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Enzyme Linked Immunological Studies of Cholinesterases in Human Blood

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Enzyme Linked Immunological Studies
of Cholinesterases in Human Blood

JEFFREY WYNN JONES

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fulfilment of the requirements of
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Awards for the degree of Doctor of
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List of Abbreviations

ACH _E	Erythrocyte Acetylcholinesterase
ATI	Acetylthiocholine Iodide
BSA	Bovine Serum Albumin
BTI	Butyrylthiocholine Iodide
BzCh	Benzoylcholine
Ch _E	Plasma Cholinesterase
DN	Dibucaine Number
DTNB	5,5'-Dithio-bis-2-nitrobenzoic acid
ELAT	Enzyme Linked Antiglobulin Test
ELISA	Enzyme Linked Immunosorbent Assay
FN	Fluoride Number
HDN	Haemolytic Disease of the Newborn
HRP	Horse Radish Peroxidase
LISS	Low Ionic Strength Saline
OPD	o-phenylene diamine
PBS	Phosphate Buffered Saline
PNH	Paroxysmal Nocturnal Haemoglobinuria
RI	Rocket Immuno-electrophoresis
RO2N	RO2 Number
TMB	3,3',5,5'-tetramethylbenzidine
UBS	Unbuffered Saline

Enzyme Linked Immunological Studies of Cholinesterases in Human Blood
Jeffrey Wynn Jones

Abstract

A modified Ellman method using acetylthiocholine as substrate has been used to reliably measure erythrocyte AChE activity (CV=7%). Reliable immunological quantitation of this enzyme (CV=7%) was achieved using monoclonal antisera to AChE (AE-1 and AE-2) in a fluid phase homogeneous enzyme-linked antiglobulin test (ELAT).

AChE activity and antigen levels were similar for both adult male and female samples. Cord samples at term measured 60% of the adult value and reached 100% within 5 months.

An overall increase in AChE activity was observed as pregnancy progresses with patients 1 to 3 days post delivery showing significantly elevated levels of activity and antigen.

Investigation of haematological disorders revealed cases of reduced levels of activity and antigen in a variety of conditions.

An assay of plasma ChE activity using butyrylthiocholine as substrate has been developed using a microplate Ellman technique (CV=5%). An enzyme linked immunosorbent method (ELISA) using monoclonal antibodies MAb2-1 and 2-4 (CV=5%) provided a sensitive and reliable means of quantitation together with the capability of handling a large number of samples.

Normal Gaussian distribution curves were obtained for both activity and antigen levels for $E_1^u E_1^u$, $E_1^u E_1^a$ and $E_1^a E_1^a$ individuals. The approximate amount that each of the genes E_1^u , E_1^a and E_1^f contribute to the ChE activity and antigen levels is presented allowing the effective efficiency (EE) of the gene product to be calculated. Immunological measurements have aided in the segregation of the silent gene variants representing zero, very low, low and high levels of immunoreactive protein and the identification of a new gene, E_1^x . Family studies have confirmed the existence of this gene. Analysis of activity and immunological data suggest that the E_1^h and E_1^t gene could be identical.

A significant decrease in ChE activity occurs during pregnancy with even lower levels presenting during the first 2 to 3 days post delivery. Immunological measurements indicate similar reductions in the amount of ChE protein during these periods.

Introduction

Hunt and Taveaux (1906) produced evidence for the involvement of acetylcholine (ACh) and other choline esters in neurotransmission and Dale (1914) proposed that ACh was rapidly split into acetic acid and choline by an esterase. The enzyme found in horse serum (Stedman et al., 1932) was called cholinesterase and was later identified in particulate form within the erythrocyte membrane (Stedman and Stedman, 1935). Alles and Hawes (1940) indicated that the serum and cell enzymes from human blood were qualitatively different. The serum enzyme hydrolyses butyrylcholine or propionylcholine faster than acetylcholine, whereas the cell bound enzyme preferentially acts on acetylcholine at low substrate concentration (Mendel and Rudney, 1943). The serum enzyme was originally called 'nonspecific' or 'pseudocholinesterase' while the erythrocyte membrane was believed to contain the specific or true cholinesterase, the latter being the same type of enzyme as present in nerve and muscle tissue (Nachmansohn and Rothenberg, 1945). Augustinsson and Nachmansohn (1949) introduced the term acetylcholinesterase (AChE) for this enzyme and restricted the term cholinesterase (ChE) to the 'nonspecific' enzyme, also referred to as butyrylcholinesterase, propionylcholinesterase or plasma cholinesterase.

The Enzyme Commission have given AChE and ChE the systematic names acetylcholine acetylhydrolase (E.C. 3.1.1.7.) and acylcholine acylhydrolase (E.C. 3.1.1.8.) respectively.

Erythrocyte Acetylcholinesterase

Although, AChE was first detected in the human erythrocyte in 1928 (Galehr and Plattner, 1928) its function in red cells remains unclear. The enzyme is also found in platelets (Zajicek, 1957), lymphocytes (Szelenyi et al., 1982) and possibly granulocytes (Zajicek, 1957).

