.2, p. 46), that serum cholinesterase stabilises LDL, slowing its metabolism and hence increasing its half-life and plasma concentration. It was further proposed that pre-beta lipoproteins might be increased by a 'log-jam effect', explaining the observed increase in triacylglycerols.

Kutty *et al.* (1975) reported the investigation of a patient accidentally poisoned by the organophosphate, Parathion. The serum cholinesterase, LDL and cholesterol were measured on admission and two-hourly to 12 hrs and then at 24, 48 and 96 hrs. The LDL fell to a trough at 6-3 hrs and then rose through the rest of the period. The serum cholesterol reached a minimum at 10 hrs before making a similar recovery. The serum cholinesterase activity was zero on admission. By two hours it was partially restored following a dose of the

cholinesterase reactivator pralidoxime chloride on admission. It then fell again to 12 hrs before also recovering. It was felt that the coincident fall of cholinesterase activity and LDL could not be due to liver damage as recovery was too rapid and, taken along with the finding that serum cholinesterase and beta-lipoprotein levels were both depressed in guinea-pigs at one hour after dosage with the organophosphate phospholine iodide, these results were taken to indicate that cholinesterase has a function in the synthesis of LDL from VLDL.

Kutty *et al.* (1981a) proposed the use of a complementary risk factor (CRF), serum cholinesterase activity/HDL-cholesterol, to improve prediction of risk of cardiovascular disease based upon the established risk factor (ERF), total cholesterol/HDL-cholesterol. They investigated all the correlations between CRF, ERF, 'total LDL' (LDL + VLDL), serum triacylglycerol and cholesterol in 290 adults. CRF was more highly correlated with ERF than with the other lipid indices. It has a much smaller coefficient of variation than ERF which might suggest that it would be less useful, but it behaved differently. ERF correlated best with 'total LDL', then with triacylglycerols, and only slightly less well with total cholesterol. CRF correlated better with triacylglycerols than with 'total LDL' and much better with either than with total cholesterol. Except for the finding that ERF correlated better with triacylglycerol than with total cholesterol, which might not have been guessed, these results are what would be expected from the fact that total cholesterol is the numerator of ERF and from the relationships of cholinesterase with lipid indices found by Cucuianu and colleagues discussed above. They thus support Cucuianu's findings. Jain, Kutty *et al.* (1983) compared CRF with ERF in hyperlipoproteinaemic patients and controls. ERF was highest in type IIb hyperlipoproteinaemia, followed by IIa, and much less elevated in type IV, whereas CRF was almost identically and impressively elevated in Types IIb and IV, but only just higher than controls in type IIa. The investigators concluded that the use of ERF or CRF alone could predict the risk of ischaemic heart disease in 45% of the cases whereas the combination improved the predictive value to 65%. The relationships of the levels of serum

cholinesterase in the different groups and controls in this study were similar to those found in the Cucuianu series of papers except that the mean activity was rather higher in type IIb than in type IV, whereas Cucuianu *et al.* consistently found it slightly higher in type IV.

Kutty *et al.* (1987) measured the cholinesterase, 'total LDL', cholesterol and triacylglycerols in the serum of 66 patients with confirmed ischaemic heart disease and 83 controls. 34 of the patients had serum cholinesterase activities greater than the 95th percentile of the controls and all of them had activities greater than the control mean. 50% of the patients were hypercholesterolaemic and 80% of them were hypertriacylglycerolaemic. Serum cholinesterase was positively correlated almost equally with 'total LDL' and triacylglycerols, and only a little less with cholesterol. More interesting however was the finding that 18% of the ischaemic heart disease patients with significantly raised serum cholinesterase had normal lipid levels. The authors suggested that this could be explained by their theory (mentioned above) that cholinesterase stabilises LDL and suggested that the clearance of LDL could be sufficiently impeded by elevated cholinesterase, even in normolipidaemic individuals, to engender atherosclerotic plaque formation. They proposed the posibility that people at high risk of developing atherosclerosis might fall into two categories: those with hyperlipidaemia (and raised serum cholinesterase) in whom susceptibility is multifactorially determined and dietary excess is a major risk factor, and others with inherited hypercholinesterasaemia who might be at even greater risk even though diet and blood lipids are well controlled. A subsequent investigation found that the C_5 variant, which has aproximately 30% higher activity than the usual variant (see Section 1.5, p. 14) was not more common in hyperlipidaemic subjects than in controls (R.H. Payne, personal communication).

A few other authors have published on clinical studies of cholinesterase in hyperlipidaemia. The paper of Myrtek *et al.* (1978) - referred to earlier in connection with cholinesterase reduction with diet - showed correlations between serum cholinesterase and lipids but differed from all other reports in finding a

greater correlation of cholinesterase with cholesterol than with triacylglycerol. A slight positive correlation with phospholipids before rehabilitation was not significant but when figures from before and after the rehabilitation programme were combined the correlation increased and became significant at the 0.001 level, but was still less than that with cholesterol. A table of baseline correlations is given between 23 measurements, including lipids, proteins, electrolytes, blood cells, catecholamines, uric acid, maximal oxygen consumption, and weight. Amongst the enzymes, glutamate-oxaloacetate transaminase (GOT) and, weakly, glutamate-pyruvate transaminase (GPT) correlated with triacylglycerols and phospholipids, but neither correlated with cholinesterase. Apart from lipids, the only strong correlations of cholinesterase were with weight, blood sugar, and serum creatinine. Perhaps the later might be related to cholinesterase through common association with hypertensive renal damage. When results before and after rehabilitation were combined, correlations of cholinesterase with GOT and GPT appeared because those enzymes also decreased with weight loss and increase of maximal oxygen consumption.

The largest single study - of 1023 individuals - has been published by Schriewer *et al.* (1985). Most of these subjects (824) proved to have normal serum lipid levels but like Cucuianu *et al.* and Kutty *et al.*, Schriewer *et al.* did find serum cholinesterase activity to be raised in hypertriacylglycerolaemia and mixed hyperlipoproteinaemia (higher in the latter) compared to normolipidaemic subjects and intermediate levels in individuals with pure hypercholesterolaemia. They also divided the subjects into three categories with regard to HDL-cholesterol, with more similar numbers in the groups, and found an inverse relationship with serum cholinesterase. However, on regression analysis they found that the inverse relationship of cholinesterase with HDL-cholesterol disappeared when triacylglycerol was taken into account but that there was a positive correlation of cholinesterase with triacylglycerol which did not disappear when HDL-cholesterol was taken into account and therefore decided that the primary association was with triacylglycerol or VLDL metabolism. They also

scored their subjects on a nine-point coronary risk scale based on serum triacylglycerol, relative body weight, smoking and diastolic blood pressure, and found a clear correlation of serum cholinesterase with this index. They remarked that the question of whether raised serum cholinesterase is itself a coronary risk factor could only be decided by a prospective study, but said that if the serum cholinesterase is very high it would be worth looking for the presence of other risk factors.

Reuter and Geus (1987a,b) published results on 206 individuals divided into four groups by serum lipids (normolipidaemic, cholesterol raised, triacylglycerol raised or both raised) and divided into two groups by weight (normal or increased). In all four serum lipid groups, cholinesterase was higher in overweight subjects, and in both weight groups cholinesterase was higher in hypercholesterolaemic subjects than in the respective normolipidaemics, but significantly raised in hypertriacylglycerolaemic subjects, and more so in those with mixed hyperlipidaemia. Lipid concentrations were not given and there was no indication of measurement of lipoproteins, but the authors considered that raised cholinesterase activity is caused by increased VLDL secretion and, citing their earlier work (Geus, 1980; Geus and Reuter, 1982 - not seen), suggested the following possible mechanisms: nonspecific induction of protein synthesis; simultaneous synthesis of cholinesterase and 'VLDL-apoprotein'; induction of cholinesterase by intermediates of triacylglycerol metabolism.

Lehtonen *et al.* (1986) measured or calculated total cholesterol LDL-cholesterol, VLDL-cholesterol, HDL, HDL-cholesterol, triacylglycerols, ApoA-I, ApoB and serum cholinesterase in 83 patients with 3-vessel coronary artery disease and controls (matched for age, sex, weight and smoking). They examined every factor and various combinations as discriminators. Cholinesterase was found not to be a good discriminator on its own, but ApoA-I/cholinesterase and HDL/cholinesterase (nearly the reciprocal of 'CRF' of Kutty *et al.*, 1981a) detected 77.1% and 71.1% of the patients respectively. For comparison, the best single-index discriminators were HDL-cholesterol (74.1%)

and ApoA-I (73.9%) and one other good two-index discriminator was HDL/total cholesterol (nearly the reciprocal of 'ERF', 73.6%).

Magarian and Dietz (1987) measured cholinesterase and triacylglycerols, total cholesterol, VLDL-cholesterol, LDL-cholesterol and HDL-cholesterol in hypertensive patients in a cross-over study of therapy with α-blocker, β-blocker or placebo. They found that cholinesterase correlated negatively with HDL-cholesterol and positively with all the rest. The correlations were as good or better than those found in other studies, but the authors failed to recognise the check and balance that produced these results. The correlations were doubtless decreased because β-blockers significantly decrease HDL-cholesterol and significantly increase the other lipid indices (Magarian *et al.*, 1987) while competitively inhibiting serum cholinesterase (Whittaker *et al.*, 1981). However they were increased by internal controlling because the 117 measurements consisted of 6 or more measurements on each of the 16 patients (2 whilst on each drug and 2 or more in the intervening period on placebo).

At the beginning of this section it was mentioned that diabetes mellitus is associated with raised serum cholinesterase. Hyperlipoproteinaemia is known to be common in diabetes. Eight of the hyperlipidaemic patients included in the study of Cucuianu *et al.* (1968) were diabetic but he never studied diabetics as a separate group. Most of the work on association of serum cholinesterase and lipids in diabetes has been done in animals and will be discussed below. Venkatakrishnan (1990) reports measurements of serum cholinesterase, triacylglycerols, cholesterol, 'total LDL' (LDL + VLDL), HDL-cholesterol and LDL-cholesterol in type I and in Type II diabetic patients. She found significant positive correlations between cholinesterase and triacylglycerol in both types; correlations with other lipid indices were not significant.

Finally, in the early stages of alcoholism fat accumulates in the liver and hyperlipidaemia may also be found. Lieber (1989) has reviewed work on this. Ethanol displaces fat as a source of energy for the liver. This block in fat

oxidation favours fat accumulation. In addition, the altered redox state secondary to the oxidation of ethanol promotes lipogenesis, for instance, through an increase of α-glycerophosphate and enhanced formation of acylglycerols. The depressed oxidative capacity of the mitochondria injured by chronic alcohol feeding also contributes to the development of the fatty liver. The accumulation of fat in the liver acts as a stimulus for the secretion of lipoproteins into the bloodstream and the development of hyperlipidaemia. Hyperlipidaemia may also be caused by the proliferation of the endoplasmic reticulum after chronic ethanol consumption and the associated increase of enzymes involved in the assembly of triacylglycerols and lipoproteins. The propensity to enhance lipoprotein synthesis is offset, at least in part, by a decrease in microtubules and the impairment of the secretory capacity of the liver. The level of blood lipids depends upon the balance between these processes. At at the early stage of alcohol abuse, when liver damage is small, hyperlipidaemia will prevail, whereas the opposite occurs with severe liver injury.

Cucuianu *et al.* (1976) included 37 alcoholics in their study. Only 8 of them had hyperlipidaemia (7 type IV and 1 type V) and none of them had raised cholinesterase. Vincent *et al.* (1982) found the mean serum cholinesterase in chronic alcoholics to be nearly three times the normal. They also studied subjects brought into hospital in a state of alcoholic intoxication and found the mean to be raised in them too, but not so much. They quoted other authors with similar results. They also quoted several groups who had studied the effects of alcohols on serum cholinesterase activity *in vitro*. They confirmed previous findings with ethanol, showing that with benzoylcholine as substrate, serum cholinesterase activity increased with increasing concentration of ethanol in the medium up to a maximum between 6 and 7% alcohol, thereafter declining and becoming progressively inhibited beyond 12% alcohol, whereas with acetylcholine as substrate the activity was inhibited from the lowest concentration of alcohol. They made it clear that their findings of raised serum cholinesterase in chronic alcoholic and intoxicated patients could not be due to the effect of alcohol

present in the blood at the time of sampling because they used acetylcholine as the substrate. They also measured serum γGT and found that during 'drying-out' patients' γGT fell very dramatically from very high levels while the serum cholinesterase changed very little. They mentioned however that the level fell progressively during the pathological progression of alcoholic cirrhosis and could then be used as an indicator of prognosis. They concluded (translation) 'The activating effect of alcohol on serum cholinesterase appears connected in alcoholics to an action secondary to enzymatic induction on the liver and hepatic biosynthesis of serum cholinesterase and corresponds, it seems, to a process of steatosis (fatty liver) and hypertriacylglycerolaemia', citing Cucuianu *et al.* (1976). They did not however measure the serum lipid levels themselves.

The patients of Cucuianu *et al.* (1976) had all been known to have been consuming large quantities of alcohol for at least five years. Most of them had enlarged livers. Only 10 had raised alanine aminotransferase (same enzyme as GPT). The liver is usually enlarged with fatty infiltration but tends to shrink in cirrhosis. Alanine aminotransferase activity is usually mildly elevated in acute alcoholic hepatitis but can be normal in cirrhosis. Cirrhosis might therefore have been the cause of the non-elevation of the cholinesterase in some of the cases, but is otherwise mysterious. It was noted that the nutritional condition of the alcoholic subjects was not as good as that of the control subjects or of the non-alcoholic hyperlipidaemic subjects, but this might also have applied to the patients of Vincent *et al.* (1982).

1.7.2 *In vitro* studies

Lawrence and Melnick (1961) applied 11 histochemical enzyme stains to human serum following immunoelectrophoresis and found localization of all of them in the beta-lipoprotein precipitin line. They then separated beta-lipoprotein from serum and plasma samples by ultracentrifugation, measured the activities of 12 enzymes in the beta-lipoprotein fraction. Following ultrasonication the activity of 10 of the enzymes, including serum cholinesterase, increased considerably.

Freezing and thawing, addition of dinitrophenol, ether extraction and bubbling oxygen through the preparation had the same effect to varying degrees. The authors interpreted their findings as demonstrating that beta-lipoproteins form physical complexes with various proteins thereby carrying them in inactive states. R.H. Payne (personal communication) has tried to replicate the ultrasonication experiment without success. Dubbs (1966) has been quoted as providing support for this theory with respect to cholinesterase but his work did not actually do so. He subjected serum to ultrasonication and then electrophoresis and found striking increase in the cholinesterase C_3 band and lesser increases in the C_1 and C_2 bands - and eventual disappearance of all bands. From what is now known of the structure of serum cholinesterase (see Section 1.3, p. 6) this merely indicated fragmentation of the tetramer into dimer and monomer before further denaturation.

Kutty and Jacob (1972) incubated human serum samples with different concentrations of isoniazid, which proved, as they hoped, to be a cholinesterase inhibitor. They found a linear relationship between percentage inhibition of serum cholinesterase and fall in LDL concentration while the total serum protein concentration remained constant and they suggested that the fall in LDL might be caused by the inhibition of the cholinesterase. Kutty and Acharya (1972) reported similar experiments using single concentrations of isoniazid or eserine. Both cholinesterase inhibitors apparently caused beta lipoprotein concentrations to fall. Ultrasonication of serum for 25 minutes caused an increase in pre-beta lipoprotein and decrease in beta lipoprotein, as estimated by dextran precipitation and cellulose acetate electrophoresis (and an increase in cholinesterase C_3). Incubation of LDL with 'isolated' rabbit or human serum cholinesterase for six hours at pH 6.5 'appeared to stabilize the lipoproteins as assessed by agar-gel electrophoresis'. K.M. Kutty (personal communication) has kindly provided the details of this experiment not given in the abstract. The cholinesterases were isolated by eluting them from starch gel after electrophoresis. On the agar gel LDL without additions was compared with LDL incubated with either of the

preparations of cholinesterase and with LDL incubated with the solution used to elute the enzymes. The lane loaded with the LDL incubated with the elutant solution showed only about 20% of the amount of LDL as in the lane of the unincubated LDL, whereas in the lanes containing enzyme about 80% of the LDL remained.

Kutty *et al.* (1973) incubated isolated human serum beta-lipoprotein with phospholipase D^2. Electrophoresis then showed replacement of the beta-lipoprotein band of the untreated lipoprotein preparation with a band of prebeta mobility and the appearance of a strong cholinesterase band where little had been evident in the untreated material. Thus in this series of experiments Kutty and colleagues had not only shown that addition of cholinesterase to LDL appeared to stabilize the LDL but that inhibition, destruction or 'release' of cholinesterase (by phospholipase D) from LDL seemed to cause a reversion of LDL to a particle with pre-beta mobility. They formed the theory (Kutty *et al.*, 1973) that cholinesterase was associated with the LDL particle, but chemically rather than physically, as Lawrence and Melnick (1961) had proposed. They suggested that cholinesterase might bind by its active site to the phosphorylcholine moiety of lecithin on the surface of LDL, stabilising the LDL and inactivating the cholinesterase and that the action of lipoprotein lipase on the triacylglycerols of VLDL produces a pre-LDL (which one might now identify as IDL) and that the binding of cholinesterase to this formed 'true' LDL.

Kutty *et al.* (1977) prepared beta-lipoprotein from the serum of 33 patients by heparin-calcium-chloride precipitation. After washing the precipitates in 1% triton X-100 in saline they measured the cholinesterase activity and found that it was approximately proportional to the concentration of beta-lipoprotein in the samples from which it had been prepared. As previous users of this method of preparation of beta-lipoprotein (Buckley *et al.*, 1968; Eaton and Kipnis, 1969) had examined their products for contamination with albumin (by gel-, paper- or immuno-electrophoresis) and found little or none, Kutty *et al.* took the finding of cholinesterase in the lipoprotein to indicate a structural association between the

two. It should be noted however that enzymatic staining is much more sensitive than protein staining and is also more sensitive than visually examined unstained immunoprecipitation, as results to be presented here have shown.

Kutty and colleagues (Chu *et al.*, 1978) then measured cholinesterase, cholesterol and triacylglycerols in sera sent for lipid screening and in LDL precipitated from those sera. The cholinesterase was increased in both sera and LDL fractions in type IIa hyperlipoproteinaemia, and more so in types IIb and IV (slightly more in IV) compared with the normolipidaemic patients. However, in type IIa the elevation reached statistical significance in the LDL fraction but not in the whole sera and they took this as support for their theory. They also divided the data into four groups by LDL concentration and found the pattern of rise in triacylglycerols, cholesterol and cholinesterase with LDL to be very similar in the precipitated LDL to that in the whole sera. They proposed that there are two pools of cholinesterase activity in the serum, one free and the other bound to LDL. No significant difference was found in mean cholinesterase activity between whole sera and supernatants from the precipitation of LDL. The mean was just less in the supernatants. Actually, if cholinesterase is bound inactive in LDL one might expect the result to be lower in the whole serum since it is diluted by LDL, but this difference might not be detectable. The activity in the precipitated LDL was less than one tenth of that in serum. Cholesterol, triacylglycerols and cholinesterase were also compared in serum and LDL fractions of obese subjects with and without hyperlipidaemia. In the LDL, as in whole serum, cholinesterase was higher in hyperlipidaemia. The overall conclusion was that cholinesterase might play a rôle in both lipoprotein synthesis and structure.

In an abstract, Puhakainen, Ryhänen and Penttilä reported measurement of cholinesterase in serum, HDL and 'VHDL'. They found most activity in the 'VHDL' and noted that patients with low HDL concentrations tended to have high total serum cholinesterase activity and *vice versa*. Ryhänen *et al.* (1982) enlarged on this. They pooled serum from 80 healthy normolipidaemic adults and measured cholinesterase, cholesterol and triacylglycerols in the whole serum and in VLDL,

LDL, HDL and VHDL fractions. No cholinesterase was detected in the VLDL. The activity in the HDL (expressed as IU/L) was an order of magnitude greater than that in the LDL, and in the VHDL it was another order of magnitude greater and about three times the activity of whole serum. The fractions were then washed and the measurements made again. The cholesterol concentration of all fractions was increased by the washing. The cholinesterase activity in the HDL was halved, and reduced slightly in the VHDL, but in the LDL it apparently increased slightly. The latter finding was supported by the finding on electrophoresis of one of the minor cholinesterase bands that was not detected in the unwashed fraction. This was interpreted not as showing that cholinesterase was truly an integral part of LDL and consequently increased in concentration by washing as was cholesterol, but as indicating the removal of inhibitory substances. Inspection of the results reveals that albumin was detected in all the fractions - in the same rank order but not the same proportions as the cholinesterase - and that it too was not all removed by the washing, though it never actually increased. Citing their abstract, these workers came to the conclusion that LDL increases serum cholinesterase activity and that HDL inhibits it.

1.7.3 Animal studies

Much of the animal work on serum cholinesterase and lipids has been done in rats, so it is worth noting that sex and specific differences in induced changes in cholinesterase activity have been found. Harrison and Brown (1951) reviewed some such findings and reported a study of rats fasted for six days. Serum cholinesterase cholinesterase activity at the start was almost six times higher in virgin females than in males. During the fast the activity fell continuously in the females but remained constant in males while the serum albumin and total protein decreased gradually in both sexes, slightly more in females. Body and liver weights fell continuously in both sexes but per gram of liver the cholinesterase remained constant in males, while in females it fell sharply for two days and then levelled.

It has long been known that accumulation of fat in the liver occurs when the diet is inadequate with respect to its content of 'lipotrophic factors': choline, betaine and methionine (see references in Hawkins and Nishikawara, 1951). This type of fatty liver can occur in starvation and during the feeding of high-fat diets. Choline can be synthesized using labile methyl groups donated by methionine, and one theory for the mechanism of fatty liver is that lack of choline impairs synthesis of phospholipids (Mayes, 1990). Fatty liver also occurs in alcoholism, as was mentioned above.

Hawkins and Nishikawara (1951) measured serum cholinesterase activity in rats fed a hypolipotrophic diet with or without supplements of lipotrophic factors. They expected that animals showing fatty liver would have a lowered serum cholinesterase since the enzyme is synthesized in the liver. However, in male adult and weanling rats, serum and liver cholinesterase was significantly raised in animals fed a hypolipotrophic diet compared with those on diets suplemented with any of the lipotrophic factors. Female rats on the hypolipotrophic diet had significantly lower serum cholinesterase than those given a supplement of choline chloride. The authors concluded from this that the hypolipotrophic diet was not directly responsible for the elevation observed in males. Osada *et al.* (1989) fed rats a high-fat diet and compared them with others on a control diet, or returned to the control diet for a period after the high-fat diet or on either diet supplemented with lipotrophic factors. The mean serum cholinesterase was lowered in two groups given the high-fat diet alone compared with all the other groups. All the animals were male. The serum lipid levels were unfortunately not measured in these studies.

Kutty and Jacob (1972) induced hyperlipidaemia in rabbits by intravenous injection of the lipopolysaccharide of E. coli. (The mechanism by which this occurs is unknown.) Twenty-four hours after injection the mean concentrations of total serum lipid and 'total LDL', and the cholinesterase activity, were all raised. The factors by which they were raised were about 4, 11 and 2 respectively. Kutty *et al.* (1973) extended this study. They investigated similarly treated rabbits

before injection and at 24, 48 and 72 hrs. Serum cholesterol, triacylglycerols and 'total LDL' were all markedly increased at 24 hrs. before falling to intermediate levels by 48 hrs., which were maintained at 72. Quantitative measurements of the individual lipoproteins had not been developed, but their relative concentrations were observed by inspecting electron micrographs of the sera and counting the particles of different sizes, and by examining the bands on electrophoresis. Both of these methods showed a massive increase of VLDL by 24 hrs. which was partially replaced by LDL by 48 hrs. and almost entirely so by 72 (the HDL falling and then rising again in the meantime). The serum cholinesterase activity was not measured, but it was examined by electrophoresis to look at the bands, whose meaning had not been established at the time. The results showed a large increase in enzyme activity by 24 hrs., a lower level at 48, and an activity back to control or below by 72. These results very nicely bear out the predominant impression from the clinical studies that increase in cholinesterase activity is related to the appearance of VLDL in the serum and is not directly related to absolute levels of cholesterol, triacylglycerols or LDL. The investigators were however more interested in the possibility of the chemical association of cholinesterase with the LDL particle, one of the *in vitro* studies described above having been reported in the same paper.

Kutty *et al.* (1977) investigated the possible role of cholinesterase in lipoprotein synthesis in rats by measuring the incorporation of ^{3}H-lysine into lipoprotein fractions with or without treatment with the cholinesterase inhibitor neostigmine. The neostigmine, which reduced the serum cholinesterase activity by about 50% caused a fall in labelled beta lipoprotein of about 75%, a slight increase in labelled pre-beta lipoprotein and about a 50% increase in labelled alpha lipoprotein. Serum glutamate-pyruvate transaminase activity and cholesterol ester/cholesterol ratio were unaffected. The results were taken as support for the hypothesis of stabilization of LDL by cholinesterase. However, since lipases are esterases, it is also possible that the neostigmine might have inhibited lipoprotein lipase which converts VLDL to IDL. The increase in labelled

HDL is more of an enigma. It could be due to a reduction in HDL clearance, due to some other effect of neostigmine, but it could just be a result of the reciprocal relationship observed to occur between LDL and HDL which is not properly understood.

Similar results were obtained by Ryhänen *et al.* (1984) in a study of poisoning by the organophosphate dichlorvos in rabbits. They found that serum cholinesterase activity fell to a minimum at 4 hrs. after treatment, returning to normal by 72 hrs. γGT and LCAT were affected in the same way, though the changes in the latter were not statistically significant. LDL showed a very dramatic parallel fall and rise, and HDL moved in the opposite direction though the changes were much smaller. VLDL was unaffected. Cholesterol fell and rose slightly with LDL. Triacylglycerols and esterified fatty acids rose slightly to peaks at 8-24 hrs. In a previous paper (Nousiainen and Ryhänen, 1984) it had been reported that (in male rats) disulphiram also inhibited serum cholinesterase but caused a rise in cholesterol and a fall in triacylglycerols. The lipoproteins were not measured. Ryhänen *et al.* (1984) claimed that this disagreed with Kutty's hypothesis and suggested that organophosphates might cause a fall in LDL by inducing LDL receptors, or by inhibiting HMG-CoA-reductase (the rate-limiting enzyme in cholesterol synthesis) by phosphorylating it. Since the VLDL concentration was unaffected, the latter explanation at least seems unlikely.

Kutty *et al.* (1981b) found that in genetically obese mice (ob/ob), heterozygotes (ob/+) with obesity induced by injection of gold thioglucose, and genetically diabetic mice (db/db) cholinesterase activity in serum and liver was 2-2.5 times what it was in heterozygous controls. All these categories of mice are known to have very high blood glucose (i.e. the obese mice are also diabetic) and the investigators found that they all had higher calorie-intakes than the controls. ob/+ mice fed a high-carbohydrate diet also had raised liver and serum cholinesterase but not as high as the other groups. Their calorie-intake and blood glucose were also found to be raised but to a lesser extent than the other abnormal groups. Diabetic mice starved for 24 hrs showed a 40% reduction in

cholinesterase activity in the liver but no reduction in the plasma, possibly due to long half-life of serum cholinesterase, though there could have been continued secretion of enzyme already synthesized.

Kutty *et al.* (1983) went on to observe increases in food-intake, weight, and serum cholinesterase in growing weanling mice. Comparison of obese (ob/ob) and lean (ob/+) mice showed that all three variables were higher in the obese mice from about 28 days onwards and steadily separated. However, from the first blood sampling at 24 days until 35 days, while food intake and weight were increasing serum cholinesterase activity fell and thereafter rose. Testing mice only 21 days old showed that the cholinesterase was initially low but that its activity transiently rose sharply on weaning from a mothers milk (high fat) to a laboratory mouse-food (high carbohydrate) diet, so the type, not just the amount of food mattered. Diabetic (db/db) mice restricted to the same amount of food as the average taken by non-diabetic mice showed a similar rise in serum cholinesterase activity to theirs from 32 days, though at a higher level, but diabetic mice allowed food *ad lib.* showed a much steeper increase. Serum lipids are known to be raised in diabetes and frequently in obesity, but they were not measured in these studies.

Kutty *et al.* (1984) then studied Zucker fat rats. These animals are obese, hyperphagic and hypertriacylglycerolaemic with hyperinsulinaemia and normal blood sugar. Homozygous fat rats were compared with homozygous lean controls and correlations were calculated with the two groups combined. Serum cholinesterase activity correlated better with serum triacylglycerols than with insulin or cholesterol, but less well than with food intake, weight or liver cholinesterase activity. However, the numbers were small (27 total). There was apparently a negative correlation between serum cholinesterase and blood glucose in these animals. In this study and that on the adult mice (Kutty *et al.*, 1981b) the propionylcholinesterase activity was also measured in adipose tissue. It apparently fell when rises occurred in serum and liver, but it was then realized (mentioned in the discussion of the 1984 paper) that this was because of the

increase in fat content of the cells and that per cell the adipose tissue cholinesterase also increased.

In the same laboratory, Venkatakrisnan (1990) has studied serum cholinesterase and lipids in diabetic animals. Induction of diabetes with streptozotocin and alloxan in rats was used as a model for type I diabetes. Serum cholinesterase, triacylglycerols, glycerol and 'total LDL' all increased with the development of diabetes, returned to normal when the hyperglycaemia was controlled with insulin, and rose again when the insulin was stopped. Total cholesterol and HDL always moved in the opposite direction to the other indices except that cholesterol did go up when diabetes was induced with alloxan rather than streptozotocin. VLDL was not measured quantitatively but electrophoresis indicated that fluctuations in it corresponded to the changes in serum triacylglycerol levels.

Gold thioglucose-treated mice were used as a model for type II diabetes. These animals show insulin resistance with hyperinsulinaemia and hyperglycaemia. Like the type I model rats they showed rises in serum cholinesterase, triacylglycerols, glycerol and 'total LDL', but in these animals the total cholesterol and HDL-cholesterol also increased. These studies thus show that serum cholinesterase activity is not directly related to total cholesterol, bearing out the suspicion expressed by Cucuianu *et al.* (1975). Furthermore elevation of serum cholinesterase occurs in Zucker fat rats along with high insulin and glucose levels that are normal but correlate negatively with cholinesterase, while in type I diabetic rats elevation of serum cholinesterase occurs with low insulin and high glucose, and in type II diabetic mice with high insulin and high glucose, indicating clearly that cholinesterase is not directly related to either glucose or insulin levels. It should also be noted that in contrast to the Zucker rats and type II diabetic mice the induction of type I diabetes in rats caused weight loss indicating that the commonly observed association of serum cholinesterase elevation with obesity is not a fast rule.

Liver propionylcholinesterase activity was increased in both the type I and type II diabetic models though not significantly in the former. The activity in adipose tissue was significantly but only slightly increased in the former but not in the latter, but as in Kutty *et al.* (1981b and 1984) these were wet-weight estimates and enzyme activity per cell or per gram of protein was not estimated but likely to be very elevated.

Iso-OMPA, which inhibits serum and liver propionylcholinesterase, caused decreases in serum glycerol, triacylglycerol and 'total LDL' levels in normal and diabetic rats, and diabetic mice, and withdrawal of iso-OMPA resulted in their increase. Changes in cholesterol were less clear. In the type I diabetic model rats it went down along with triacylglycerols but in the type II diabetic mice it went up slightly. Iso-OMPA is supposed to be a specific inhibitor of cholinesterase but it also caused significant decrease in serum glucose in the diabetic rats and lesser decreases in the control rats and diabetic mice, so the results should not be taken to prove that cholinesterase plays an active rôle in lipid metabolism. However, the fall in glycerol was taken as suggesting that serum cholinesterase might play a rôle in adipose tissue lipolysis.

Intravenous heparin injection is known to release lipoprotein lipase from blood vessel walls leading to rapid hydrolysis of plasma triacylglycerols. Administration of heparin to diabetic rats caused 85% reduction in triacylglycerols within 30 minutes but there was no change in serum cholinesterase. This was taken as indicating that the increase in serum cholinesterase in diabetic rats 'is not due to the serum level of triglycerides'. The clinical studies described above put a slightly different slant on this. They suggested not that the two are not related but that serum cholinesterase activity - and possibly synthesis - correlates with the rate of synthesis of triacylglycerols (or VLDL) rather than with the absolute level. In the diabetic rats the two go up together and the fact that cholinesterase does not fall when the rate of removal of triacylglycerols is increased does not detract from this.

Two other animal studies are relevant here. Butler *et al.* (1988) investigated the effects of the peroxisome-proliferating hypolipidaemic agents nafenopin and clofibrate on the activities of serum esterases in mice and rats of different genotypes and ages. They found that though serum arylesterase activity fell, serum cholinesterase activity significantly increased in all groups of both species. They noted however that these drugs cause hepatomegally and suggested that the increased serum cholinesterase might just be the result of increased liver mass. Udom *et al.* (1989) looked for a correlation between serum cholinesterase and LDL in Watanabe Heritable Hyperlipidaemic rabbits. They found that in contrast to a three-fold variation of LDL, serum cholinesterase activities lay within a narrow range, and there was no correlation between the two. These animals have hyperlipidaemia due to an LDL-receptor defect. The result is therefore exactly what one would expect if cholinesterase activity is correlated with VLDL synthesis and not with absolute levels or removal rate of LDL and illustrates it very well. The authors however completely missed this interpretation. They worked on the hypothesis of Kutty *et al.* (1978) of two pools of serum cholinesterase, one bound to LDL and the other free, and concluded that serum cholinesterase may be regulated so as to maintain complexation with LDL within physiologically acceptable limits.

Finally, Kutty *et al.* (1979) investigated the possible involvement of serum cholinesterase in lipid metabolism by studying atherosclerosis. They induced atherosclerosis in rabbits by feeding them cholesterol and compared frozen sections of their aortae with controls. Cholinesterase activity was found in the intima only in the aortae from the atherosclerotic rabbits and the spots of cholinesterase staining were reported to correspond to the sites of lipid staining in adjacent sections. The substrate used for cholinesterase staining was acetylthiocholine so this experiment did not prove that the enzyme responsible was one also found in serum. (Augustinsson, 1961, found the cholinesterase activity in rabbit serum to be greater, and about equal, with acetylcholine and propionylcholine than with butyrylcholine as substrate. Simeon *et al.*, 1988, found

evidence of three cholinesterases in rabbit serum, two hydrolysing both acetylcholine and butyrylcholine and one hydrolysing, of those two substrates, only butyrylcholine.) In the same paper, Kutty *et al.* reported measurement of cholesterol ester synthesis and propionylcholinesterase activity in cultured rat fibroblasts exposed to serum from the rabbits. In cells grown in hyperlipidaemic rabbit serum both were increased compared with controls, the cholesterol ester synthesis about eleven times, the cholinesterase activity about twice. The cholinesterase activity did not seem to be derived from the serum because the activity in cells grown in normal rabbit serum with additional horse serum cholinesterase was not increased significantly. Addition of neostigmine to the hyperlipidaemic serum reduced both measurements, the cholesteryl ester synthesis to about seven times control and the cholinesterase activity to a level not significantly above control. The authors concluded that cholinesterase was synthesized by the fibroblasts in the presence of hypercholesterolaemic serum and that by removing choline esters as Clitherow *et al.* (1963) had proposed (see 1.7.1, p. 31) it maintained ideal conditions for fatty acid metabolism.

1.8 Measurement

Serum cholinesterase is usually measured through its choline esterase activity. For several reasons this is not a direct measure of the concentration of enzyme protein. Firstly, there are numerous genetic variants, some of them common and most probably at least some of these have different specific activity with respect to the most commonly used substrate in measurement, butyrylthiocholine (see Section 1.5, p. 11).

Secondly, various drugs and toxins inhibit the enzyme. These include not only compounds used specifically as cholinesterase inhibitors such as neostigmine (used in the treatment of myaesthenia gravis), ecthiopate iodide eyedrops (for glaucoma, McGavi, 1965) and organophosphate insecticides, but other drugs to which more people are exposed. Propranolol (Whittaker *et al.*, 1981, and therefore probably other beta-blockers), chlorpromazine and its

sulphoxide (Erdos *et al.*, 1958, and therefore probably other phenothiazines), phenelzine and other monoamine oxidase inhibitor antidepressants (Bodley *et al.*, 1969), the antiemetic, metoclopramide (Kabam *et al.*, 1988; Kao and Turner, 1989) and the chemically similar but less used antiarrhythmic, procainamide (Kabam *et al.*, 1987), and the anti-cancer drugs cyclophosphamide, mechlorethamine (nitrogen mustard) and triethylene nitrophosphoramide ('Thio-tepa', Zsigmond and Robins, 1972) all cause clinically evident reduction of serum cholinesterase activity by prolonging the action of succinylcholine and have either been shown to inhibit serum cholinesterase *in vitro* or are likely to do because of their structures. (Other drugs known to reduce serum cholinesterase activity - contraceptive pills (Robertson, 1967) and the gluco-steroids dexamethasone and prednisone (Bradamante *et al.*, 1989) probably do so by reducing synthesis of the enzyme.)

Thirdly, one has to consider the possibility of activation of the enzyme. The insulin-dependent increase in lipoprotein lipase activity in adipocytes has been has been shown to be due to activation of pre-existing protein rather than to increased synthesis (Semenkovich *et al.* (1989) and serum cholinesterase has been shown to be activated *in vitro* by alcohols (Whittaker, 1968a).

In the work for this thesis serum cholinesterase concentration was measured to determine whether the raised activity found in hyperlipidaemia is due to increased concentration of enzyme or not, and in case some high-specific-activity variants might be found. There were reasons for believing that the high activity found in hyperlipidaemia was indeed due to increased concentration of enzyme apart from the relationships between cholinesterase activity and lipids discussed in the preceding sections.

Kunkel and Ward (1947) inhibited serum cholinesterase irreversibly by injecting diisopropyl fluorophosphate into five human subjects and observing the return of enzyme activity which could only come from synthesis of new protein. Two of the subjects had liver cirrhosis and low serum cholinesterase activities,

two had nephrotic syndrome and high activities, and one was a control. In all cases the activities returned from zero back to 80-100% of their original levels in 32 days and the curves of percentage of original level per day of regeneration were very similar. The serum lipid levels were not measured, so one cannot say that they did not behave in exactly the same manner as the enzyme and one cannot say for certain that the high activities in nephrotic syndrome were not caused by activation by one of the lipids, but the authors' conclusion that the levels reflected the rates of synthesis still seemed likely.

Further, Altland *et al.* (1971) measured serum cholinesterase concentration in 269 blood donors and found a correlation of 0.87 with activity. They did not measure serum lipids and did not study subjects with above-normal activities, but one might reasonably expect that some of their subjects would have been hyperlipidaemic, so the fact that they did not find any individuals with markedly higher specific activities suggests that lipids do not activate the enzyme. However, it seems that not everyone is convinced of this. Ryhänen *et al.* (1982) concluded that LDL cholesterol increases serum cholinesterase activity while HDL cholesterol inhibits it. Measurement of cholinesterase concentration can settle this question. Furthermore, it was felt that the examination of whether triacylglycerols or VLDL correlate better with the activity or the concentration of cholinesterase could address the question of whether the enzyme is actively involved in their metabolism or merely cosynthesized.

In the work presented here cholinesterase was purified from human serum, antibodies were raised against it, and the antiserum was used to measure the concentration of cholinesterase in human serum samples. Impurities in the antigen solution and immunological variation in the cholinesterase were found incidentally.

Chapter 2

MATERIALS AND METHODS (1)

2.1 Purification of human serum cholinesterase

2.1.1 Introduction

Das and Liddell (1970) purified human serum cholinesterase by ion-exchange chromatography on DEAE-cellulose at pH 4.0, followed by electrofocusing in a pH gradient, followed by gel filtration. The first step is effective because serum cholinesterase is one of very few serum proteins that are negatively charged at pH 4. It therefore remains in the column whilst the majority of proteins, being less acidic, pass straight through. Das and Liddell eluted the cholinesterase with an exponential gradient of sodium chloride from 0 to 0.2 M in the buffer, 0.02 M sodium acetate.

Muensch *et al.* (1976) used the same first step, but modified it by introducing the sodium chloride in a linear gradient, which they claimed resulted in higher purification. They also added 1 mM EDTA (ethylenediaminetetraacetate) and 1 mM mercaptoethanol to the buffer to improve stability of the enzyme. They replaced the other two steps with preparative polyacrylamide disc gel electrophoresis.