Approximately 13% of AChE from the human erythrocyte consists of carbohydrate (Niday et al., 1977). This corresponds to a carbohydrate/protein ratio of 0.16 with glucose (22%), galactose (38%), mannose (11%), glucosamine (16%), galactosamine (11%) and sialic acid (2%) as the sugar components.

Lipid, phosphorus and fatty acid analysis indicate phosphatidylserine and cholesterol as the major lipid components. Roberts and Rosenberry (1985) demonstrated that red cell AChE contains at least 10 different fatty acids, the majority (70%) present in the hydrophobic domain. Palmitate (16:0) is the most abundant (37%) with stearate (18:0) (11%), oleate (18:1) (12%), docosatetraenoate (22:4) (14%) and docosapentaenoate (22:5) (10%) as the other major constituents. Two moles of fatty acid are present per mole of catalytic subunit with saturated and unsaturated fatty acids present in equimolar amounts.

Human erythrocyte AChE is characterized by the presence of all the common amino-acids (Table.1). The N-terminal amino acid is blocked. Chhajlani et al. (1989) have analysed five tryptic peptides to determine the amino acid sequences. These showed no homology to those for human serum and brain butyrylcholinesterase or *Torpedo californica* acetylcholinesterase.

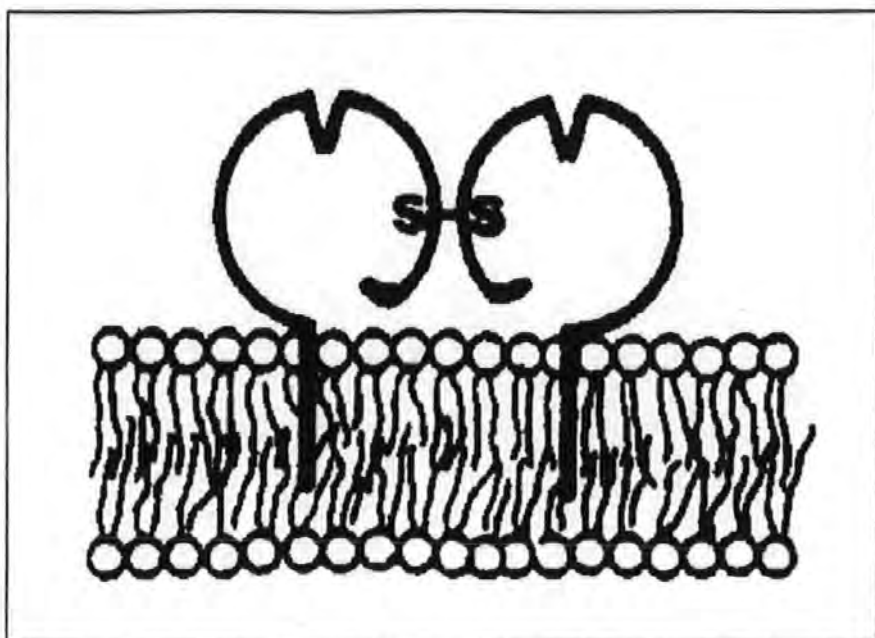


Figure 1 Diagrammatic representation of AChE from human erythrocyte membranes anchored in the lipid bilayer (Weitz et al., 1984).

Table 1 Amino Acid Composition of Human Serum Cholinesterase
and Human Erythrocyte Acetylcholinesterase

Amino Acid	Percentage of total Amino Acids	
	AChE ¹	ChE ²
Alanine	9.7	5.9
Arginine	6.8	4.0
Aspartic acid	7.4	4.2
Cysteine	1.0	1.4
Glutamic acid	9.7	6.4
Glycine	9.9	8.0
Histidine	2.3	1.4
Isoleucine	2.1	4.7
Leucine	10.1	8.7
Lysine	1.7	6.1
Methionine	2.2	1.9
Phenylalanine	4.9	6.8
Proline	7.8	5.2
Serine	5.8	6.4
Threonine	4.5	6.1
Tryptophan	1.9	3.1
Tyrosine	3.4	3.5
Valine	8.6	5.6

¹ Rosenberry and Scoggin (1984)

² Lockridge et al. (1987)

Structure

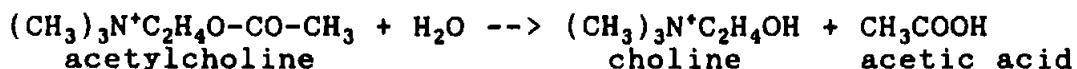
The enzyme is an integral part of the red cell membrane extending to the outside surface (Heller and Hanahan, 1972). This was confirmed by the agglutination of human red cells by monospecific antiserum to AChE (Niday et al., 1977). The basic functional form is a dimer of molecular weight 160,000 containing a single intersubunit disulphide bond (Rosenberry and Scoggin, 1984). Approximately 700-800 AChE dimers per erythrocyte have been estimated, corresponding to 40µg/ml of membrane volume (Ott et al., 1982).

Erythrocyte AChE is a globular protein (G2) (fig.1). It has a high lateral mobility within the membrane and shows little or no interaction with the cytoskeletal network (Weitz et al., 1982). It is an amphipathic protein, each subunit having a large hydrophilic domain orientated towards the outside of the membrane and a smaller hydrophobic domain (less than 10 kD) localized at the carboxy terminus anchoring the enzyme into the lipid bilayer of the red cell membrane (Dutta-Choudhury and Rosenberry, 1984). Evidence by Haas et al. (1986) indicated that the membrane-binding domain is a covalently linked glycolipid at the C-termini of the subunits and has the sequence HIS-GLY-ethanolamine-Z, where ethanolamine is in amide linkage to glycine and Z is a partially characterized structure containing carbohydrate and fatty acids. The lack of hydrophobic amino acids indicate that other components are responsible for the hydrophobicity of the membrane-binding domain of red cell AChE. Virtually all of the fatty acids which are covalently linked to red cell AChE are localized in the hydrophobic domain fragment

and participate in the membrane attachment of the enzyme. The current structure proposed indicates that Z consists of phosphatidylinositol (PtdIns) which is attached via a glycosidic linkage to a glucosamine residue which is attached to an oligoglycan. This oligoglycan is linked to the ethanolamine residue. Studies have shown a reduced expression of PtdIns-anchored proteins in the rare acquired haemolytic disorder paroxysmal nocturnal haemoglobinuria (PNH) (Low, 1987).

Enzyme Activity

AChE catalyzes the hydrolysis of acetylcholine to choline and acetic acid.



The interaction between ACh (Beveridge et al., 1971) and AChE (Nolte et al., 1980) is one between two highly polar reactants. However, extensive non-polar hydrophobic areas (Kabachnik et al., 1970; Narayanan and Balaram, 1981) channel the ACh molecules towards the active centre, which is comprised of an anionic and esteratic site. Once within range the electrostatic attraction between the onium head of ACh and the anionic site takes over resulting in their binding followed by interaction by hydrogen bonding of the ether-oxygen of ACh with the acid group of AChE (fig.2) (Beckett and Albadr, 1975). The nucleophilic serine residue at the esteratic site reacts covalently with ACh allowing hydrolysis to proceed.

Interaction with the anionic subsites of the active centre involves only two of the three N-methyl groups of ACh and possibly the N-methylene group as well (fig.2). The acid group of AChE is located 3-4Å away from the centre of the anionic site in a three dimensional structure to which the ACh molecule can be attached (Ngiam and Go., 1987). Evidence has also been provided for the existence of peripheral anionic sites (P1, P2, P3 and P4) on AChE that may regulate activity at the catalytic site (Changeaux 1966).

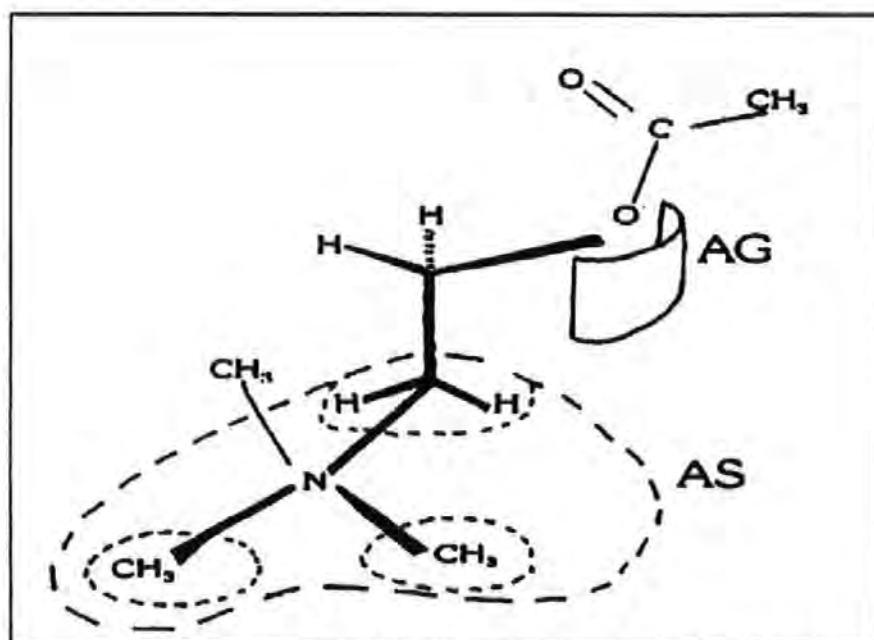


Figure 2 Model of AChE/substrate interaction (Ngiam and Go, 1987). AS = anionic site which is made up of three anionic subsites; AG = acid group.