Lockridge and La Du (1978) used the same first step, with the modifications of Muensch *et al.*, and followed it with affinity chromatography on procainamide-Sepharose 4B at pH 6.9. Subsequently (Lockridge *et al.*, 1979) they omitted the mercaptoethanol from the buffers, because they found that the enzyme was more stable in its absence, and they operated the affinity column at pH 7.0. Later (Lockridge *et al.*, 1987a) they added a third stage, consisting of ion-exchange chromatography again, this time at pH 7.0, but the reason for this was not given.

In this work, the method of Lockridge and co-workers was used with several modifications. First, the removal of cholinesterase from the ion-exchange column

was different. In the absence of an apparatus to produce a linear gradient, cholinesterase, with other very acidic proteins, from batches of up to 500 ml of human serum was eluted with 1 M sodium chloride after washing all other proteins out of the column with buffer. Then cholinesterase-containing fractions from all batches were pooled and put back onto the column, and the serum cholinesterase eluted with 0.06 M sodium chloride, the concentration found to remove it by Lockridge and colleagues. Second, choline was used to elute the cholinesterase from the affinity column, instead of sodium chloride. Third, affinity chromatography was followed, not by further ion-exchange chromatography, but by gel filtration. This easily separated the cholinesterase from the single other remaining protein detected on electrophoresis, which was of much lower molecular weight than cholinesterase.

2.1.2 Detection of cholinesterase and other proteins during purification

2.1.2.1 Polyacrylamide gel electrophoresis (PAGE)

Serum and effluent and eluates from columns were examined and compared by PAGE (T = 6.43%; C = 2.6%, bis) in a vertical slab-gel apparatus (Hoefer Scientific Instruments Vertical Slab Gel Unit, SE 600 series) with gel cross-section 3 × 142 mm, run at constant power of 15 Watts. The details were as follows:

Acrylamide solution (amounts for 500 ml)

Acrylamide	73.05 g
N,N′-methylene-bis-acrylamide (bis)	1.95 g

Buffers (Ashton and Braden, 1961)

Gel buffer × 5 (amounts for 1 L)

citric acid	7.2 g
tris base	27.9 g

boric acid	5.9 g
LiOH	0.6 g
bromophenol blue	trace

Vessel buffer (amounts for 1 L)

boric acid	11.8 g
LiOH	1.2 g

Making the gel:

Put together gel apparatus.

Pour 30 ml acrylamide solution into a 50 or 100 ml measuring cylinder.

Add 14 ml 5 × gel buffer.

Add 1 drop of TEMED (N,N,N',N'-tetramethylethylenediamine).

Make volume up to 45 ml with 5 × gel buffer. Mix.

Weigh out 40 mg of ammonium persulphate and disolve in 25 ml deionized water.

Add the ammonium persulphate solution to the acrylamide/buffer.

Mix and pour immediately into the gel former through a funnel.

2.1.2.2 Stains

2.1.2.2.1 Serum cholinesterase stains

2.1.2.2.1.1. Specific stain

The usual method of detection of cholinesterase used employs S-butyrylthiocholine iodide as substrate in a solution also containing copper sulphate, sodium citrate, and potassium ferricyanide, as described by Karnovsky and Roots (1964). In the presence of cholinesterase, thiocholine is released, and this is precipitated as colourless cupric thiocholine. The thiocholine reduces the ferricyanide to ferrocyanide, and the latter combines with the copper ions to form

the insoluble copper ferrocyanide. The citrate chelates the copper ions in solution, preventing the formation of copper ferricyanide. The recipe is:

Stock solutions

Solution A:

Per litre:

maleic acid 11.61 g

NaOH 6.0 g

Adjust to pH 6.0 if necessary.

Solution B:

Sodium citrate 0.87 g in 30 ml water

copper sulphate 0.37 g in 50 ml water

Disolve separately and mix.

Prepare freshly

Solution C: 8 mg potassium ferricyanide in 10 ml water

Solution D: 30 mg butyrylthiocholine iodide in 40 ml of solution A

Mix in order: solution D

solution B (8 ml)

solution C

Then pour over gel.

2.1.2.2.1.2 Non-specific stain (α-naphthyl propionate)

Sometimes, for the purpose of illustration, cholinesterase was demonstrated with a non-specific esterase staining method, in which the substrate is α-naphthyl propionate. In the presence of cholinesterase, α-naphthol is released, and this forms a complex with the diazonium dye, Fast Red TR Salt (4-chloro-o-toluidine diazotate). This method gives a darker stain, which does not fade. The recipe is:

Disolve 10 mg α-naphthyl propionate in 1 ml ethanol.

Disolve 10 mg Fast Red in 100 ml water.

Mix and leave to stand.

Filter just before use (because α-naphthyl propionate breaks down spontaneously to α-naphthol and that reacts with the Fast Red).

2.1.2.2.2 Protein stains

2.1.2.2.2.1 Amido Black

Other proteins were usually stained with 0.01% Amido Black in 5% acetic acid, and if necessary to discern faint bands, or for illustration, the gel was destained with several washes with 10% acetic acid.

2.1.2.2.2.2 Coomassie Blue

When greater sensitivity was required, protein was stained with Coomassie Brilliant Blue R. The recipe is:

Stain

methanol	42 ml
Coomassie Brilliant Blue R	100 mg

Mix well. Then add

water	42 ml
glacial acetic acid	16 ml

Mix well and filter.

Pour over gel, cover, place in oven at 50°C for several hours.

Wash

2-propanol	10%
acetic acid	12.5%

in water.

Change wash several times until background is clear.

2.1.3 Preparation of the Affinity Column

The affinity ligand, procainamide, was coupled to the gel, CH Sepharose 4B (ω-aminohexyl-sepharose 4B, Pharmacia Fine Chemicals, Inc.) with the coupling agent, EDC (1-(3-dimethylaminopropyl)-3-ethyl-carbodiimide hydrochloride). 1 gram of the gel was used. The manufacturer states that the amount of coupled spacer groups in CH Sepharose 4B is 10 - 14 μM/ml of swolen gel, and 1 g of dry gel expands to about 4 ml when wet. Therefore the maximum amount of spacer groups is about 60 μM. The molecular mass of procainamide hydrochloride is 271.8, so 60 μM is about 16 mg. This would be the minimum amount required to occupy all available positions on the gel. 100 mg was used, giving approximately a six-fold excess. The carbodiimide should be in ten to a hundred-fold excess so about 600-6000 μM was required. The molecular weight of EDC is 191.7, so the required amount was in the range of 115 mg to 1.15 g. 1 g was used.

The gel was allowed to expand in 0.5 M sodium chloride, and then washed on a sintered glass filter with the rest of 200 ml of 0.5 M sodium chloride, and then with 50 ml of water. It was then rinsed into a beaker containing the procainamide disolved in a small amount of water. Then the EDC, also disolved in a little water, was added. The pH was adjusted to about 4.6 with hydrochloric acid. The total volume was adjusted to about 50 ml so that the EDC would be at about the recommended concentration of 0.1 M. The reaction mixture was stirred for about 25 minutes while the pH was monitored to see that it remained in the range 4.5-6.0; it drifted up slightly to 4.7. Then it was left on a reciprocating shaker for about 24 hours.

After this, the gel was washed with five washes each of alkaline buffer (0.1 M sodium bicarbonate, 0 5 M sodium chloride, pH 8.3) and acid buffer (0.1 M sodium acetate, pH 4.0) alternately, about 40 ml each time. It was then packed in a column and equilibrated with 0.02 M potassium dihydrogen phosphate, pH 7.0, containing 1 mM EDTA (hereafter referred to as affinity column buffer).

2.1.4 Small-scale Trials

Before attempting purification of serum cholinesterase from large volumes of human serum, trials were done with 1 ml volumes. In the first trial, the serum was diluted to 10 ml with the affinity column buffer (see above) and applied straight to the affinity column in the hopes of bypassing the ion-exchange step. A creamy layer appeared in the top of the gel. The column was rinsed with 25 ml of buffer. The creamy layer in the gel did not move. Then elution of cholinesterase was attempted. 0.4 M NaCl in buffer was used, as Lockridge and La Du (1978) found that the usual enzyme eluted between 0.2 and 0.4 M. Cholinesterase was not detected in twenty 1 ml fractions. It was not found in the initial effluent or washings, so had to be still on the column. 1.5 M NaCl in 0.01 M sodium phosphate, pH 7.4, was then added to the column, and 1ml fractions were collected. The creamy material in the top of the gel immediately started to move. After collecting five fractions it was cleared, and after the seventh, collection was stopped. Polyacrylamide gel electrophoresis showed that there was a trace of cholinesterase in fraction 4, and much more in fractions 5, 6, and 7. Staining for protein showed that the protein also first appeared in fraction 4, but had its greatest amount in that fraction and decreasing amounts in the subsequent fractions to a trace in fraction 7. It was encouraging that the cholinesterase came off the column a little more slowly than most other proteins, but it was clear that the ion exchange step was required.

About 3 ml of DEAE-Sephacel (Pharmacia Fine Chemicals, Inc.) was packed into a small column for tests, and equilibrated with the buffer used by Lockridge *et al.* (1979) and used in the DEAE-Sephacel columns throughout in this work, i.e. 0.02 M sodium acetate with 1 mM EDTA, pH 4.0. 1 ml of serum was diluted to 25 ml with buffer and run into the column. It soon began to run very slowly, as did more buffer added after it. Das and Liddel (1970) dialysed serum repeatedly against the buffer until the pH reached 4.0, and then removed precipitate by centrifuging. Presumably the column had become blocked with protein precipitated by low pH and low ionic strength.

The column was washed out and the gel replaced, and this time 1 ml of serum was again diluted to 25 ml with buffer, but filtered through fine-grade filter paper (Whatman 42) before puting it onto the column. No running problem was encountered this time. 25 ml of plain buffer was passed through the column after the diluted serum, to rinse out proteins that had not bound, and then elution of serum cholinesterase was attempted. 0.06 M NaCl in buffer was used, as Lockridge and La Du (1978) reported that the enzyme eluted between 0.03 and 0.06 M. Twenty 1ml fractions were collected. Some cholinesterase was detected in all the fractions, but nearly all of the activity was in just four fractions (9-12). No protein was detected in any of the fractions after staining the polyacrylamide electrophoresis gels in Amido Black for 24 hrs.

The four fractions containing the bulk of the cholinesterase activity were pooled and dialysed against the affinity column buffer (0.02 M potassium phosphate, 1 mM EDTA, pH 7.0) and run into the affinity column. After rinsing the column with buffer, elution of serum cholinesterase was attempted with 0.4 M NaCl in affinity buffer, as before. No cholinesterase (and no other protein) was detected in twenty 1 ml fractions. Then 0.7 M NaCl in buffer was tried. A peak of cholinesterase (ascending and descending activity) was found in six 1 ml fractions, 5-10, and further fractions were not examined. Some other protein was detected, despite the fact that none had been detected in the DEAE-Sephacel column eluate that was put onto the affinity column. It was nearly all in fraction 5, but a trace was seen in fraction 6.

The cholinesterase thus eluted from the affinity column between 0.4 and 0.7 M NaCl. To narrow this, another 1 ml of serum was processed in the same way, and this time elution from the affinity column with 0.5 M NaCl in buffer was tried. As in the previous run, no protein was detected in the fractions from the ion-exchange column with Amido Black. In case this was due to diffusion of protein whilst waiting for the cholinesterase stain to appear, before staining for protein, duplicate gels were run with fractions collected from the affinity column, and one was stained immediately for protein. The cholinesterase did elute with this salt

concentration, but was spread over many fractions. It first appeared in fraction 5, peaked in fraction 10, and was still present in the last fraction tested, 20. Other protein was detected only in fraction 5, and only in the gel stained immediately for protein, the duplicate, which was stained for cholinesterase overnight, was also stained for protein after about 18 hrs, but showed nothing.

Apparently, 0.4 M NaCl in buffer had not eluted cholinesterase or other proteins from the column, but 0.5 M NaCl in buffer had eluted both. At this stage it was decided to try displacing cholinesterase from the affinity column with choline at a concentration that should leave other proteins on the column.

Another serum sample was processed by ion-exchange chromatography and dialysis as before, and this time, after running the pooled dialysed cholinesterase-containing fractions into the affinity column, the column was rinsed with 0.3 M NaCl in buffer and then elution of cholinesterase was attempted with 0.3 M choline. Twenty 1 ml fractions were collected. Cholinesterase was detected in fractions 9-20, but the stain appeared slowly, and was still not very intense after about three hours. It was therefore decided to try running 0.4 M choline through the column to see whether there was any cholinesterase left on it. Cholinesterase was indeed detected in all of thirty fractions collected. From this, two conclusions were drawn. Firstly, 0.4 M choline should be used directly next time, and secondly, since the cholinesterase stain depends upon enzymatic activity and will eventually detect even minute traces of the enzyme if allowed long enough to do so, gels should be read after a fixed time period to avoid misleading results. One hour was chosen.

When the next serum sample had been loaded onto the test DEAE-Sephacel column, and fractions eluted with 0.06 M NaCl in buffer, the gels were stained for protein after one hour in cholinesterase stain. For the first time after this stage, some protein was detected amongst the cholinesterase-containing fractions. (Cholinesterase appeared in fractions 6-12, mainly in 8-11, and a very little other protein was detected in fraction 9.) After the pooled dialysed

cholinesterase-containing fractions had been loaded onto the affinity column, the said column was rinsed with 0.4 M NaCl in buffer, and then twenty 1ml fractions were collected with 0.4 M choline in buffer. A sharp peak of cholinesterase was found in fractions 3-6. It was suspected that the choline solution had not been completely mixed, so that the first few ml added to the column may have had a higher concentration of choline, which might have accounted for the prompt appearance of the cholinesterase and the lack of tailing. However, the latter could also be accounted for by the limiting of the time of incubation with the stain. No other protein was detected in any of the fractions. 0.5 M NaCl in buffer was then applied to the column and twenty more 1 ml fractions collected to see the protein come off the column, and to check that no cholinesterase had been left on it. There was no cholinesterase, but no other protein was detected either. 20 ml of 1 M NaCl was applied to the column and more fractions collected. again, no protein was detected. The only explanation therefore was that the protein had come off the column with the 0.4 M-NaCl-in-buffer wash that was passed through the column before elution of the cholinesterase with the choline. The effluent had not been saved, as that concentration of salt had not been found previously to move the proteins. At this stage it was felt that the time had come to proceed to a larger-scale trial.

2.1.5 Full-scale Purification

2.1.5.1 Material

Das and Liddell (1970) and Lockridge and La Du (1979) purified their human serum cholinesterase from plasma from time-expired blood donations. The Red Cross Blood Transfusion Service here is able to use all this plasma, so it was not available, but it kindly supplied out-dated bags of concentrated platelets ('platelet-enriched plasma'), which contained an average of about 54 ml each. Each bag contained material from a single donor. The plasma was pooled for cholinesterase extraction, and no attempt was made to distinguish cholinesterase variants before pooling, as the aim was to raise polyclonal antibodies to any human serum cholinesterase.

Fibrinogen and platelets were removed by adding 1 ml of 1 M calcium nitrate per 100 ml of the pooled platelet-enriched plasma and incubating at 37°C until clot had formed, and then at 4°C overnight to shrink the clot. The clot was removed by straining through muslin. Das and Liddell (1970) centrifuged to remove remaining clot, but this was not done in this work, as filtration was going to be necessary after bringing the pH down to 4 for ion-exchange chromatography anyway. The serum was divided into portions and frozen.

2.1.5.2 Ion-exchange chromatography

DEAE-Sephacel was packed in a column of about 2.5 cm internal diameter. The preservative alcohol was run out and the gel rinsed with deionized water and equilibrated with buffer (0.02 M sodium acetate, 1 mM EDTA, pH 4.0). The gel settled to a height of about 47 cm (a volume of about 230 ml). Whilst the alcohol was coming out, it was noted that it running very slowly, so a peristaltic pump was attached. This was always used thereafter. With the pump at the same setting (full speed) the flow-rate was found to vary according to the material passing through the column, but was at best about 22-23 ml/hr.

120 ml of the serum was used for the first trial run of larger-scale purification. 10.9 M sodium acetate, pH 4, was added to bring down the pH. 1.4 ml brought it down from 7.2 to 5.0. A further 5.2 ml was required to bring the pH down to 4.4 and no more was added. The serum was centrifuged at 1500 r.p.m. for 45 min. but most of the precipitated protein remained in suspension. It was decided not to centrifuge again, as more protein would be precipitated on reduction of the ionic strength anyway. The serum was dialysed against the DEAE-Sephacel column buffer. The pH of the last lot of buffer at the end of dialysis was 4.02 but, when the serum was released from the dialysis bags, it was found to have a pH of 4.4. Dialysis had not reduced the pH at all, though it had no doubt reduced the salt concentration. However, it was decided to proceed without further dialysis. The serum was filtered through coarse filter paper (Canlab F2402) followed by fine paper (Whatman 42), and loaded onto the column.

The effluent was collected in tubes with an automatic fraction- collector set to 8 min/tube. The yellow colour of the serum appeared in fraction 26, indicating a bed-volume of around 75 ml. The serum was followed by 100 ml of buffer to flush out non-adherrent proteins, and then elution of cholinesterase was begun with 0.06 M NaCl in buffer. The fraction-collector was reset to start collecting from the time that the elutant started entering the gel. Alternate fractions were checked for the presence of cholinesterase and other proteins from no. 24 onwards. The cholinesterase eventually appeared in fractions 178-208 with most of it in 186-190, the peak at 188. Other proteins were detected in fractions 186-192, also having their peak in no. 188. It was decided that since the cholinesterase took such a long time to elute, future batches would be eluted with molar NaCl in buffer and that any extra protein accumulated by so doing could be separated by puting all the pooled cholinesterase-containing fractions back onto the column at the end and eluting with 0.06 M NaCl then. Fractions 183-195 were pooled and frozen.

In the next run, 250 ml of serum was used. This time glacial acetic acid was used to bring the pH of the serum right down to 4.0. 7 ml was required. Serum that had been kept in a refrigerator for three weeks without freezing, serum that had been frozen and thawed but not acidified, and the serum that had been adjusted to pH 4 were examined qualitatively by PAGE. All showed plenty of cholinesterase activity. The 250 ml of acidified serum was dialysed (without prior centrifugation or filtration) for two days in three 2 L batches of buffer. It was then filtered through coarse and fine paper, as before, and run into the column. It was followed by 100 ml of buffer and then 1 M NaCl in buffer was put onto the column and fractions were collected as before.

This time the cholinesterase appeared much earlier, and spread over less fractions. Alternate fractions were examined and it was detected in nos. 60-76, with the most in 64-70. Muensch *et al.* (1976) and Lockridge and La Du (1978), using sodium chloride gradients, found that cholinesterase eluted in a small protein shoulder preceeding the main protein peak, so it was to be expected that

more protein would be found in the cholinesterase-containing fractions this time. This was indeed the case. The protein was found in more of the fractions (62-72), though cholinesterase was more bunched, and the staining was heavier.

The cholinesterase-containing fractions were again pooled and frozen. It was noticed that they were opalescent, and that opalescence tailed away in the fractions on either side. Inspection of the rest of the fractions revealed opalescence in the early ones too, attenuating, and disappearing at about fraction 40. It was therefore decided to check these fractions for cholinesterase activity along with a sample of effluent collected whilst the serum was passing through the column. This sample was collected just after the last of the serum had passed into the top of the gel, i.e. when there was about 75 ml left to come through. The fraction-collection started 100 ml later, when the last of the buffer entered the gel and the 1 M NaCl in buffer started to enter. The sample of effluent contained just a little cholinesterase, indicating that not all of the enzyme was binding to the gel; there was, as expected, a lot of other protein. In the opalescent fractions there was a little cholinesterase in the first six (and a little other protein in the first five) indicating that a little cholinesterase was being flushed out of the column with the plain buffer.

Because of these findings, it was decided to collect all the effluent in fractions from the time that the serum started entering the gel and to check right through for cholinesterase. 250 ml was used again. The first 22 fractions of effluent were opalescent. Fractions 23-29 were full of white precipitate despite the facts that the serum had been filtered after dialysis and the column had been washed since the previous run. The next two fractions were opalescent/yellow, and following ones were clear yellow. No cholinesterase was detected in the fractions showing opalescence or precipitate. The enzyme started to appear somewhere between fractions 38 and 48, i.e. after about 110-140 ml of effluent had come out of the column after the serum started entering the top. A small amount of cholinesterase was detected in all the rest of the effluent from the serum. It stopped coming off the column within the first 50 ml of the buffer rinse,

as before. With 5 μl samples for PAGE there was only faint staining throughout. It did not increase to the intensity of stain seen with samples of serum, so it was deduced that most of the enzyme was binding throughout the time that serum was flowing through the column and that therefore the amount of serum loaded did not need to be decreased.

The cholinesterase peak, eluted with 1 M NaCl in buffer, was restricted to even fewer fractions than in the previous run. It was decided to dialyse the next batch of serum against buffer without adjusting the pH first. 250 ml was used again. As in the previous run, the effluent was collected in fractions from the time that the serum started entering the gel. The appearance of the effluent fractions was similar to that seen in the previous run. The opalescence extended from fraction 2 up to about fraction 45, the yellow colour appearing in fraction 33, immediately following a tube of white precipitate. Cholinesterase was first detected in fraction 67. It was detected throughout the effluent from there on, but only on staining for about two hours, and the staining was only faint. This time, gels were also stained for protein. The protein appeared somewhere between fractions 30 and 40. Presumably, the material causing opalescence and frank precipitate in the early part of the effluent was aggregated protein that had passed through the filter paper but would not enter the polyacrylamide gel. Its appearance in the effluent very soon after puting the serum onto the column and well before the yellow colour (albumin bound to bilirubin) would be explained by its inability to enter the DEAE-Sephacel beads . The eluted cholinesterase peak seemed to contain more this time in that more fractions showed heavy staining on PAGE. Fractions beyond the cholinesterase - and completely overlapped narrower peak of other proteins - were examined for the presence of another protein peak beyond. No such peak was found, confirming that all the proteins remaining on the column after rinsing with the (pH 4.0) buffer were lifted off with the cholinesterase by 1 M NaCl in buffer.

In future, all serum was dialysed against buffer directly, without adjusting the pH first. In the next run 500 ml was tried. After dialysis it was filtered through

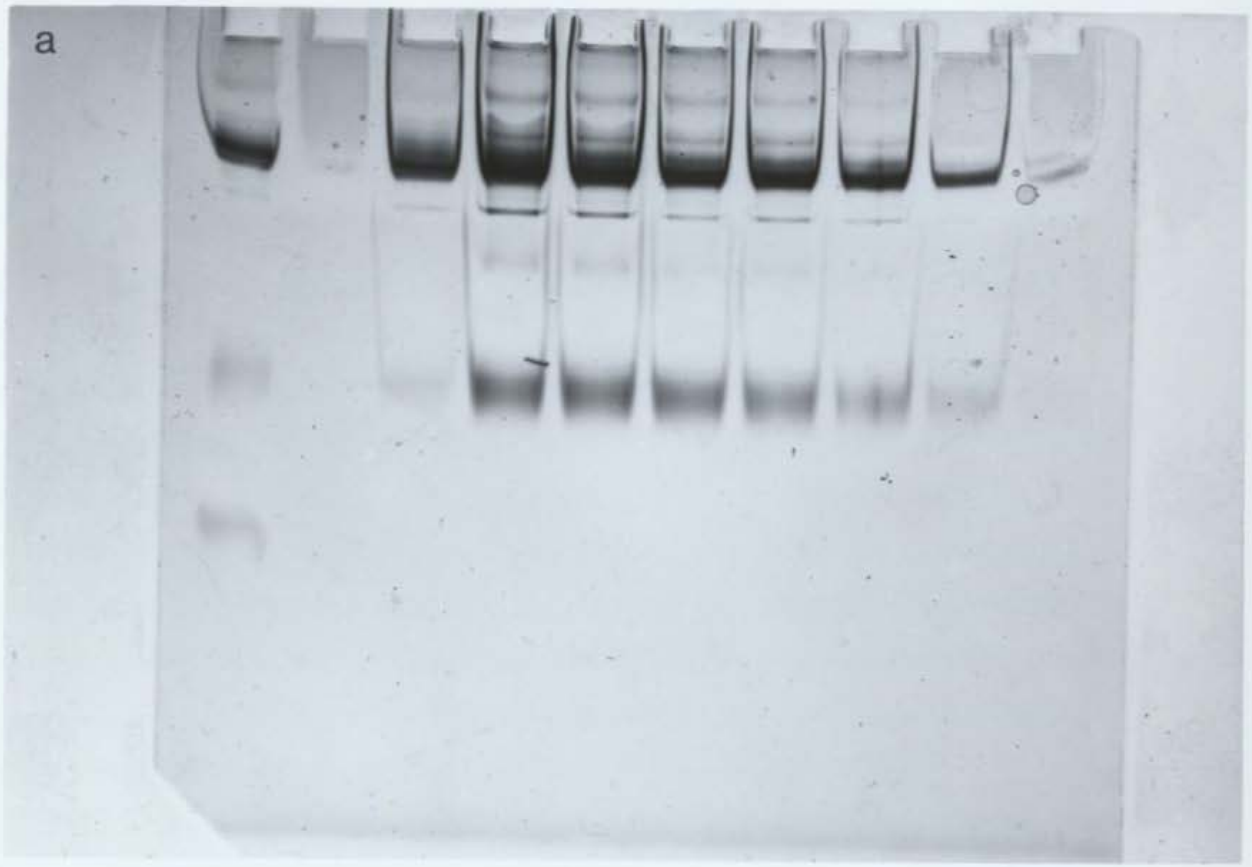
coarse filter-paper, laid on scintered glass and layered with diatomaceous earth, using low vacuum. The effluent from the column was again checked for evidence of cholinesterase overflow. The intensity of cholinesterase staining was compared with that from serum on the same gel. After the usual blank volume, a slight trickle of the enzyme was detected throughout the effluent, as before. It did not increase in concentration towards that of the serum, so the capacity of the column did not seem to have been exceeded and a further three batches of 500 ml of serum each were processed.

As was mentioned earlier, opalescence was noted not only in the early part of the effluent when running the serum into the column, but in a portion of the eluate coinciding with the cholinesterase peak. This proved to be useful in identifying which fractions to examine to find the cholinesterase. With 500 ml batches of serum, the pattern was very clear-cut. A clear fraction would be followed by the most densely opalescent one, and in following fractions the opalescence would gradually fade away. The first fraction staining heavily for cholinesterase was always either the one with maximum opalescence or the next one, though the one showing the most cholinesterase staining would be a few tubes later. The detection of cholinesterase and other proteins in the eluted fractions is illustrated in Figs 1a and 1b, in which duplicate gels have been stained for cholinesterase and protein respectively. The first lane was loaded with 5 µl of serum and the others with 5 µl each of representative fractions across the cholinesterase peak. It is clear that the peak fractions contain more cholinesterase and much less protein than the serum.

After the fourth batch of 500 ml of serum had been processed, the cholinesterase-containing fractions saved from all runs were pooled. Some white material was noticed on the bottom of the bottle containing the fractions from the very first run. In case this was DEAE-Sephacel beads to which cholinesterase would bind when the salinity was decreased, the whole pool was filtered through coarse filter paper. It was then dialysed against the DEAE-Sephacel column buffer to remove the 1 M NaCl. Despite the fact that all this material had been

Fig. 1. Polyacrylamide gel electrophoresis of serum and cholinesterase-containing fractions eluted from the DEAE-Sephacel column

The figure shows duplicate gels: (a) stained for serum cholinesterase with α-naphthylpropionate/Fast Red, (b) stained for protein with Coomassie Brilliant Blue R. The lane on the far left contains 5 μL of human serum and the others contain 5 μL each of fractions 68, 69, 72, 75, 78, 81, 84, 87 and 90 counting from the entry of the eluting 1 M NaCl into the top of the column. The fractions were collected over 8 min each at a flow-rate of about 22-23 ml/hr. In (a) the fastest band in the serum lane is albumin, which shows slight esterase activity with α-naphthylpropionate. The other bands in (a) are all serum cholinesterase. (See Section 1.5, p. 11) for a disccussion of the multiple bands of serum cholinesterase.) The sharp leading edge to the serum cholinesterase peak is illustrated by the difference between the adjacent fractions 68 and 69. Fraction 72 contains much more cholinesterase and there is a long tail.


a


b

dialysed against this buffer before (when it was serum) more precipitation occured in the dialysis bags this time. The solution was therefore filtered again (through Whatman No. 2 filter paper) before being applied to the column again. In the meantime, of course, the 1 M NaCl had been washed out of the column with buffer. The cholinesterase solution was followed with more buffer, and then elution with 0.06 M NaCl in buffer was begun. The flow-rate, measured when the solution was going into the column, was 22.54 ml/hr. All the effluent was kept and tested in case of column overflow, but no cholinesterase was detected, despite the fact that there had been a steady small leak of cholinesterase when serum was being loaded onto the column. During elution, 12-minute fractions were collected, starting at No. 1 again from the time that the 0.06 M NaCl started entering the column. The cholinesterase appeared in fraction 109, was most abundant in fractions 112-121 and diminished in a long tail. Fraction 193 was the last in which the enzyme could be detected within one hour. Testing every third tube, protein staining with Amido Black was only seen in fractions 112 and 115, which showed lightly-staining bands of low molecular weight. After approximately 918 ml of 0.06 M NaCl in buffer had passed into the gel and no second peak of protein had been detected, 1 M NaCl in buffer was run through. No more protein was found. Fractions 109-163 were pooled and dialysed against affinity column buffer.

2.1.5.3 Affinity chromatography

The cholinesterase solution (about 250 ml) was loaded onto the affinity column and all the effluent was saved in case of overflow. As all the effluent was collected in one beaker, which would lead to dilution of any cholinesterase that might overflow from the column towards the end of loading, 5, 25 and 50 µl volumes were examined (by PAGE and staining as usual) and the staining with the larger volumes at one hour was considered sufficient to warrant keeping the effluent to pass through the column again.

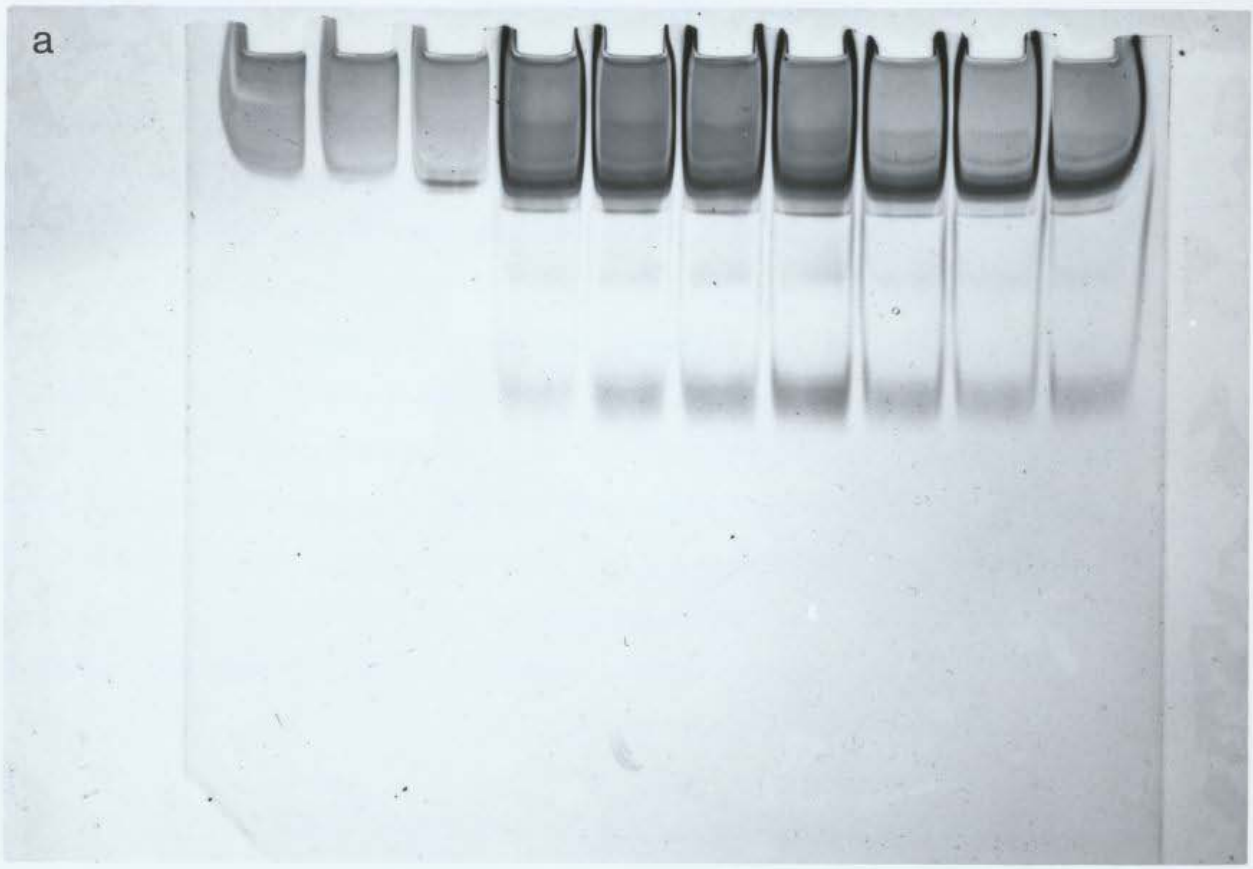
Twenty 1 ml volumes of 0.4 M NaCl in affinity column buffer were put onto

the column and twenty 1 ml fractions collected, the expectation from the small-scale trials being that this would elute other proteins but leave the cholinesterase on the column. Then twenty-five 1ml volumes of 0.4 M choline were put onto the column to elute the cholinesterase and an equal number of fractions collected. Cholinesterase was present in all fourty-five fractions. Disappointingly, much of the enzyme eluted with the sodium chloride - the 7th to 20th fractions showing staining in only three minutes. Other proteins were indeed eluted. They were seen in the 4th to 8th fractions only, with only a trace in the 8th. See Fig. 2. In the fractions collected after changing the elutant to choline, cholinesterase decreased in the first three, then increased again, being greatest in the next two fractions, and then tailed down again. Slight protein staining was seen in the 4th to 6th of these but the position of the staining suggested that this was cholinesterase. The 4th to 8th fractions eluted with NaCl were put aside for further treatment and all of the other fourty fractions were pooled. Then the choline was rinsed out of the column with 10 ml of buffer and ten more 1 ml fractions were collected. Cholinesterase was detected in the first five of these and they were added to the pool.

The effluent containing cholinesterase was then passed through the column again. Again all the effluent was collected, but no overflow was detected. This time a more cautious approach was adopted in elution. As before, fractions were collected after puting each 1 ml of elutant onto the column. First 20 ml of 0.1 M NaCl in buffer were passed through, then 20 ml of 0.2 M and then 20 ml of 0.3 M NaCl in buffer. In fractions 1-20, a very little cholinesterase was detected in 4 and 5, but other proteins were found in fractions 4-7. In fractions 21-40, there was a little cholinesterase in 25-27, but other protein in 24-27, though not nearly as much as in the previous peak. In the next twenty, alternate fractions were sampled and cholinesterase was seen in fractions 46-58. There was more than in the previous two little peaks, though still not very much, but no other protein was detected. Another 20ml of 0.3M Nacl in buffer was passed through the column but no more cholinesterase or other protein was found.

Fig. 2. PAGE of the first 10 fractions eluted from the affinity column with 0.4 M NaCl

The figure shows duplicate gels: (a) stained for *serum cholinesterase* with α-naphthylpropionate/Fast Red, (b) stained for protein with Amido Black. The fractions were of 1 ml each. Each lane was loaded with 5 μL.


a


b

Then fractions were collected with 20 ml of 0.3 M choline in buffer. Alternate fractions were sampled. Cholinesterase staining appeared in fractions 86-92 within nine minutes, and in all fractions sampled by one hour. No other protein was detected in any of the fractions sampled. Rather than collecting a tail of cholinesterase with this concentration of choline, with diminishing returns, twenty fractions (101-120) were collected with 0.4 M choline in buffer. Sampling of alternate fractions showed cholinesterase staining in 106 and 108 at 4.5 minutes and in all fractions by one hour, with a peak in 106. Fractions 81-120 inclusive were added to the cholinesterase pool and fractions 1-80 were discarded. The column was then rinsed with 4ml of affinity column buffer and the effluent run straight into the pool. It was then rinsed with a further 25 ml of buffer to flush out any remaining choline. The effluent was checked for cholinesterase and found negative. The column was then ready for reprocessing the fractions saved earlier.

The five cholinesterase-bearing fractions from the first pass not put into the pool contained the most cholinesterase but also the most protein. They had been eluted with 0.4 M NaCl (in 0.02 M buffer) so the 5 ml volume was made up to 100 ml with water to reduce the molarity to approximately 0.02 M, and then passed back through the column. 50 and 100 µl portions of the effluent were inspected by PAGE. Traces of protein were detected, but no cholinesterase. Twenty 1ml fractions were collected from the column with 0.2 M NaCl in buffer, to elute the other proteins, and then twenty 1 ml fractions were collected with 0.4 M choline to elute the cholinesterase. In the first twenty fractions proteins were detected with amido black in 4-7, but cholinesterase was also found, starting in fraction 3, coming to a peak, visible within ten minutes, in 5 and tailing away by 17, in which none was detectable at one hour. In the second twenty fractions alternate ones were sampled and cholinesterase staining appeared in 24 and 26 in 3 minutes and in all fractions tested by 10 minutes. There was enough cholinesterase in fraction 24 for it to be detectable with amido black, but no other protein was found in these fractions. Fractions 3, 8-11 and 21-40 were added to the cholinesterase pool.

The cholinesterase solution (114 pooled fractions) was first dialysed against 10mM ammonium bicarbonate. The idea behind this was that the solution could then be freeze-dried and that the ammonium bicarbonate would evaporate (as ammonia, carbon dioxide and water) leaving the cholinesterase to be redisolved in a much smaller volume. However, as the frozen solution became more concentrated, it melted. The aim of purification was of course to ensure that when the solution was used to immunize a rabbit the overwhelming majority of anti-human antibodies were against cholinesterase rather than contaminating proteins. The concentration achieved by the freeze drying could have elevated any contaminants from undetectable to detectable concentrations. A small drop of the concentrated solution was taken and serial one-in-ten dilutions were made with electrophoresis buffer and examined by PAGE and staining with amido black, the idea being to estimate the number of orders of magnitude that the cholinesterase was more concentrated than any contaminants. However, a single cholinesterase band was seen in the first lane and no bands were seen at any of the dilutions. (A duplicate gel was stained for cholinesterase activity. The stain appeared in the first two lanes within 4 min., and in decreasing intensity down to 10^{-7} by 1 hr.)

The solution was then dialysed against 'normal saline' (0.9% NaCl). In the meantime a gel was prepared to illustrate the single cholinesterase band in the solution of purified enzyme beside the multiple bands of untreated serum. When it was destained to remove background stain, it became apparent that there was another faint band, indicating a low molecular weight contaminant in the cholinesterase solution. See Fig. 3.

2.1.5.4 Gel filtration

The cholinesterase in normal saline solution was freeze-dried again, and redisolved in a small volume of deionised water for application to a gel-filtration column. A Sephadex G75 column, 16 × 1 cm was equilibrated with 200 ml of 0.9% NaCl. The cholinesterase solution was then applied to the column and

Fig. 3. PAGE of partially purified cholinesterase after affinity chromatography compared with untreated serum

Left, human serum; right, partially purified serum cholinesterase. The gel is stained for protein with Amido Black.

eluted with further 0.9% NaCl. On electrophoresis, no protein was detected apart from the cholinesterase in the cholinesterase-containing fractions, and they were pooled.

2.2 Estimation of the concentration of the purified enzyme

This was done in two ways. One was the Bio-Rad Protein Assay Kit No. 1 'standard assay procedure' (Bio-Rad Laboratories (Canada) Ltd.). This assay is based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm when binding to protein occurs. The other method was the absorbance method which was used by Lockridge and La Du (1978) for their purified cholinesterase. In this the protein concentration is calculated from the absorbance at 280 nm using an extinction coefficient of 1.8 cm^{-1} for a 1 mg/ml solution. Presumably by sheer chance the results by these two methods were the same in the first three places of decimals: 1.072 mg/L. The spectrophotometer used was a Philips Pye Unicam SP6-550. With the more advanced Beckman Du-70 spectrophotometer used later to look at the UV spectrum of the protein (see Chapter 3, p. 96) the result was 0.988 mg/ml. As neither method warrants this kind of accuracy, the round figure of 1 mg/ml was adopted.