Factors Affecting Erythrocyte AChE Activity

Alterations in AChE activity have been noted to parallel changes in lipid content in human erythrocytes (Kamber et al., 1984). There is a decrease in the cholesterol:phospholipid ratio as the cell ages and this is followed by a decrease in the activities of membrane bound enzymes, including AChE.

AChE activity appears to be dependent on the surface pressure of the surrounding bilayer. Drugs have been shown to inhibit the activity of membrane-bound AChE. Lignocaine is a lipophilic local anaesthetic that reversibly inhibits AChE activity of erythrocyte membranes possibly by increasing the lipid fluidity of the membrane (Laity and Whittaker, 1971).

Variation in Cellular Activity

AChE has been studied in the erythroid cells of human bone marrow (Keyhani and Maigne, 1981). The enzyme has been found by a cytochemical method in human basophilic and polychromatophilic erythroblasts, in which it occurs in the nuclear membrane, endoplasmic reticulum and golgi structures. In more mature orthochromatophilic erythroblasts the enzyme was found only in the storage compartment (the golgi apparatus), suggesting the end of its biosynthesis at this stage of maturation.

Reticulocytes which appear during the physiological response following acute blood loss have an elevated erythrocyte AChE activity while those present in pathological reticulocytosis occurring in leukaemia, Hodgkins disease, multiple myeloma and myelofibrosis, show decreased red blood cell AChE activity (Sabine, 1965). Reticulocytes of the former type contain

approximately three times as much AChE as mature erythrocytes with a progressive fall in enzyme activity with increasing age (Sabine, 1965).

Burman (1960) recorded the mean cord blood AChE activity to be 53% of the adult mean which was in general agreement, although slightly lower than that reported by other workers (Sabine, 1955; Pritchard and Weismann, 1956). The adult blood level appears to be reached between 3 and 5 months of age. Lower levels of AChE in the neonates may be explained by the phenomenon of stress reticulocytosis, in which foetal erythropoiesis is accelerated close to term. It has been suggested that normoblasts 'skip' a generation, perhaps at the stage when most AChE is synthesized (Lawson and Barr, 1987). The possibility of accelerated enzyme inactivation has not been eliminated (Herz and Kaplan, 1973).

Electrophoretic patterns of adult and cord red cell AChE indicate more anodal migration of the foetal band. Neuraminidase treatment, which removes sialic acid, reduces the migration of both adult and foetal enzymes, which become electrophoretically identical. This treatment however, did not influence the catalytic activity. The electrophoretic difference was confirmed by isoelectric focusing where the pI of the adult enzyme was 4.80 while the foetal enzyme was 4.62 (Garre et al., 1980). The pI of cord and adult AChE following neuraminidase treatment was 5.67. These observations indicated that the amount of sialic acid in these bloods was not identical.

AChE is also found on human T cells (Topilko and Caillou, 1985) and cultured human leukaemia/lymphoma cell lines (Ajmar et al., 1983; Rubinstein et al., 1984). These observations aid the

characterization of lymphocytes, since B cells have no detectable AChE activity.

In mice, AChE is a specific and early marker of the thrombocytic series and is a constituent of the platelet membrane (Paulus et al., 1981). The release of AChE, by maturing megakaryocytes and platelets, may establish a feedback inhibition loop whereby the level of acetylcholine available to precursor cells depends on megakaryocyte age and/or numbers. The level of AChE could therefore control megakaryocytopoiesis by a similar regulatory role in human erythropoiesis (Keyhani and Maigne, 1981).

Erythrocyte AChE in Clinical Conditions

Shanor et al. (1961) reported no variation in erythrocyte AChE due to the age (after 3-5 months) or sex of an individual. However, AChE is increased in the red cells in hereditary spherocytosis, in which molecules appear more fully exposed at the cell surface, probably related to the physical state of the lipid environment (Frenkel et al., 1980; Streichman et al., 1983) and a change in the membrane surface pressure.

Paroxysmal nocturnal haemoglobinuria (PNH) is a rare acquired haemolytic disorder in which clones of red cells exhibit increased susceptibility to complement-mediated lysis and a deficiency of AChE activity (Dacie, 1967; Rosse and Parker, 1985). Patients with PNH generally have a population of apparently normal cells (PNH I cells, which have normal amounts of AChE and are resistant to the haemolytic action of complement) that coexist with a population of complement sensitive cells (PNH

III cells, which lack AChE). Another population of cells, called PNH II cells, may be present along with or instead of the PNH III clone. PNH II cells show only intermediate sensitivity to complement, but also lack AChE. Radioimmunoassay, radioimmunoprecipitation (Chow et al., 1985) and immunodisplacement techniques (Brimijoin et al., 1986) employing monoclonal antibodies indicate that AChE is absent from complement sensitive PNH cells and present in complement insensitive PNH cells. Clonal cultures of bone marrow from two patients with PNH have shown dual populations of erythroid 'bursts' (BFU-E) (Rotoli et al., 1984). Normal colonies were seen as well as complement sensitive colonies, the latter being deficient in AChE. This deficiency seems only to be a marker for the disease.

In most cases of ABO haemolytic disease of the newborn the red cell AChE activity is below normal (Herz et al., 1972; 1973). Kaplan et al. (1964) showed that the AChE activity of red cells in ABO disease are significantly lower than those in rhesus disease and in normal newborns.

Erythrocytes coated with IgG 'warm' antibodies with or without complement, in autoimmune haemolytic anaemia (AHA), are more likely to have reduced AChE activity than red cells sensitized by complement binding cold antibodies (Sirchia et al., 1970).

Some decrease of AChE may also occur in disordered erythropoiesis such as myelofibrosis with myeloid metaplasia, erythroleukaemia and refractory anaemia (Herz and Kaplan, 1973). AChE has also been reported to be decreased in Down's syndrome (Kedziora et al., 1982).

Johns et al. (1962) reported a family showing reduction in erythrocyte AChE activity. Genetic variation in erythrocyte AChE was also reported by Coates and Simpson (1972).

Plasma Cholinesterase

Human ChE hydrolyses many choline and non-choline esters, its optimal substrate being butyrylcholine and unlike AChE it hydrolyzes benzoylcholine. The enzyme is found in plasma and in most tissues, but not in human erythrocytes.

Plasma ChE is of clinical importance in the hydrolysis of many muscle relaxants (Kalow and Genest, 1957; Brown et al., 1981), ester-type local anaesthetics and certain other drugs (La Du, 1971). The genetic variants of human serum ChE were discovered after succinylcholine gained widespread use as a muscle relaxant.

Succinylcholine or suxamethonium is the dicholine ester of succinic acid and has a short duration of action due to its rapid enzymic destruction by plasma cholinesterase. About 95% of an intravenous injection is hydrolyzed in the blood before reaching the patient's muscle. It was noticed that 1 in 2000 patients with lower levels of cholinesterase activity developed a prolonged apnoea after a standard dose of the drug (Kalow and Gunn, 1959). Evidence indicated the low enzymic activity to be inherited and a significant number of the healthy relatives had reduced levels of activity suggesting a serum protein polymorphism. It was tentatively proposed that the sensitive individuals were homozygous for an abnormal gene and that the inheritance was recessive (Lehmann and Ryan, 1956). Evidence by Stovner (1955) using a variety of substrates and inhibitors indicated that the cholinesterase of suxamethonium sensitive individuals appeared to be qualitatively different from normals and was called atypical.

Two genetically independent loci, the first involving four

allelic genes have been linked to alterations in serum cholinesterase activity (Whittaker, 1980). Currently, four main systems of nomenclature for the first locus are in use (Table 2). The classification of Motulsky has been widely applied and has been adopted in this thesis.

Table 2 Alternative Nomenclature for the Genetic Variants of human plasma cholinesterase coded for by the four alleles at locus 1

Genotype				Phenotype		
Motulsky (1964)	Goedde and Baisch (1964)	Lehmann and Liddell (1965)	Shows et al (1979)	Motulsky (1964)	Goedde and Baisch (1964)	
$E_1^u E_1^u$	$Ch_1^U Ch_1^U$	NN	CHE1*U/CHE1*U	U U	Ch1(UU)	
$E_1^a E_1^a$	$Ch_1^D Ch_1^D$	DD	CHE1*A/CHE1*A	A A	Ch1(DD)	
$E_1^f E_1^f$	$Ch_1^F Ch_1^F$	FF	CHE1*F/CHE1*F	F F	Ch1(FF)	
$E_1^s E_1^s$	$Ch_1^S Ch_1^S$	SS	CHE1*S/CHE1*S	S S	Ch1(SS)	
$E_1^u E_1^a$	$Ch_1^U Ch_1^D$	ND	CHE1*U/CHE1*A	UA I	Ch1(UD)	
$E_1^u E_1^f$	$Ch_1^U Ch_1^F$	NF	CHE1*U/CHE1*F	UF UF	Ch1(UF)	
$E_1^u E_1^s$	$Ch_1^U Ch_1^S$	NS	CHE1*U/CHE1*QO	US U	Ch1(US)	
$E_1^a E_1^f$	$Ch_1^D Ch_1^F$	DF	CHE1*A/CHE1*F	AF AF	Ch1(DF)	
$E_1^a E_1^s$	$Ch_1^D Ch_1^S$	DS	CHE1*A/CHE1*QO	AS A	Ch1(DS)	
$E_1^f E_1^s$	$Ch_1^F Ch_1^S$	FS	CHE1*F/CHE1*QO	FS F	Ch1(FS)	

Plasma Cholinesterase Variants

E_1^a gene

Kalow and Genest (1957) showed that the inhibition characteristics of the enzyme with dibucaine differed in sensitive and non-sensitive individuals. A fixed concentration of dibucaine ($10^{-5}M$) was used and the percentage inhibition measured using $5 \cdot 10^{-5}M$ benzoylcholine as substrate was defined as the dibucaine number (DN) under standard conditions of temperature and pH. Two allelic genes were postulated. The E_1^u gene is responsible for the biosynthesis of the usual form and the E_1^a gene for the atypical form of the enzyme. Sensitive individuals $E_1^aE_1^a$ have a DN about 20, $E_1^uE_1^u$ have a DN about 80 and some relatives of the sensitive patients were identified as $E_1^uE_1^a$ with a DN about 60 (Table 3). Maximum rates of hydrolysis of choline esters by the usual enzyme were two to four times greater than those obtained with the atypical enzyme (Davies et al., 1960). All Michaelis constants for the normal enzyme were lower than those for the atypical enzyme.