2.3 Raising antiserum

Antiserum was raised in a 4 Kg female New Zealand white rabbit. The initial dose was 1 mg of the purified human serum cholinesterase in 1 ml 0.9% NaCl in an emulsion with 1.25 ml Freund's complete adjuvant administered in two i.m. gluteal injections. A booster of 1 mg cholinesterase in alum suspension was given i.v. after 16 weeks and a further booster of 1 mg cholinesterase in an emulsion with 1 ml Freund's incomplete adjuvant in 2 i.m. gluteal injections after a further 7 weeks. The antiserum used in this work was taken 5 weeks after the second booster.

2.3.1 Preparation of antigen emulsions

The emulsions of the cholinesterase solution with Freund's adjuvant (complete or incomplete) were formed by forcing the two liquids to and fro between two glass syringes though a narrow connector. In order to produce a water-in-oil emulsion it is necessary to add a volume of the aqueous antigen solution equal to about half the volume of the oily Freund's adjuvant at first, only adding more antigen solution after thorough mixing, and then in at least two portions. In all, the liquid has to be passed though the connector several hundred times. The formation of a water-in-oil emulsion was tested by putting a small drop of the emulsion onto the surface of water in a beaker. If a water-in-oil emulsion has been formed the drop will remain discrete, but if emulsification has not been successful (i.e. an oil-in-water emulsion has formed) the drop will rapidly spread. If all the required antigen solution had been added and a water-in-oil emulsion had not formed after prolonged mixing, a little more of the Freund's adjuvant was added.

2.3.2 Preparation and administration of antigen-in-alum suspension

The suspension was prepared as follows. 1 ml of the 1 mg/ml serum cholinesterase solution was mixed with 2 ml of 0.5 M sodium bicarbonate and then 1 ml of 10% aluminium hydroxide was added drop-by-drop with constant mixing. The mixture was then left at 4°C overnight and formed a gel. The gel was washed three times with phosphate-buffered saline (PBS: 0.8% NaCl, 0.05 M Na_2HPO_4), 5-10 ml each time, by mixing, centrifuging and removing supernatant. The gel was resuspended in about 1 ml of PBS.

Before i.v. administration of the suspension, prophylaxis against anaphylactic shock was given. The antihistamine tripelennamine hydrochloride ('Vetastim') was used at a dose of 1 mg/Kg i.v.

2.3.3 Use of Ouchterlony gels to monitor antibody titre

Ouchterlony gels were used to monitor antibody titres. These were composed of 1% agar in PBS poured onto a glass plate. (15 ml was used on a plate 1.25 inches square.) The plate fitted into a device which enabled wells to be bored at regular intervals in a honeycomb pattern. Antiserum could thus be compared with up to six dilutions of human serum in surrounding wells and different batches of antisera, each with surrounding wells of diluted human serum, could be compared on the same gel. The same volume of antiserum or diluted human serum was used in each well. The gels were kept in moist air in a closed container and incubated at room temperature for up to four days. Between any pair of wells, the higher the antibody titre in the antiserum the nearer would the precipitin line be to the serum well, and the sooner would the line become visible. After the second booster, the titre continued to rise for the first four weeks. The titre at five weeks was slightly less than that at four weeks (though greater than that at three weeks) and the rabbit was exsanguinated the following day.

2.4 Radial immunodiffusion

In this technique gels are made which contain antiserum. Solution containing antigen is placed in wells in the gel and antigen diffuses out until it is all immobilized by immunoprecipitation. At this time the area of the immunoprecipitin ring is proportional to the amount of antigen in the innoculum (Mancini *et al.*, 1965). Agar must be brought to the boil to melt it but the temperature would denature the antibodies so the hot gel has to be cooled to a temperature at which its solution is still liquid but which will not denature the antiserum.

2.4.1 Method of making gels

Agar was used for the first radial immunodiffusion gels but, while working on the technique, agarose was tried and found to be easier to handle, disolving much more readily, and was always used thereafter. The method used was as

follows. 2% agarose was made up in 0.15 M NaCl containing 1% sodium azide and disolved by bringing to the boil in a microwave oven. Enough agarose for many gels could be made up in a conical flask and stored in a refrigerator, melting in a microwave oven on subsequent occasions. A beaker was tared on a balance with a sheet of polystyrene foam on the pan for heat insulation. The appropriate amount of 2% agarose for the number of gels to be poured was then weighed into the beaker (plus a little extra because some would remain on the sides of the beaker - 4% extra was found to be enough), covered, and left to cool in a 54°C waterbath. (A plastic Petri dish served well as a beaker lid.) A second, slightly heavier beaker was then weighed without changing the taring of the balance. This was used to contain a solution of antiserum of twice the concentration required in the gel and exactly the same volume as the weighed 2% agarose, including the extra 4%. The solution was made with the same 0.5 M NaCl, 1% NaN_3 as for the agarose. As the bottle of this saline solution was kept refrigerated a little less than the required amount was weighed out, warmed slightly in the microwave oven, and made back to the same weight with drops of deionized water from a Pasteur pipette to replace evaporation. The appropriate amount of antiserum was then added and the solution made up to the required volume with the saline from a Pasteur pipette. This beaker was then covered also and placed in the same waterbath to warm.

After half an hour the beaker containing the antiserum solution was lifted from the waterbath, its outside dried, and its contents poured into the other beaker and the solutions were mixed with a spatula warmed by running hot tap-water. Condensed water on the sides of the beaker was returned to the mixture as much as was practical. Then the beaker containing the mixture was removed from the bath, its outside was dried, it was placed on the balance and the evaporative loss made up with deionized water. It was then rapidly returned to the bath, stirred and covered again. The mixture now contained 1% agarose and the required concentration of antiserum. The balance, still bearing the sheet of polystyrene foam was now tared to the weight of a plastic Petri dish. The gels

were then poured. The dishes were $3^{7}/_{16}$ inches (8.75 cm) internal diameter and 8 g of gel was weighed into each dish. The dishes were pre-warmed in a 37°C oven. The procedure for each was as follows. The beaker of gel was removed from the bath and its outside dried to prevent bath-water from running into the dish. The gel was weighed into the dish and the beaker covered again and returned to the bath. Then the dish was removed from the balance, tilted in a rotary manner to spread the gel evenly, and placed on a table to cool, partially covered by its lid. The next dish was then taken from the oven to the balance and the tare adjusted.

When the gels had set, wells were bored. If the wells were to be bored the same day, the gels were left in a refrigerator for at least half an hour as the gel was soft at first and tended to tear. If kept overnight, they were stored in a moist atmosphere to prevent drying (a screw-topped jar with wet paper at the bottom). 19 wells of 3 mm diameter were cut in a hexagonal array as in Fig. 7 (p. 105, legend not relevant yet) with 1.5 cm between the centres of the wells. The wells were bored manually, with the layout of the wells on a piece of paper under the dish as a template, and the cores were removed by connecting a syringe to the end of the borer.

2.4.2 Dispensing samples

A Ziptrol 'delivery system' (Drummond Scientific Company, Broomhall, Pensylvania) was used for loading the wells. Consisting of a glass capillary tube, a wire piston and a scale (1-5 µL), this device is very simple and very accurate. It has the advantage over diposable-tip micropipettes of having direct contact between liquid and piston, and the advantage over the Hamilton syringe of having no dead-space. Before dispensing each sample the capillary tube was wiped to remove drops on the outside. After dispensing it was wiped again before inserting into the next serum sample. It was then rinsed with the new sample three times to remove any traces of the previous sample on the inside surface of the tube.

2.4.3 Measurement of ring area

Mancini *et al.* (1965), the authorities on radial immunodiffusion, projected the magnified silhouettes of the precipitate rings onto cardboard paper, drew round them with a pencil, cut them out and weighed them. They said that this was better than merely recording diameters because the precipitates may depart from ideal circular shape. It is however very time-consuming and impractical if many measurements are made. In this study the diameters of the precipitates were measured in two directions at right angles and the mean taken. The measurements, in tenths of a millimetre, were made with a 'calibrating viewer' (Transidyne General Corporation, Ann Arbor, Michigan). To calculate the 'area' the mean diameter was squared and the square of the diameter of the well (i.e. 900 square tenths of a millimetre) subtracted. Division of the diameter by 2 before squaring, and multiplication by π, were unnecessary as they apply to all measurements equally. The reason for subtracting the (constant) area of the well is given in Section 4.2.1, p. 136.

2.4.4 Other details

The concentration of antiserum in the gels, time and temperature of incubation, and time of staining are given in Chapter 3 after description of preliminary experiments. Sample volume, calculation of concentration of cholinesterase, control and error estimates are discussed in Chapter 4.

2.5 Immunoprecipitation

Immunoprecipitation of protein in human serum with the rabbit anti-human-serum-cholinesterase antiserum was carried out essentially by the method described in Davis *et al.* (1986). The procedure was as follows. In each of two tubes 5 µl of human serum were mixed with 10 µl of preimmune rabbit serum and 35 µl of immunoprecipitation buffer (see below), the tubes were centrifuged to bring the mixtures back to the bottoms of the tubes, and incubated for ten minutes at room temperature. Then 20 µl of an approximately 50/50 v/v suspension of protein A-Sepharose 'beads' (Pharmacia Fine Chemicals, Inc.) in

RIPA buffer (Perbal, 1984) was added to each tube to remove any resultant nonspecific immunoprecipitates and the tubes were incubated for 30 min at 4°C on a slowly-turning rotor to invert them gently. Then they were centrifuged and the supernatants removed to fresh tubes. This step was repeated. To one tube 40 µl of rabbit antiserum was then added and to the other a further 40 µl of preimmune rabbit serum and the contents were mixed, spun down, and incubated overnight at 4°C. For removal of the immunoprecipitates after incubation a further 20 µl of the protein A-Sepharose suspension was added to each tube and they were again incubated on a rotor at 4°C for 30 min. Before electrophoresis the supernatants were removed from the protein A-Sepharose beads and saved and the beads were washed 7 times, with 250-500 µl of immunoprecipitation buffer each time, before being resuspended in sample buffer (see next section) like all the other samples.

The constitution of the immunoprecipitation buffer was 5 mM tris(hydroxymethyl)aminomethane (hereafter referred to as 'tris'), pH 7.5, 0.15 M NaCl, 1% Nonidet P-40, 2 mM EDTA. 'Nonidet P-40' is a non-ionic detergent, used to stop protein aggregation which could cause inappropriate precipitation. The EDTA is included to chelate divalent cations.

2.6 Sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

The method of Laemmli (1970) was used with a vertical slab-gel apparatus (gel cross-section 1.5 × 140 mm). The acrylamide concentration was 3% (w/v) in the stacking gel and 10% (w/v) in the separating gel. The sample buffer contained 2% SDS, 5 % 2-mercaptoethanol, 10% glycerol, 50 mM Tris-HCl, pH 6.8 and 0.1% bromophenol blue at final volume. The samples were heated to 100°C for 4 min before electrophoresis at 20 V overnight. The molecular mass markers used were those of the Pharmacia High Molecular Weight Calibration Kit. The other solutions used were as follows:

Acrylamide solution: see 2.1.2.1, p. 62

'Lower tris (×4)': 1.5 M Tris-HCl, pH 8.8 + 0.4% SDS

'Upper tris (×4)': 0.5 M Tris-HCl, pH 6.8 + 0.4% SDS

***Tris-glycine reservoir buffer* (×4)**: 12 g tris base + 57.6 g glycine made up to 1 L with deionised water. 0.1% SDS is included when diluting to ×1.

Ammonium persulphate solution: 10% w/v, made up immediately before use

The gels were made up as follows:

Lower gel

Mix 13.3 ml acrylamide solution

5.0 ml lower tris (×4)

2.8 ml water.

'Degas' the solution under vacuum.

Add 5 μL TEMED.

Make up 10% ammonium persulphate and add 0.6 ml.

Mix. Pour gel. Layer isobutanol on top of gel while preparing upper gel. (N.B. The isobutanol is stored with water. The isobutanol is the top layer.)

Upper gel

Mix 1.5 ml acrylamide solution

1.87 ml upper tris (×4)

100 μL 0.1% bromophenol blue

2.82 ml water.

Degas. When lower gel has set, remove isobutanol.

Add 7.5 μL TEMED

112 μL 10% ammonium persulphate.

Mix. Pour gel. Insert comb.

A 20-toothed comb was used. The lanes were loaded palindromically, so that one half was the mirror image of the other (for contents of the lanes, see Section 3.4.1, p. 107). After electrophoresis the gel was cut in half using the teeth of the upper gel as a marker. Then the upper gel was removed. The left half of the lower gel was then stained with Coomassie blue for 7 min and destained in three lots of 10% acetic acid, 20% methanol over about 20 hrs. During staining of the left half, the right half was bathed in transfer buffer (see below) prior to western blotting.

2.7 Western blotting

Western blotting (Burnette, 1981) was carried out using nitrocellulose filter (Schleicher & Schuell, Inc., Keene, NH 03431, USA) and a current of 150 mA for about 18 hrs. 10% goat serum in PBS was used for blocking, and goat-anti-rabbit-Ig antibodies linked to horse-raddish peroxidase (Jackson ImmunoResearch Laboratories, Inc, West Grove, PA 19310, USA) as the indicator system. The protein marker lane on the blot was cut off and stained with amido black before the rest of the filter was exposed to antibodies. R_f values were calculated for the marker bands from the blot and the gel and averaged to plot a calibration curve from which molecular masses of proteins on gel and blot were estimated. Further details were as follows:

Transfer buffer: dilute 1 L of tris-glycine reservoir buffer (×4) as above to 4 L but add enough SDS solution (e.g. 10 ml of 5%) to make final (5 L) volume only 0.01%. Add 1 L methanol.

After transfer (blotting) the gel was stained with Coomassie blue for 2 min and destained to check that transfer had been complete. No protein was left in the gel except a little at the origin in some lanes. After blocking the filter for 1 hr (in a bag containing 10 ml of the 10% goat serum, on a rotor, at room temperature), 50 μL of the rabbit anti-human-serum-cholinesterase antiserum was added to the bag

and incubated for 1 hr (on the rotor, at room temperature). The filter was then given 4 ten-minute washes, the first, second and fourth of 100 ml PBS each, the third of 100 ml triethanolamine buffer (50 mM triethanolamine-HCl pH 7.4, 100 mM NaCl, 2 mM EDTA, 0.5% Triton X-100, 0.1% SDS, Ottaviano and Gerace (1984), which is more stringent). It was then blocked again with the 10% goat serum, incubated with the secondary antibody solution (above), 20 μL in the 10% goat serum, and washed again before staining for peroxidase thus: weigh 24 mg of 1-chloro-4-methyl naphthol into a tube; add 8 ml methanol; pour into 40 ml PBS; add 20 μL of H_2O_2 and pour onto filter immediately.

2.8 Subjects

The Janeway Hospital Chemistry Laboratory receives blood samples for estimation of serum lipid profiles from a large area and has for a number of years measured serum cholinesterase activity of these samples routinely. The staff kindly saved samples for this study after these estimations had been made and checked. 282 of these patients were included in the study and their ages ranged from 8 to 86 years. Further description will be given in Chapter 5.

For comparison, 117 blood donor samples were also assayed. When blood is donated, the collecting-bag contains anticoagulent but at the end of collection the tube is clamped and blood is milked from the tube into a container without anticoagulent and tested for evidence of HIV, hepatitis and syphylis. These samples were released for use in this study immediately after testing. The ages of the individual donors were not available, but the limits for donors are 17 - 70 years (up to the 71st birthday).

Pooled serum from another 56 blood donors was used as a secondary standard in estimation of serum cholinesterase concentration.

2.9 Statistics

The calculations of statistics and displays of data used in interpretation were made with the use of the computer software 'Minitab', VAX/VMS version (various editions, Minitab Inc.).

Chapter 3

RESULTS (1)

Cholinesterase impurities, antiserum specificities and immunological variation of serum cholinesterase

3.1 Initial inspection of the purified serum cholinesterase

On PAGE of the product of the last stage of purification, only one protein band was seen (Fig. 4). The ultraviolet absorbance spectrum was then examined with a Beckman DU-70 spectrophotometer (Fig. 5). There was a peak at 275.6 nm, a trough at 251.1 nm, and a small shoulder at 289.5 nm. This result was very similar though not identical to that obtained by Lockridge and La Du (1978). They reported a peak at 280 nm, a trough at 254 nm and a small shoulder at 290 nm.

3.2 Initial trials of the antiserum for serum cholinesterase concentration measurement

3.2.1 Immunoturbidity

It was originally hoped to use the antiserum to measure serum cholinesterase concentration by immunoturbidity with the IL Monarch autoanalyser with which cholinesterase activity and serum lipids were measured (for details see Chapter 4, p. 128). There are kits for measuring the concentrations of several other proteins on this machine and the manual gives details of how to set up new tests. An initial attempt was made to develop such a method for serum cholinesterase. The details will not be given here because the method was not subsequently used, but the attempt was important because it led to an investigation of the purity of the purified cholinesterase and the specificity of the antiserum. Very poor correlation was found between the immunoturbidity and activity measurements of serum samples and after carefully examining the

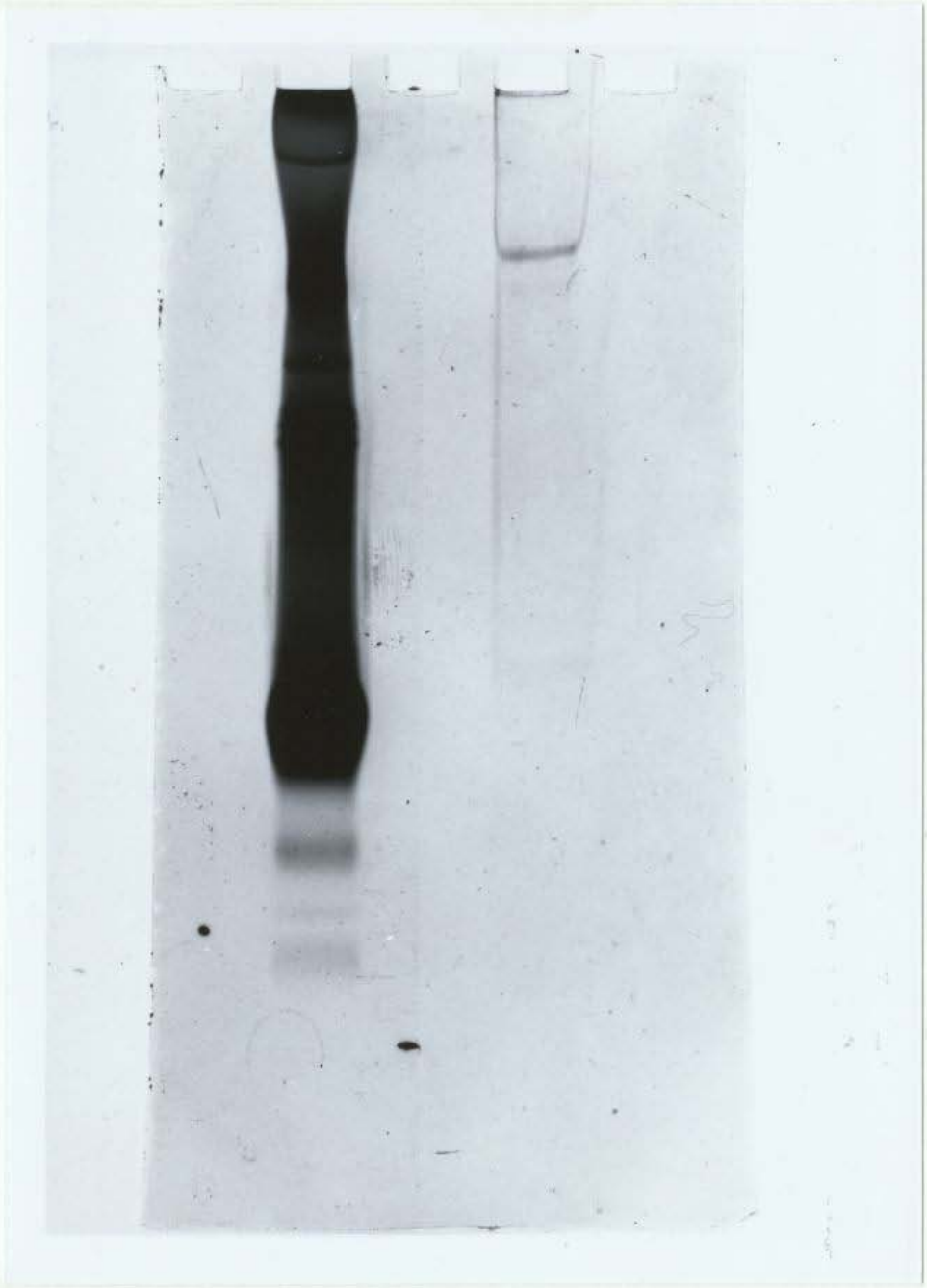


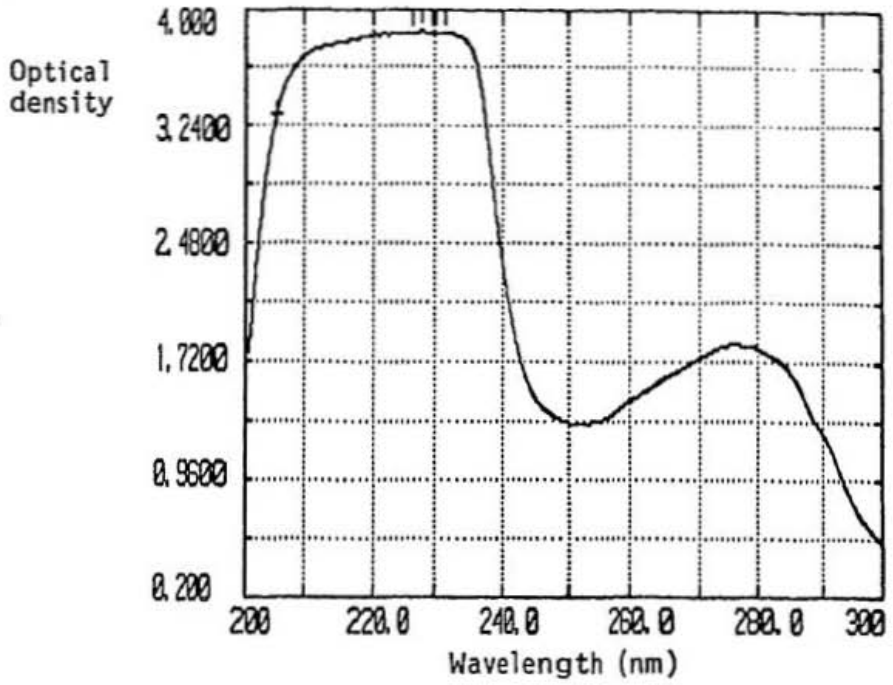
Fig. 4. PAGE of the serum cholinesterase after gel filtration compared with human serum

Left, 10 μL of a 50/50 mixture of human serum and 20% PVP; right, 5 μg of the purified human serum cholinesterase (5 μL, 1 mg/ml) after gel filtration. Both lanes are overloaded to improve detection of faint bands. The stain is Coomassie Brilliant Blue R.

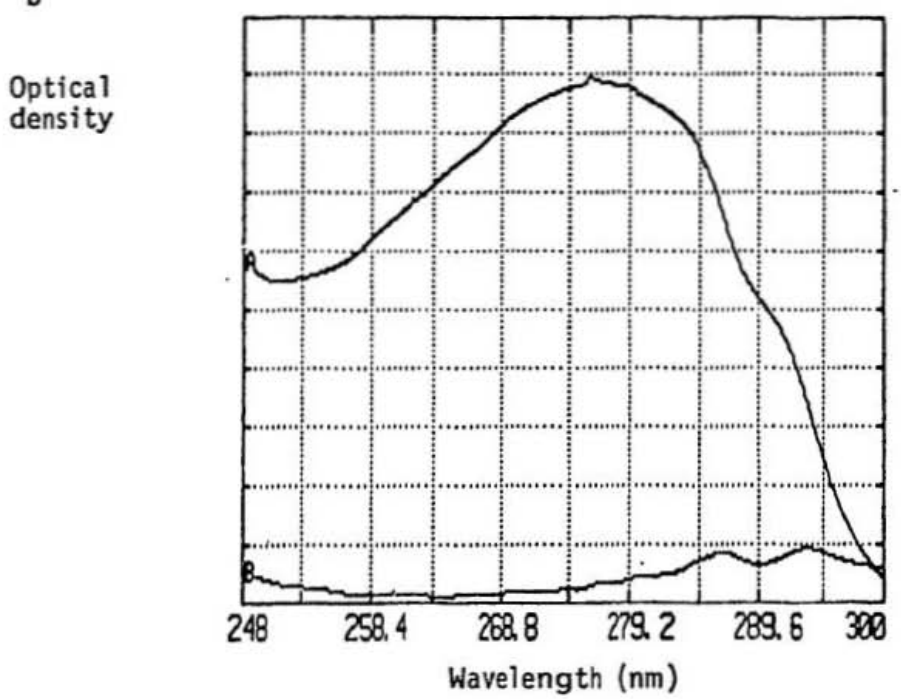
Fig. 5. Ultraviolet absorption spectrum of the purified serum cholinesterase

Panel a shows the spectrum from 200 to 300 nm. The large peak on the left is due to absorption by the peptide bonds; the top is truncated. The peak on the right is due mainly to aromatic rings and is generally more useful in distinguishing different proteins. Panel b shows the second peak on a larger scale (line A) and the first differential (line B). The vertical scale runs from 0.500 to 2.000 for A. For some reason not explained in the manual of the spectrophotometer, this instrument plots the differential in reversed sense, i.e. upside down, and zero for that curve does not correspond to one of the lines on the grid. However, the shape of the curve is otherwise correct and serves to define the position and single nature of the small shoulder.

a


Optical density
4.800
3.2400
2.4800
1.7200
0.9600
0.200
200
220.0
240.0
260.0
280.0
300
Wavelength (nm)

b


Optical density
248
258.4
268.8
279.2
289.6
300
Wavelength (nm)

method for other sources of error it was decided to check the antiserum by trying another method.

3.2.2 Radial immunodiffusion

The antiserum taken five weeks after the final booster was chosen for the initial trial as it would have a higher antibody titre than the serum from the final bleed and there was more of it than the slightly higher titre serum collected the previous week. This antiserum was subsequently used in all the rest of the work. Two of the factors that come into play in deciding the precise parameters of materials in radial immunodiffusion are balancing the wish to conserve antiserum with the need to produce a visible immunoprecipitate and the need to produce precipitin rings large enough to give a reasonably small measurement error but not so large as to interfere with one another. As antigen diffuses out from the well and antibody becomes incorporated into precipitate, a zone surrounding the developing immunoprecipitate forms that is relatively deficient in antibody and as precipitin rings from neighbouring wells approach one another they eventually come into a region mutually depleted and the rings will bulge towards one another and, if there is enough antigen, fuse (Heremans, 1971). Variations were made in amount of gel in the dishes, size of well, volume of inoculum, dilution of antiserum and dilution of serum in order to find the optimum parameters for the antiserum used.

The first satisfactory rings with blood donor sera were produced at ratios of concentration of antiserum to concentration of serum inoculum of 1:4 to 1:8. It was then seen that around most wells there were two rings. In a few cases only one ring was visible, and in a few cases three. There was no correlation between the serum cholinesterase activity and the area of the outer ring, and the correlation with the area of the inner ring was only 0.21 ($p < 0.05$). However, both ring areas correlated with the immunoturbidity, with $r = 0.61$ for the outer, 0.48 for the inner, both $p < 0.001$. The obvious conclusion was that the antiserum contained antibodies against two non-cholinesterase human serum proteins,

despite the fact that serum cholinesterase was the only protein detected in the purified product with which the rabbit had been immunized. This prompted further investigation.

3.3 Simple tests of antiserum affinity

The immediate questions were of whether the antiserum contained any antibodies against serum cholinesterase and, if so, whether they inactivated the enzyme. Three experiments were set up. The Ouchterlony gel used to monitor the antibody titre in the antiserum was stained for cholinesterase, a radial immunodiffusion gel was stained for cholinesterase, and human serum reacted with antiserum or pre-immune rabbit serum was subjected to electrophoresis to see whether the cholinesterase activity was removed.

The Ouchterlony gel was washed in four changes of 1M NaCl over 5 days to remove unprecipitated proteins before staining. The result is illustrated in Fig. 6. It shows that the precipitated cholinesterase is confined closer to the serum wells than the precipitin line previously presumed to be cholinesterase. The photograph has detected another precipitin line much closer to the antiserum well, though this had not been noticed with the naked eye. A precipitin line is not the boundary of diffusion of antigen from the serum well; it represents the point of equivalence, where the concentrations of antigen and antibody (diffusing in opposite directions) are equal and the greatest precipitation occurs. The cholinesterase stain appears wherever there is an active molecule of the enzyme so is visible further away from the serum well than the precipitin line would have been if it could have been seen. This is undoubtedly the reason why the stain overlaps the most prominent precipitin line in some places. The two precipitin lines and the cholinesterase front are all different shapes, related to their relative distances from the serum and antiserum wells. These explanations are obvious with hindsight, but at first the appearance was interpreted as meaning that the gel had not been washed for long enough.

The similarly washed and stained radial immunodiffusion gel is illustrated in

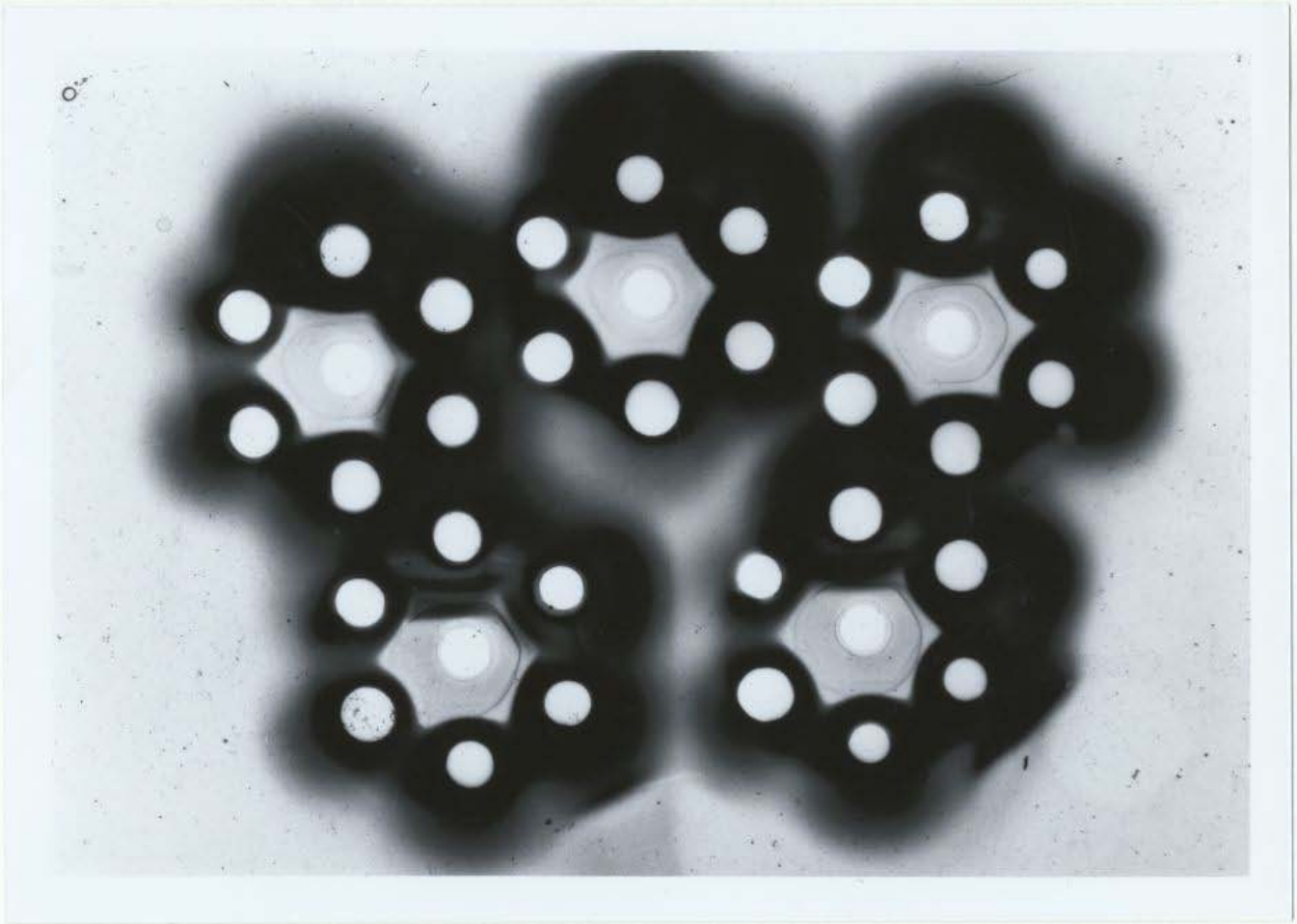


Fig. 6. Ouchterlony gel stained for serum cholinesterase

The central well in each rosette contains rabbit antiserum from successive weeks after booster immunization; top left one week to bottom right five weeks. The peripheral wells contain dilutions of human serum with isotonic saline. The top well in each rosette contains undiluted serum and then in clockwise order 1/2 dilutions to 1/32.

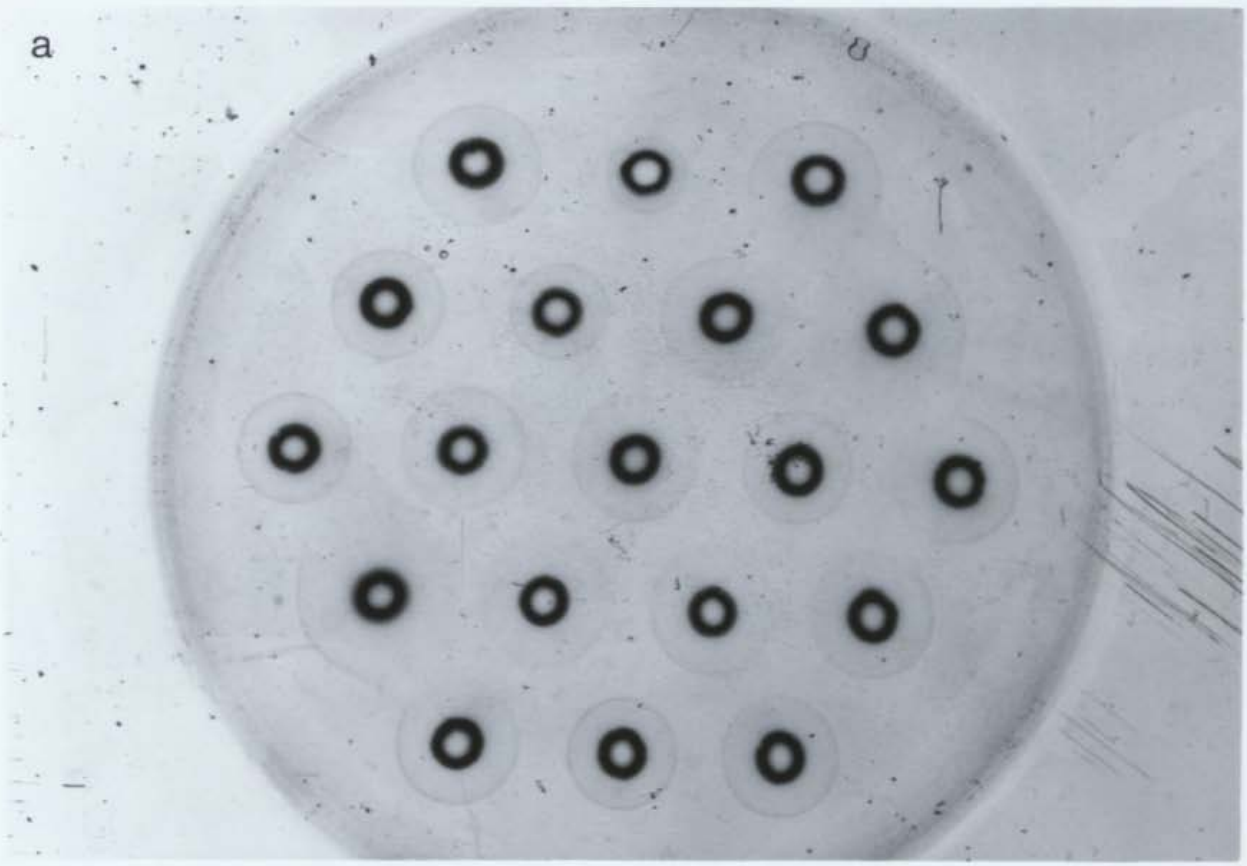
Fig. 7. The cholinesterase stain was confined to a thin line round each well. In (a) the photographic sensitivity was increased to show the outer non-cholinesterase precipitin ring. (The inner ring had faded by the time the gel was stained and photographed.) At this sensitivity the ring of stain appears much more substantial than it did to the naked eye. The actual appearance was more like that of the ring in the centre of the top line in the less sensitive photograph (b). In case the stain represented enzyme which had been too highly polymerized to enter the gel or that had not been bound by antibody but by ionic binding to the agar which had been used for this gel, a control gel was set up with samples in the wells but no antiserum in the gel. When this had been incubated, soaked in saline and exposed to stain, no staining was seen. This showed that the antiserum did contain antibodies against serum cholinesterase.

For the electrophoresis, 10 μL of human serum was mixed with 40 μL of the immune or pre-immune rabbit serum and incubated at 37°C for 4 hr and then at 4°C overnight. Then approximately 50 μL of 20% polyvinylpyrrolidine was added to each tube, and to tubes containing 50 μL of human or pre-immune rabbit serum and the contents were mixed and centrifuged at 14,000 r.p.m. for 5 min. Then the gel was loaded with 10 μL of the supernatants from the tubes containing both human and rabbit serum, 2 μL from that containing only human serum and 8 μL from that containing only rabbit serum so that the control lanes contained the same amounts of the sera as the reaction lanes. The gel, stained for serum cholinesterase, is illustrated in Fig. 8. It shows that human serum cholinesterase (lanes 1 and 5) was unaffected by pre-immune rabbit serum (lane 2) but was removed (by precipitation) by the antiserum (lane 4), confirming the result of the radial immunodiffusion gel. (One would expect to see rabbit serum cholinesterase in lanes 3 (pre-immune rabbit serum) and 4 but none is visible. Rabbit serum cholinesterase activity is more specific for acetyl- and propionylcholinesterase than for butyrylcholinesterase, which was used in staining the gel, and even with those substrates rabbit serum cholinesterase activity is lower than that of humans (Augustinsson, 1961). The gel was already

Fig. 7. 1 in 8 antiserum RID gel stained for serum cholinesterase

Two photographs of the same radial immunodiffusion gel with different sensitivity. The gel contains 1 in 8 rabbit antiserum. The wells contain human sera. Photograph (a) is more sensitive and shows the Petri dish and the outer one of the non-cholinesterase precipitin rings. The dark ring round each well is serum cholinesterase stained with specific stain. Because of the increased sensitivity these rings appear much broader than they did to the naked eye. Photograph (b) more closely approximates the naked eye appearance.

a



b

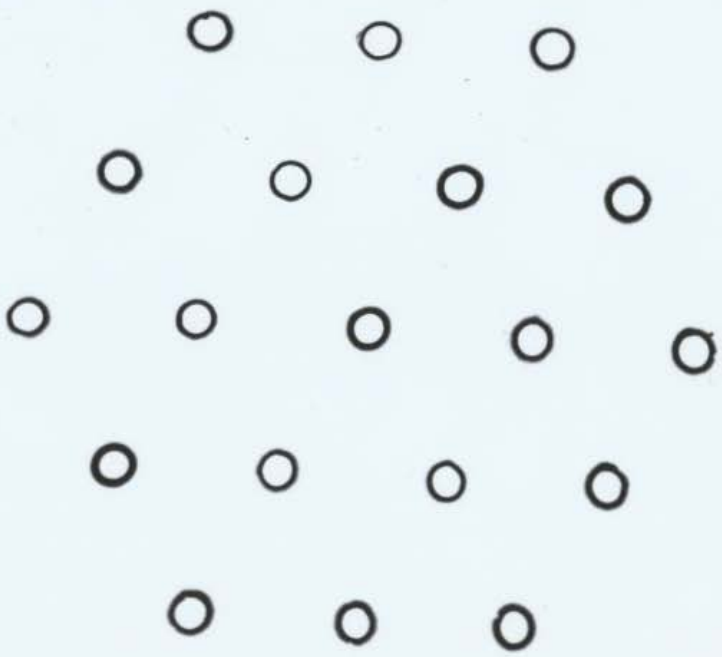


Fig. 8. PAGE showing removal of human serum cholinesterase by rabbit antiserum

Lanes 1 and 5, human serum; Lane 3, preimmune rabbit serum; Lane 2, supernatant of human serum incubated with preimmune rabbit serum; Lane 4, supernatant of human serum reacted with rabbit antiserum. The gel is stained specifically for serum cholinesterase.

slightly overstained at the time of photography. Several hours after rinsing stain off the gel a cholinesterase band appeared in lane 3 but no band appeared in lane 4. It appeared from this that the rabbit may have developed autoimmune acholinesteraseaemia.)

These experiments showed that the antiserum did bind serum cholinesterase but did not inactivate it. It was subsequently found that cholinesterase precipitin rings suitable for measurement (up to about 1 cm in diameter) could be produced with undiluted serum by reducing the antiserum concentration in the radial immunodiffusion gels to only 0.15%. They were invisible unstained but could be visualized by specific staining. As only serum cholinesterase was stained it was not necessary to wash the gels in changes of saline to remove the other proteins.

(This called into question the rabbit's autoimmunity status. The first 1 in 8 antiserum gel stained was pink all over. This was taken to be so because of rabbit serum cholinesterase in the antiserum, hence the washing in saline of another gel before staining to produce the result shown in Fig.7 (p. 105). With only 0.15% antiserum in the gels but the same quanities of human serum in the wells there was no background staining despite the fact that the gels were not washed in saline. Therefore the pink staining seen in the first gel must have been due to unprecipitated rabbit serum cholinesterase. The non-appearance of a rabbit serum cholinesterase band on PAGE is thus rather puzzling.)

Having discovered two trace impurities in the cholinesterase preparation, it was decided to explore this further by western blotting.

3.4 SDS-PAGE and western blotting

3.4.1 Results

The results of SDS-PAGE and western blotting are shown in Fig. 9. The purified cholinesterase lane in the gel (lane 1) shows two bands which presumably correspond to the monomer and dimer. Lockridge *et al.* (1979) found

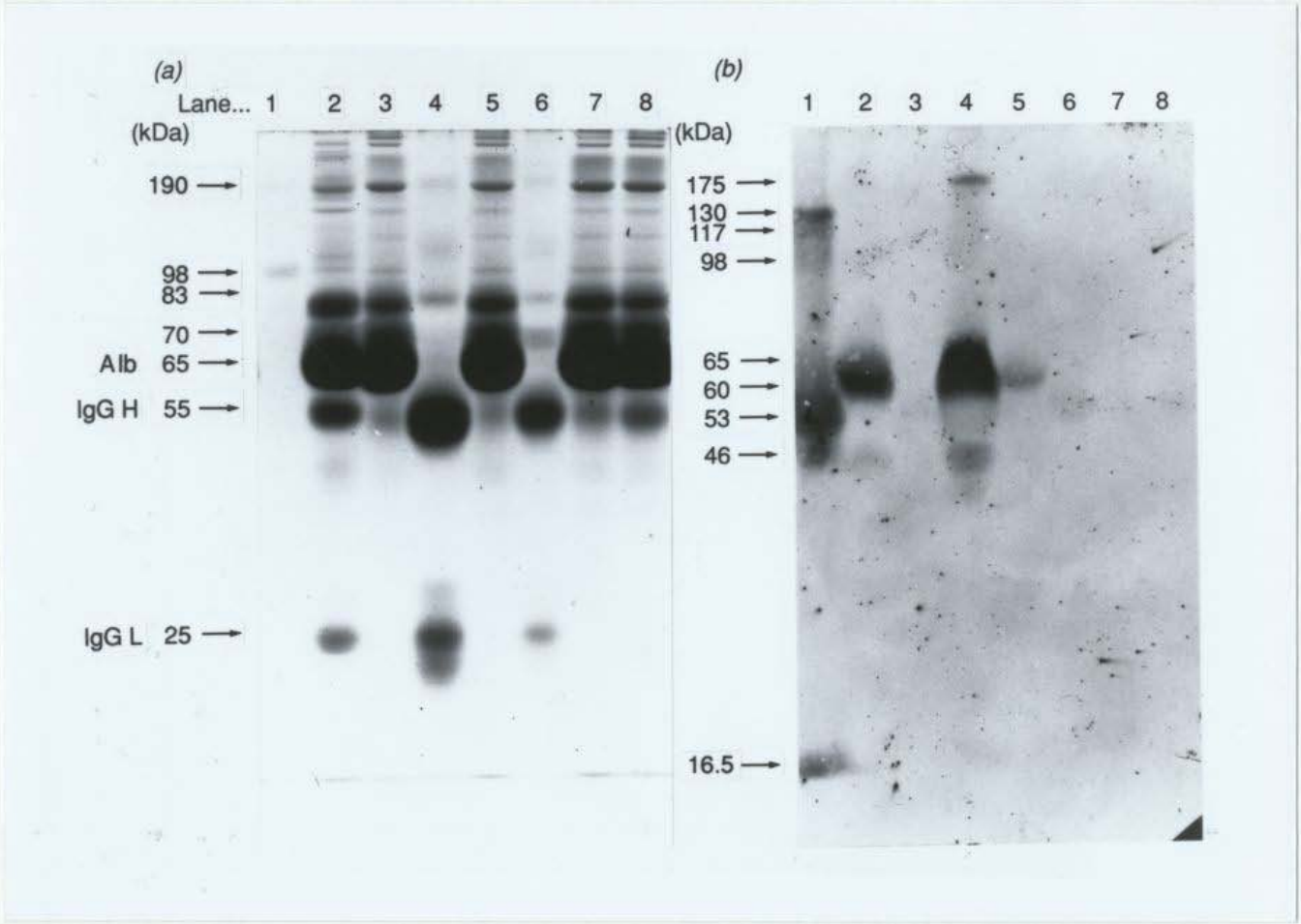


Fig. 9. SDS-PAGE and corresponding western blot

Lanes from one half of the gel (a) are shown, stained with Coomassie Blue. The other half was used for a western blot (b) with the rabbit anti-human-serum-cholinesterase antiserum in the primary reaction. Lanes are: lane 1, purified human serum cholinesterase; lane 2, human serum; lanes 3 and 4, supernatant and immunoprecipitate respectively from reaction of human serum with rabbit anti-serum-cholinesterase antiserum, prepared as described in Section 2.5, p. 90; lanes 5 and 6, supernatant and precipitate from control reaction of human serum with pre-immune rabbit serum; lane 7, pre-immune rabbit serum; lane 8, rabbit anti-serum-cholinesterase antiserum. Labeled bands are: Alb, albumin; IgGH, IgG heavy chains; IgGL, IgG light chains.

two bands of unequal intensity on SDS/PAGE, the dimer and a trace of monomer in the absence of reducing agent, or the monomer and a trace of dimer in the presence of dithiothreitol or mercaptoethanol. The sample buffer used in this work contained 2-mercaptoethanol. The estimates for the molecular masses were 98 kDa for the dark band and 190 kDa for the faint band. The estimates of Lockridge *et al.* (1979) from SDS/PAGE were 90 and 180 kDa, but revised estimates from the complete amino-acid sequence and estimated carbohydrate content are about 85.5 and 171 kDa (Lockridge *et al.*, 1987a).

Lane 4 of the gel, was loaded with immunoprecipitate from 10 times the volume of serum used in lane 2 but the serum cholinesterase monomer band seen in lane 1 is not visible here. In addition to the IgG light and heavy-chain bands two other major bands of higher molecular mass are seen. The estimates of their molecular masses are 190 and 83 kDa. These bands are also present in the protein A-Sepharose precipitate from the reaction of human serum with preimmune rabbit serum (lane 6) so are presumably also immunoglobulin subunits. Since the cholinesterase is not completely broken down into monomer it seems reasonable that not all of the S-S bonds in the immunoglobulins would have been broken either. The heavier band might be a single IgM subunit, which has a molecular mass of about 180 kDa, and the lighter band might be a half IgG (one light and one heavy chain), molecular mass about 80 kDa.

On the western blot, lane 1 shows only a faint smudge in the position in which the major protein band is known to lie from lane 1 in the gel, indicating that the human serum cholinesterase monomer is not very immunogenic to the rabbit. There is a very faint line corresponding in position to the much less abundant dimer. There is however a dark band between these positions. Its estimated molecular mass is 130 kDa. It is possible that this represents the serum cholinesterase C_2 compound. The immunoprecipitate lane, lane 4, shows a lone band of high molecular mass. The estimate was 175 kDa. It seems likely that this is the serum cholinesterase dimer.

In addition to the above-mentioned bands, the western blot reveals that the rabbit antiserum contains antibodies to at least five other proteins. More confidence can be placed on the estimates of the molecular masses of these proteins as the log molecular mass vs. R_f graph was linear in this molecular mass range and the human albumin and immunoglobulin bands in the gel give checks of accuracy. Two of these proteins, 65 kDa (probably albumin) and 60 kDa, are seen as dark bands in the lanes of human serum and immunoprecipitate from human serum (2 and 4) but are barely present in the purified cholinesterase (lane 1). Two others, 53 and 46 kDa, are seen as dark bands in the purified cholinesterase lane but only lightly in the human serum and immunoprecipitate lanes, and a further band, 16.5 kDa, is seen only in the purified cholinesterase lane. The blot shown is a repeat of an earlier blot not used because some of the other lanes were seen to be overloaded in the corresponding gel. The earlier blot clearly shows the same three dark bands present in the purified cholinesterase lane in positions in which no protein is detectable with Coomassie blue staining in the corresponding lane in the gel.

Lane 3 contains supernatant from the immune reaction. The protein pattern in the gel is that of a mixture of human and rabbit serum proteins with the immunoglobulins removed. On the blot no bands are seen, confirming the removal of all proteins that react with the antiserum to the immunoprecipitate (which was run in lane 4).

Lanes 5 and 6 contain supernatant and precipitate respectively from the control reaction with preimmune rabbit serum. In the gel the appearance of lane 5 is about the same as that of lane 3, indicating no discernible difference due to proteins precipitated from one but not from the other. In lane 6 one sees, as expected, immunoglobulin bands in lesser density from preimmune rabbit serum than from the serum of the immunized rabbit, but one does not expect to see any other bands. The appearance therefore of an extra pale broad band is puzzling. Its average molecular mass is about 70 kDa which is the size of the IgM heavy chain. It seems possible that this is indeed what it represents and that it has

largely disappeared after three immunizations when the immune system has gone over largely to the production of IgG.

The blot appearance of lanes 5 and 6 is easily explained. Since the preimmune rabbit serum should fail to precipitate the protein precipitated by the antiserum, one expects the appearances of lanes 5 and 6 to be the reverse of those of lanes 3 and 4. In lane 5, however, only one pale band is visible. The explanation is in the relative amounts of material used. The limits to amounts used to avoid distortion due to overloading were deduced from an earlier gel. The whole of the immunoprecipitates from the reactions detailed in Chapter 2 were used, divided between the duplicate lanes, but only about 1% of the supernatants were used, containing material from about 10% of the amount of human serum run in lane 2. This reduction was necessary because the reaction mixtures also contained rabbit serum of total volume equal to ten times that of the human serum (see Section 2.5, p. 90). The lanes contained material derived from the following volumes of human serum: lanes 2, 0.25 μl each; lanes 3 & 5; 0.025 μl and lanes 4 & 6, 2.5 μl. Lanes 1 contained 2.5 μl of purified serum cholinesterase and 0.25 μl of rabbit serum was used in lanes 7 & 8.

No bands are seen on the blot in lanes 7 and 8. This shows that the goat anti-rabbit-Ig antibodies do not bind to denatured rabbit immunoglobulins and therefore that none of the bands in the other lanes are due to rabbit immunoglobulin.

3.4.2 Discussion

3.4.2.1 Impurities in purified serum cholinesterase

Western blotting clearly showed that the purified human serum cholinesterase contained at least three proteins (53, 46 and 16.5 kDa) that were highly immunogenic yet were present in concentrations below the level of detection by Coomassie blue staining of the gel. The highlighting of two other proteins (65 and 60 kDa) in human serum demonstrates that the antiserum contains antibodies against these two further proteins and therefore presumably

that the purified cholinesterase contains these proteins even though only weak staining is seen on the blot in this region in lane 1 (Fig. 9, p. 108).

Radial immunodiffusion of human serum demonstrated at least two protein components precipitated by the antiserum whose concentrations did not correlate with serum cholinesterase activity and were therefore presumably not breakdown products of the cholinesterase. Furthermore, the fact that they required for their containment on radial immunodiffusion antiserum a concentration about two orders of magnitude higher than that required to limit serum cholinesterase to rings of about the same size suggests that these were proteins that are present in human serum in much higher concentrations than serum cholinesterase. Direct comparison of concentrations of the proteins however cannot be made since the ratios of the concentrations of the antibodies to the different proteins are unknown.

Both of these proteins were much more variable in concentration than is albumin (which might be the 65 kDa antigen). It is probable that the larger and more easily seen of the non-cholinesterase-staining rings represented the 60 kDa protein as it was the only one present in high enough concentration to be seen in lane 5 on the blot. The 46 kDa protein presumably accounts for the other ring since it is the only other contaminant that is abundant in the immunoprecipitate. It may be α_1-proteinase inhibitor (α_1-antitrypsin). α_1-proteinase inhibitor is acidic (pKa 4.6) making it quite a good candidate to be copurified with serum cholinesterase in the first stage of purification at pH 4. It is also abundant in serum at around 1.3 g/L and as an acute phase protein its concentration is known to be highly variable. Its molecular mass has been estimated as 47-55 kDa (Carrell and Owen, 1979). α_1-acid glycoprotein is more acidic and is also abundant in serum (around 1 g/L) and an acute phase protein, but it has been found to be a poor antigen (Jager, 1953) unless its sialic acid moieties are removed (Athineos, 1962), and its molecular weight is somewhat lower, the highest estimate being 44.68 kDa (Oss and Bronson, 1974) but probably the best 39.5 kDa (Schmid, 1975).

Further speculation as to the identity of the contaminants seems pointless, but it is of interest to look to see whether one of the impurities was of the right molecular mass to be dipeptidyl peptidase IV. In 1982, Lockridge reported hydrolysis of substance P by highly purified human serum cholinesterase. Hydrolysis was found to occur at both the N- and C-terminus. Nausch and Heymann (1985) showed clearly that the aminopeptidase activity could be accounted for entirely by the trace presence of dipeptidyl peptidase IV (also known as post-proline dipeptidyl aminopeptidase, dipeptidyl aminopeptidase IV and originally as glycylpropyl β-naphthylamidase). They found that dipeptidyl peptidase IV eluted from the procainamide-gel affinity column behind the serum cholinesterase peak but overlapped it, and that passing the cholinesterase peak fractions through the affinity column a second time reduced the dideptidyl peptidase IV activity to a level undetectable by incubation with substance P for 48 hr at 37°C.

Human dideptidyl peptidase IV was purified by Ōya *et al.* (1971), who estimated its molecular mass by gel filtration to be approximately 225 kDa. Yoshimoto and Walter (1977) purified the enzyme from lamb kidney and estimated its molecular mass by the sedimentation equilibrium method and by SDS/PAGE to be 230 ± 15 kDa and found it to consist of two identical subunits of 115 kDa. As described in Chapter 2, p. 61, the method of purification of serum cholinesterase was derived from that of Lockridge but included gel-filtration after the affinity chromatography. On the blot there is a possible band, estimated at 117 kDa. If this is indeed dipeptidyl peptidase IV, it strengthens the estimate of 130 kDa for the presumed cholinesterase band.

(The investigation of the apparent carboxypeptidase activity of purified serum cholinesterase is not yet complete. Boopathy and Balasubramanian (1987) found that the two activities coeluted from a procainamide-Sepharose affinity column with NaCl and still coeluted when passed through a second time and eluted with 0.05 M procainamide. They also coeluted from a concanavalin-A-Sepharose column and a Sephadex G-200 gel-filtration column and

comigrated on PAGE. Subsequently, Rao and Balasubramanian (1990) reported isolation of the peptidase activity to a 50 kDa peptide fragment produced by limited α-chymotrypsin digestion of purified serum cholinesterase and the sequence of the five N-terminal amino-acids of this fragment corresponded to part of the cholinesterase sequence. However, Checler *et al.* (1990) showed that different preparations of purified serum cholinesterase differed in their peptidase activities and that monoclonal antibodies against serum cholinesterase could remove the cholinesterase activity and electrophoretic bands without affecting the peptidase activity. This evidence seems conclusive but the enzyme responsible for carboxypeptidase activity has yet to be identified.)

Comparison of lanes 1 in the gel and the blot makes it very clear that all of the contaminants noted are more immunogenic than the serum cholinesterase monomer since though they cannot be seen on the gel their labelling is at least as dark as that in the position in which the monomer lies. The nucleotide sequence of the rabbit 'butyrylcholinesterase' gene has now been published (Jbilo and Chatonnet, 1990). The amino-acids show 91.6% identity with those of the human protein.

3.4.2.2 Nature of the immunogenic serum cholinesterase

The results raise two major questions about the cholinesterase itself: in lane 1, why is the darkly-staining high-molecular-mass band on the blot in a different position (corresponding to 130 kDa) from the major protein band in the gel (corresponding to 98 kDa), and why are the bands in different positions in the purified cholinesterase and immunoprecipitate lanes on the blot?

With regard to the first question, firstly, the portion of gel shown and the part used for blotting were parts of the same gel. There was no distortion of the dye-front (which is seen to be straight in the portion shown) and indeed the cholinesterase lane was next to the protein marker lane in each half and the two halves were of identical appearance before blotting. Secondly, the two lanes 1 were loaded from the same reaction tube. There can be no doubt then that very few antibodies have bound to the free serum cholinesterase.

The darkly-staining band can therefore only be either yet another contaminant or a combination of the serum cholinesterase monomer with another molecule. The obvious candidate for the latter possibility is the C_2 compound (see pp. 14-15). Its mass is about 150 kDa (Masson, 1990), whereas the estimate for the band on the blot was 130 kDa. However, as will be shown later (Fig. 11, p. 123, lanes 1 and 2), in PAGE the purified cholinesterase migrates more rapidly than the cholinesterase in serum, presumably because of the retardant effect of a large amount of albumin in the serum. This was a consistent finding in PAGE. Assuming that the same occurred in the SDS-PAGE, the purified cholinesterase would have an apparent molecular mass a little less than the same molecule in serum. The molecular mass markers accurately identified the molecular masses of the IgG light and heavy chains in the serum so it seems quite possible that the band in question could represent a molecule a little heavier than estimated. If the band on the blot does correspond to C_2, this could explain why there appear to be so many antibodies to the 65 kDa protein seen in lanes 2 and 4 even though there is little if any of this protein free in the purified cholinesterase preparation, i.e. antibodies to the albumin moiety of C_2 bind to the albumin in the serum.

If the estimate 130 kDa is correct, this rules out a combination of serum cholinesterase monomer with albumin and the protein has to be either another contaminant or a combination of the monomer with another, smaller molecule. The plasma from which the cholinesterase was purified came from 67 donors so almost certainly contained C_5 (see pp. 14-20). A C_5 band was not seen on PAGE of the purified cholinesterase but, as the contaminants have shown, this does not preclude its presence at a concentration too low for detection by Coomassie blue but detectable immunologically. There was therefore a possibility that the band in question could represent a partial-reduction breakdown-product of C_5. As the band was not seen in the serum sample used for the blot PAGE was performed on that to make sure that it did not contain C_5. It did not exhibit C_5 but surprisingly showed two other low-mobility bands (Fig.

11, lane 6). Whether these represent higher polymers of a variant cholinesterase or combinations of cholinesterase with other proteins, it appears that they were dissociated by reduction as no bands heavier than 65 kDa appear in the serum lane on the blot.

α_1-antitrypsin has a single free thiol group and can link with the free thiol group of κ light chains *in vivo* as well as *in vitro*, and normally about 1% of the α_1-antitrypsin in plasma occurs as a complex with IgA, linked by disulphide bridging to the heavy chain (Carrell and Owen, 1979). It is possible that serum cholinesterase can also form a disulphide bond with κ light chains because an extra serum cholinesterase band has been observed on electrohporesis in some patients with myeloma (Gallango and Arends, 1969). The molecular mass of a combination of the κ chain and the serum cholinesterase monomer would be about 110 kDa, which is too light to account for the band on the blot, but the molecular mass of a combination of the cholinesterase monomer with α_1-antitrypsin would be about 140 kDa. This and other similar combinations could possibly occur.

Specific enzyme staining is of course much more sensitive than general protein staining. Though the gel in Fig. 11 is slightly overstained with specific stain for serum cholinesterase, neither C_5 nor C_2 is seen in the purified cholinesterase (lane 1). On using 25 times as much purified cholinesterase, a strong C_2 band was seen, but there was no distinct C_5 band or any unusual band. The C_2 compound thus seems the most likely explanation for the '130' kDa band on the blot.

With regard to the second question, the high-molecular-mass band in lane 4 on the blot is well placed to be the serum cholinesterase dimer. If the dimer is antigenic enough to attract this density of labelling then the question arises as to why the dimer band just visible in lane 1 in the gel is not labelled. There does actually appear to be a trace of labelling in this position on the blot. It may be that there was much more dimer in the immunoprecipitate. In defence of this

hypothesis, it may be noted that there is no labelling in lane 4 in the places expected for the monomer or the 130 kDa protein so the cholinesterase might nearly all have been in the dimer form in this tube. The gel does not contradict this. There is a band in the right position and it is much heavier than the dimer band in lane 1. There is, however, the likelihood that at least part of the protein in this band is immunoglobulin because of the presence of a weaker band in the same position in the control lane 6. However, if as discussed earlier, this band in lane 6 corresponds to one subunit of IgM we should expect little of this in lane 4 if the interpretation of the 70 kDa band is correct, suggesting that there is much less IgM in the antiserum than in the preimmune rabbit serum. It still has to be explained however why the cholinesterase should be less dissociated in lane 4 than in lane 1. The solutions were all boiled in the same waterbath at and for the same time. Since some dimer remained in the purified cholinesterase solution after 4 minutes boiling with 5% mercaptoethanol it seems that it takes some time for all disulphide bonds to be reduced. Unlike the protein in the purified cholinesterase tube, the protein in the immunoprecipitate tube was bound to antibodies bound to protein A-Sepharose at the beginning of the 4 minutes so may have required longer for reduction.

An alternative possibility raised by the finding of extra bands in the serum on PAGE is that the '130 kDa' band relates to one of them, but the need for an explanation for the non-appearance of this band in the serum lane on the blot is the same.

3.5 Adoption of radial immunodiffusion

For the immunological measurement of protein concentrations in clinical laboratories, radial immunodiffusion has now largely been superceded by automated immunoturbidity and immunonephalometry. They require absolutely monospecific antibody preparations. The raw antiserum was clearly not suitable for these techniques. However, the combination of radial immunodiffusion with specific staining for serum cholinesterase obviates the need for a monospecific antiserum and it was decided to use this technique.

Fig. 10. Human serum cholinesterase radial immunodiffusion rings

(a) The appearance of the rings of about 75% of samples. (b), (c) Two examples showing ring E. The division of ring D into D_1 and D_2 is just visible in (a) and (b) but is much more easily seen in (c). (d) A specimen showing very broad D rings. Tnere is no definite ring E but there is a trace of staining round the well.

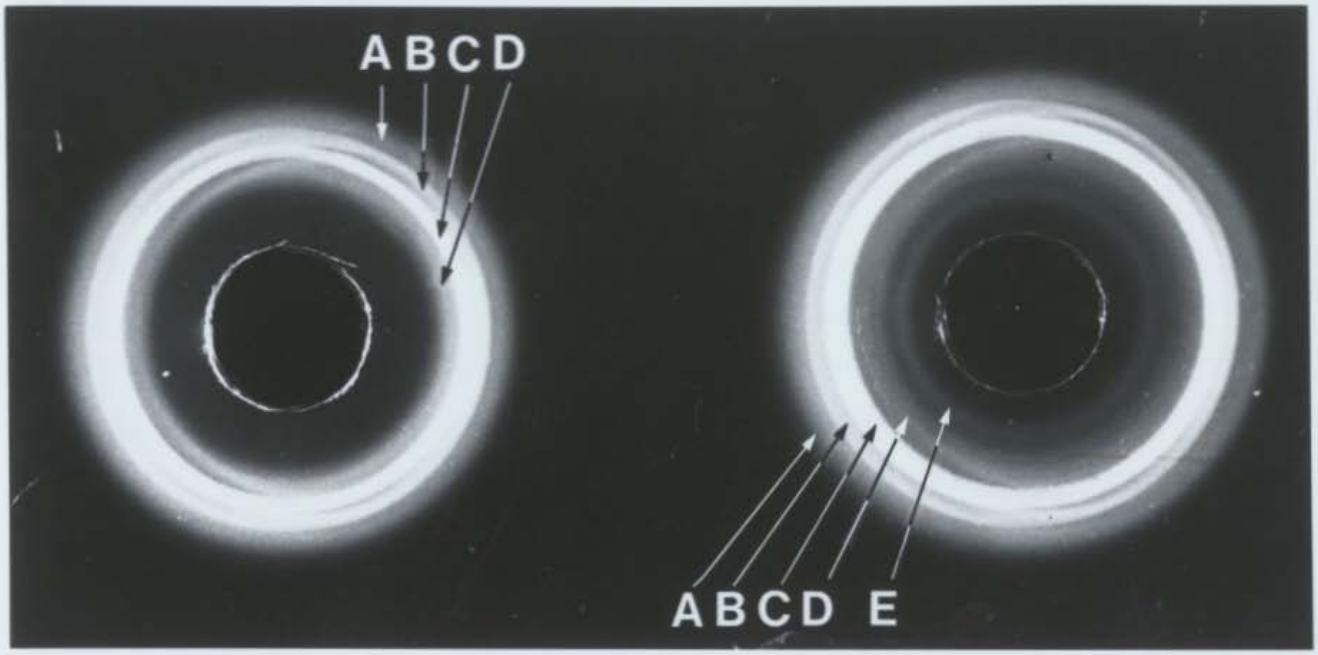
3.6 Time and temperature of incubation of radial immunodiffusion gels

Mancini *et al.* (1965) showed that the immunoprecipitates grow to a maximum size dependent upon the concentrations of antigen and antibody, after which no further growth occurs. Only when the maximum size has been reached is the area (or square of diameter) of the precipitate linearly proportional to the concentration of antigen. Before that time, measurements have to be referred to a complex calibration curve and accuracy is severely reduced. The final size is independent of temperature, but is reached sooner at higher temperatures. They also found that the time required for a precipitate to achieve its final size increases as a function of molecular mass, disc sizes of about 1 cm in diameter being achieved at 37°C in less than 24 hr by Bence-Jones proteins (25 kDa), 3 days by albumin (65 kDa, Heremans, 1971, Mancini's co-author, says 4 days), 7 days by IgG (160 kDa) and 10 or more days by antigens as large as IgM (900 kDa). The molecular mass of the serum cholinesterase tetramer is about 342 kDa (Lockridge *et al.*, 1987a). It was therefore decided to conduct a small trial to establish the appropriate incubation time. An RID plate (i.e. a radial immunodiffusion gel, as described in Section 2.4.1, p, 87, in a Petri dish) was inoculated with 5 μL of pooled human serum in each well and incubated at 37°C. The gel surrounding two wells was cut out daily and stained for cholinesterase and the rings measured, and it was found that ring growth ceased after about seven days. On this basis, plates were always incubated at 37°C and no plates were incubated for less than 7 days, though occasionally for convenience they were left a day longer.

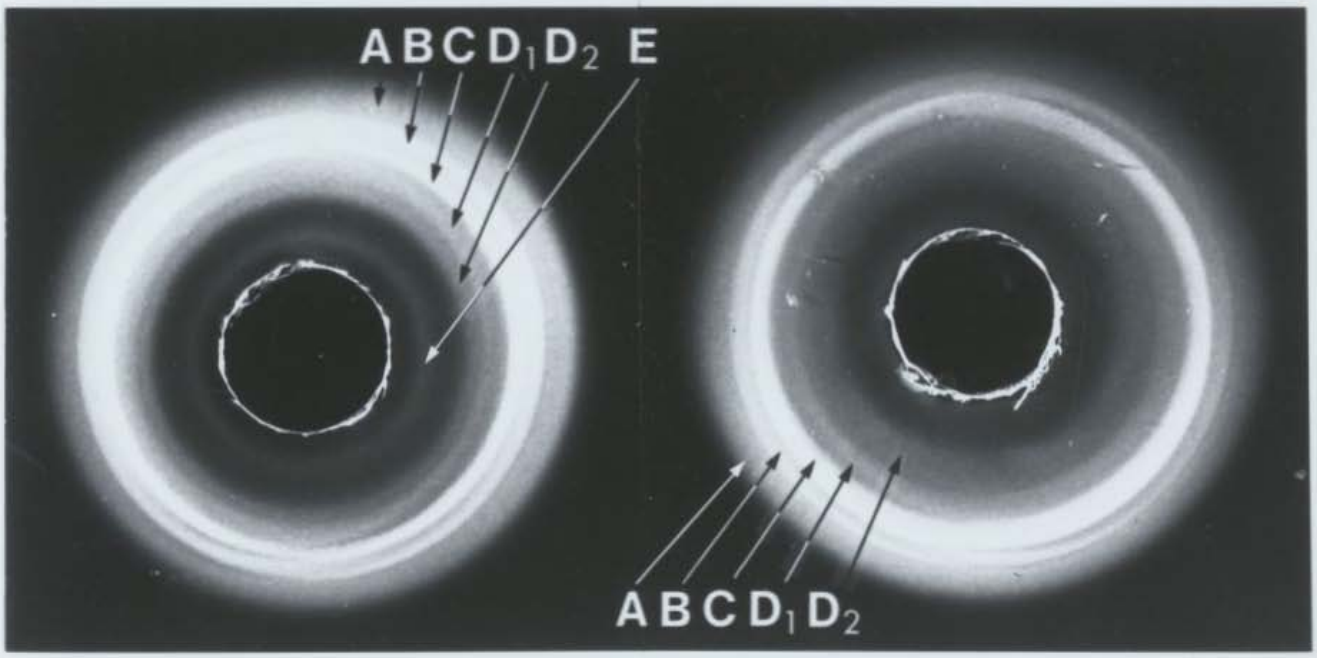
3.7 Immunological variation of serum cholinesterase

3.7.1 Observations

On staining plates inoculated with sera of different individuals for serum cholinesterase for 1 hr, it was noticed that the immunoprecipitates were composed of a set of five concentric rings which varied between individuals. The rings were designated A-E (Fig. 10).



a *b*



c *d*

Ring C was nearly always the darkest and both its inner and outer edges were sharp. The main variability in ring C was in its width, i.e. distance between inner and outer edges. The main variability in the other four was in their darkness, and indeed, whether they could be seen at all. A might be darker than B or *vice versa* and only one might be visible at first, but with sufficient staining both could be demonsted in all specimens. This was not so for D and E. Ring E was sometimes a very distinct darkly-staining annulus with fairly well-defined inner and outer borders and sometimes a pale disc surrounding the well, but was only seen in any form in about 25% of sera. Ring D was sometimes discrete, in which case it was usually nearer to C than to E but sometimes nearer to E, often abutted C and sometimes abutted E as well, and was sometimes not discernible at all.

The exact appearance was found to depend upon the time of staining. Eventually, pale rings will become darker until the divisions between the rings can no longer be seen, but the above description comes from comparisons of specimens on the same gel. On repeating radial immunodiffusion several times with some specimens it was found that the most striking feature, the presence or absence of ring E is consistent. On photography it was discovered that ring D is subdivided into two (D_1 and D_2); this is more easily seen in some specimens than in others. Fig. 10 shows examples. The specimen in Fig. 10d is described later in this section. There seemed to be two main possibilities as to the origin of these rings.

The first was that the rings might correspond to the bands seen on electrophoresis (see p. 14 *et seq.*). The position of an immunoprecipitin ring at the termination of diffusion is the position of equivalence of antigen and antibodies. It is possible that the different size isomers of serum cholinesterase might precipitate in different positions because with increasing polymerization there should be less different epitopes exposed. The association of the enzyme with another protein, as in C_2, would also be likely to produce a ring in a different position by this means even if there were no antibodies against the other protein.

The second possibility came from the description of Heremans (1971), who reported that the distribution of protein within a disc of immunoprecipitate in single radial immunodiffusion is far from uniform, even at termination of diffusion. The protein accumulates along the rim of the precipitate while moving out of the centre of the system. His diagram showing the change in distribution of human albumin precipitated with rabbit antiserum with time illustrates this and shows that at termination there is a small step in concentration on the trailing edge, and his photograph shows that this gives the appearance of a second ring. Probably the ring appearance described here is due to a combination of these factors. Mancini *et al.* (1965) say that sudden changes in temperature can cause concentric striae in precipitin discs, but the gels were incubated in a 37°C incubator and gels incubated at different times all showed the same ring structure.

The areas of all of the rings were correlated with one-another and with the serum cholinesterase activities of the samples. Rings A and C gave the best correlations with activity, both greater than 0.9.

Initially C_5 seemed the most likely explanation for ring E because it is a common variant. Its occurrence varies widely between populations but its incidence is about 10% in the British Isles (Whittaker, 1986), the ancestral origin of most Newfoundlanders. This is considerably less than the frequency with which we found ring E, but it should be remembered that Simpson (1972) reported that though polyacrylamide gel electrophoresis detected 33% more cases than starch gel electrophoresis it still failed to detect the band in 50% of sera typed as negative by the starch method but expected to have the band from family information.

PAGE of 9 sera that showed well-defined E rings and 9 that showed no E rings revealed an extra band in just one specimen, the specimen whose radial immunodiffusion pattern is shown in Fig. 10b (p. 120). The band, illustrated in Fig. 11, lane 5, was not C_5. The serum chosen for use in the western blot was

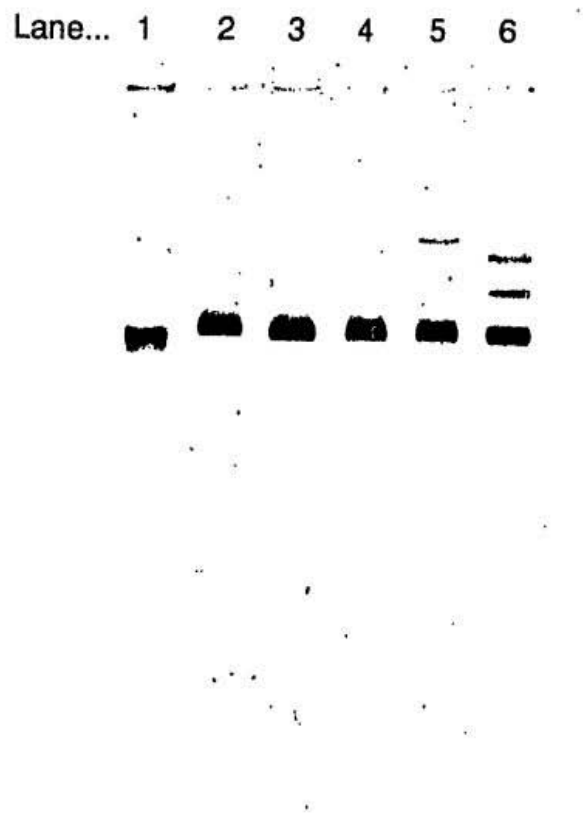


Fig. 11. PAGE of serum samples showing extra RID rings and/or extra PAGE bands, and controls, stained for serum cholinesterase

Lanes are: lane 1, purified human serum cholinesterase; lane 2, fresh human serum (The other samples had all been frozen. This sample was taken just before running the gel as a control in case the other specimens showed storage bands. Radial immunodiffusion had not been performed with it.); lane 3, serum that showed no radial immunodiffusion ring E (This is the specimen used in Fig. 10a., p. 120); lanes 4, 5, sera which did show ring E (The one in lane 5 is the one in Fig. 10b.); lane 6, the serum used on the western blot and in Fig. 10d. The major band present in all lanes is C_4. C_1, C_2 and C_3 are not visible.

that of the blood donor with the highest serum cholinesterase activity out of 117 tested. The activity was 5195 U/L (mean of all the donors 3562, S.D. 704). As already mentioned, PAGE of this specimen showed two extra bands, Fig. 11, lane 6. The radial immunodiffusion pattern of this specimen is shown in Fig. 10d. There is no definite ring E, but ring D is unusually broad.

3.7.2 Discussion

While it has been demonstrated that the presence or absence of ring E is repeatable in individual specimens, it is not known whether this is a constant finding in serum from the same subjects at different times. It remains possible therefore that this is some kind of physiological phenomenon, but if it is genetic then the majority of cases with ring E must be due to variants which are immunologically but not electrophoretically distinct from the usual variant. The high frequency of ring E then has to be explained. Two points can be deduced at the outset from the ring appearance alone. One is that if it is genetic the cases so far seen are heterozygotes since the rings A-D are present. The other is that since ring E is smaller than the other rings, either the compound responsible for it is less abundant than those responsible for the other rings or there are more antibodies in the antiserum directed against the ring E compound than against the others. In the latter case the only candidate could be a cholinesterase molecule which on reduction produced a component responsible for the ?>130 kDa band on the blot that we have already decided is probably C_2. In the former case there are other possibilities.

Now that butyrylcholinesterase cDNA and portions of the genomic DNA have been cloned, an increasing number of variants at the DNA level is being found (Bartels *et al.*, 1989; Arpagus *et al.*, 1990). It is likely that some variants will be found that have amino-acid changes which do not affect substrate/inhibitor affinity characteristics or electrophoretic mobility but yet could be immunologically distinct. There is however already a known variant which is abundant enough as a heterozygote to be a candidate. As mentioned in the introduction the K variant

(Rubinstein *et al.*, 1978) has been estimated to have an allele frequency of 0.115 (Evans and Wardell, 1984), giving a heterozygote frequency (2pq) of 20%. Bartels *et al.* (1989) have reported a DNA polymorphism at nucleotide 1615, resulting in an amino-acid polymorphism (ala/thr) at position 539. Their estimate for the frequency of the heterozygote is 22% and they are investigating the possibility that this could be the polymorphism responsible for the K variant. The K variant was found to be a quantitative one, reducing the number of cholinesterase molecules by 33%, either by reduced synthesis or increased degradation (Rubinstein *et al.*, 1978). This variant is thus not only of the right frequency to correspond to Ring E but could account for ring E being smaller than the rings due to polymers formed by the product of the other allele. The variant was originally thought to reduce the number of usual cholinesterase molecules, but if the studies of Bartels *et al.* show that their polymorphism does correspond to the K variant, this could explain the immunological difference.

Serum cholinesterase concentration was first measured by radial immunodiffusion followed by specific staining for the enzyme by Altland and Goedde (1970) and subsequently by Rubinstein *et al.* (1976) and Eckerson *et al.* (1983). None of these authors described the multiple rings. This may be because they did not incubate their gels as long as was done in this work.

Fahey and McKelvey (1965), measuring immunoglobulins by radial immunodiffusion, incubated for 24 hr at 4-10°C (i.e. a much shorter time than that required to reach 'equivalence') and found a linear relationship between the diameter and the log of the antigen concentration. Rubinstein *et al.* (1976), measuring serum cholinesterase, used the latter method, except that they incubated at room temperature. Their photograph shows little evidence of concentric rings. Eckerson *et al.* (1983) used the relationship of concentration to area but incubated for only 48 hr at room temperature; they give no illustration. Altland and Goedde (1970) incubated for two days at 37°C. Their photograph shows at least two concentric rings with most samples and possibly three with some, but no gaps can be seen between the rings.

Rubinstein *et al.* (1976) quote Berne (1974) in support of the method they used. Actually, after a detailed examination of several possible methods of estimating antigen concentration by radial immunodiffusion, Berne concluded that the Mancini method was the only reliable one. This was because smaller circles cease growth first and therefore intermediate readings produce plots that are partially straight and partially curved and critically dependent on time and temperature. Also, the range of diameters is greatest at equivalence (termination).