E_1^f gene

The fluoride resistant gene E_1^f was recognised by using sodium fluoride as differential inhibitor (Harris and Whittaker, 1961). The fluoride number (FN) was defined as the percentage inhibition by $5 \cdot 10^{-5} M$ sodium fluoride of the rate of hydrolysis of $5 \cdot 10^{-5} M$ benzoylcholine under standard conditions (temperature, pH etc). The E_1^f gene is allelic with the usual and atypical genes.

Silent Genes

Further studies in the 1960's indicated the existence of a silent gene where no cholinesterase activity could be demonstrated (Liddell et al., 1962; Hart and Mitchell, 1962). These individuals were homozygous $E_1^s E_1^s$, the silent gene being allelic with the usual gene (Simpson and Kalow, 1964).

Goedde et al. (1965) described two silent gene sera showing 2% activity with benzoylcholine as substrate and immunological reactivity was demonstrated using rabbit antisera to the usual human plasma cholinesterase. A structural alteration of the enzyme protein was assumed. Five other cases studied by Goedde et al. (1965, 1968) showed that 2 had no detectable enzymic or immunological activity.

A large number of silent homozygotes were found among the Eskimos of Western Alaska (Gutsche et al., 1967; Scott and Wright, 1967). One group (Type I) was found to have about 1% and the other group (Type II) about 3% of the usual activity. Acrylamide gel electrophoresis showed that Type I sera produced two bands of activity, one faster than any found in the usual enzyme, whereas Type II sera had five bands of activity, all corresponding to those found in the usual enzyme (Rubinstein et al., 1970). No reaction was detected with Type I sera when tested with an antisera to usual cholinesterase, whereas Type II did react with the antibodies. These workers concluded that the Type I serum cholinesterase is quantitatively and qualitatively different from both usual and Type II cholinesterase, whereas Type II and usual cholinesterase differ only quantitatively. Type I (E_1^s) and Type II (E_1^t) silent genes are allelic with each other

and with the E_1^u gene.

A third type of serum cholinesterase deficiency has been reported in Eskimoes having less than 10% of usual cholinesterase activity with a major band that migrates slightly faster than the major band of usual cholinesterase following starch gel electrophoresis at pH 5.3. This variant is allelic to the E_1 locus and the gene has been designated E_1^r (Scott and Wright, 1976). An individual with genotype $E_1^rE_1^s$ or $E_1^rE_1^t$ has less than 10% of usual cholinesterase activity.

Considerable confusion still exists over the heterogeneity of sera showing little or no cholinesterase activity. Further evaluation of these sera comparing enzymic and immunological reactivity should provide further insight and clarity into the characterization of these variants.

E_1^j and E_1^k

Garry et al. (1976) reported anomalous dibucaine numbers obtained with some sera which led to the postulate of a new gene, E_1^j . The $E_1^aE_1^j$ heterozygotes can be detected by inhibitors, but the other E_1^j variants can only be identified by genetic analysis. It was proposed that the usual enzyme, as produced by the E_1^u gene, was still being produced in these phenotypes, but the level of the circulating enzyme was reduced by about 66% (Rubinstein et al., 1976).

Using the inhibitor RO2 0683 (dimethylcarbamate of (2-hydroxy-5-phenyl(benzyl)-trimethyl-ammonium bromide) Evans and Wardell (1984) concluded that the E_1^j gene is probably rare in the general Caucasian population and that the similarity of DN and

FN values for $E_1^a E_1^j$ and $E_1^a E_1^f$ could lead to confusion in genotyping using only dibucaine and fluoride as differential inhibitors.

Rubinstein et al. (1978) described another variant E_1^k in two families, segregating for the E_1^a allele, from unusual dibucaine numbers. Evans and Wardell (1984) found the genotype $E_1^a E_1^k$ to be relatively common amongst Caucasians, but the possibility existed for many to be incorrectly phenotyped as $E_1^u E_1^a$. In addition, at present it is impossible to separate $E_1^u E_1^k$ and $E_1^k E_1^k$ from the usual homozygote $E_1^u E_1^u$, or from the heterozygote $E_1^u E_1^f$ by RO2 inhibition. Rubinstein et al. (1978) indicated that the E_1^k gene causes a reduction in enzymic and immunological activity of about 33% which could therefore be used to discriminate these genotypes. However, a more recent survey found $E_1^u E_1^k$ genotypes to have an average of 91% of the activity of the usual homozygote (Whittaker and Britten, 1985) and the flat Gaussian distribution curve of enzymic activities for the $E_1^u E_1^k$ heterozygote is not clearly separated from the similar distribution curve for the usual homozygote. Until the genotypes $E_1^u E_1^j$, $E_1^u E_1^k$ and $E_1^j E_1^k$ can be resolved from $E_1^u E_1^u$ the genetics of the E_1^j gene, as well as the E_1^k gene, will present problems. Some workers have observed enzyme activities within the normal range for some $E_1^u E_1^j$ genotypes (Whittaker and Britten, 1989).