3.8 Choice of ring and time of staining

Having incubated the gels for 7 days and found multiple rings it had to be decided which ring to measure. At first the outermost diameters were measured and the multiple rings ignored. However, it soon became apparent that this was leading to reduced correlation between serum cholinesterase activity and the areas so obtained because in some cases ring A had been measured and in other cases, ring B, because ring A was very faint or invisible. Ring C, because of its darkness and sharpness of edge in most cases, gave the best correlation with cholinesterase activity, but it was obscured in some cases. It was therefore decided that it would be best to stain long enough for ring A to be seen in all cases and to measure that. This usually required staining for 2 hr and sometimes it was necessary to make up a fresh batch of stain and wait longer. Long staining eventually obscures divisions between concentric rings. Because an enzymic activity stain will eventually render even a few enzyme molecules detectable, the final size visualized is dependent on time of staining and therefore it is important to have controls on every plate as it is also to control for small differences in thickness between gels.

INVESTIGATION OF CONCENTRATION OF SERUM CHOLINESTERASE IN BLOOD-DONORS AND PATIENTS

Chapter 4

MATERIALS AND METHODS (2)

including results of investigations of measurement errors

4.1 Estimation of serum lipid indices and cholinesterase activity

Serum cholesterol, triacylglycerols, 'total LDL' (LDL + VLDL), HDL-cholesterol, and cholinesterase activity were measured on an IL Monarch^{TM} 2000 chemistry autoanalyser (Instrumentation Laboratory, Lexington, MA, USA). The LDL-cholesterol was calculated from the results of the cholesterol, triacylglycerol and HDL-cholesterol measurements. In addition, lipoprotein electrophoresis was carried out, followed by densitometry, and percentages of α, pre-β and β lipoproteins were derived from this, along with chylomicrons if present. The results of lipoprotein electrophoresis were used in combination with the assay results in the classification of individual sets of results into Fredrickson hyperlipidaemia types and in the calculation of LDL, VLDL and HDL concentrations from the 'total LDL' concentration.

Below, the details of lipoprotein electrophoresis and densitometry will be given first, followed by details of the autoanalyser-measured lipid concentrations and then the derived indices and classification. After this the details of the serum cholinesterase activity measurement will be given before going on to further details of the estimation of the concentration of the enzyme by radial immunodiffusion.

4.1.1 Serum lipoprotein electrophoresis and densitometry

Agarose-gel electrophoresis of serum lipoproteins was carried out with equipment and reagents comprising the Paragon Lipoprotein (Lipo) Electrophoresis Kit (P/N 655910, Beckman Instruments, Inc., Fullerton, CA, USA) and repeat supplies from the same source. 0.5% agarose gels are supplied ready-made in sealed sachets. The gel is a thin layer on the surface of a flexible plastic sheet. A gel, removed from its sachet, is blotted to remove excess buffer on the surface and a flexible plastic 'template' is laid across the free surface, aligned by means of dots on the suporting sheet, and gently rubbed to ensure sealing with the gel. 5 µL volumes of serum samples are placed in slots in the template and allowed to stand for 5 min to diffuse into the gel. The template is then blotted to remove excess serum and is itself removed. The electrophoresis tanks are on the same horizontal level and the gel on its suporting plastic sheet is fitted onto a frame ('the gel bridge assembly') which holds the gel in an arc with opposite ends in the two tanks, with the positions of the absorbed samples parallel to the tanks but out of the buffer on the positive side. Electrophoresis is then carried out with barbital buffer (10 mM 5,5-diethylbarbituric acid, 50 mM 5,5-diethylbarbiturate sodium, pH 8.6) at 100 V, constant voltage, for 30 min. The gel is then fixed in 30% reagent alcohol, 10% acetic acid, aq. for 5 min, dried with a gel-dryer, stained in 0.07% Sudan Black B for 5 min, destained with 3 washes of 45% reagent alcohol, rinsed in deionised water and dried again. The 'reagent alcohol' is 95% ethanol denatured with methanol.

The intensities of the lipoprotein bands are then estimated with a Beckman Appraise™ densitometer by absorption at 600 nm. The instrument scans the gel and measures the areas under the absorption peaks and calculates their individual percentages of the total area under the curve.

4.1.2 Total cholesterol concentration

This was measured with reagents of the IL Test™ Cholesterol Kit (Instrumentation Laboratory). The method is a modification of that of Allain *et al.* (1974). Cholesterol esters are hydrolysed with a microbial cholesterol esterase and the released cholesterol, along with previously free cholesterol, is then oxidized with a microbial cholesterol oxidase to cholest-4-en-3-one and hydrogen peroxide. In the presence of horse-radish peroxidase the hydrogen peroxide then reacts with 4-aminophenazone (4-aminoantipyrene) and phenol to produce the red coloured 4-(p-benzoquinone-mono-imino)-phenazone (quinoneimine) and water. The original total cholesterol concentration is then proportional to the optical density at 500 nm.

4.1.3 Triacylglycerol concentration

This was measured with reagents of the 'Triglycerides GPO-PAP' test (Boehringer Mannheim Canada, Dorval, Quebec). Triacylglycerols are hydrolysed by a lipase to fatty acids and glycerol. The glycerol is then phosphorylated by glycerol kinase and ATP to glycerol-3-phosphate (and ADP). The glycerol-3-phosphate is oxidized by a glycerolphosphate oxidase to dihydroxyacetone phosphate and hydrogen peroxide. The last stage is then the same as that used in cholesterol measurement except that 4-chlorophenol is used instead of phenol and HCl appears as an additional product.

The measurement of triacylglycerols by hydrolysing them with lipase and estimating glycerol was suggested by Wahlefeld (1974). He suggested using the method of glycerol estimation proposed by Eggstein and Kuhlmann (1974). They suggested the conversion to glycerol-3-phosphate but their next stage was the production of pyruvate and subsequently lactate and measurement by decrease in optical density due to oxidation of NADH. The estimation of hydrogen peroxide production by reaction with 4-aminoantipyrene and phenol was first proposed by Trinder (1969) for estimation of blood glucose. The modification of the last stage presumably improves sensitivity since when 4-chlorophenol is used, one

molecule of quinonimine is produced for every molecule of hydrogen peroxide whereas when phenol is used one molecule of quinoneimine is produced for every two molecules of hydrogen peroxide.

As in cholesterol measurement, the concentration of triacylglycerols is then estimated by measuring absorption due to quinoneimine at 500 nm.

4.1.4 'Total LDL'

The total heparin-precipitable lipoprotein concentration was measured by the method of Burstein and Samaille (1974), adapted locally for the autoanalyzer. ApoB-containing lipoproteins form insoluble complexes with heparin in the presence of calcium ions and the rate of change of turbidity is measured at 620 nm. The standard used to calibrate the machine when the test was set up was LDL prepared by ultracentrifugation as described by de Lalla and Gofman (1954) and modified by Ewing *et al.* (1965).

4.1.5 HDL-cholesterol

Chylomicrons, VLDL and LDL were precipitated from serum samples with 'HDL Cholesterol Precipitant' (Boehringer Mannheim Canada) which contains phosphotungstate and magnesium ions. This is part of the method of isolating HDL described by Burstein *et al.* (1970). The HDL remains in the supernatant. The HDL-cholesterol was then measured by measuring the cholesterol in this supernatant by the method described above (4.1.2).

4.1.6 Quality control of autoanalyzer methods

As previously stated (2.8, p. 94) the serum samples (from patients) provided for cholinesterase concentration measurement in this project were samples for which serum lipid profiles had been requested. The lipid analyses by the methods given above had already been performed and the coefficients of variation given below are derived from lipid estimations of quality-control standards carried out at the same time. Serum cholesterol, triacylglycerol and 'total LDL' concentrations (and cholinesterase activity) were measured in the

same 'run' of operation of the autoanalyzer on up to 30 specimens. In each run the laboratory included one or two of each of at least two of three different serum-based quality-control standards. The standards were 'Precinorm', 'Precilip' and 'Precilip EL', Boehringer. HDL-cholesterol estimation was run separately and a different quality-control, produced by Sigma, was used, though in the second month (see below) Precinorm was also used.

Lipid measurements on these controls were recorded and means and standard deviations calculated at the end of each month. At the end of each run the results of each lipid index in the controls were compared with the previous results, and if for any particular index more than one control result was outside the 95% confidence limits for the previous month, that test was repeated on all the samples in the run. Within-run and within-day variation was not estimated. The specimens used were measured in a period which overlapped two months. The coefficients of variation for the above lipid indices for the two months were:

Precilip cholesterol	4.75%, N = 19 and 4.62%, N = 53
Precinorm cholesterol	4.89%, N = 20 and 4.35%, N = 44
Precilip EL cholesterol	2.76%, N = 19 (second month only)
Precilip triacylglycerols	6.22%, N = 17 and 5.94%, N = 53
Precinorm triacylglycerols	8.37%, N = 18 and 6.22%, N = 47
Precilip EL triacylglycerols	4.37%, N = 21 (second month only)
Precilip 'total LDL'	7.83%, N = 16 and 11.95%, N = 53
Precinorm 'total LDL'	2.89%, N = 18 and 4.85%, N = 43
Precilip EL 'total LDL'	5.24%, N = 18 (second month only)
Sigma HDL	5.45%, N = 31 and 7.75%, N = 9
Precinorm HDL	8.32%, N = 41 (second month only)

These results come from the time when the laboratory first started to keep regular monthly statistics. It is known that the results from some runs were not recorded, particularly in the first month, and that results from some runs that were rejected were included, so the variation in the accepted results was somewhat less than indicated by some of these figures.

The lipid assays on the blood donor specimens were all carried out in the first of these months except for the first 25 specimens, which were assayed in the preceding month. The control results for these 25 specimens were however within the same ranges as the other specimens.

4.1.7 Formulae for calculated lipid indices

LDL-cholesterol was calculated thus:

LDL-cholesterol = total cholesterol − HDL-cholesterol − triacylglycerol/2.183

Where all terms refer to concentrations in mmol/L. This is 'the Friedewald equation' of Friedewald *et al.* (1972) converted to S.I. units. The original equation predicted LDL-cholesterol in mg/100 ml and the triacylglycerol concentration was then divided by 5. Friedewald *et al.* found that there were three circumstances in which their equation would not give accurate predictions: in the presence of chylomicrons, in type III hyperlipoproteinaemia, and when the triacylglycerol concentration was greater than 400 mg/100 ml (4.56 mmol/L). Note was therefore taken of these conditions in analysis of the data.

The other three calculated indices are all based on the relative proportions of the lipoproteins on electrophoresis.

LDL = 'Total LDL' × %β/(%β + %pre-β)

VLDL = 'Total LDL' × %pre-β/(%β + %pre-β)

HDL = 'Total LDL' × %α/(%β + %pre-β)

Again, these equations refer to concentrations in mmol/L.

4.1.8 Classification into hyperlipoproteinaemia types

Classification into the Fredrickson hyperlipoproteinaemia types was carried out with the results of the serum lipid assays and the information from lipoprotein electrophoresis by the criteria laid down in a memorandum of the World Health Organization (Beaumont *et al.*, 1970) except that the acceptable limits of serum lipids used were those more recently laid down in a position statement of the Canadian Society of Clinical Chemists (Dinwoodie *et al.*, 1988).

4.1.9 Cholinesterase activity

The method was a modification of that of Dietz *et al.* (1973) using S-butyrylthiocholine iodide as substrate instead of S-propionylthiocholine iodide and adapted locally for the autoanalyzer. The serum cholinesterase hydrolyses the butyrylthiocholine to form butyrate and thiocholine and the thiocholine then reacts with 5,5'dithiobis-(2-nitrobenzoic acid) to form 5-thio-2-nitrobenzoate and 2,2'dithiobischoline. (The buffer used here is 0.05 M tris, pH 7.4 rather than the phosphate buffer pH 7.6 used by Dietz *et al.*) The thionitrobenzoate has a yellow colour. The machine measures absorbance at 405 nM at 15-second intervals and determines the maximum rate of reaction. The enzyme activity is expressed in units per litre (U/L) where 1 unit represents 1 μmol thiocholine released per minute.

4.1.9.1 Linearity of activity measurement *vs.* concentration

Table I shows activity results on five dilutions of purified cholinesterase. In the first run it was seen that the activity of the second concentration was very close to twice that of the first, and that of the third close to three times the first, but that the activity of the fourth was much less than four times the first and the fifth less than the preceding two. Because of this, the run was repeated. The results were similar. This suggested that the activity assay was not reliable at high activities so the top two concentrations were diluted 1 in 2 and remeasured. When these results were multiplied by 2 they were found to be very close to what would have been expected. From these results it was concluded that the assay

Table I. Linearity of serum cholinesterase activity assay

		Relative concentration				
		1	2	3	4	5
Activity	1st run	1712	3458	5016	5258	3916
	2st run	1654	3310	5038	5382	4545
	means of runs	1683	3384	5027	5320	
	repeat at 1/2 dilution				3431	4297
	repeat result × 2				6862	8594

Activity measurements (U/L) on dilutions of purified serum cholinesterase to determine the upper limit to linearity of the assay.

became non-linear above about 5,000 U/L. Therefore, when the serum cholinesterase concentrations of the human serum samples had been measured and an initial regression equation had been derived, the activities were remeasured at half dilution on all samples for which activity was predicted to be over 5,000 U/L. Then, if the new estimate was closer to the predicted result than to the original result, the new estimate was accepted, but if not then the original result was retained.

4.2 Further details of cholinesterase concentration measurement

4.2.1 Derivation of relative concentration from ring measurement

Mancini *et al.* (1965) ensured that all their gels were exactly the same thickness by pouring them between two spaced glass plates. As the gels used in the work reported here were weighed into Petri dishes it was necessary to control for small differences in thickness of gel between dishes. This is because the gel contains the antiserum and it is the number of antibodies rather than their concentration that counts. A thicker gel will contain more antibodies and so restrict the diameter of immunoprecipitate to a smaller size. Since a control is required for between-day variation in amount of antiserum due to measurement error in making up gels, as well as for differences in size of precipitate visualized because of between-plate variation in staining (see Section 3.8, p. 126) this did not require any extra diversion of wells from holding samples.

Pooled human serum was used as a (secondary) standard against which to compare the precipitin rings of the samples measured. One way of making this comparison would be to use several different amounts of the pooled serum and to draw (or calculate by regression) a separate calibration line for each plate. If this was done it would be unnecessary to subtract the well 'area' (square of diameter, see Section 2.4.3, p. 90) from the square of the overall diameter, but it would require using several of the 19 wells on every plate, and more labour in pouring more gels and more calculating. The alternative method adopted was to

have pooled serum in one well on each plate and to express the areas of the rings of the samples as a percentage of that of the pooled serum. This requires subtraction of the well area because a proportion is calculated and having a constant added to the numerator and the denominator would obviously cause distortion. The division of the diameter by 2 and the multiplication by π to get the real area (πr^2) is rendered redundant because these factors would cancel out. The defensibility of this method will be discussed further in Section 4.4, p. 154. The pooled serum was always put in the central well. This proved to be very important (see Section 4.3.2.1, p. 147).

4.2.2 Calibration of secondary standard

In order to calculate the actual cholinesterase concentrations of the serum samples it was necessary to use the purified cholinesterase (the primary standard) to measure the concentration of cholinesterase in the pooled serum (the secondary standard). For this estimation alone, a calibration line *was* used. It was decided that this should be done with all dilutions of calibrator and calibrated on the same gel to avoid between plate variation. However it was not known what size the rings produced by any dilution of the purified cholinesterase might be and it was important to avoid ring interference. Because of this, and rather than make multiple large dilutions of the purified cholinesterase, integer volumes of the pooled serum from 2 to 10 µL (whose precipitate sizes were predictable) were used to constuct a calibration line with which to calculate their own concentration in reverse from three spaced dilutions of the primary standard. The correlation of the squares of the ring diameters with the serum volumes was 0.999 and the derived concentration of serum cholinesterase in the pooled serum was 23.7 mg/L. To express the sample concentrations in mg/L instead of as a percentage of that of pooled serum it was then only necessary to multiply the ratio (sample ring diameter squared - well diameter squared)/(pooled serum ring diameter squared - well diameter squared) by 23.7 instead of multiplying by 100.

Several estimates of the concentration of cholinesterase have been made

before and these have recently been reviewed by Brock *et al.* (1990). They ranged between 5 and 15 mg/L. Brock *et al.* comment that this 'consensus' is not surprising since all the studies were based on the same polyclonal antiserum. They present their own result. They have a monoclonal antibody for serum cholinesterase and have used it to purify the enzyme by immunoaffinity chromatography and to measure concentrations in serum by enzyme-linked immuno-sorbent assay (ELISA). They obtained a mean serum cholinesterase concentration in 33 blood donors of 4.51 mg/L.

The estimate reported here is more than five times that of Brock *et al.* (1990). This difference is probably largely due to the different methods used for measuring the protein concentration of the primary standard. Brock *et al.* used the Lowry method (Lowry *et al.* (1951) with bovine serum albumin as the reference protein whereas the method used here was that of the Bio-Rad Protein Assay Kit No. 1 with bovine gamma globulin as the reference. The Bio-Rad kit information booklet lists various advantages over the Lowry method likely to increase accuracy. It also gives a table comparing estimates of the concentrations of 23 proteins by the two methods. Even though in this comparison the same reference protein (bovine gamma globulin) was used for both methods, the concentration estimates for some of the proteins differed by as much as three times. The estimates reported here for serum cholinesterase by the Bio-Rad kit method and the absorbance method agreed very closely. Certainly the impurities found in the purified cholinesterase in this study could not account for such a large difference between estimates of the serum concentrations, since they were only present as traces. The concentrations reported in the figures in this work are given in mg/L rather than as a percentage of the secondary standard but the actual concentrations are not important to the findings. The correlations of serum cholinesterase concentration with serum cholinesterase activity and with lipid indices are unaffected.

4.2.3 Linearity of concentration *vs.* ring-area graph

It was necessary to determine how large the cholinesterase precipitin rings could grow before interference between rings from adjacent wells reduced accuracy and how much serum should be used to produce rings of optimum size. Six gels were inoculated with 2, 3, 4, 5, 6 and 7 μL of pooled human serum in every well. On the gel with 5 μL per well all the rings had sharp edges. On the one with 7 μL per well all the rings had faint fluffy edges and some were obviously beginning to merge. On the one with 6 μL per well most rings had sharp edges but a few showed some loss of definition; these were ones slightly larger than the others due to within-plate variation (see 4.3.2.1, p. 147). The means of the squares of the diameters of the rings on each plate were calculated, and correlations with volume of inoculum computed for the first four plates (2 to 5 μL), the next four (3 to 6 μL) and the last four, and for the first five and for all plates. (There is no need to subtract the well for correlation purposes.) Using the means of all rings on each plate eliminates error due to within-plate variation and considerably reduces error from measurement of ring diameters. The results are given in Table II. They again show very good correlation between ring area and amount of cholinesterase, but do show a reduction in correlation with the larger rings. The different volumes of serum of the same concentration of cholinesterase were of course used to simulate the precipitates that would be produced by samples all of the same volume but with different concentrations of cholinesterase. On these trial plates all the rings on any plate were of similar size. On plates of individual samples the precipitates would all be of different sizes. Based more on the appearance of the rings than on the figures, it was decided that 5 μL should be the maximum inoculum volume and that estimates of concentration in samples giving rings over about 100 tenths of a millimetre in diameter should be repeated with a smaller volume of inoculum unless all the adjacent rings happened to be rather smaller and there was no sign of bulging or fraying of the edges of the precipitates.

Table II. Linearity of serum cholinesterase concentration estimation

a	Volume of serum (μL)	2	3	4	5	6	7
b	Mean ring diameter (mm/10)	65.08	76.83	86.35	94.85	104.4	107.9
c	Square of diameter	3339	5006	6562	8103	10005	10755
Correlations (r) of c with a							
Series used		2-5	3-6	4-7	2-6	2-7	
r		>0.999	0.999	0.987	0.999	0.996	

Relationship of mean serum-cholinesterase-stained radial immunodiffusion ring area to number of microlitres of serum used on a series of gels, each containing 19 wells

4.2.4 Sample volumes

Serum cholinesterase concentrations of the blood donor samples were estimated using 5 μL volumes and a few were repeated using 3 μL. As serum cholinesterase activity tends to be high in hyperlipidaemia it was anticipated that many of the samples from patients might produce large precipitin rings that would interfere with one-another. These samples were therefore divided on the basis of their cholinesterase activity results. For those with activities greater than the 95th percentile of the activities of the donors 3 μL volumes were used. For hyperlipidaemic patients with normal cholinesterase activities, 5 μL volumes were used at first. The rings of the latter were found to be large and very difficult to measure because of indistinct edges. It was realized that though their activities were within the normal range they tended to be towards the top end of the range so that their mean was higher than that of the donors, and it was clear that interference was affecting estimation of concentration. For this reason they were all retested at 3 μL.

In order to make sure that any difference in specific activity that might be found between hyperlipidaemic patients and blood donors could not be due to the fact that their concentrations were measured with different sample volumes, the estimates of 50 blood donors were repeated at 3 μL. In keeping with the finding of excellent linearity of ring area *vs.* amount of cholinesterase, no significant difference was found between the means of the two sets of estimates. The mean ring diameters at 5 and 3 μL were 96.5 and 77.0 mm/10 respectively.

4.3 Error in cholinesterase measurements

4.3.1 Activity

4.3.1.1 Within-run variation

This was estimated by putting portions of the same serum sample into 25 cups of the IL-Monarch and running activity measurement twice. The results are shown in Table III. In each run, one result (the minimum in each case) stood out from the rest of the group on casual inspection. This was not the same cup of

Table III. Within-run variation of serum cholinesterase activity measurement

	Mean	S.D.	C.V. (%)
Run 1	3969.2	61.8	1.56
Run 2	3975.3	69.2	1.74

Results for two runs of the same 25 identical cups of human serum. S.D., standard deviation; C.V. coefficient of variation.

serum in the two runs, showing that this was due to random measurement error rather than to a specific problem related to one cup of serum. As the coefficient of variation is derived from the standard deviation and it is variances, not standard deviations that are additive, we have to take not the mean but the root mean square of estimates of the same coefficient of variation. In this case the result is 1.65%.

4.3.1.2 Between-run variation

The serum cholinesterase activity was initially measured at the same time as the lipids on all specimens. In each run the laboratory included one or two of each of at least two of three different serum-based quality-control standards. The standards were 'Precinorm', 'Precilip' and 'Precilip EL', Boehringer. These standards were used routinely by the laboratory for lipid analysis runs, in which the cholinesterase was also measured. They contained serum cholinesterase but the manufacturer did not market them for cholinesterase standardisation. When the serum cholinesterase concentrations had been determined, specific activities (activity/concentration) were calculated to see whether the raised activity in any cases of hyperlipidaemia could be due to increased specific activity. The specific activities of the patients showed a bimodal distribution, the group with higher specific activity containing a greater number than the other. The specific activities of the blood donors, however, had a roughly normal unimodal distribution, the mean corresponding to the lower peak of the patients' results. This was a very exciting result, suggesting a distinct difference between the specific activity of serum cholinesterase in the majority of hyperlipidaemic patients and that of controls. The data were however examined carefully to see whether there might be some other explanation.

Investigation of the distribution of different types of hyperlipidaemia in the two peaks of the distribution showed no tendency of any type to cluster in one or other peak, immediately raising the suspicion that the result was an artifact. Plotting specific activity against the order in which the specimens had been

assayed showed that specific activity made fairly sudden shifts up and down with relatively level stretches in between. Calculation of mean specific activity of samples measured in different runs showed significant differences between runs.

Attempts were then made to correct for between-run variation by using the quality control standards. Taking first one standard, the mean cholinesterase activity result for that standard in all the runs was calculated, and then the activity results for all the specimens in any particular run were multiplied by the mean of that standard for all runs divided by the mean of the usually two results of that standard in the particular run. Whichever standard was used the distribution of specific activities of the hyperlipidaemic patients became roughly normal and the mean considerably *lower* than that of the donors, with small differences depending upon which standard was used.

The patient specimens saved by the laboratory for this study had not been assayed at the same time as the donor samples, so the question arose as to whether this result was real or due to a change in batch of reagent or quality controls in the meantime. The laboratory records were inspected and the means of serum cholinesterase activity were calculated for 50 each of patients found to have normal lipid levels and ones with type IIa, type IIb and type IV hyperlipidaemia and compared with equivalent groups from the saved specimens. The means of all groups from the time when the donor samples were measured were lower than those of the saved samples. Using the means of all 200 samples at the two times to correct the donor activity results, instead of using the quality control results, brought the mean specific activity of the donors closer to that of the hyperlipidaemic patients, but it was still very significantly different. The quality control records were then examined and it was found that in the intervening period between the times when the donor and saved patient samples were measured there was a sudden step in cholinesterase activity results of Precilip but no change in the lipid results for the same standard. The obvious explanation was that there had been a change in batch of Precilip and that the manufacturer controlled lipid levels in different batches but not serum

cholinesterase. The laboratory did not have any record of times of changing to new batches. It was therefore decided that cholinesterase activity measurements of all specimens from patients and donors should be repeated but that the lipid results could stand.

Donor and patient samples were put in the same runs so that between-run variation which had been observed amongst the previous patient measurements, could not be held to account for any differences between the two groups. In every run, two portions of each of 'Precinorm', 'Precilip' and 'Precilip EL', were included, one of each at each end of the series of samples. All the reported measurements of serum cholinesterase activity were made with standards from the same batch of each.

Because of the previously observed between-run variation, attempts were again made to correct for this. Several runs were repeated *in toto*. The two sets of activity results of the human samples were then plotted against the same concentration estimates to see whether between-run variation caused change in the intercept or of the slope of the plot. Adding or subtracting a small amount from every result would be the appropriate means of correction for change in intercept, whereas multiplication of all results by a calculated factor would be the right method if the slope changed. The investigation showed that both slope and intercept could vary. The six quality-control results from each run were then compared. It was found that they were quite good at predicting changes in slope but poor at predicting changes in intercept. Several methods of using the quality control results to correct the activity measurements were tried but in every case the adjustment led to slight decrease in the correlation between activity and concentration. It was therefore decided to use the activity results uncorrected and to be aware of the error.

Table IV shows the variation of measurements of the three standards in the same 15 runs. It can be seen that the variation in estimates of cholinesterase in Precilip was greater than that in the other two standards. This was also seen in

the estimation of within-run variation. The means of the two Precinorm cholinesterase estimates in the two runs of table III (p. 142) were 3329 and 3328, whereas those of Precilip were 2429 and 2459.5. The means for cholesterol, triacylglycerols and total LDL respectively for a representative month in the laboratory were for Precinorm 4.57, 1.45 and 2.64, for Precilip 3.98, 2.21 and 1.74, and for Precilip EL 9.36, 3.73 and 5.89. Thus the difference in error of measurement of cholinesterase activity between the standards cannot be attributed to interference with the cholinesterase assay by lipids. As the reason is unknown, the root mean square of all six estimates of coefficient of variation was taken. This was 2.76%. As single samples measured in different runs are subject to both within- and between-run variation, this is the overall variation due to both sources of error. The coefficient of variation due to between-run variation alone is the square root of $2.76^2 - 1.65^2 = 2.20\%$.

4.3.2 Concentration

4.3.2.1 Within-plate variation

Originally, one plate was inoculated with 5 μL of pooled serum in every well for this estimation, but the plates subsequently prepared to check the limit to linearity of the amount of cholinesterase with immunoprecipitin ring area are also useful. Random error in measurement of the diameters of the rings with the calibrating viewer will be a larger percentage of the diameter the smaller the ring so the relevant plates to look at are those with rings of about the same size as those of the samples. For the blood donors, the 5 μL plates are appropriate. The mean ring diameter of the 111 high-activity samples measured was 89.0 mm/10, which is slightly larger than that of the 4 μL pooled serum rings, and the mean ring diameter of the 117 normal-activity hyperlipidaemic samples was 80.6 mm/10, which is between those of the 3 and 4 μL rings. Though in fact all the wells contained the pooled serum secondary standard, the concentrations given by each of the 18 peripheral rings were calculated using the central ring as standard just as for all the plates of clinical samples. The coefficients of variation of these concentrations from the 3, 4, and two 5 μL plates were respectively 6.26, 6.49, 5.62 and 5.83 percent, root mean square 6.06%.

Table IV. Between-run variation of serum cholinesterase activity measurement

		Mean	S.D.	C.V.(%)
Precinorm	Start	3369.5	93.0	2.76
	End	3385.4	62.8	1.86
Precilip	Start	2516.9	78.0	3.10
	End	2472.9	87.1	3.52
Precilip EL	Start	2145.8	58.2	2.71
	End	2158.7	48.8	2.26

Results for three different commercial quality-control preparations at the beginning and end of each of 15 activity assays

This is quite a large error. The two components of this error that might come to mind first are in pipetting the serum samples into the wells and in measuring the diameters of the precipitin rings. The Ziptrol device used for pipetting is so simple that very little of the error is likely to come from this source. The measurement error was not large either. At first, rings were measured twice in each of two directions at right angles. For 67 pairs of measurements (the two measurements in each pair made on different days) the differences were calculated. Then all negative signs were changed to positive, each difference expressed as a percentage of the first measurement of the pair, and the mean of these percentages taken. The result was 1.34%. The diameters recorded were small because at the time ring C was being measured. By the time the decision had been made to measure ring A the rings were only being measured once in each direction. The mean of the diameters from which this result was derived was 78.12 mm/10 for the first measurement and 77.66 for the second. For the larger rings A, the differences would have been a smaller percentage.

Two other sources account for most of the error. These were revealed only by noting the positions of rings of different sizes on the plates. The first is termed here 'the position effect'. An impression was gained early in the measurement of the samples that the rings might be larger towards the outside of the dish. It was to examine this possibility that the first plate with 5 μL of pooled serum in every well was prepared. The arrangement of the wells on the plates has a six-fold symetry and there are four classes of position with respect to distance from the centre of the plate: the central well, the six wells of the inner hexagon, the six wells in the middles of the outside edges, and the six outer corner wells (see Fig. 7, p. 105, again). On the trial plate the (ring C) diameters of the central ring and the means of the six rings in each of the other classes of position in the same order were respectively: 70.25, 72.71, 76.71 and 76.88 mm/10. On this plate (which later proved to show an exceptionally large position effect) the mean of the outer corner well diameters was 109.4% of the mean of measurements of the central well diameter. Using the figures from this plate it was decided to correct

the calculated serum cholinesterase concentrations for the 117 blood donor samples according to the position that each sample had occupied. When this was done, it was found that the corrected concentrations correlated slightly less well with the cholinesterase activities than did the uncorrected results.

Looking at the problem another way, it was decided that the results of the specimens could be used to look for evidence of the effect even though their cholinesterase concentrations were all different. The central wells always contained the pooled serum standard so the samples were in three classes of position. It would be possible for the mean concentration of the samples in one position class to be different from that of the samples in another position by chance, but this could be controlled for by looking at the independently-measured serum cholinesterase activities of the same samples. In the blood donors, the mean activity of the samples in the corners was by chance higher than that of the samples in the inner hexagon with the mean of the activities of the samples in the middles of the outsides intermediate. It was no surprise therefore to find that the mean concentrations were distributed in the same way. When the activities were divided by the concentrations it was revealed that there was no significant difference in the mean specific activities in the three position classes.

Calculation of the means of the ring diameters in the four different position classes on the six pooled serum plates used to look at linearity showed that on some plates the rings were smaller in the middle than at the outside and in some *vice versa*, and that all the differences were much smaller than on the first pooled serum plate. It seemed then that chance operating on small numbers could be responsible. This was because the effect had been assumed to be due to some kind of interference because the central and inner hexagon wells are each surrounded by six other wells whereas those in the middles of the outsides have only four adjacent wells and those on the corners only three. By this explanation the gradation in ring size should always be in the same direction. In retrospect another mechanism can be proposed. The Petri dishes are made of plastic. Around the edge underneath there is a little ridge about 0.5 mm high. It is

therefore possible for the centre of the plate to buckle slightly, either upwards or downwards, without drawing attention by causing the plate to rock. This could perhaps occur when the 'hot' (54^0C) gel is poured into the dish. If the centre sags the gel will be a little thicker there so there will be more antibodies and the precipitates will be restricted to a slightly smaller diameter than those further out, and if the centre buckles upwards the reverse will hold.

It is thus possible for the position effect to cause error without being apparent in the addition of a series of plates because of cancelling. It would seem however that the buckling is more often downwards than upwards. If the rings are smaller in the centre the calculated concentrations will be larger in the outer samples and so these outer samples will apparently have lower specific activities. No position trend in specific activities was found in the results of 48 normolipidaemic patients. The apparent mean specific activities of the outer hexagon samples were slightly smaller than those of the inner hexagon in the normal-cholinesterase-activity hyperlipidaemic patients at both 5 and 3 μL but this was not statistically significant. There was however a statistically significant difference in the same direction between the means of the corner and inner hexagon samples in both the donor samples repeated at 3 μL and in the high-cholinesterase-activity patients. The greatest difference was in the latter, the mean of the corner samples being 6.6% lower than that of the inner hexagon samples. The mean error from this source over samples in all three classes of position would of course be less. Because this error will affect the mean concentration estimate of samples on a plate it contributes not only to within-plate error but to between-plate error. The lesson from this discovery would seem to be to use glass.

The other source of error was discovered from the linearity plate data only after writing out the results for each plate in the same pattern as they appeared on the plate. It was noted that the largest of the 19 rings was nearly always on the outside and that the smallest ring was also nearly always on the outside, and that these two rings were always on opposite sides of the plate, though in every

case the direction across the plate was different. The obvious explanation was that the table on which the dishes had been laid while the gels set was not perfectly level, so that the gels were very slightly thicker on one side than on the other. This was indeed found to be the case. Because the pooled serum standard was always in the central well, this does not affect the mean concentration but only increases the standard deviation. By the time this error was realized, all the specimens had been measured and the nonetheless good correlations (see Chapter 5, p. 157) were known, and it was decided to interpret the results in the full knowledge of the errors rather than repeat all of the work.

4.3.2.2 Between-plate variation

Extra controls - apart from the pooled serum - were not included on the plates for the purpose of calculation of between-plate variation because it had been intended to eliminate this source of error by calculating the concentrations of the samples on each plate with respect to the pooled serum standard on the same plate. With the discovery that there was a position effect which could make all the sample rings larger or smaller with respect to the central ring made it necessary to devise a way of assessing the magnitude of this error. Two methods were used.

The first was to extract the information from the data of the clinical samples in a similar way to that used for the position effect. By this time, any samples whose results had appeared to be affected by interference had been retested. Also, for any sample in which cholinesterase concentration and activity had been found to be at variance, one, or usually both estimates had been repeated to check this. Thus the first thing to do was to go back to the original results and look at the data from each individual plate. Secondly, it would obviously be pointless to compare the mean concentration of a plate of blood donor samples with that of a plate of hyperlipidaemic patients since the latter were known to have higher serum cholinesterase activities and, by this stage of the investigation, concentrations also. Like would have to be compared with like.

However, if one could assume that there was no difference in specific activities of cholinesterase between different groups of subjects one could use the activities to control for such differences in concentration, leaving only the variation attributable to between-plate error, and could use all the plates for the estimation. Analysis of the data separated into plates led to the conclusion that one could not make this asumption (see Section 5.4, p. 161). Thus like would still have to be compared with like. Amongst the patient samples there were ones in which repeat testing showed that their specific activity was considerably lower than that of the others. These results disturbed the within-plate means. For this reason the estimation of between-plate variation was restricted to plates of blood donor samples.

There were seven plates on which the donor samples had been tested with 5 μL and another three plates on which samples had been repeated at 3 μL. Amongst the donors it was still true that the mean concentrations of the samples on two plates could be different due to chance, so the system of controlling for this with activity by calculating specific activities was used. The specific activity of each sample was calculated (using the best estimate of activity if activity had been repeated, but of course using the original concentration result) and the mean specific activity on each plate found. Calculation of the means eliminated the within-plate variation so that comparison of the ten means as individual data would give the between-plate variation. The coefficient of variation was therefore calculated from the mean and standard deviation of these means. It was 2.68%. This was however the between-plate variation of specific activity estimates and to find the variation in concentration alone it would be necessary to remove the variation derived from activity measurement. This however is more easily said than done. The samples on each plate did not all have their activities measured in the same run and there were not the same number of samples in a run as in a plate, but calculation of the *mean* specific activity on each plate should remove most of the error from measurement of activity leaving most of the between-plate variation from activity as chance variation (sampling error). The best that can be

said is therefore that the between-plate variation in concentration is probably a little less than 2.68%.

The other method was to look at the means of the concentrations calculated from the rings on the plates of pooled serum used to investigate within-run variation. In the latter work the 2 μL plate was excluded because the rings were small and the within plate error therefore likely to be larger than in the plates of clinical samples (the CV was actually 7.7%). This should not affect the mean, so it could be used for the between-plate examination. The 6 and 7 μL plates were not used however as the linearity analysis had shown that their means were affected by interference. Thus there were five useful plates: the 2, 3, 4, and two 5 μL plates. The coefficient of variation of the mean concentrations on these plates was 3.55%. This however is based on an extremely small number of data. In that light it is remarkably close to the other estimate and the best estimate is that this source of error contributes variation of about 3% (CV).

Because measurements were repeated when suspected to be incorrect, for example because of poor correspondence between activity and concentration, the actual mean deviations of the final data from their true values are probably a little less than those indicated by the error calculations.

4.3.3 Limitation of interpretation of results imposed by measurement errors

In the next chapter it will be shown that the overall variation in serum cholinesterase activity and concentration in the samples was much larger than that due to errors, and that very good correlations between the two measurements were still found despite these errors, as well as interesting correlations with lipid indices. However, specific activity is calculated by dividing activity by concentration and the errors are compounded. In the course of the study of the errors in both measurements it was found that small differences in means due to between-run or between-plate variation could cause statistically significant differences in specific activity between runs or between plates when

no such difference was likely in reality. It was therefore concluded that statements about quantitative differences in specific activity could not be made without considerably more precise measurements of the components and that any such comparisons should be restricted to well-considered qualitative ones.

4.4 Justification of the use of well diameter in calculation of serum cholinesterase concentration

The method of calculation of the cholinesterase concentrations of samples from ring diameters with reference to the concentration and ring diameter of the pooled serum standard has been explained. It involves subtracting the square of the well diameter from the square of the ring diameter. This implies the assumption that if the square of ring diameter is plotted against amount of cholinesterase in the sample the intercept on the ordinate will be the square of the well diameter, 900 $(mm/10)^2$. The findings of Mancini *et al.* (1965) indicated that this is usually not exactly so but that the intercept is slightly larger than the well. They proposed that the two were related by the equation

$$S_o = p + q.S_w$$

where S_o is the area of the intercept and S_w that of the well. Unfortunately they did not give values to p and q and they never gave any units for their areas from which these values might have been derived. (Probably they were in mg of cardboard or something similar and so not relatable by the reader to the well diameters.) They also found that the amount of solvent diluting the same amount of antigen appears to affect slightly not only the size of the intercept but the area of the precipitate, both being larger by the same amount for any given increase in volume of diluent. This means that the use of different volumes of undiluted serum to simulate different amounts of cholinesterase in serum samples all of the same volume is not exactly correct - though making dilutions would have introduced an additional source of error - and that the calibration of serum samples, which all have different ratios of solute to solvent, is not exact.

It is the purpose here to show that the figure of 900 $(mm/10)^2$ is probably not far from the true value, and that altering it would have very little effect upon the correlations between the calculated concentrations and other measurements. For a start, it was noticed that slight alterations of the position of points used to calculate a regression line may have large effects upon the intercept calculated whilst barely altering the slope. In looking at the position effect, the means of the squares of the diameters of the six rings in each of the three sample position classes were calculated for each of the plates used to look at linearity. Regressions against volume of inoculum were calculated for the squares of the central well rings for the first four plates (2 to 5 μL) and for the first five plates, and for the means of the squares of the rings in the different position classes for the same series of plates. Thus eight regressions were calculated and the ratio of solute to solvent and the well size was constant. The correlation coefficients were very high: 0.997 in one, 0.999 in two and >0.999 in the other five. The coefficient of variation of the slopes was 5.62%. The result for the intercepts was 39.4%, the range being 334 to 1296. However, the mean of the eight intercepts was 898.2 which could hardly be closer to 900. (This little investigation very nicely explains why the results of the quality control samples in the activity measurement were quite good at predicting slope but useless for predicting intercept in between-run variation.)

One other little test was done at an early stage. For 102 of the blood donors, correlations were calculated between their original activity measurements and concentrations calculated using 900 and a range of other intercepts. The correlation was 0.912 using 900 but 0.911 at 1000 and fell to 0.910 by 1400. Going the other way, it remained at 0.912 down to 500, below which, surprisingly, it gradually increased, reaching 0.916 at -4,000 and remaining at that down to at least -200,000. It was decided to use the theoretical value of 900 derived from the well diameter.

4.5 Summary of method of cholinesterase concentration measurement

The concentration of the enzyme was measured by radial immunodiffusion in Petri dishes containing 8ml of 1% agarose containing 0.15% rabbit anti-serum-cholinesterase antiserum. In each gel 19 wells of 3 mm diameter were cut in a hexagonal array with 1.5 cm between the centres. The central well on every plate was loaded with a standard, consisting of pooled human serum from 56 donors which was calibrated against the purified cholinesterase, and 18 samples to be measured were placed in the other wells. 5 µl volumes were used for samples expected to have normal concentrations and 3 µl where concentration was expected to be high from knowledge of the activity. The gels were incubated at 37°C for 7 days and then stained specifically for serum cholinesterase for at least 2 hrs. The diameters of the discs of immunoprecipitate were then measured with a calibrating viewer in two directions at right angles. Concentrations were calculated in relation to the standard on the same plate by the formula:

$$\text{Conc. (mg/L)} = 23.68(\text{sample diam.}^2 - 900)/(\text{standard diam.}^2 - 900)$$

where 23.68 is the concentration of the standard in mg/L, diam. is diameter in tenths of a millimetre, and 900 is the square of the diameter of the well. Any samples creating precipitin rings of diameters greater than about 10 mm or showing any sign of interference with other rings were retested with a smaller volume of serum.

Chapter 5

RESULTS (2)

5.1 Further details of the subjects

It is only too easy to derive a falsely high correlation coefficient by selecting samples with results towards both ends of the distributions of the variables under study. The patient samples used in this study were already from a selected group in that their physicians had had some reason to request serum lipid profiles, for example because of family history or because the patients had conditions with which hyperlipidaemia is associated, or because they had previously been found to have hyperlipidaemia and were on treatment. That bias could not be removed, but further bias could be avoided by not using further selection in the samples used for correlation calculations. In this study there was particular interest in cases in which serum cholinesterase and/or lipid indices were at the extremes, but for the purpose of correlation calculations, only consecutively-received samples of the same age-range as the blood donors were used. Of the 282 patient samples in whom serum cholinesterase concentration was measured, 209 fitted these criteria. They comprised 35 who had normal lipid profiles, 72 with type IIa hyperlipidaemia, 70 with type IIb, I type III, 30 type IV and 1 type V. Their mean age was 44.3 years. The other 73 patients comprised ones selected because of high serum cholinesterase activity or low or normal lipid levels as well as ones excluded from the consecutive series on the basis of age. They were composed of 1 hypolipidaemic patient, 2 with hypo-beta-lipoproteinaemia, 18 normolipidaemic, 17 type IIa hyperlipidaemia, 21 type IIb, 1 type III, 12 type IV and 1 type V.

5.2 Variation between samples

The variation in serum cholinesterase for the blood donors and consecutive age-matched patients is shown in Table V. The total variation in both concentration and activity estimates is much larger than that due to errors.

Table V. Variation in serum cholinesterase for donors and patients

		Mean	S.D.	CV(%)	Min.	Max.
Blood donors N = 117	activity (U/L)	3562	702	19.8	2112	5195
	concentration (mg/L)	25.1	4.6	18.4	14.9	35.7
Matched patients						
All N = 209	activity (U/L)	3715	807	21.7	1778	6542
	concentration (mg/L)	27.7	5.8	20.8	15.4	50.0
Female N = 112	activity (U/L)	3513	802	22.8	1778	6542
	concentration (mg/L)	26.2	5.6	21.4	15.4	50.0
Male N = 97	activity (U/L)	3949	751	19.0	1846	5806
	concentration (mg/L)	29.5	5.5	18.6	15.4	47.7

Although many of the additional patient samples were at the upper and lower ends of the range, none were beyond the extremes of the consecutive age-matched patients. For the patients the sexes are known and the means of both activity and concentration are significantly higher for men, $p = 0.0001$ for activity and even less for concentration. For the donors the distribution of activity and concentration measurements were slightly positively skewed. For the matched patients however, the distributions were normal for the whole data and for the sexes separately.

5.3 Correlation between serum cholinesterase activity and concentration

Serum cholinesterase activity is plotted against its concentration for blood donors and for all the patients in Fig. 12. The correlation coefficient, r, for the blood donors was 0.95, $p < 0.001$. but for the consecutive age-matched patients 0.88, $p < 0.001$. The regression equation calculated from the donors was

$$\text{Activity (U/L)} = -80 + 145 \times \text{concentration (mg/L)}.$$

Ideally this line should pass through the origin. It is close. The intercept is only about 2.25% of the mean activity of the donors.

The regression line has been drawn through both plots. For the donors there was only one individual whose result stood out noticeably from the rest. The serum cholinesterase concentration of this sample was 22.7mg/L. The observed activity was 2329 U/L but the activity predicted from the concentration on the basis of the regression was 3209.4 ± 45.5 U/L (95% confidence interval). The residual (i.e. difference from expected) was thus −880.4 and the standard residual (i.e. number of standard deviations of difference from expected) was −3.99. Both activity and concentration estimates were repeated and confirmed that this was an unusual result. No clinical details are known except that the individual was accepted as a blood donor.

For the patients it can be seen that there are many more below the donor regression line than above, and that some are very much lower than expected.

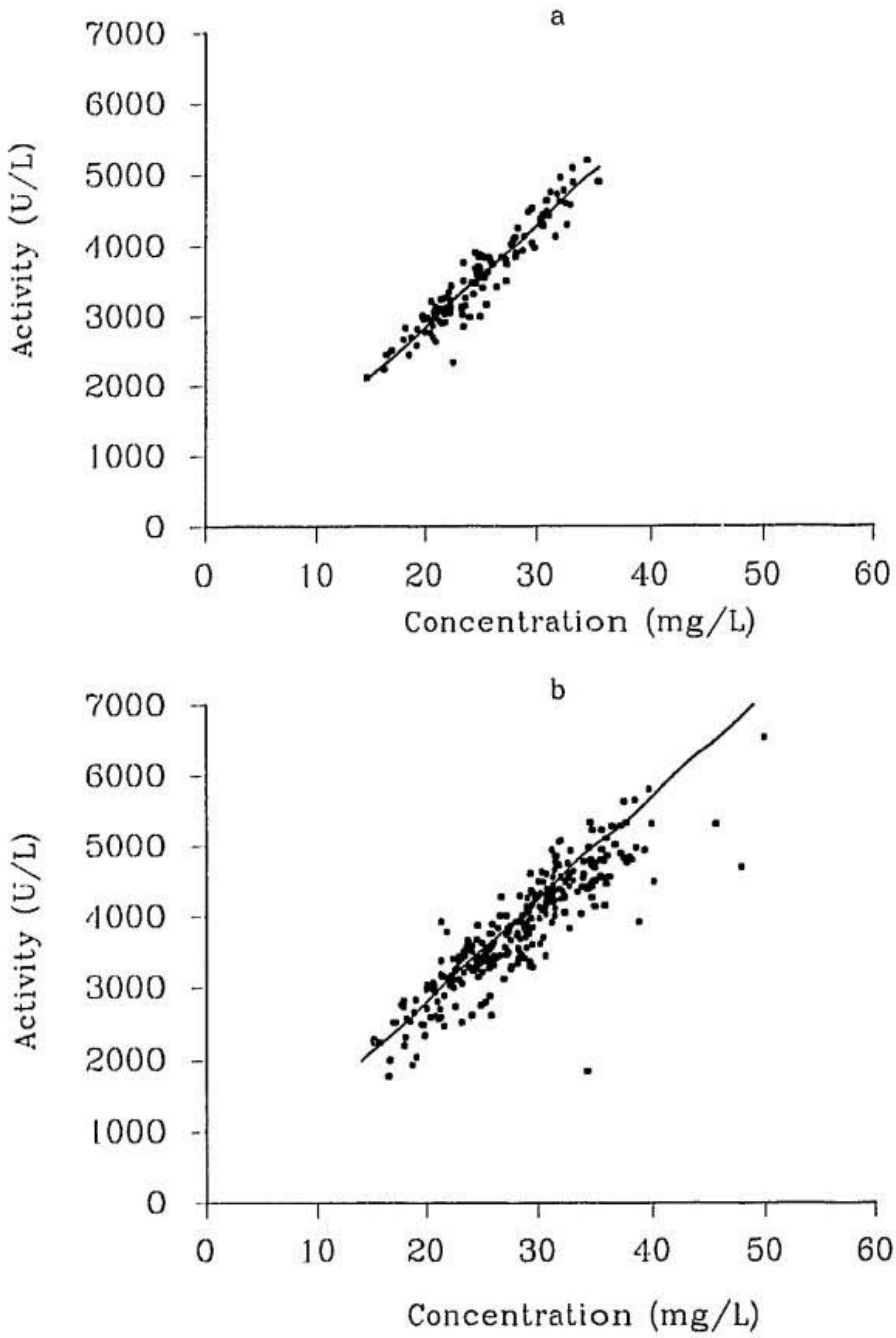


Fig. 12. Activity plotted against concentration for serum cholinesterase

(a) 117 blood donors: (b) 282 patients for whom serum lipid profiles had been requested. The regression line calculated from the blood donor results has been drawn on both graphs.

Samples standing out, and those furthest from the line on the border of the main group were retested. The original estimates were always kept for all future calculations unless the repeat result was substantially closer to the expected value, in which case that result was substituted. The figure shows the results after this had been done. Thus the results appear to show that the mean specific activity of serum cholinesterase for the patients was lower than that for the blood donors and that for some individuals it was much lower. The next two questions then had to be of whether there was a real difference in specific activity and, if so, what the cause might be.

5.4 Comparison of specific activities

The mean specific activities (1 standard error in parentheses) of the 117 donors and the 209 matched patients were 141.7 (0.83) and 134.3 (0.90). Applying Student's t test, $t = 5.98$, $p < 0.0001$. However, this result is not valid because it assumes that the individual measurements were independent, and of course they were not. Activity measurements were grouped in runs and concentration measurements were grouped in plates. As has been mentioned in Section 4.3.1.2 (p. 143) between-run variation in the initial measurements of activity of the patient samples resulted in statistically significant differences in mean specific activity between runs though none was likely in reality. When the activity measurements were all repeated, donor and patient samples were put into the same runs so that between-run variation could not be held to account for a difference between the groups. The concentration measurements, however, had been made with donor and patient samples on different plates and so provided a possible source of difference in specific activity estimates.

The specific activity estimates for patients and blood donors derived from the concentration results on the individual radial immunodiffusion plates, as described in Section 4.3.2.2 (p. 151), are compared in Table VI. The two plates of normolipidaemic patients had mean specific activities within the range of the means of the donor plates but 9/12 of the plates of hyperlipidaemic patients had

Table VI. Comparison between blood donors and patients of mean specific activity estimates of samples whose serum cholinesterase concentration was measured on the same gel

Blood donor plates using 5 μL serum			
N	Mean	S.D.	C.V.(%)
18	140.9	8.3	5.9
14	141.0	8.4	6.0
18	150.7	11.0	7.3
18	138.2	9.6	7.0
18	146.6	5.6	3.8
18	138.6	12.7	9.1
16	143.4	9.3	6.5
Blood donor plates using 3 μL serum			
N	Mean	S.D.	C.V. (%)
18	143.8	9.7	6.8
18	144.0	8.3	5.8
14	140.4	10.4	7.4

Normolipidaemic Patients (5 μL serum)			
N	Mean	S.D.	C.V.(%)
18	137.2	9.8	7.1
18	148.1	5.8	3.9
Hyperlipidaemic patients with normal ChE activity (3 μL serum)			
N	Mean	S.D.	C.V.(%)
18	128.5	12.5	9.7
18	138.8	10.6	7.6
18	128.9	6.3	4.9
18	127.5	8.5	6.7
16	124.1	15.4	12.4
14	131.8	14.2	10.8
Patients with high serum cholinesterase activity (3 μL serum)			
N	Mean	S.D.	C.V.(%)
17	142.0	10.4	7.3
15	131.7	9.1	6.9
18	143.1	8.7	6.1
16	136.6	15.4	11.2
18	131.4	9.0	6.8
18	131.7	7.7	5.8

In those cases where less than the 18 possible samples on a plate are recorded, this is because the other wells were either irregular and could not be used or contained samples in a different category. If less than 14 of the samples were of the same category the plate was excluded from this comparison. For example, 12 samples from normolipidaemic patients were on a plate with 3 samples selected because of low lipid concentrations, 2 with low cholinesterase activity and 1 blood donor sample. (ChE = serum cholinesterase.)

means below the lowest one of the 10 donor plates. This strongly suggests that the difference between patients and donors cannot be attributed to chance, despite the size of between-plate variation. It may also be noted that the coefficients of variation are generally larger in the hyperlipidaemic patient plates as might be expected from the distributions in Fig. 12 (p. 160). The two donor plates with the highest variation are those on which the one outstanding sample (see p. 159) appeared.

5.5 Investigation of reduced correlation in patients

In order to determine whether the reduced correlation for patients could be caused by one of the serum lipids or lipoproteins inhibiting the enzyme or interfering with the activity assay, the expected activities of all the patient samples were calculated from the donor regression equation. The differences between observed and expected were then compared with the lipid indices. No correlation was found between the difference and any lipid index.

Another possibility was that the reduced, and more variable, specific activity in the patient samples could be caused by aging of the samples. The blood donor samples were handed over for this study one or at most two days after they were taken, being refrigerated till then and frozen from then onwards. The patient samples however were kept by the hospital laboratory for several weeks before being handed over (the longest seven weeks) and were only refrigerated during that time. However, serum cholinesterase is a very stable enzyme. Whittaker (1986) reviewed reports on this. The enzyme has been found to be stable for at least several weeks at 0-5°C and several years when frozen. As a check, 55 blood donor samples refrigerated at 4°C were compared with the 117 (frozen) samples used in this study. The concentrations were estimated when the samples were 7 months old and the activities were assayed when they were a year old, the assays being done at the same time that the patient and donor specimens used in this study were remeasured. The results are shown in Table VII.

Table VII. Serum cholinesterase ageing at 4°C

		N	Mean	S.D.	S.E.	t	p	DF
CONCENTRATIONS	Frozen	117	24.93	4.63	0.43	0.29	0.77	84
	Refrigerated	55	24.66	6.05	0.82			
ACTIVITIES	Frozen	117	3562	704	65.1	2.58	0.01	104
	Refrigerated	55	3262	715	96.4			

Comparison of the serum cholinesterase concentrations and activities of blood donor samples kept refrigerated with ones kept frozen. The concentrations were measured when the refrigerated samples were 7 months old and the activities when they were 1 year old.

Key: t, two-sample t similar to Student's t; p, probability of means being as different by chance; DF degrees of freedom. The DF are different in the two comparisons despite the numbers being the same because in this test they are calculated from the variances of the samples.

As the concentrations of the two sets of samples are the same, the figures suggest that the mean activity diminished. The activities of the old donor specimens were measured when they were fresh but those results could not be compared with the later results because of between-run variation and change in batches of quality-controls (see Section 4.3.1.2, p. 143) so this comparison between the two sets of donor samples is the best that could be done. Just as there is a small possibility that the apparent difference in specific activity between donors and patients was caused by between-plate variation in concentration, there is a possibility that there was actually no detctable loss in activity of the old donor samples but that there was a difference in mean concentration which the concentration assay failed to detect. This possibility is greater because there were less old donor samples than patient samples. However, if we assume that there was real loss of activity, this loss comes to 8.42% in 357 days. If this loss was linear then in 41 days this would have been 0.97%. Actually the loss should be exponential, but with an asymptote of zero this would make virtually no difference over such a short period in comparison to the obviously long *in vitro* half-life of the enzyme.

Thus our estimate is that slow decay of serum cholinesterase activity at 4°C might account for an approximately 1% difference in mean specific activity between the blood donor and patient samples. This is not enough to account for the observed difference, so again we come to the conclusion that there probably was a real difference between patients and donors. An additional factor is that some of the patient samples come to the laboratory by post. Whittaker (1986) says that 'plasma cholinesterase is able to withstand the rigours of most postal services', but is possible that the lower correlation of cholinesterase activity with concentration for patients than for donors could be due to this. It might also be part of the explanation for the lower correlations of cholinesterase activity than cholinesterase concentration with serum lipid indices (see 5.7.1, p. 173) but it will also be shown that the concentration of the enzyme correlates less well with serum lipids for patients than for blood donors and that for this there is a different explanation.

One other source of variation for which to check was a sex-difference in correlation in the patients. The correlation between activity and concentration in the women was 0.93 but in the men 0.80. Plotting the two measures for the two sexes, Fig. 13, shows the reason. By chance presumably, all of the four results furthest below the regression line were from male patients. The important question was of whether there was any difference in specific activity between the sexes. Specific activities were calculated for all the individuals and the means calculated (rather than dividing mean activity by mean concentration). The results are given in Table VIII. They show no difference in specific activity between the sexes. This is not subject to between-plate error since the samples from the male and female patients were interspersed at random on the same Petri dishes for concentration measurement but, as discussed above, the actual size of the difference in specific activity between patients and blood donors, if real, should not be judged from these figures. It is evident that the points furthest from the line disturb the correlation result more than the mean and it should be noted that for the women patients, not just for the men, the standard deviation of specific activity is considerably larger than that found for the donors.

5.6 Serum cholinesterase in hyperlipidaemia types

The mean cholinesterase results of the consecutive age-matched patients categorized by hyperlipidaemia types are shown in Tables IX and X. The relationships between the means of concentration in the different groups closely mirror those of the activity means. The blood donors had not been asked to fast overnight before the blood was taken, as is usual for serum lipid profiles, but their serum lipids were examined. Only cholesterol, triacylglycerols and 'total LDL' were measured. The Janeway Hospital Laboratory was not asked to perform lipoprotein electrophoresis and type classification on the samples, but rough classification was possible on the basis of the indices measured. The same maxima and minima of serum cholesterol and triacylglycerol for the groups were used as for the patients. The results for the donors are given in Tables XI and XII.

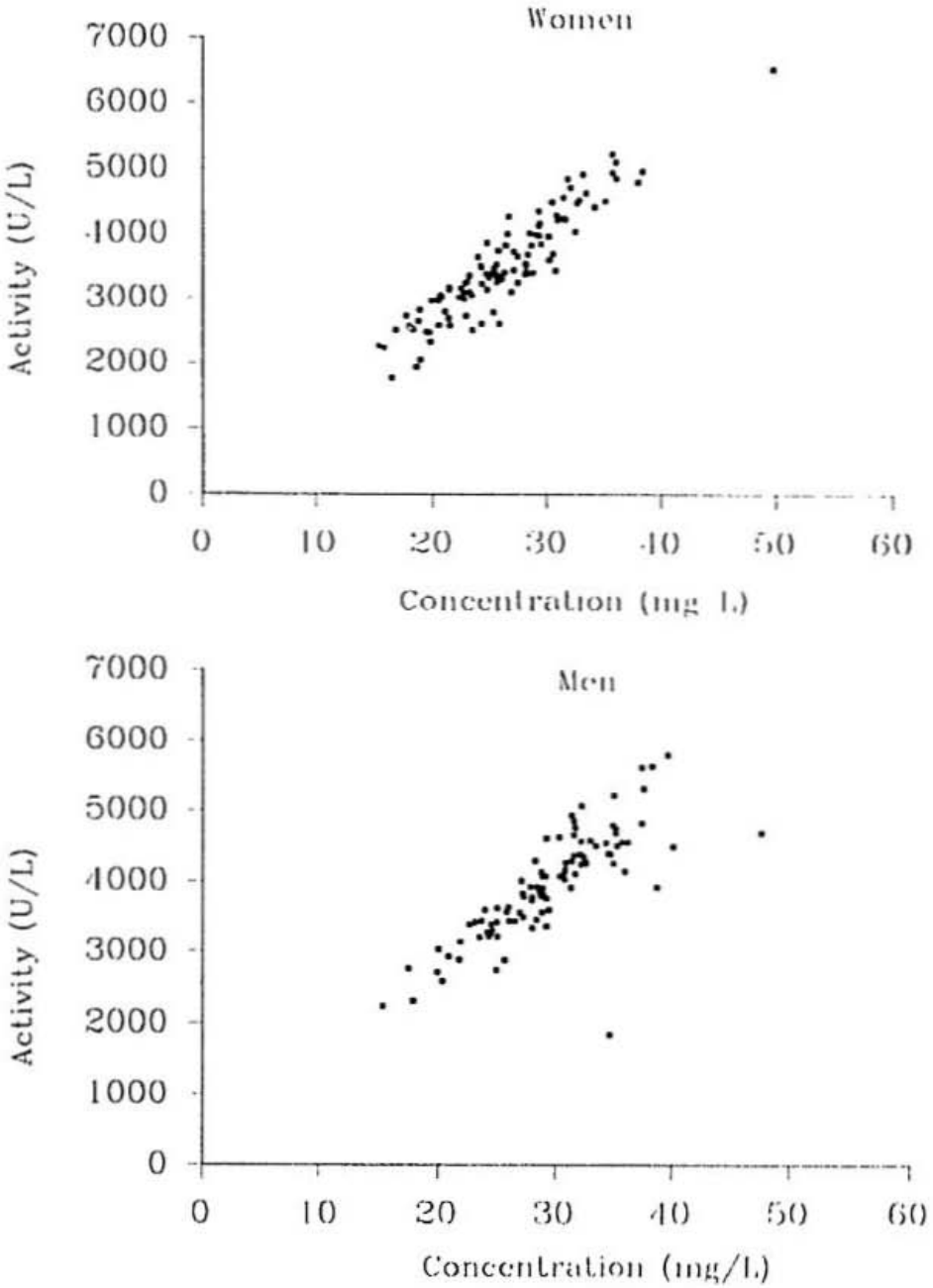


Fig. 13. Activity plotted against concentration for serum cholinesterase for the matched patients divided by sex

Table VIII. Specific activities of serum cholinesterase for male and female patients compared with blood donors

	N	Mean	Min.	Max.	S.D.	S.E.
Blood donors	117	141.7	102.6	158.7	9.0	0.83
Female patients	112	134.3	101.1	160.2	12.1	1.14
Male patients	97	134.4	53.3	157.6	14.2	1.44

Table IX. Serum cholinesterase activity in the common serum lipid categories drawn from the age-matched patients

Type	N	Mean	Min.	Max.	S.D.	S.E.	Difference between means	Sig.
Norm	35	3186	2242	4635	560	94.6	Norm & IIa	0.0004
IIa	72	3666	1778	5228	771	90.9	IIa & IIb	N.S.
IIb	70	3868	2051	5806	761	91.0	IIa & IV	N.S.
IV	30	4037	2482	6542	950	174	IIb & IV	N.S.

Norm:normolipidaemic Significance test: two-sample t. N.S.: not significant.

Table X. Serum cholinesterase concentration in the common serum lipid categories drawn from the age-matched patients

Type	N	Mean	Min.	Max.	S.D.	S.E.	Difference between means	Sig.
Norm	35	22.2	15.4	30.4	3.75	0.63	Norm & IIa	<0.0
IIa	72	28.0	16.6	40.2	4.84	0.57	IIa & IIb	001
IIb	70	28.9	18.0	47.7	5.39	0.64	IIb & IV	N.S.
IV	30	30.4	17.6	50.0	6.68	1.22	IIb & IV	N.S.

Table XI. Serum cholinesterase activity in the common serum lipid categories drawn from the blood donors

Type	N	Mean	Min.	Max.	S.D.	S.E.	Difference between means	Sig.
Norm	56	3265	2112	5195	602	80.4	Norm & IIa	N.S.
IIa	26	3477	2657	4959	603	118	Norm & IIb	<0.0001
IIb	24	4036	2834	5084	596	122	IIa & IIb	0.0019
IV	11	4236	2897	4898	696	210	IIa & IV	0.0062
							IIb & IV	N.S

Table XII. Serum cholinesterase concentration in the common serum lipid categories drawn from the blood donors

Type	N	Mean	Min.	Max.	S.D.	S.E.	Difference between means	Sig.
Norm	56	23.3	14.9	34.9	3.96	0.53	Norm & IIa	N.S.
IIa	26	24.4	17.9	33.0	4.16	0.82	Norm & IIb	<0.0001
IIb	24	28.1	18.1	33.5	3.85	0.79	IIa & IIb	0.0017
IV	11	29.7	21.6	35.7	4.24	1.28	IIa & IV	0.0024
							IIb & IV	N.S.

In the donors too, the concentration means closely mirror the activity means. Logically speaking, one should say that activity mirrors concentration, but historically, activity was studied first. It was noted that there is a difference in pattern between the patients and the donors. In both, the means of serum cholinesterase activity and concentration ascend in the order normolipidaemics, type IIa, type IIb, type IV, but whereas in the donors the means in type IIa are closer to those of the normolipidaemics, as found by most other investigators, in the patients the type IIa means are highly significantly higher than in the normolipidaemics and not significantly lower than those of type IIb and IV. The patient data were therefore divided to see whether there was a sex difference in this. The results are shown in Table XIII.

In activity and concentration measurements the mean for every group is higher for men than for women. In concentration for both sexes and in activity for women the mean for type IIa is significantly different from that for normolipidaemia. In activity for men the mean for type IIa is closer to that of the normolipidaemics than to that of type IIb but neither difference reaches significance. Between the hyperlipidaemic groups, the means for type IIb are the same as those of IIa for women but the same as those of type IV for men, but the differences between IIa and IV are not significant. Partitioning data in several ways at once always leads to reduction in numbers in individual cells so that eventually statistically meaningful conclusions cannot be reached. Having eliminated the data of patients outside the age-limits and then partitioned by lipidaemia type and by sex the numbers in the groups are rather small.

The term 'significance' used in this section refers to probability calculated by Student's t test. The rationale for accepting the validity of the test for these data is that sera from individuals belonging to the different lipidaemia types were distributed at random in the runs and plates of activity and concentration measurements, and the same goes for the sexes. This is completely true of the blood donors, but in the patient group the one exception is that the cholinesterase concentrations of the normolipidaemic patients were measured on

Table XIII. Comparison of mean serum cholinesterase activities and concentrations in different serum lipid groups between sexes for patients

		Women									
		Activity					Concentration				
Type	N	Mean	S.D	S.E.	Difference between means	Sig.	Mean	S.D.	S.E.	Difference between means	Sig.
Norm	24	3080	512	104	Norm & IIa IIa & IV	0.0023 N.S.	21.3	3.4	0.7	Norm & IIa IIa & IV IIb & IV	<0.0001 N.S. N.S.
IIa	44	3590	807	122			27.2	4.9	0.7		
IIb	34	3598	121	121			27.1	4.3	0.7		
IV	9	3826	1279	426			29.4	9.6	3.2		
		Men									
		Activity					Concentration				
Type	N	Mean	S.D	S.E.	Difference between means	Sig.	Mean	S.D.	S.E.	Difference between means	Sig.
Norm	11	3417	614	185	Norm & IIa Norm & IIb IIa & IIb	N.S. 0.0048 N.S.	24.1	3.8	1.2	Norm & IIa IIa & IV	0.0022 N.S.
IIa	28	3786	707	134			29.1	4.6	0.9		
IIb	36	4124	729	121			30.7	5.8	1.0		
IV	21	4128	792	173			30.8	5.2	1.1		

different plates from the rest. However, as we have seen, the pattern of concentrations between lipidaemia groups was the same as for activities (Tables IX and X) so this does not appear to have made a serious difference.

5.7 Cholinesterase and lipid indices

5.7.1 Correlations

The correlations between the serum cholinesterase measurements and the serum lipid indices are shown in Table XIV. For the patients with the sexes pooled the best correlations were with 'total LDL' (LDL + VLDL) and within that were better with VLDL than with LDL. The correlations were lower with triacylglycerols than with VLDL and about the same with total cholesterol as with LDL and with LDL-cholesterol. There was no significant correlation with HDL and there were negative correlations with HDL-cholesterol. The correlations were in almost the same order with concentration as with activity and in every case were better with the former even though the measurement error was greater with concentration.

It should be remembered that the LDL-cholesterol concentration was calculated by the Friedwald equation (4.1.7, p. 133) which is not accurate for cases with triacylglycerol concentrations over 4.56 mmol/L or those with chylomicrons or type III hyperlipoproteinaemia. These restrictions required the exclusion (for this index only) of 25 of the 209 patients, one with type III and 24 with high triacylglycerols, of whom one was type V. When the calculation was made for the excluded patients, two sets of data gave negative results, one with type IV and a triacylglycerol of 9.3 mmol/L, the other with type V and a triacylglycerol of 15.5 mmol/L. If only these two were excluded the correlations fell to 0.21 and 0.27 with the activity and concentration of cholinesterase respectively. However, it will be shown below that the exclusion of individuals with high triacylglycerols improves the correlations not only of LDL-cholesterol with serum cholinesterase but of triacylglycerols with cholinesterase. The reason for this will be discussed later.

Table XIV. Correlations of serum cholinesterase with lipid indices for patients and blood donors

	Patients (N = 209)				Blood Donors (N = 117)			
	Activity		Concentration		Activity		Concentration	
	r	p	r	p	r	p	r	p
LDL	0.36	<0.001	0.40	<0.001	-	-	-	-
VLDL	0.43	<0.001	0.45	<0.001	-	-	-	-
'Total LDL'	0.46	<0.001	0.51	<0.001	0.60	<0.001	0.61	<0.001
Triacylglycerols	0.38	<0.001	0.42	<0.001	0.51	<0.001	0.52	<0.001
Cholesterol	0.35	<0.001	0.41	<0.001	0.48	<0.001	0.48	<0.001
LDL-cholesterol *	0.33	<0.001	0.41	<0.001	-	-	-	-
HDL-cholesterol	-0.29	<0.001	-0.30	<0.001	-	-	-	-
HDL	-0.05	N.S.	-0.01	N.S.	-	-	-	-

Correlation coefficients (r) and respective probabilities (p) of serum lipid indices with serum cholinesterase activity and concentration in the blood donors and matched patients. (* For LDL-cholesterol N = 184 after exclusion of cases in which the Friedwald formula cannot give accurate predictions. See 4.1.4)

For the blood donors the correlations with the three lipid indices measured were in the same order as for the patients but were all greater. The correlations between each lipid index and the two cholinesterase measures were much closer than for the patients, being only slightly better with concentration for 'total LDL' and triacylglycerol.

When the patient data was divided by sex - Table XV - the correlation results were found to be considerably better for women than men. The reason for this is apparent from examination of the plots of serum cholinesterase against lipids (next section). The order of size of the correlation coefficients is the same as in the whole data except that, in women only, the results for LDL-cholesterol are much higher. Inclusion of the only six excluded patients who were women reduces the correlations of this index with cholinesterase activity and concentration to 0.34 and 0.42 respectively, i.e. to less than the correlations of total cholesterol with cholinesterase, as for men. For men, inclusion of the seventeen excluded patients whose results were not negative reduces the correlations to 0.11 and 0.19 respectively, neither of which is significant.

5.7.2 Distributions

Taking the two measures with the best correlation, serum cholinesterase concentration is plotted against 'total LDL' for the patients in Fig. 14. The addition of the 73 extra patients excluded from the correlation calculations (b) helps to fill some of the gaps in the distribution and does not alter the shape. We now see why the correlation is not higher; the relationship is not linear. With low levels of 'total LDL' the cholinesterase concentration tends to be low. With increasing 'total LDL' there is a steady slow increase in the minimum cholinesterase found whereas the maximum cholinesterase found rises steeply up to about 3 g/L of LDL (still a low level) and then remains approximately constant except for three very high cholinesterase results. Thus, though with the lowest 'total LDL' levels the cholinesterase is low, with moderate levels of the former there is a wide range of cholinesterase concentrations. This range is progressively diminished

Table XV. Correlations of serum cholinesterase with lipid indices for male and female patients

	Women (N = 112)				Men (N = 97)			
	Activity		Concentration		Activity		Concentration	
	r	p	r	p	r	p	r	p
LDL	0.41	<0.001	0.47	<0.001	0.31	<0.01	0.34	<0.001
VLDL	0.43	<0.001	0.49	<0.001	0.37	<0.001	0.37	<0.001
'Total LDL'	0.49	<0.001	0.56	<0.001	0.39	<0.001	0.43	<0.001
Triacylglycerols	0.42	<0.001	0.47	<0.001	0.31	<0.01	0.35	<0.001
Cholesterol	0.42	<0.001	0.49	<0.001	0.32	<0.01	0.37	<0.001
LDL-cholesterol*	0.45	<0.001	0.56	<0.001	0.24	<0.05	0.37	<0.01
HDL-cholesterol	-0.26	<0.01	-0.31	<0.001	-0.18	N.S.	-0.15	N.S.
HDL	+0.08	N.S.	+0.11	N.S.	-0.04	N.S.	+0.03	N.S.

Correlation coefficients of serum lipid indices with serum cholinesterase activity and concentration in the matched patients divided by sex. (* N = 106 for women and 78 for men for LDL-cholesterol after exclusions. See Table XIV legend.)

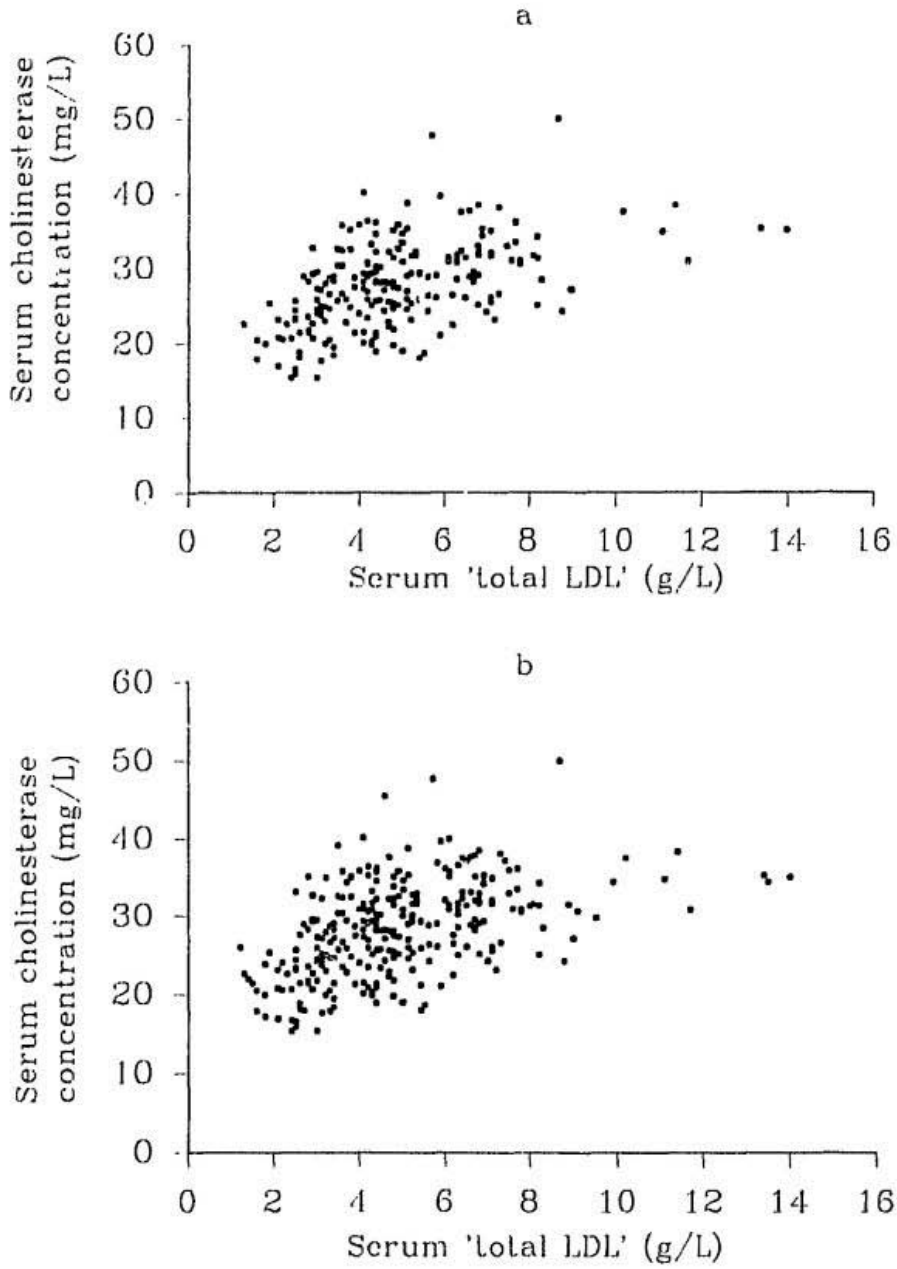


Fig. 14. Distribution of serum cholinesterase concentration against serum 'total LDL' concentration in patients

(a) the 209 consecutive samples for patients in the same age range as the donors; (b) all of the 282 patients measured

from the lower end as 'total LDL' increases so that with the highest lipid levels only high cholinesterase is found.

The plots of cholinesterase concentration against other lipid indices and the plots of cholinesterase activity against the lipid indices are similar, the shape tending to be lost as the correlation coefficient diminishes. Two examples are shown in Fig. 15.

When the data is plotted for the sexes separately, Fig. 16, it is seen that all the individuals with the highest 'total LDL' happened to have been men. The distribution is thus truncated in women and appears more linear. The same expianation applies to the higher correlation in blood donors, whose plot is shown in Fig. 17. Inspection of plots of cholinesterase against LDL-cholesterol showed that the reason for the improvement in the correlations on exclusion of individuals with high triacylglycerol concentrations was that the excluded results tended to lie in the top left quadrants of the plots. The excluded individuals tended to have high cholinesterase - which we have seen is associated with high triacylglycerols - and low LDL-cholesterol results, no doubt related to the fact that the triacylglycerol term in the equation is subtracted. The effect of exclusion of these individuals was thus to make the plots less triangular and more linear. However, the reason for the correlations of LDL-cholesterol with the two cholinesterase measurements being greater for women than for men or for the sexes combined, and better than most other indices in women but not in men was not apparent. The results of the sexes are compared in Fig. 18. Because of uncertainty as to the actual LDL-cholesterol concentrations of the excluded individuals, this index is not considered further.