E_1^h

Whittaker and Britten (1987) reported unusual inhibition characteristics in two unrelated suxamethonium sensitive individuals. The dibucaine and fluoride numbers suggested the phenotype $E_1^a E_1^a$, but multiple estimations of the RO2 number were unique. In addition, enzymic activity such as that found in some of the silent genes were noted (Whittaker, 1986). Since the latter are not known to exhibit unusual inhibition characteristics a new allele E_1^h was proposed.

Table 3 Activity and Inhibition Numbers of the Genotypic Variants of Serum Cholinesterase

Genotype	Relative Enzymic Activity ¹	DN	FN	Frequency
$E_1^u E_1^u$	100	77-83	56-66	96%
$E_1^u E_1^s$	50	77-83	56-68	1 in 190
$E_1^u E_1^f$	86	70-83	46-54	1 in 200
$E_1^u E_1^a$	77	48-69	44-54	1 in 25
$E_1^a E_1^f$	59	45-59	28-39	1 in 20,000
$E_1^f E_1^f$	74	64-69	34-43	1 in 154,000
$E_1^f E_1^s$	37	64-69	34-43	1 in 150,000
$E_1^a E_1^a$	43	8-28	10-28	1 in 2000
$E_1^a E_1^s$	22	8-28	10-28	1 in 29,000
$E_1^s E_1^s$	Activity too low to measure			1 in 100,000

¹ Benzoylcholine as substrate

Electrophoresis

Human serum cholinesterase exists in multiple molecular forms. Four bands may be demonstrated in fresh plasma, using starch gel or polyacrylamide electrophoresis at alkaline pH with α -naphthylacetate as substrate and about 90% of the activity is present in the C4 band. LaMotta et al. (1965) showed that the isoenzymes are interconvertible and that the multiple forms of plasma cholinesterase are molecular aggregates. It was found that the C4 enzyme is a tetramer whose subunits are equal in size and charge with a molecular weight comparable to the C1 component of the native enzyme.

An extra slow migrating cholinesterase band (C5) may be demonstrated in some Caucasians following starch gel electrophoresis at pH6 and its presence, C₅+ phenotype, was shown to result in 30% more cholinesterase activity than with the C₅- phenotype. The gene coding for this band, designated the E2+ gene (its allele is called the E2- gene), is located at the second locus for cholinesterase and is not allelic to the E1 locus (Table 4). The C₅+ phenotype has been found only in association with the E₁^uE₁^u and E₁^uE₁^a genotypes suggesting the extra band is a hybrid or complex of the enzyme protein from the E₁ locus with a second complementary protein from the E₂ locus (Scott and Powers, 1974; Muensch et al., 1978).

The recorded studies of the E₂ allele indicate that some children have shown the E₂+ phenotype even when both parents were E₂- (Harris et al., 1963; Ashton and Simpson, 1966; Goedde et al., 1973).

Neitlich (1966) reported four family members with

cholinesterase activities several times normal, an extra slow migrating band being present on starch gel electrophoresis. This variant was slower than the C_3+ band coded for at the E_2 locus. It was named Cynthiana after its place of origin.

An unusual patient with leiomyoma has also been described, sensitive to suxamethonium and exhibiting only 2% normal cholinesterase activity, but after six months, activity slowly increased with an increase in the slow migrating bands on PAGE until the usual major band was observed after about a year. It is believed that initially the serum contained two factors, one a neuraminidase-like enzyme responsible for altering the electrophoretic mobility of cholinesterase by cleaving various amounts of sialic acid and the other a proteolytic enzyme for reducing the cholinesterase activity. The proposed mechanism was called the 'epigenetic modification' hypothesis (Ogita, 1975). The neuraminidase-like enzyme is believed to be genetically controlled, but not expressed except under appropriate physiological or pathological conditions by a gene called N^R . Its allele, N^U is associated with its absence.

Table 4 Alternative nomenclature for the electrophoretic variants of human plasma cholinesterase coded for by two common alleles at locus 2

Genotype		
Harris et al (1963)	Motulsky (1964)	Shows et al (1979)
C ₅ -	E ₂ -	CHE2*C5-
C ₅ +	E ₂ +	CHE2*C5+

Chromosome Assignments

Genetic linkage studies suggest that the first locus of cholinesterase, E1 is situated on the long arm of chromosome 3 (Arias et al., 1985), at location 3q21-q26 (Huerre et al., 1984), by linkage with the caeruloplasmin gene and the transferrin receptor gene (Kidd and Gusella, 1985). Studies have shown that there are three introns at nucleotides 93, 1433 and 1600 (Kott 1987). The first of these is in front of the leader peptide sequence, while the other two are near the 3' terminus of the coding region.