5.8 The effect of using logarithms

Cucuianu *et al.* (1975) stated that because of very accentuated skew in the distributions of serum triacylglycerols and prebeta lipoprotein concentrations these data were transformed to their logarithms for statistical purposes. They quoted the correlations of serum cholinesterase activity with the logarithms of

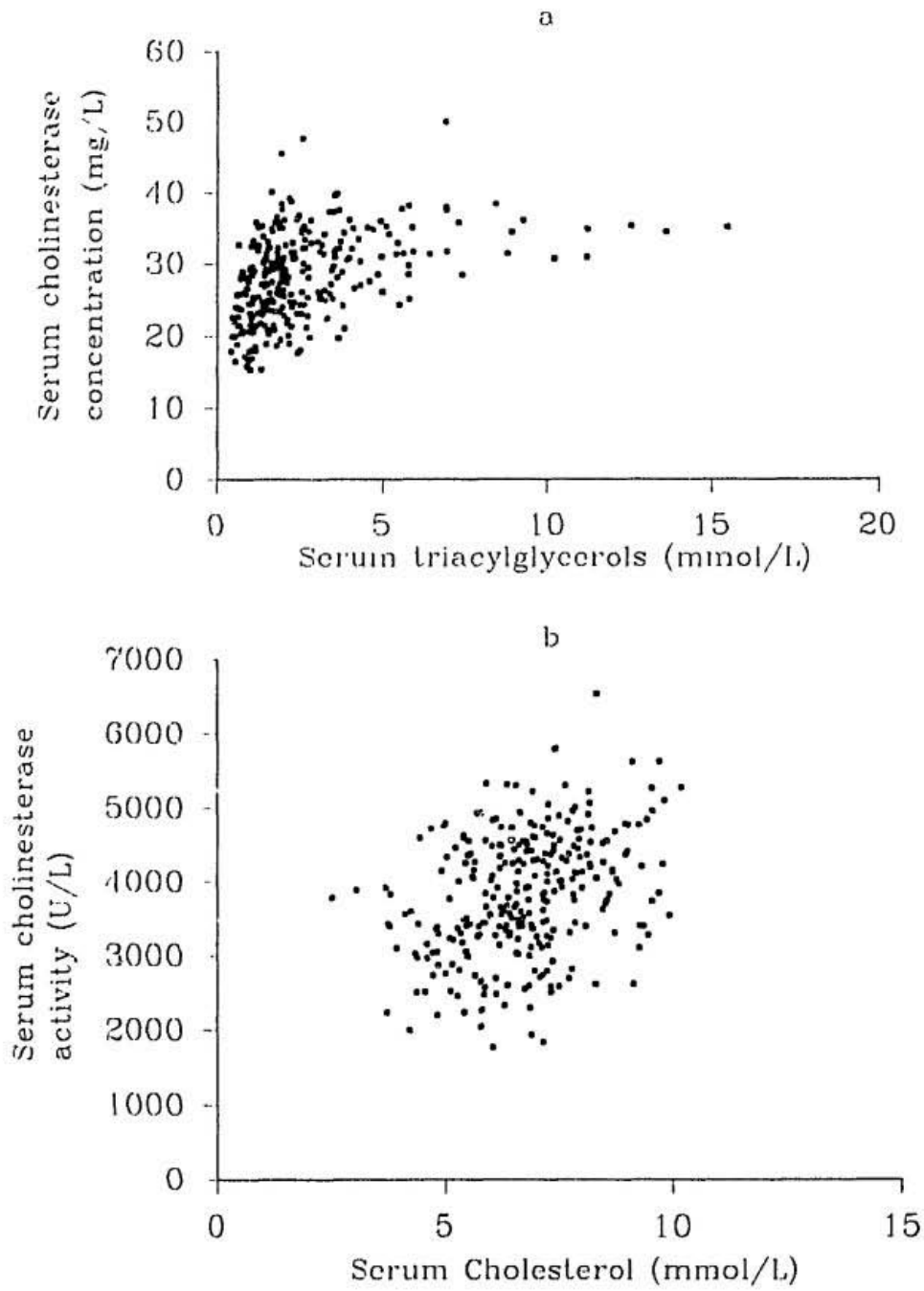


Fig. 15. Other examples of plots of serum cholinesterase against lipid indices

(a) concentration of the enzyme against triacylglycerol concentration; (b) cholinesterase activity against cholesterol concentration

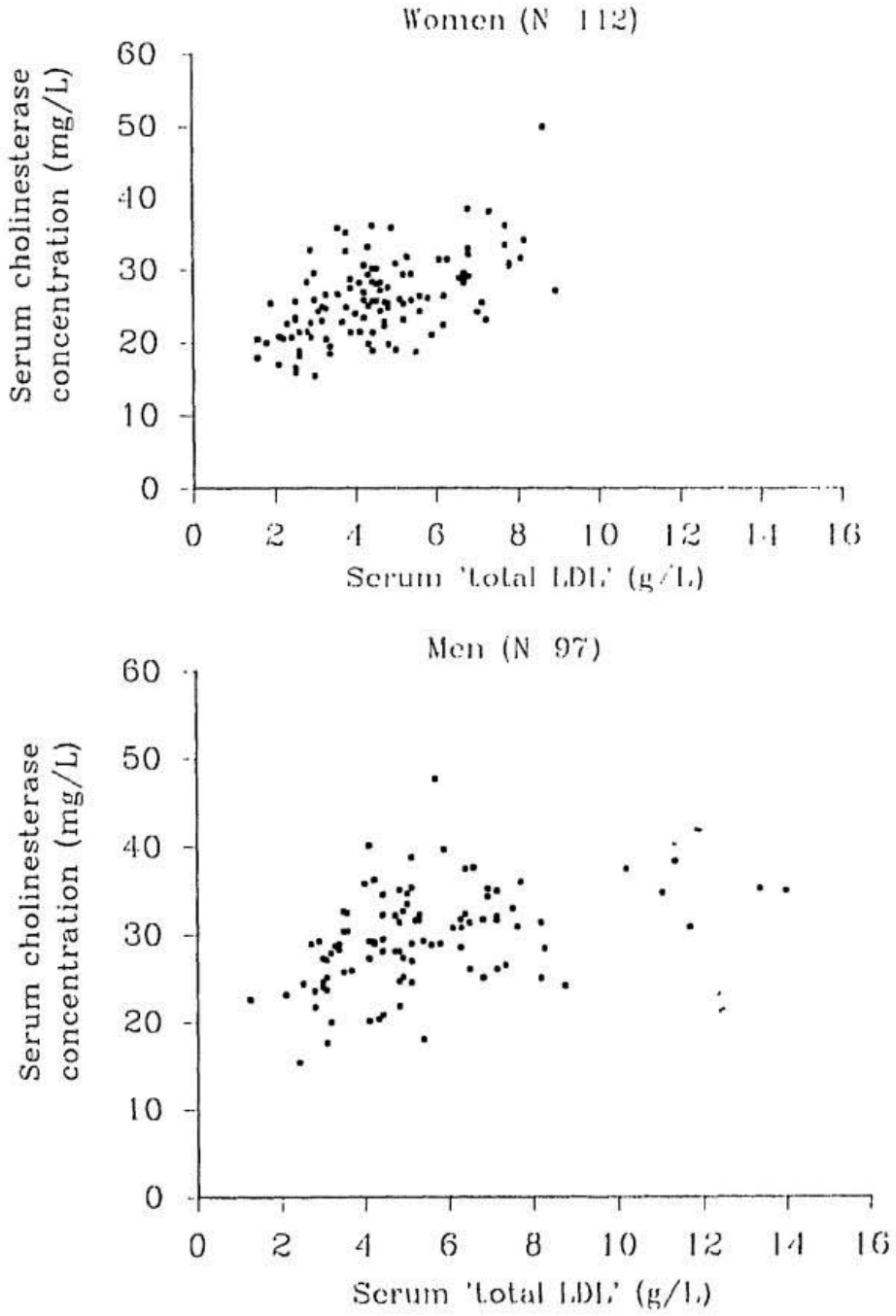


Fig. 16. Distributions of serum cholinesterase concentration against serum 'total LDL' concentration for the 209 patients divided by sex

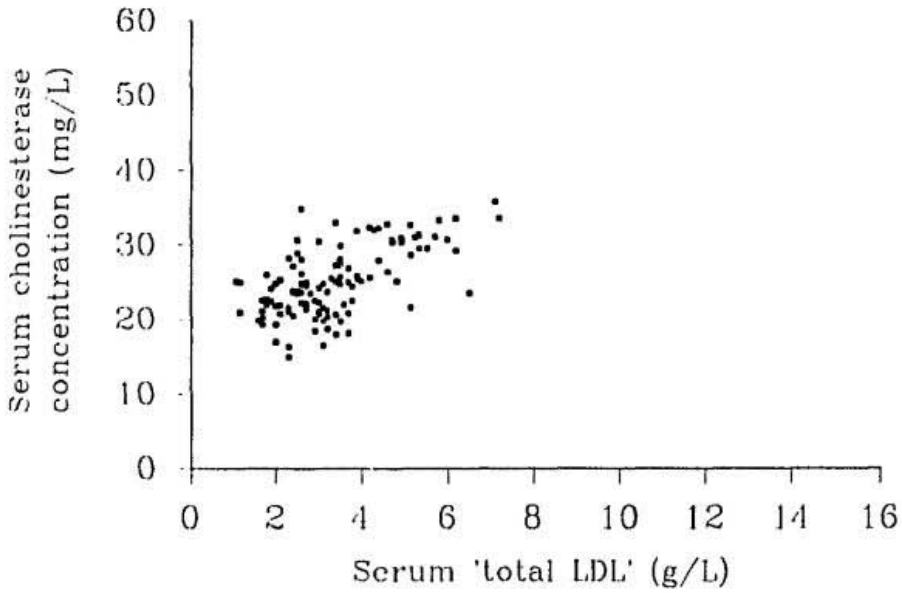


Fig. 17. Distribution of serum cholinesterase concentration against serum 'total LDL' concentration for the blood donors

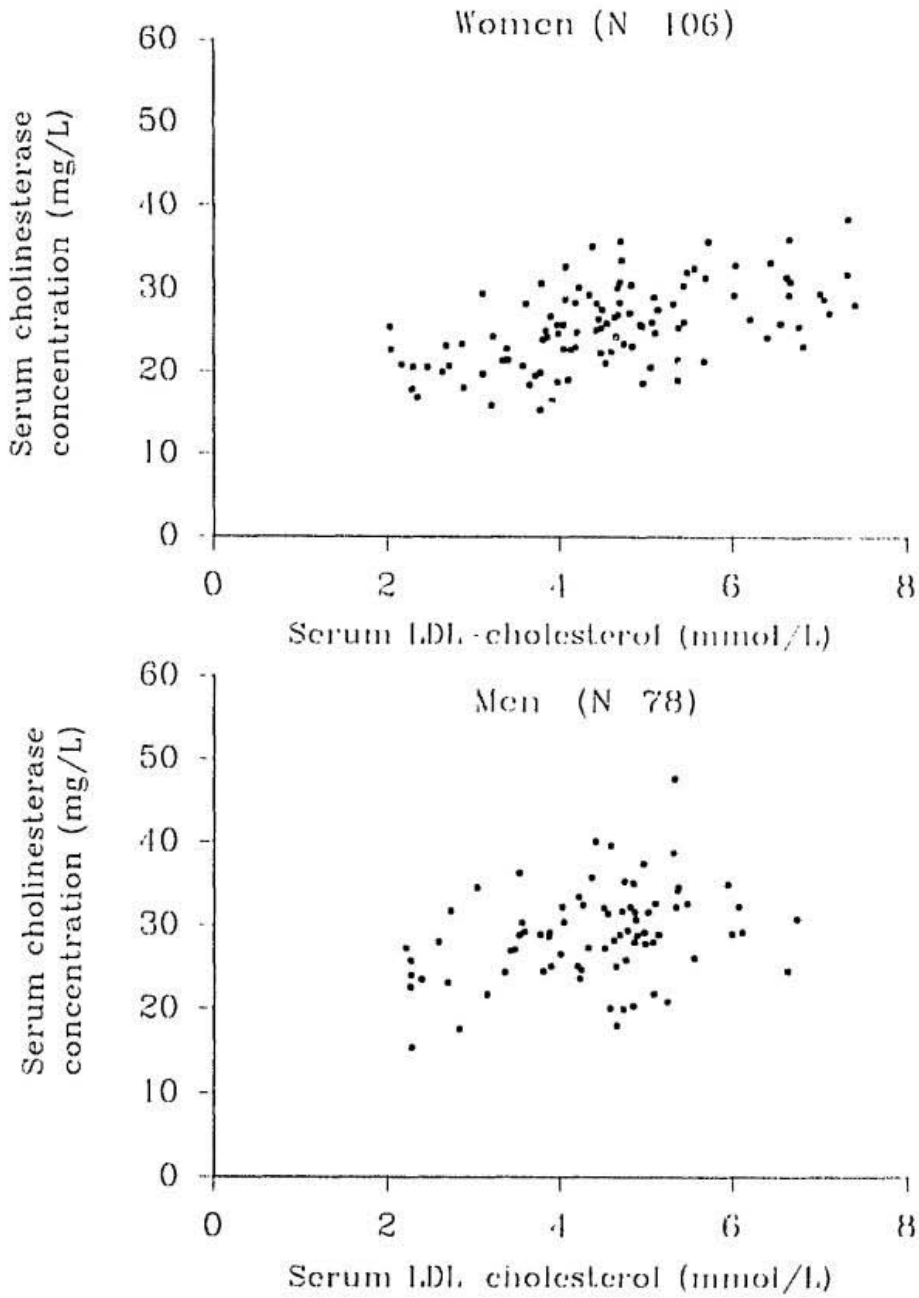


Fig. 18. Distributions of serum cholinesterase concentration against serum LDL-cholesterol concentration calculated by the Friedwald equation

these lipid indices. They also mentioned that the increase of serum cholinesterase activity tended to flatten with increasing triacylglycerol. They said that it was their belief that the cholinesterase activity was correlated with triacylglycerol secretion rate and that the flattening occurred because of high triacylglycerol concentrations due to decreased removal rather than increased secretion.

Cucuianu *et al.* (1976) gave correlations of cholinesterase activity with serum triacylglycerol and prebeta lipoprotein and their logarithms side by side, showing considerable improvement in the correlation coefficients on using the logarithms (0.252 to 0.447 for triacylglycerols and 0.252 to 0.505 for prebeta lipoproteins). They also gave a graph of cholinesterase activity against triacylglycerol to show the flattening to which they had referred. They did not show the raw data, as in Figs. 14 - 17 (pp. 177, 179, 180 and 181), but divided the results into quintiles of triacylglycerol and calculated the means for each quintile. The result was a convex curve with a fairly linear increase in cholinesterase activity with triacylglycerol turning to a plateau at higher levels. Their work prompted an investigation of the effect of taking logarithms on the data presented here.

Fig. 19 shows histograms of the five lipid indices correlating best with cholinesterase. Only the patients used for the correlation calculations are shown. Triacylglycerols and 'total LDL' show skewing which is more marked in the patients than in the donors. The cholesterol distributions are not significantly skewed. The VLDL distribution is more skewed than that of LDL. The effects of taking logarithms of these indices on their correlations with the measures of serum cholinesterase are shown in Table XVI.

As expected from its normal distribution, the correlations with cholesterol do not improve with logarithms. The effects on the other indices are much more slight than found by Cucuianu *et al.*, (1976) some only appearing in the third place of decimals (not given). For the patients the changes are at least in the

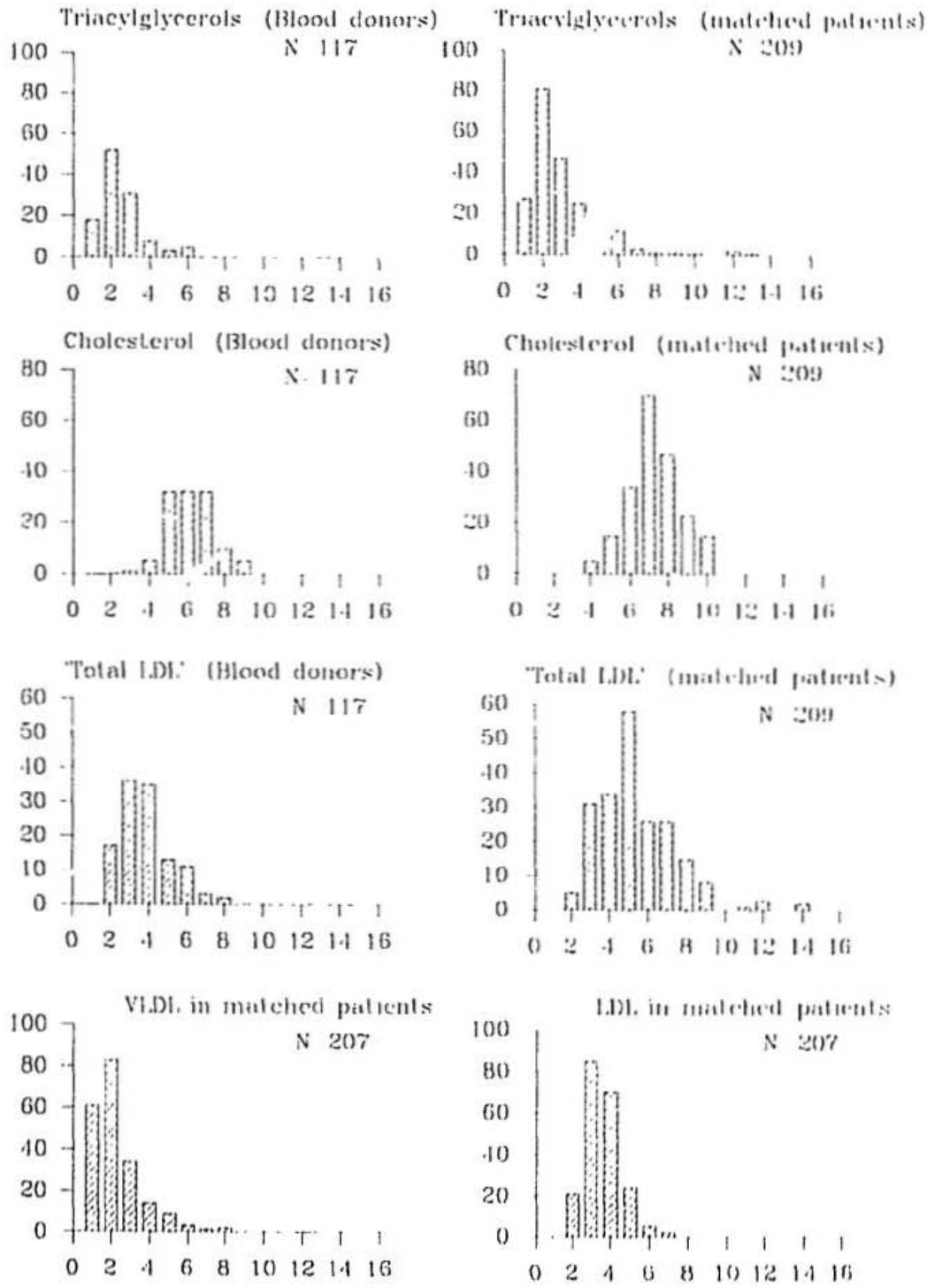


Fig. 19. Histograms to show distributions of the serum lipid and lipoprotein indices that correlate best with serum cholinesterase

Table XVI. Effect of logarithms on serum cholinesterase-lipid correlations

		LDL	Log. LDL	VLDL	Log. VLDL	tLDL	Log. tLDL	Trig	Log. Trig	Chol	Log. Chol
Activity	Patients	0.36	0.37	0.43	0.43	0.46	0.47	0.38	0.42	0.35	0.35
	Donors					0.60	0.56	0.51	0.49	0.48	0.45
Concentration	Patients	0.40	0.44	0.45	0.48	0.51	0.54	0.42	0.47	0.41	0.41
	Donors					0.61	0.56	0.52	0.51	0.48	0.45

Comparison of correlation coefficients (r) of serum cholinesterase activity and concentration with serum lipid indices against those with the logarithms of the lipid indices. tLDL = 'Total LDL'. Trig = triacylglycerol. Chol = cholesterol. All results $p < 0.001$.

same direction as those found by Cucuianu *et al.*, but for the donors the correlations become worse.

The following explanation seemed likely for these results. A convex curve is brought closer to a straight line by taking logarithms of the variable on the abcissa. The original line is curved because the individuals with the highest triacylglycerol levels do not have much higher cholinesterase than those with a little lower triacylglycerol for whatever reason. The blood donors do not include individuals with very high triacylglycerols so their curve should be much more nearly straight in the first place. The effect on a straight-line graph of taking logariths of the variable on the abcissa is to make it a concave curve. This will reduce the correlation. The theory provides an explanation of why Cucuianu *et al.* (1976) found greater improvement in correlation than was found with the data presented here. Cucuianu *et al.* had relatively more subjects with high triacylglycerol and LDL levels; Their numbers of normolipidaemic, type IIa, type IIb, type IV and Type V subjects were 24, 22, 31, 42 and 11 whereas in the series used for correlation calculations in this work the numbers were 35, 72, 70, 30 and 1.

The theory was tested. Because the best correlation was between serum cholinesterase concentration and 'total LDL' that was used instead of cholinesterase activity and triacylglycerols, and as the investigation was into the shape of the curve rather than calculation, the whole 282 patients were included. By the quintiles method, Cucuianu *et al.* (1976) arranged their subjects in order of their serum triacylglycerols. They then took the 20% with the lowest triacylglycerols and calculated the means of triacylglycerol and cholinesterase activity, and did the same for each successive 20%. With the data used here this method does not adequately represent the shape of the distribution; even with the extra data, subjects with the higher lipid levels are still relatively less represented than in the data of Cucuianu *et al.* (1976). Individuals in the top quintile of 'total LDL' - with results from 6.8 to 14 g/L - occupy more than 50% of the whole range, 1.2 to 14 g/L. To cope with this problem, instead of dividing the

number of individuals into five, the *range* of 'total LDL' was divided into fifths. This inevitably meant that the top two fifths contained small numbers of individuals, but it did serve to illustrate the shapes of the curves.

Fig. 20 shows the effect of using logarithms of 'total LDL' in the patients and donors. It illustrates that the explanation for the changes in correlation results was indeed correct. It would seem that Cucuianu *et al.* biased their correlations (unintentionally) by selecting extra subjects with high triacylglycerol levels and by using logarithms, a practice which they continued in subsequent papers.

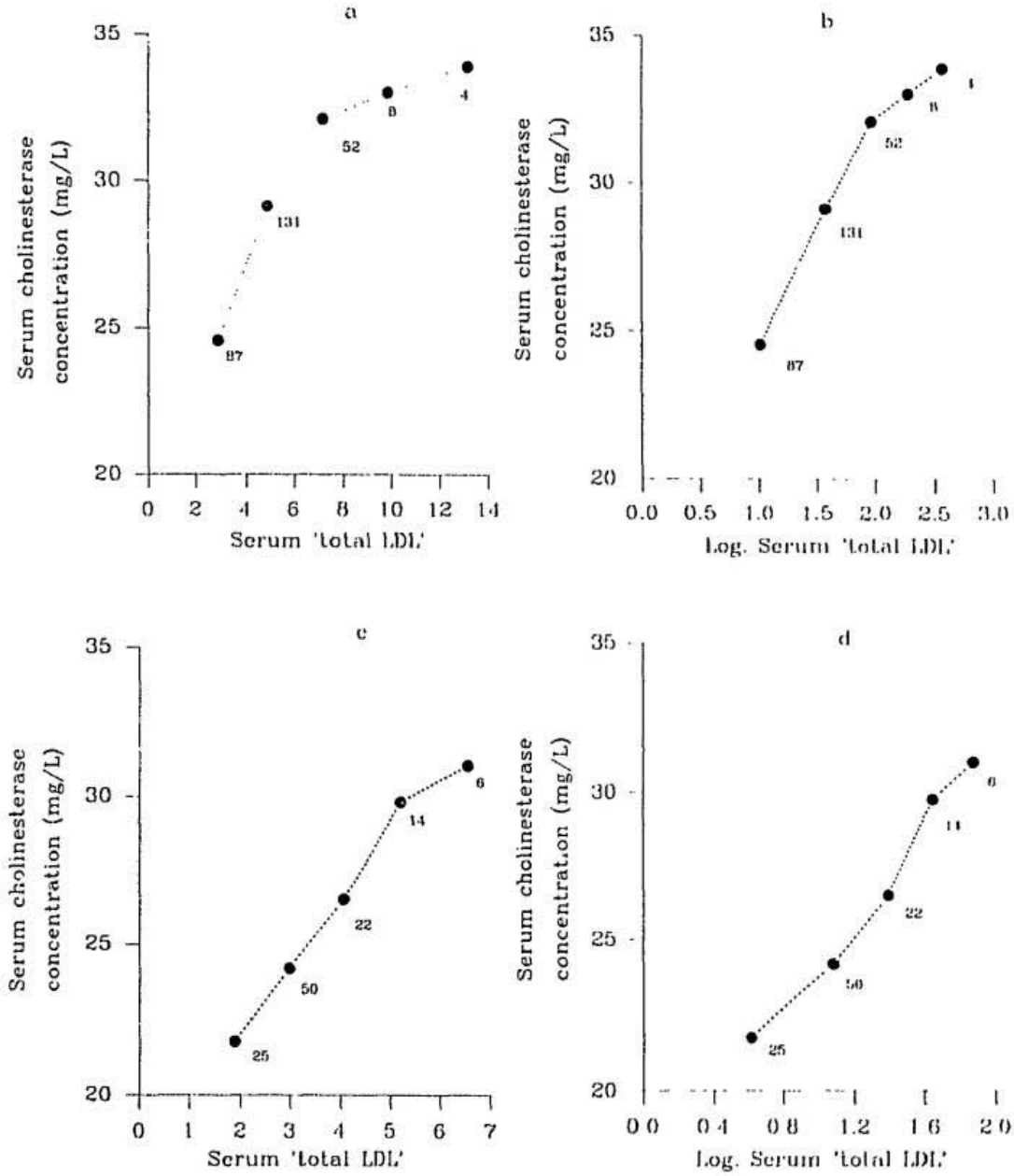


Fig. 20. The effect of taking logarithms of 'total LDL' concentration on cholinesterase-'total LDL' plot shapes

Serum cholinesterase concentration in relation to serum 'total LDL' (a,c) or its logarithm (b,d). The points are the means of the results falling into sucessive fifths of the ranges of serum 'total LDL' concentration for all the 282 patients (a,b) and blood donors (c,d). The numbers under the points represent results in that fifth of the range. Note that the horizontal scales are different for patients and donors.

Chapter 6

6.1 DISCUSSION

6.1.1 Preliminary remarks

6.1.1.1 Reliability of cholinesterase measurements

Very careful assessment was made of the errors involved in the two cholinesterase assays in order to be fully aware of the limitations to interpretation of results. Though, with hindsight, some of these errors could have been reduced, the correlation between concentration and activity compares well with with the results of the two other groups who have published correlation coefficients. Aitland *et al.* (1971), who used radial immunodiffusion (Altland and Goedde, 1970) to measure concentration and the method of Kalow and Lindsay (1955) for activity and claimed that the reproducibility of both their measurements was better than 3%, found a correlation (r) between the two of only 0.87 in 269 blood donors. Brock *et al.* (1990) measured concentration by ELISA. They measured the cholinesterase concentration of all their 33 blood donor samples and calibrators in duplicate on a single microtitre plate and quoted a within-run C.V. of 1.36% judged from the deviations of duplicates. The activity was measured by an autoanalyser method (Brock and Brock, 1990) for which their estimates of CV_{total} were 1.34%, 1.92% and 1.36% with different lots of quality control material. For the correlation between activity and concentration they quoted $r^2 = 0.90$. In the work presented here, the error in concentration measurement was certainly larger than those claimed by the other authors, even after repeating a few measurements, yet the finding in 117 blood donors was $r = 0.95$, $r^2 = 0.90$.

6.1.1.2 Sex difference in serum cholinesterase

A significant sex-difference in human serum cholinesterase activity, the mean usually higher in males, has often been reported. Propert and Brackenridge (1976) reported such a finding and reviewed other reports from

1951 onwards, some finding a difference and some not. Reports of a difference have continued (Al-Azzawi *et al.*, 1984; Yücel *et al.*, 1988; Brock, 1989; Brock and Brock, 1990), but the most pertinent report is probably that of Simpson (1966). She found that the activity in serum was significantly affected by haematocrit and weight and that when these were taken into account there was no sex-difference. Unfortunately these variables cannot be eliminated from the data presented here as they are not known, but comparison of the means of serum cholinesterase measures in the different hyperlipidaemia types, and of the orders of correlations with lipid indices in the two sexes, shows that differences in these relationships are minor and could be accounted for by chance. Thus it is safe to pool the sexes in consideration of the relationship of serum cholinesterase with serum lipids.

6.1.1.3 Normality of cholinesterase distribution

Serum cholinesterase activity was shown to have a normal (Gaussian) distribution in 451 individuals of the 'usual' phenotype (402 C_5- and 49 C_5+) by Harris *et al.* (1963). Altland *et al.* (1971) found a symetrical distribution of cholinesterase concentration in their 269 blood donors, of whom 9 had the UA genotype and 22 were C_5+. However, Yücel *et al.* (1988) found that the histograms of activity were positively skewed in both sexes (75 of each) of Turkish blood donors. In the present study the consecutive matched patient data showed normal distributons of activity and concentration in both sexes but the donor results showed a positive skew. Since the independent measures of activity and concentration agreed in this it was presumably real but, assuming that the patient data represent the true picture, the donor distribution could easily be explained by the chance occurrence of more women than men in the sample. If this was the case, the mean cholinesterase activity and concentration are a little lower than they would have been if the sexes were equally represented, but, from the evidence of the patients, this should not affect conclusions about the relationship of cholinesterase to lipids.

6.1.2 The relationship of serum cholinesterase to serum lipids

6.1.2.1 Serum cholinesterase in hyperlipidaemia types

Within the hyperlipidaemia classification groups the means of serum cholinesterase activity ascend in the same order (normolipidaemics, type IIa, type IIb, type IV) for the patients and the donors. However, for the patients the type IIa mean is highly significantly higher than that of the normolipidaemics and not significantly lower than those of type IIb and IV whereas for the donors the mean for type IIa is not significantly different from that of the normolipidaemics and is highly significantly different from those of types IIb and IV. This difference is maintained by the concentration means, and within the two sexes. The means of activity and concentration for men are higher than those of women in every group but, with minor differences attributable to smaller numbers, show the same pattern. (The only other authors to split their data by types and sexes were Schriewer *et al.* (1985) but they had very small numbers in some categories.)

All the publications giving mean serum cholinesterase activity for the hyperlipidaemia types agree that the mean is considerably higher for types IIb and IV than for subjects with normal lipid levels and is intermediate in type IIa. Where they differ is in whether the mean is higher for type IIb or type IV, and in whether that for type IIa is closer to that for normolipidaemics or the other hyperlipidaemic groups. Our patient data was classified with the benefit of lipoprotein electrophoresis while the donor data was not, but this does not seem to account for the difference. Cucuianu *et al.* (1975, 1976, 1978, 1985a, b) found the same pattern as in our blood donors and they did use electrophoresis. Schriewer *et al.* (1985) also found the activity mean for IIa closer to that for normolipidaemics but that for type IIb greater than that for type IV, and they did not do electrophoresis. Reuter and Geus (1987a, b) found IIa closer to IV, and IIb higher than IV, but did not give their lipid methods. Jain *et al.* (1983) found the mean for type IIa almost half way between those for normolipidaemia and type IV (slightly nearer to normolipidaemia) and also found the mean higher for IIb than

for IV, but their work was done in the same laboratory in which the cholinesterase activity and lipid measurements presented here were done. It would seem therefore that these differences would not be resolved without large numbers of subjects and are not substantial enough to be important. The main conclusions that can be made are that the differences in means of activity between groups are underlain by differences in concentration and that though men have higher serum cholinesterase than women the enzyme varies in a similar way with respect to lipid levels.

6.1.2.2 Correlation of serum cholinesterase with serum lipids

Serum cholinesterase concentration and activity correlated almost equally well with serum lipids in the blood donors. In the patients, activity correlated less well with concentration than it did in the donors, and less well with lipids than did concentration. Probably the reason for both of these was the same. If the only reason is that there had been some decay in activity *in vitro* then we can say nothing about whether cholinesterase might be actively involved in lipid metabolism from this. However, one difference between the patients and blood donors is that the donors would not have been taking medications whereas some of the patients would have been. Some of these would have been likely to have been drugs known to cause partial inhibition of serum cholinesterase, such as beta-blockers, whose uses include the treatment of hypertension, angina, and cardiac dysrrhythmias and secondary prevention of myocardial infarction. This is particularly relevant since most of the patients either had or had had hyperlipidaemia, with which cardiovascular disease is known to be associated.

Even if drugs only account for part of the difference between the correlations of cholinesterase concentration and activity with lipids, this would be evidence that the association of serum levels of cholinesterase and lipids is not due to active involvement of the enzyme in lipid metabolism. Since the measurement error was greater with concentration than with activity one would

expect the correlations of concentration with lipids to be poorer than those of activity with lipids in the donors. The fact that this is not so lends support to the idea.

The lower correlations of cholinesterase concentration with lipids for the patients than for the donors are explainable entirely by the fact that the blood donors do not include any individuals with very high serum lipids. The effect of such individuals on the correlation coefficients is demonstrated very well by the large differences in correlations for male and female patients (Table XV, p. 176), the men showing poorer correlations and individuals with much higher lipids than had any woman (Fig. 16, p. 180). The reason that these individuals have such an effect is that serum cholinesterase reaches a plateau with higher lipid levels (Fig. 20a, p. 188).

In their comparison of the correlations of the activities of serum cholinesterase and γGT with serum lipids, Cucuianu *et al.* (1976) noted that serum cholinesterase was known to decrease in chronic liver disease, advanced heart failure and in the acute phase following surgery or myocardial infarction, and they excluded such patients. Levels of the enzyme are also known to be affected by other conditions, there being for instance a fall in pregnancy and the puerperium (Shnider, 1965). It was not possible to exclude such conditions from the present data for lack of clinical information. It is by no means certain however that excluding such conditions is correct or helpful. If serum cholinesterase and lipid levels are truely related then they should go up or down together in different conditions.

6.1.2.3 Previous work on the relationship

Having explored all the caveats we are now in a position to examine the actual nature of the association of serum cholinesterase and lipids. The work (described in the introduction) with activity assays of the enzyme established that there are stronger associations with triacylglycerols and VLDL than with cholesterol and that that with the latter is secondary to the others. The

impression of most authors was that synthesis or secretion or activity of the enzyme is related to synthesis or secretion of triacylglycerols or VLDL. The finding of Kutty *et al.* (1973) that cholinesterase increased with VLDL in rabbits treated with E. coli lipopolysaccharide, and fell as the VLDL was replaced by LDL supports this. Cucuianu *et al.* (1975, 1976) suggested that the flattening of the increase in cholinesterase with the higher values of triacylglycerol could be explained by the latter being due to decreased removal from the blood rather than increased synthesis. The failure of cholinesterase to fall significantly in treatment of hyperlipidaemia with clofibrate (Haragus *et al.*, 1973), which seems to lower triacylglycerols mainly by enhancing their removal from the serum, was cited (Cucuianu, 1988) as evidence that the enzyme might be coinduced with lipoprotein synthesis rather than induced by serum lipoprotein concentrations. The finding of Venkatakrishnan (1990) that serum cholinesterase does not decline when triacylglycerol concentration is lowered by releasing lipoprotein lipase with heparin might similarly be taken as support for the contention that the enzyme is not related to the concentration of triacylglycerol as such, as might the absence of correlation between serum cholinesterase and LDL in rabbits with hyperlipidaemia due to an LDL-receptor defect (Udom *et al.*, 1989).

6.1.2.3.1 Serum lipids in cholinesterase poisoning

The evidence from poisoning of cholinesterase slightly militates against active involvement of the enzyme in lipid synthesis. Kutty *et al.* (1975) found that serum cholesterol and 'total LDL' fell in a patient poisoned with the organophosphate parathion, but the lipid levels started to recover before the cholinesterase. Kutty *et al.* (1977) found that when the enzyme was inhibited with neostigmine in rats there was a decrease in incorporation of ^{3}H-lysine into LDL, but a slight increase in incorporation into VLDL. Similarly, Ryhänen *et al.* (1984) found that when the enzyme was inhibited by dichlorvos in rabbits the serum LDL fell dramatically (much more than the cholinesterase activity) but the VLDL was unaffected. These findings could be explained by the drugs inhibiting lipoprotein lipase which converts VLDL to IDL but, if so, the failure of VLDL to fall

does not necessarily exclude cholinesterase from its synthesis since its level would be expected to stay up if it was no longer being removed.

6.1.2.3.2 Lipids in thyroid disorders

Evidence has been cited in the introduction that serum cholinesterase and triacylglycerols go up together in nephrotic syndrome and diabetes mellitus. Cucuianu *et al.* (1968) noted the occurrence of high cholinesterase with low cholesterol in hyperthyroidism and low cholinesterase with high cholesterol in hypothyroidism. They explained the former observation by increased mobilization of lipids but even faster removal of lipids and the latter (in their 1975 paper) by decreased removal of lipids. The data given in their 1968 paper tell more than the authors have mentioned in their discussion. They have a plot of serum cholinesterase activity *vs.* cholesterol in which normal-body-weight subjects, obese subjects, patients with hyperthyroidism and ones with hypothyroidism are shown with different symbols. By inspection it is apparent that in all four groups serum cholinesterase activity increases with increasing cholesterol and that parallel or nearly parallel regression lines could be drawn through the four groups of points. Thus the findings of high cholinesterase with low cholesterol and low cholinesterase with high cholesterol in thyroid patients is not paradoxical; there is a positive correlation between the two indices in each of the dysthyroid states, but at different levels.

For any given serum cholesterol level, hypothyroid patients have the lowest serum cholinesterase, then come the normal weight subjects then obese subjects and then hyperthyroid patients with the highest cholinesterase values. This might be interpreted as indicating that serum cholinesterase does not play an active part in lipid metabolism but is synthesized in parallel with it, and that synthesis occurs at the lowest rate in hypothyroidism, and in the other groups at higher rates through to the highest in hyperthyroidism. Looked at the other way, the results show that for any given serum cholinesterase level the hyperthyroid patients have the lowest cholesterol, then come the obese subjects, then the

normal weight subjects, and then the hypothyroid patients with the highest serum cholesterol. If we assume that the serum cholinesterase level is more closely associated with the rate of synthesis of cholesterol then results tell us that cholesterol is removed from the bloodstream more rapidly in hyperthyroidism than in obesity, more rapidly in obesity than in normal weight subjects, and least rapidly in hypothyroidism.

Actually it is now known that expression of the LDL receptor is reduced in hypothyroidism (Brunzell, 1988) and is increased in hyperthyroidism (Staels *et al.* (1990) so this is a partial answer. However, the correlation of cholinesterase with cholesterol seems to be a secondary one through the correlation of both with triacylglycerol concentration. Unfortunately Cucuianu *et al.* apparently did not measure serum triacylglycerols in their thyroid patients. It is however known that hyperlipidaemia is uncommon in hyperthyroidism and that serum triacylglycerol concentration is increased in hypothroidism due to reduction of lipoprotein lipase (Brunzell, 1988). Putting this together with the findings of high cholinesterase in hyperthyroidism and low cholinesterase in hypothyroidism an inference that could be drawn is that serum cholinesterase is not directly related to the serum concentration of triacylglycerols and therefore is probably related to their rate of synthesis. However, this would imply that triacylglycerol synthesis is increased in hyperthyroidism and decreased in hypothyroidism. Other possibilities are that serum cholinesterase synthesis *is* induced by serum triacylglycerol concentration but is 'downregulated' in hypothyroidism and 'upregulated' in hyperthyroidism, by an effect of thyroid hormone on the gene, like the synthesis of lipoprotein lipase and the LDL-receptor, or that if cholinesterase concentration were measured, rather than activity, and haematocrit were taken into account, the mean concentrations would be found to be the same or even reversed.

6.1.2.3.3 Other conditions lowering serum cholinesterase

Cucuianu *et al.* (1985b) included in their data 23 patients with decompensated portal cirrhosis, 10 with cholestasis and 12 postoperative patients. The mean serum cholinesterase activities in these groups were respectively 33.8%, 34.8% and 62.4% of that of normal weight normolipidaemic controls. The mean serum triacylglycerol was not significantly different from control in any of these groups but as cholinesterase was not plotted against triacylglycerol it is not possible to say whether the two were correlated within the groups. The serum cholesterol was significantly lower in the cirrhotic and postoperative patients and not significantly higher in the cholestatic patients, and the lipoproteins were not measured.

6.1.2.4 Could serum cholinesterase be cosynthesized with ApoB?

The evidence discussed so far then largely supports the idea that serum cholinesterase is cosynthesized or cosecreted with triacylglycerol or VLDL and is not directly related to absolute levels of serum lipids. The rank order of the correlations found in the data presented here ('total LDL' > VLDL > triacylgiycerols, total cholesterol, LDL-cholesterol and LDL) suggests that the enzyme could be cosecreted with apoB100. However, all this would at first seem to be overturned by the conclusion of Sorci-Thomas *et al.* (1989) that 'dietary factors that increase LDL concentrations act by reducing clearance of apoB-containing particles rather than by increasing production of these lipoproteins'. If serum cholinesterase is not related to the absolute levels of serum lipids, and if their production does not vary, it is hard to see how the levels of cholinesterase and lipids could be related at all. But surely there is a relationship. A closer look is required.

Sorci-Thomas *et al.* (1989) started by citing various pieces of evidence for down-regulation of hepatic LDL-receptors by dietary cholesterol and saturated fats and suggested that reduced clearance of LDL precursors could yield a more

efficient conversion of apoB100 particles into LDL through the delipidation cascade. They allowed that in some cases higher LDL concentrations might result from increased LDL production as well, though this would not necessarily be due to increased apoB production. They then cited their earlier work of which they said that results from liver perfusion studies had indicated that alterations in dietary cholesterol and fat do not alter hepatic production of apoB100-containing particles.

In the first cited, Johnson *et al.* (1983), they fed African green monkeys for four months on high-cholesterol (test) or control diets, during which those on the test diet had higher mean concentrations of plasma cholesterol, LDL and apoB and had higher LDL particle size. Then they removed their livers and perfused them by recirculation with an initially lipoprotein-free medium for 4 hrs. and measured the accumulated cholesterol and the size and composition of the LDL particles. They found that the cholesterol secretion was greater from the livers of the animals fed more cholesterol, and correlated with the cholesterol concentration and LDL size in their plasma. All lipoprotein particles of LDL or lower density from the perfusate from livers of the test-fed animals had 5- to 15-fold more cholesteryl ester than the corresponding subfractions from the control-fed monkeys' liver perfusates. The triacylglycerol and free cholesterol were increased too, but not as much. The authors also noted differences between the LDL in the perfusate and that in the plasma and found that the LCAT in the perfusate was inactive. They deduced that the cholesteryl esters in the particles came from liver secretion rather than modification in the recirculating perfusate, and that very large LDL particles found in plasma but not perfusate were derived from intravascular modification of cholesteryl-ester-enriched particles. Neither the number of particles nor rate of secretion of apoB100 were measured. The amount of protein in the subfractions was however measured. In the VLDL particles it was the same in the two groups but in the IDL fractions and LDL it was greater in amount - though not in percentage - in the test-fed group.

Johnson *et al.* (1985) reported a similar study in which African green

monkeys were given 40% of their calories as butter or safflower oil. Again they found that the rate of accumulation of cholesterol in the liver perfusates correlated with the size of the LDL particles in the animals' plasma, but for any given rate the LDL and VLDL particles were smaller from the safflower group and the VLDL contained more cholesteryl esters and less triacylglycerols than those from the butter group. The percentage of protein in all subfractions of lipoprotein was the same in the two groups. The amount of protein was the same in VLDL but in the 7 succeeding subfractions up to and including LDL the amount was more in the particles from the butter group in the first 4 and more in those from the safflower group in the heavier 3 subfractions.

Johnson *et al.* (1986) (abstract) found that cynomolgous macaques develop higher plasma LDL levels and more severe atherosclerosis than do African green monkeys fed the same diet. They found that the rate of hepatic apoB secretion was only half as much in macaques for a given cholesterol secretion rate. Looked at the other way, their data showed that the VLDL of macaques contained twice as much cholesterol per apoB molecule (i.e. per particle) than did the VLDL of African green monkeys. Also, 68% of the apoB was in VLDL in the macques whereas the figure was only 33% in African green monkeys, indicating a difference in efficiency of conversion of VLDL to LDL or of removal of LDL.

Then Sorci-Thomas *et al.* (1989) measured apoB and LDL-receptor mRNAs in African green monkeys that had been fed high- and low-cholesterol diets with high and low saturation of fats for five years. They found no significant effect of dietary fats on apoB100 mRNA abundance in the liver while animals fed the higher cholesterol diets had about 50% less LDL-receptor mRNA in the liver. They also perfused livers from monkeys fed the high-cholesterol diets and measured secretion of apoB100 and cholesterol. The rate of apoB100 secretion was linear and identical in the two groups. There was no correlation between hepatic apoB secretion rate and the plasma LDL-cholesterol concentration but there was a positive correlation between cholesterol secretion and plasma LDL-cholesterol.

It would seem then that the rate of synthesis of apoB, and hence of VLDL particles, is not affected by diet (at least in African green monkeys) but that when dietary fat is higher the apoB100-bearing particles are larger and contain more cholesterol and triacylglycerol. Thus it would seem that it is still possible for serum cholinesterase secretion to be correlated with triacylglycerol secretion. In all three papers dicussed the hepatic cholesterol secretion rate was said to correlate with the animals' plasma total cholesterol or LDL-cholesterol. This makes the authors' claim that 'diet-induced changes in plasma cholesterol concentrations are primarily due to effects on catabolism of apoB-containing lipoproteins rather than on synthesis and secretion of these lipoproteins from liver' seem a little silly since they have left out cholesterol secretion. The authors never gave figures for the correlations but in Sorci-Thomas *et al.* (1989) the indvidual data were given for hepatic cholesterol secretion rate and plasma LDL-cholesterol for 17 animals so it is possible to calculate. The correlation coefficient is 0.71, so about half of the variation in LDL-cholesterol concentration is due to rate of hepatic cholesterol secretion. Hepatic triacylglycerol secretion rate and plasma triacylglycerol concentration were apparently not measured.

The earlier quotation above, regarding LDL concentrations *may* be correct. However, Payne *et al.* (1991) have measured the serum apoB in 76 overnight-fasting subjects for whom lipid profiles had been requested. They have found that the serum apoB concentration can be predicted to within 4% from a combined regression of the serum cholesterol and triacylglycerol concentrations. If serum cholesterol correlates with cholesterol secretion but apoB secretion does not vary and its concentration is solely dependent upon LDL-receptor regulation, it seems paradoxical that serum cholesterol and triacylglycerol can predict the apoB concentration so accurately. It is worth adding here that Sorci-Thomas *et al.* (1989) admit that hepatic LDL-receptor mRNA was similar in abundance in polyunsaturated and saturated-fat-fed animals, suggesting that the difference in plasma cholesterol concentration between these groups is not mediated via effects on the LDL-receptor. Further work is needed.

6.1.2.5 The new light shed by the work presented

We can now return to the results of this study and ask what they can tell us that has not been shown by earlier work. This is the first time that serum cholinesterase concentration has been compared with serum lipids and lipoproteins. The results show that the raised serum cholinesterase activity found in hyperlipidaemias is indeed due to increased concentration of the enzyme rather than to increased specific activity, such as might have been caused by an allosteric interaction with one of the lipids. Also, at least in these subjects, there is no subpopulation with a high-specific-activity variant. It has also been shown that, at least in these patient samples, concentration is a more reliable measure - for whatever reason - for determining the relationship of serum cholinesterase to the lipids. More important, perhaps, this appears to be the first time that anyone has taken notice of the actual shapes of the plots of cholinesterase against lipids.

6.1.2.6 The meaning of the shape of cholinesterase-lipid plots

6.1.2.6.1 Preliminary remarks

Before considering the meaning of these shapes, it is worth emphasizing two points. One is that in comparing the serum concentrations of two components one must remember that both can vary in the rate of entry and the rate of leaving the circulation. The other is that serum cholinesterase is in the serum, or more properly when considering what might be happening *in vivo*, the plasma. This point is made to deal with the suggestions that serum cholinesterase activity might correlate with lipid synthesis. Since the lipids and lipoproteins are synthesized in the liver, and 'serum' cholinesterase is in the plasma, measuring the enzyme in the blood would then only be useful as a predictor of the butyrylcholinesterase activity in the liver. Waterlow (1950), Kutty *et al.* (1981b, 1984) and Venkatakrishnan (1990) have shown that serum and liver cholinesterase activities tend to go up together, but Kutty *et al.* (1984) showed that serum cholinesterase activity correlated better with serum triacylglycerol concentration than with liver cholinesterase activity in Zucker fat rats.

6.1.2.6.2 The correlation coefficient is an inadequate measure

Correlation coefficients between serum cholinesterase and the serum lipid indices were calculated mainly because other workers had done this before, but what the plots of cholinesterase with those indices that give the best correlations show is that this statistical test is not really appropriate because the relationship is not linear. The distribution of points is triangular. Because of this it is possible that the lipid index which is the most closely related to serum cholinesterase (assuming that there is a substantive relationship) is not the one that gives the highest correlation coefficient. It is possible that the true relationship is with triacylglycerols rather than 'total LDL'; compare the outlines in Figs. 14b (p. 177) and 15a (p. 179). This would make much more sense in terms of the results of Sorci-Thomas *et al.* (1989) discussed above.

6.1.2.6.3 Logarithms and the abstracted cholinesterase-lipid curve

If serum cholinesterase was really related to the logarithm of the triacylglycerol concentration this would say something about the kinetics of the relationship and it should still be true if only part of the range of variation was studied. The fact that the logarithm gave a poorer correlation in the blood donors is somewhat against this. The shape of the distribution shows that the relationship of cholinesterase to triacylglycerol or 'total LDL' is not a straight line which curves over and reaches a plateau with the higher lipid values; that description is only an abstraction which may not represent the true nature of affairs. The plots show that both the lower and upper borders are approximately straight.

6.1.2.6.4 The gap between Cucuianu's findings and the new results

Why is it then that at low serum concentrations of triacylglycerol or 'total LDL' a wide range of cholinesterase concentrations is found but that at high lipid

concentrations only high cholinesterase is found? The hypothesis of Cucuianu *et al.* (1975) was that elevation of serum cholinesterase activity 'could be connected to mechanisms leading to an increased secretion rate of lipoproteins' and that the graph of mean cholinesterase against mean triacylglycerol levels out because some of the highest triacylglycerol concentrations are due to decreased removal rather than increased production. On its own, this does not explain why some individuals with low serum triacylglycerol or 'total LDL' have cholinesterase concentrations as high as those with high concentrations of triacylglycerol. It also predicts that there should be some individuals with high triglyceride who do not have high serum cholinesterase. No such individuals occur in the data presented here so it is worth inspecting their composition.

6.1.2.6.5 Could the data include hyperlipidaemia due to decreased clearance alone?

Amongst these data there are two individuals whose serum lipoprotein electrophoresis suggested that they had type III hyperlipoproteinaemia. This is a condition (also called remnant removal disease or dysbetalipoproteinaemia) in which there is decreased clearance of VLDL remnants due to defective binding of apoE to the LDL receptor. About 1 in 100 people have two alleles producing abnormal apoE. Most of these do not have hyperlipidaemia and tend to have low cholesterol and LDL levels but about 1 in 100 of them have hyperlipidaemia, apparently because of an additional cause of hypertriacylglycerolaemia. The two cases in these data did not have very high lipid concentrations. Their cholinesterase, triacylglycerol and 'total LDL' concentrations were respectively 36.2 and 34.2 mg/L, 2.02 and 3.23 mmol/L, and 4.4 and 6.9 g/L and by inspection of Figs. 15a (p. 179) and 14b (p. 177) it can be seen that they both lie quite close to the top left corners of the distributions, i.e. the serum cholinesterase concentrations are quite high even though the triacylglycerol and 'total LDL' concentrations are not very high.

In the plot of cholinesterase concentration against triacylglycerol the two

most extreme right points represent the two patients with type V hyperlipoproteinaemia and all the other individuals in the top half of the range have type IV (the highest triacylglycerol concentration amongst the type IIb patients being 6.97 g/L). In the plot of cholinesterase *vs* 'total LDL' the top right corner of the distribution includes the cases of type V and cases of type IV, but also some with type IIb hyperlipoproteinaemia, the highest 'total LDL' concentration amongst the latter in these data being 10.2 g/L. In type IIb cholesterol is principally raised but triacylglycerols are also elevated; LDL and VLDL are raised. In types IV and V triacylglycerols are principally raised but cholesterol tends to be raised too in type IV and is always raised in type V. In type IV, VLDL is raised and in type V, chylomicrons are found in the blood in the fasting state as well as raised VLDL. These types are only phenotypes and all have more than one cause.

Inherited causes of hypertriacylglycerolaemia include 'familial hypertriglyceridaemia', familial combined hyperlipidaemia and disorders in which lipoprotein lipase function is defective. In familial hypertriglyceridaemia, there is increased triacylglycerol synthesis and removal seems to be normal. Cholesterol synthesis may also be increased. VLDL concentration is increased but LDL levels are normal. It can appear as type IV or type V. The incidence of this condition is thought to be about 1/200 and it would be more common amongst people selected to have serum lipid profiles. Familial combined hyperlipidaemia appears to be due to increased synthesis of apoB and can manifest as type IIa (raised cholesterol only), IIb or IV, even in the same individual at different times. (The reference used for the hyperlipidaemia types (Brunzell, 1988) was written before the paper of Sorci-Thomas *et al.* (1989) but their work could not contradict this anyway). The incidence is thought to be about 1/100. Defective lipoprotein lipase function causes hyperlipoproteinaemia type I (chylomicronaemia only) or type V. It can be due to a deficiency of the enzyme (which hydrolyses triacylglycerols in VLDL and chylomicrons) in some or all tissues or a deficiency of apoCII (which activates it) or a familial inhibitor. This cause of

hypertriacylglycerolaemia then is due to defective removal, but it is very rare. There have been other reports of familial type V of unknown aetiology and cases of type IV noted to have defects in VLDL removal not characterised by one of the above defects in lipoprotein lipase function.

Acquired hypertriacylglycerolaemia occurs in diabetes mellitus (due to increased triacylglycerol synthesis), uraemia (which appears to diminish lipoprotein-lipase removal of triacylglycerols), and minimally in obesity, oestrogen therapy and alcoholism (which all seem to hepatic VLDL secretion). Also, diuretics and beta-blockers tend to increase LDL (mechanism not given) and there are other, rare, causes of acquired hypertriacylglycerolaemia.

Type V hyperlipoproteinaemia is usually due to the interaction of two common forms of hypertriacylglycerolaemia, usually one genetic and one acquired. Untreated symtomatic diabetes is frequently a cause in the presence of familial hypertriglyceridaemia, familial combined hyperlipidaemia, or less commonly remnant removal disease. Oestrogens, diuretics, beta-blockers, alcohol, and glucocorticoids often markedly exaggerate pre-existing hyperlipidaemia due to these inborn errors of metabolism. Hypothyroidism and uraemia occasionally contribute. Most cases of type V then are due to increased production of triacylglycerols. However, at high synthesis rates in type V and type IV the removal mechanism may become overloaded.

Acquired causes of type IIb hyperlipoproteinaemia include hypothyroidism, nephrotic syndrome and glucocorticoid excess either due to Cushing's syndrome or steroid therapy. In hypothyroidism, as already mentioned, LDL-receptor expression is reduced - resulting in accumulation of chylomicron and VLDL remnants and LDL - and the lipoprotein-lipase level is low. In nephrotic syndrome there is increased hepatic lipid synthesis and defective catabolism of triacylglycerol-rich lipoproteins, the latter possibly due to urinary loss of cofactors for lipoprotein-lipase. In glucocorticoid excess there are increased VLDL and/or LDL levels (mechanism not stated).

A cause of hyperlipidaemia not mentioned above is LDL-receptor deficiency which of course increases plasma LDL. It occurs in the heterozygous form with a frequency of about 1 in 500 and accounts for at least 1 in 5 cases of high serum cholesterol. Cases of this defect may well occur in the data therefore, but they usually have type IIa hyperlipoproteinaemia (with normal triacylglycerol levels) and type IIa does not occur amongst the people with very high 'total LDL' in these data.

In cases in which high serum triacylglycerol or 'total LDL' concentrations are due to defective removal without increased synthesis the theory of Cucuianu *et al.* would predict that they would have serum cholinesterase concentrations resembling those of people without raised serum lipids, i.e. that they could have a concentration anywhere in the range, not just at the high end. From the above, most cases seem to be due to increased synthesis with only untreated hypothyroidism, uraemia, and rare genetic defects causing high levels purely by decreased catabolism, while in nephrotic syndrome and in some cases of type IV and V hyperlipoproteinaemia removal may be reduced in conjunction with high synthesis, in which case serum cholinesterase would be expected to be high anyway. It is possible therefore that in the data presented here there are no cases of high serum triacylglycerol or 'total LDL' due to decreased removal alone and that the theory of Cucuianu *et al.* (1975) is not contradicted. There is however another possible explanation for the shape of the distributions.

6.1.2.6.6 An alternative hypothesis

It is clear that individuals with normal serum lipid levels have a wide range of serum cholinesterase concentrations. The observed shape of the plot against triacylglycerol or 'total LDL' would occur if serum concentrations of these entities induced increased synthesis of cholinesterase from its normolipidaemic level but that there is in most individuals approximately the same maximum possible serum concentration either due to a maximum rate of synthesis or to induction of serum cholinesterase catabolism. The abstracted plot of mean cholinesterase

against mean triacylglycerol or 'total LDL' concentration, as in Fig. 20a (p. 188), would then be curved purely because though the minimum cholinesterase increases with with increasing triacylglycerolaemia the maximum remains the same. There is then no need to invoke a different mechanism of hyperlipidaemia to account for the curvature.

6.1.2.6.7 Counters to the previous arguments

If serum cholinesterase synthesis is induced by the serum concentration of triacylglycerol or apoB-bearing lipoprotein rather than being linked to their synthesis or secretion, we have to counter the evidence for the latter. First there is the evidence that serum cholinesterase activity did not fall when triacylglycerol was lowered with clofibrate. In the 20 patients treated by Haragus *et al.* (1973) the mean serum triacylglycerol fell by 31.3%. The mean serum cholinesterase activity fell by 7.2% but this was not statistically significant. Because of the shape of the distribution and abstracted plot of means, we do not expect the mean cholinesterase to change very much for quite a large fall in triacylglycerol in the top part of the range. However, the means of serum triacylglycerol before and after treatment of the patients of Haragus *et al.* were 198 and 136 mg/100 ml (2.24 to 1.54 mmol/L in S.I. units). These values are both quite low. To find out what change in cholinesterase might be expected it is therefore necessary to make some calculations from the data used in this thesis.

It is worth noting here that the relative variation in serum cholinesterase is much less than that of triacylglycerol, the range of triacylglycerol in the 282 patients being 0.45 to 15.48 mmol/L whereas that of serum cholinesterase activity was 1778 to 5806 U/L, excluding the single activity result (6542 U/L) that stood well above the rest. In the triacylglycerol distribution the results are even more clustered at the lower end of the range than are the 'total LDL' results (compare Figs. 14b (p. 177) and 15a (p. 179)). The method of dividing the range into fifths proves unsatisfactory for prediction of changes in cholinesterase between such low triacylglycerol concentrations, the mean triacylglycerol in the

first fifth of the range (N = 218) being 1.73 mmol/L and that in the second fifth of the range (N = 48) 4.50 mmol/L. Taking 'quintiles' instead with alternately 56 and 57 individuals in each, the means of triacylglycerol concentration and serum cholinesterase activity in the second quintile (N = 57) were 1.46 mmol/L and 3727 U/L, for the third quintile (N = 56) 1.98 mmol/L and 3859 U/L, and for the fourth quintile (N = 57) 2.88 mmol/L and 3921 U/L. Interpolating between these points, the predicted fall in serum cholinesterase activity between triacylglycerol concentrations of 2.24 and 1.54 mmol/L would be from 3876.9 to 3742.7 U/L or just 3.46%. This is only about half the drop that Haragus *et al.* obtained so this shows that it would not be right to interpret their figures as showing that cholinesterase does not respond to serum triacylglycerol concentration and must rather be related to rate of synthesis.

The second piece of evidence mentioned was that Venkatakrishnan (1990) obtained a drop of 85% in serum triacylglycerol concentration within 30 minutes after intravenous injection of heparin into rats but found no fall in serum cholinesterase activity. The answer to this is that the *in vivo* half-life of serum cholinesterase is much too long to respond so rapidly. Estimates of the half-life of human serum cholinesterase have lain between 2 and 16 days (Whittaker, 1986). From this it is clear that whether serum cholinesterase correlates with the rate of synthesis or the serum concentration of triacylglycerols, it could only correlate *well* with the mean (of triacylglycerol synthesis or concentration) over at least several days.

The finding of Udom *et al.* (1989) that serum cholinesterase did not correlate with LDL concentration in rabbits with LDL-receptor deficiency also does not really stand against the hypothesis that the enzyme might be induced by serum triacylglycerol concentration since the latter was not measured and is not usually raised in that condition.

One other observation put forward by Cucuianu *et al.* (1975) was that a few patients with excessive hypertriacylglycerolaemia had normal serum

cholinesterase activity. Inspection of the records of this laboratory shows that here too not all individuals with very high triacylglycerol levels have had high serum cholinesterase activities. However, the work presented here has shown that activity measurements are not as reliable as those of concentration. If activity is used then samples sent by post to the laboratory should be excluded, as should samples from individuals taking drugs which inhibit cholinesterase activity. Samples should be frozen or activity should be measured when they are fresh, and when the activity is expected to be high - because of high triacylglycerol concentration - activity measurement should be repeated at 1 in 2 dilution.

6.1.2.6.8 A compromise hypothesis

Between the suggestions that, on the one hand, serum cholinesterase synthesis might have a stimulus in common with VLDL or triacylglycerol synthesis, or that the enzyme might even be connected with their synthesis in some way, and on the other hand the suggestion that serum cholinesterase synthesis is induced by serum triacylglycerol concentration, there is a middle way. It could be that the synthesis of the enzyme is induced by the secretion of VLDL into the plasma or by rising plasma concentration of VLDL or triacylglycerol. If so, a high serum cholinesterase concentration could indicate a high rate of triacylglycerol synthesis even in an individual in whom the triacylglycerol removal system was maintaining the serum level within the normal range.

6.1.2.7 Association of cholinesterase with the LDL particle

Since serum cholinesterase concentrations at the upper end of the normal range have been observed in association with low-normal LDL concentrations the data cannot support the hypothesis of Kutty *et al.* (1973) and subsequent papers that the enzyme prolongs the half-life of LDL. However, this does not exclude the possibility that the enzyme could be chemically associated with LDL in the way that they propsed.

6.1.3 The possible function of serum cholinesterase

The possibility that the synthesis of serum cholinesterase might be regulated by the arrival of triacylglycerols or VLDL in the plasma, is an opening to a discussion of the possible function of the enzyme in the blood. It seems highly likely that in the nervous system and muscles butyrylcholinesterase hydrolyses acetylcholine at concentrations that would inhibit acetylcholinesterase (see introduction). The enzyme hydrolyses butyrylcholine faster than acetylcholine. The argument that neither acetylcholine nor butyrylcholine occur in the plasma and that there is therefore no apparent function for the enzyme in the blood may be a rather blinkered one. For a start, the occurrence of the enzyme in the blood in many species makes it unlikely that it does not have a function in the blood. Secondly, other plasma esterases are found in increased concentrations in hyperlipidaemia. Beynen *et al.* (1987a) showed that a high-cholesterol, high-cholate diet caused a significant increase in plasma total esterase activities in 6 out of 7 strains of mice and Beynen *et al.* (1987b) showed the same in 4 out of 4 strains of rat. Patel *et al.* (1990) have shown that in streptozotocin-diabetic rats there is not only an increase in serum cholesterol, triacylglycerols and cholinesterase but in carboxylesterase and paraoxonase too.

In human serum the presence of a carboxylesterase was published only as recently as 1978 by Somorin and Skorepa. In its purified state this enzyme (EC 3.1.1.1) hydrolyses short-chain fatty-acid esters and scarcely hydrolyses long-chain fatty-acid esters. It therefore had at first no apparent function because short-chain fatty-acid esters are not normally found in human serum. However, Shirai *et al.* (1988) investigated the effects of phospholipids on the substrate affinity of the enzyme. They showed that cardiolipin increased triolein hydrolysis by the enzyme and reciprocally decreased its hydrolysis of tributyrin. Phosphatidylserine and phosphatidylinositol also enhanced the carboxylesterase-catalysed hydrolysis of triolein but not as much as did cardiolipin. Phosphatidylethanolamine and sphingomyelin did not cause any enhancement. The authors pointed out that cardiolipin has three negative

charges in its polar head whereas phosphatidylserine and phosphatidylinositol have one and phosphatidylethanolamine and sphyngomyelin have none. They proposed that the enzyme might have in addition to its catalytic site a substrate-recognition site and that this site might have three positive charges. They suggested that by interacting with this site, cardiolipin would neutralize the charge and give the enzyme greater affinity for a hydrophobic ester compound. (One slight discrepancy was that phosphatidylcholine also increased the hydrolysis of tributyrin and it has no charge. However, it was not quite as effective as phosphatidylinositol and markedly less effective than phosphatidylserine, and lysophosphatidylcholine did not enhance triolein hydrolysis.)

Shirai and Jackson (1982) showed that hydrolysis by bovine milk lipoprotein-lipase of the water-soluble substrate *p*-nitrophenyl butyrate (PNPB) was enhanced by phospholipids, particularly dipalmitoyl phosphatidylcholine. This enhancement was much greater in the gel state, i.e. with phospholipid vesicles. In this case they favoured the hypothesis that the binding of the enzyme to the lipid interface, possibly through a recognition-site, caused a conformational change which increased the affinity for the water-soluble substrate. Shirai *et al.* (1982) then showed that apoCII enhances triolein hydrolysis by the enzyme and reciprocally decreases the hydrolysis of PNPB. At the time they thought that the apolipoprotein acted by causing a conformational change in the enzyme but in discussion of their work on human serum carboxylesterase Shirai *et al.* (1988) they proposed that here too the mechanism was a change in hydrophobicity.

It seems possible that serum cholinesterase could take part in lipid metabolism in the blood by means of a change in its substrate affinity by a cofactor. Three groups of workers independently produced evidence that acetylcholinesterase has an anionic position in the active centre (see Myers, 1952). Nachmanson and Wilson (1951) reviewing their own and other work on the enzyme discussed evidence for the active centre being composed of an anionic site, having a negative charge and holding the positively-charged

quarternary-ammonium choline part of acetylcholine, and an 'esteratic' (*sic.*) site (otherwise known as the esterasic site) accommodating the acetyl part. They observed that compounds containing two cationic groups at a given separation were far more effective inhibitors than the monoquarternary ions, suggesting the existence of two anionic sites, not necessarily both substrate-activating sites.

Kalow and Davies (1958) investigated the effects of inhibitors on the usual and atypical variants of serum cholinesterase and found them to fall into three groups. The majority inhibited the usual variant more than the atypical one by the same equation. The second group was of bis-quarternary ammonium compounds which caused a much greater difference in inhibition between the two variants but in the same direction. The authors suggested that this could be because these compounds had a two-point attachment to the usual esterase but only a one-point attachment to the atypical variant. Their results indicated that the inhibition of the usual variant was partially non-competitive, suggesting that one of the negative charges might not be in the active centre. They also found that every fourth inhibitor molecule appeared to block two active centres. By contrast, the inhibition of the atypical variant was completely competitive and the blocking ratio of inhibitor to active centre was 1:1, suggesting that only one quarternary nitrogen was attached to the enzyme and that the point of attachment was always the anionic site of the active centre. Thus the negative charge that was missing in the atypical variant was apparently not in the active centre but in a position that could affect it and from which a bridge could be made to another subunit of the enzyme. The third group of inhibitors, organophosphates, inhibited the two variants equally. Since these were thought to react with the esterasic site (Nachmanson and Wilson, 1951) they suggested that the esterasic sites were the same in the two variants. This was proved to be correct when McGuire *et al.* (1989) showed that the only consistent difference between the DNA sequences coding for these two variants was a single base-change which resulted in the substitution of a neutral amino-acid (glycine) for an acidic one (aspartic acid) at position 70 and hence that the esterasic sites were the same. The active site serine is amino-acid 198 (Lockridge *et al.* (1987).

Harris and Whittaker (1961) were surprised to discover that sodium fluoride could also differentiate between the usual and atypical variants and in 1963 that at much higher concentrations sodium chloride could too. However, with sodium fluoride the usual enzyme is inhibited more than the atypical, as is the case with dibucaine, whereas sodium chloride inhibits the atypical variant more. It was also found (Harris and Whittaker, 1961, 1962) that there were some individuals who could not be classified in the same way by dibucaine and fluoride, revealing new phenotypes which were shown as probably allelic (Whittaker, 1967) and that there were some who were of the usual phenotype by dibucaine and fluoride inhibition but seen to be of an intermediate phenotype when classified by chloride inhibition (Whittaker, 1968c, 1968d). Two of the latter were known to be sensitive to succinylcholine. It would thus appear that there are at least two positive charges and at least two negative charges on the serum cholinesterase monomer in positions which can affect substrate affinity.

At this point the activation of serum cholinesterase by alcohols should be mentioned. Todrick *et al.* (1961) showed that alcohols activated cholinesterases up to an optimum, and that beyond that concentration the activation continued to decline so that at higher concentrations inhibition occurred. They worked with acetylcholinesterase from rat brain and human erythrocytes, and serum cholinesterase from the horse, and with acetyl-, β-methyl- and benzoylcholine as substrates. The finding of particular relevance to substrate-specificity was that the hydrolysis of acetylcholine by the horse serum cholinesterase was inhibited at all alcohol concentrations. Thus there was a range of alcohol concentration over which the hydrolysis of benzoylcholine was increased but the hydrolysis of acetylcholine decreased. This was confirmed with human serum cholinesterase and the same substrates by Vincent *et al.* (1982).

Todrick *et al.* (1961) also showed that the optimum activating concentration of alcohols decreases as chain length increases. Main *et al.* (1961) showed that the hydrolysis of *o*-nitrophenyl butyrate (ONPB) by human serum cholinesterase was increased by butan-1-ol and Main (1961) showed that the hydrolysis of

PNPB, for which the enzyme has less affinity, was inhibited by butan-1-ol. The latter also found that activation of ONPB hydrolysis increased with increasing alcohol chain length and that acetone and other isomers of butanol would also activate the hydrolysis though not as much as butan-1-ol.

Whittaker (1968a) investigated the effects of alcohols on the usual and atypical varianis of human serum cholinesterase with benzoylcholine as substrate She found that alcohols activated the atypical variant much less than the usual one and inactivated it at lower concentrations. With increasing chain length the activation was greater, optimum concentration of alcohol lower and the concentration giving the greatest difference in percentage activation of the variants lower. Whittaker also tried varying pH and interpreted her results as showing that changes in activity were independent of pH in the range 5.9-8.45 except that no activation of the atypical variant was observed at pH values above 6.6 with butanol. Actually the results suggest that for each alcohol there is a pH optimum both for maximum percentage activation and for maximum optimum alcohol concentration. This pH optimum is higher for the atypical than for the usual variant and it appears to go down with increasing chain length of alcohol for both variants, but more data are required. She did not try varying substrate concentration.

Todrick *et al.* (1961) did vary substrate concentration. Their hypothesis was that alcohols competitively inhibit the cholinesterases and that apparent activation is due to reduction of substrate inhibition. As there is no substrate inhibition of serum cholinesterase by acetylcholine, inhibition occurs at once. From their results they concluded that this is the case. There does however appear to be a contradiction. They did demonstrate competitive inhibition at lower alcohol concentrations and an irreversible inactivation at higher concentrations undoubtedly due to denaturation. However they have a graph of activity against pS (reciprocal of the logarithm of substrate concentration) for horse serum cholinesterase with benzoylcholine as substrate and with and without 0.19M n-butanol. If their hypothesis were correct the maximum activity should not be

increased by the presence of alcohol but the optimum pS should be lower (i.e. optimum substrate concentration higher) so that at higher substrate concentrations there is more activity in the presence of alcohol but at lower substrate concentrations there is more activity without alcohol. Actually the optimum pS is unchanged and the maximum activity is greater with butanol. This is the exact opposite of what the hypothesis predicts and the authors did not address this. In the hydrolysis of acetylcholine by acetylcholinesterase the curve was shifted to the left by butanol but the peak activity was still much higher with the alcohol.

Main (1961) was apparently unaware of the conclusion of Todrick *et al.* His investigations led him to conclude that alcohols affect the activity by changing some aspect of the environment on which k_3 (the rate constant for the change of the enzyme-substrate complex to enzyme + products) is dependent. It had been suggested to him that this activation could coexist with competitive inhibition but he concluded that this was not occurring. Whittaker (1968a) did not consider the explanation of the phenomenon but made the practical observation that the differential activation of the serum cholinesterase variants by alcohols could be used as another method of ditinguishing between them. She went on (Whittaker, 1968b) to show that there were some individuals with whom enzyme activation was discrepant with the phenotype suggested by dibucaine and fluoride inhibition, revealing yet more new phenotypes.

It has been shown that the atypical variant differs from the usual one by having one less charge apparently outside the active centre but affecting substrate affinity. If this charge reduction increases the affinity of the enzyme for more hydrophobic substrates (as in the case of serum carboxylesterase) then one might expect that the atypical variant of serum cholinesterase would have greater affinity for longer-chain choline esters than does the usual variant. As it happens, the action of these two variants upon a series of choline esters was investigated by Davies *et al.* (1960) and they concluded that this did appear to be the case, though they did not offer an explanation. Their actual finding was that

relative to the respective maximum velocities of hydrolysis of butyrylcholine by each variant, pentanoyl-, hexanoyl- and heptanoylcholine were hydrolysed faster by the atypical variant than by the usual, though only pentanoylcholine was hydrolysed faster than butyrylcholine.

It may be remembered that the C_5 variant of the enzyme appears to be a combination between serum cholinesterase and another unidentified protein and that this variant has higher activity than the usual variant. This, however, appears to be because of increased concentration rather than increased specific activity. Thus we have examples that the activity and substrate-specificity of serum cholinesterase can be altered by change of surface charge and by alcohols, if not by association with another protein. It does not seem beyond the bounds of possibility therefore that there could be a cofactor which enables the enzyme to take part in serum lipid metabolism to account for the induction of the enzyme in hyperlipidaemia. The recently-noted sequence similarity with *Geotrichum candidum* lipase (Slabas *et al.*, 1990) perhaps lends some credence to this. A sequence of seven amino-acids surrounding the serine of the active site of serum cholinesterase is identical. The disulphide bonds on either side of this are preserved. As in the cholinesterases there is an aspartic acid residue immediately before the second cysteine of the first pair, thought possibly to play a part in their active site (Chatonnet and Lockridge, 1989) and there is another aspartic acid in almost the same position as the one that is absent in the atypical variant, just beyond the first cysteine. However, in *Geotrichum candidum* lipase, in the loop between the first two cysteines there are 17 extra amino-acids. Perhaps a cofactor might fill the rôle of these extra residues. The carboxy-terminal end is much less similar to the cholinesterases. As in rabbit microsomal esterase and *Drosophila* esterase-6, the third disulphide bond is absent but two histidine residues are present in similar positions to those in all the other enzymes.

Geotrichum candidum lipase hydrolyses all ester bonds in triacylglycerols and displays a high affinity for triolein (according to Shimada *et al.* (1989), the

sequencers, quoting an earlier reference). Lipoprotein triacylglycerols are thought mainly to be hydrolysed at positions 1 and 3 of the glycerol moiety by lipoprotein lipases on the capillary walls before diffusion of the free fatty acids into the cells while the resultant 2-monoacylglycerols either diffuse in also or are hydrolysed by serum monoacylglycerol hydrolase. This would not seem to leave a niche for another serum lipase. If however there is a physiological rôle for the recently-demonstrated activity of serum carboxylesterase, then there could be further niches. Shirai *et al.* (1988) found that though cardiolipin increased the hydrolysis of triolein by carboxylesterase, it did not affect the hydrolysis of diolein and decresed the hydrolysis of mono-olein. There is also the possibility that cholinesterase might take part in some other aspect of VLDL metabolism.

6.2 CONCLUSIONS

1. Measurement of the butyrylcholinesterase concentration in human serum samples has proved more reliable than measurement of the activity of the enzyme in examination of the relationship of the enzyme to serum lipids and lipoproteins despite the fact that the repeatability of the assays was better for activity. This is because (a) activity tends to decay with age of the sample while concentration changes little, (b) the activity assay becomes unreliable at high levels and probably also (c) because various drugs competitively inhibit activity.

2. Mean serum cholinesterase concentration, like activity, was lower in women than in men. Simpson (1966) showed that mean activities were the same if correction was made for haematocrit and weight. This could not be done for these data as these parameters were not known, but the cholinesterase was found to be related to serum lipids in the same way in the two sexes.

3. The serum lipid indices that correlate best with cholinesterase concentration

are first total heparin-precipitable low density lipoprotein concentration (VLDL + LDL), then VLDL alone, then triacylglycerol concentration.

4. The plots of serum concentration of cholinesterase against these lipid indices are not linear but have a well-defined triangular shape.

   With very few exceptions the maximum cholinesterase concentration is approximately the same at all values of the lipid indices, suggesting a maximum rate of synthesis or the operation of a catabolic mechanism which limits the maximum serum concentration achievable.

   The minimum cholinesterase concentration increases linearly with increases in the lipid indices until it meets the maximum. No exceptions were encountered. In the plots of cholinesterase *activity* against the lipid indices there were some exceptions (falling below the line), probably due to the reasons given in '1' above.

   The minimum and maximum lines met at approximately the maxima of 'total LDL' and triacylglycerol found in these data (14.0 g/L and 15.48 mmol/L respectively) or slightly beyond them. At even higher concentrations of 'total LDL' or triacylglycerols the cholinesterase concentration would be expected to be at the maximum.

   Thus at low-normal concentrations of the lipid indices there is a wide range of serum cholinesterase concentrations in different individuals but, as the lipid indices increase, this range is progressively reduced from the lower end while the upper end remains constant.

5. Because of this shape, the graphs of *mean* serum cholinesterase concentration against *mean* 'total LDL', VLDL or triacylglycerol ascend in a convex shape to reach a plateau.

6. The hypothesis of Cucuianu *et al.* (1975) to explain the shape of this curve was that serum cholinesterase activity correlates with triacylglycerol synthesis and that the line is not straight because in some cases very high triacylglycerol concentrations are caused or enhanced by decreased catabolism rather than increased synthesis. This explanation ignored the fact that the maximum cholinesterase concentration is the same whatever the triacylglycerol concentration. The distribution could be explained by the induction of increased serum cholinesterase synthesis or secretion by increasing serum triacylglycerol concentration - irrespective of its cause - up to a maximum serum concentration.

7. The hypothesis of Cucuianu *et al.* would be proved correct by the finding of cholinesterase concentrations below the expected minimum for their triacylglycerol concentration in some individuals with decreased triacylglycerol catabolism without increased triacylglycerol synthesis and no defect of cholinesterase synthesis. The finding by Cucuianu *et al.* of normal cholinesterase activity in some cases of high triglyceride triacylglycerol is not sufficient because they did not measure the concentration of the enzyme.

   The alternative hypothesis would be favoured by the finding that known cases of impaired triacylglycerol catabolism did not have cholinesterase concentrations below their expected minima. The data presented here may not contain any cases in which triacylglycerol removal is impaired without increased synthesis but even if there are some, this would not prove the alternative hypothesis because their cholinesterase concentrations could be in the upper end of the range by chance. Increasing numbers of such cases without the finding of any with low cholinesterase concentrations would progressively decrease the probability of the hypothesis of Cucuianu *et al.*

8. The improvement in correlation with cholinesterase by taking logarithms of triacylglycerol concentrations operates only because of the shape of the cholinesterase-triacylglycerol distribution. The improvement is greater if the data contain enhanced numbers of individuals with high triacylglycerol concentrations and it does not happen if the data do not contain such individuals. The latter finding is against the underlying relationship of cholinesterase concentration being to the logarithm rather than to the lipid concentration directly.

9. The triangular shape of the plots of cholinesterase against 'total LDL', VLDL and triacylglycerols indicates that calculation of correlation coefficients is not the appropriate statistical method to describe the relationships. It may therefore be that the index giving the greatest correlation coefficient ('total LDL') is not the one with the primary relationship to serum cholinesterase. At the very lowest concentrations of 'total LDL' and VLDL the cholinesterase concentration tends to be low so the left border of the distribution is not parallel to the cholinesterase axis but ascends to the maximum much more steeply than does the lower border. With triacylglycerol the same tendency occurs but the left border is much steeper. This causes the shape to be further from linearity. The primary relationship may be between serum cholinesterase and serum triacylglycerols, even though it does not give the highest correlation coefficient.

10. If the hypothesis that serum cholinesterase synthesis is related to VLDL or triacylglycerol synthesis rather than to their serum concentrations is correct then the primary relationship of cholinesterase cannot be to apoB (the common factor of VLDL and LDL) if the findings of Sorci-Thomas *et al.* (1989) are correct that apoB synthesis is not increased in hyperlipidaemia,

so is presumably to triacylglycerol synthesis. If the primary relationship of cholinesterase synthesis is to serum lipid concentration then it might be related either to the concentration of triacylglycerols or apoB-bearing lipoproteins. The data cannot distinguish these possibilities.

11. The maximum found in serum cholinesterase concentration contrasts with serum γ-glutamyl transferase, the mean activity of which was shown to increase continuously with increasing serum triacylglycerol concentration by Cucuianu *et al.* (1976).

12. Radial immunodiffusion has shown repeatable individual variation in the patterns of butyrylcholinesterase-staining immunoprecipitin rings. Family studies would be required to determine whether these are inherited.

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