Genetic linkage studies have suggested a possible linkage between the E2 gene and the α -haptoglobin gene (Lovrien et al., 1978), which maps in a region on chromosome 16, distal to the fragile site 16q22 (Simmers et al., 1986). However, recent evidence (Eiberg et al., 1988) indicates tight linkage of the E2 locus to the gamma-crystallin gene cluster (CRYG) locus located quite distally on chromosome 2 long arm (2q33-q35).

Possible Role of Plasma Cholinesterase

The physiological function of cholinesterase is unknown, but may include a role in the transmission of slow nerve conduction (Bergmann and Wurzel, 1954), a protective role by removal of choline esters which would inhibit acetylcholinesterase (Lehmann and Silk, 1953; Mezincesco and Ghetie, 1974), involvement in lipid or phospholipid metabolism (Clitherow et al., 1963; Kutty, 1980), regulatory involvement of choline homeostasis in plasma (Funnell and Oliver, 1965) and involvement in permeability control and transport of sodium and other ions across membranes (Duncan, 1967).

Composition and Structure

Plasma cholinesterase is biosynthesized in the liver, but its concentration there is low because it is released into the plasma immediately following synthesis. The enzyme is a glycoprotein. Nine carbohydrate chains appear to be attached to each asparagine residue at positions 17, 56, 106, 241, 256, 341, 455, 481 and 486 (Lockridge et al., 1987) consistent with the report by Haupt et al. (1966) that 23.9% of the weight of human serum cholinesterase is due to carbohydrate. These chains are the complex type terminating in sialic acid. The amino acid composition of the purified enzyme (usual phenotype) has been determined (Muensch et al., 1976; Soreq and Gnatt, 1987; Lockridge et al., 1987) (Table 1). In addition, the complete amino acid sequence has been reported by Lockridge et al. (1987).

Atypical serum cholinesterase has been shown to be due to a point mutation in the gene which changes aspartate 70 to glycine

(McGuire et al., 1989). The mutation occurring in nucleotide base 209, which changes codon 70 from GAT to CGT, was detected in all five atypical cholinesterase families examined. This mutation (acidic to neutral amino acid substitution) accounts for the reduced affinity of atypical cholinesterase for choline esters, indicating that Asp 70 must be an important component of the usual cholinesterase anionic site.

Lockridge et al. (1979) showed that highly purified usual human cholinesterase is a tetramer of molecular weight about 340,000 with identical subunits approximately 85,500 in molecular weight (Lockridge et al., 1987). The molecule consists of a dimer of dimers (Lockridge et al., 1979) with each dimer containing only one interchain disulphide bond between two subunits and 3-5 pairs of intrachain disulphide bonds found in each subunit. The four subunits are held together by non-covalent bonds located near the surface of the molecule when the interchain disulphide bonds are broken. Human plasma cholinesterase consists of 574 amino acids per subunit, with each subunit containing one active centre.

As with acetylcholinesterase there are two active sites, the anionic and the esteratic active site. The amino acid sequence of the esteratic site for $E_1^aE_1^a$, $E_1^aE_1^s$ and $E_1^uE_1^u$ was found to be Gly-Glu-Ser-Ala-Gly-Ala-Ser-Ala-Val-Ser-Leu (Lockridge and La Du, 1986). The catalytically active serine residue underlined is the 198th residue from the amino terminus (Lockridge et al., 1987).

Cholinesterase levels in the Plasma

Apart from pregnancy and iatrogenic causes plasma cholinesterase activity in healthy adults varies very little over long periods of time. However, activity can vary with physiological factors such as age and sex and with other parameters such as body fat, plasma lipids or lipoprotein fractions. Males have higher cholinesterase activities than females (Probert and Brackenridge, 1976).

At birth, levels are approximately 30-50% of adult values (Zsigmond and Robbins, 1972), there being a significant increase from the first week of life to 4 months. This rapidly increases during the first 3-5 years of childhood and subsequently falls to the adult levels at puberty (Hutchinson and Widdowson, 1954).

The half life of cholinesterase appears to be in the range 2-16 days (Neitlich, 1966; Hall et al., 1984).

Cholinesterase in Clinical Conditions

Decreased ChE Activity

Plasma ChE activity decreases, sometimes substantially, in acute hepatitis, cancer and burns patients. Sequential determination of activity can be an index of a patients prognosis.

There is a rapid decrease in cholinesterase activity of about 30% during the first trimester of pregnancy which is maintained until delivery. A further decrease in enzymic activity occurs during the early puerperium reaching a minimum at about the third day, after which the activity returns to normal after 3-6 weeks post-partum (Robson et al., 1986). Several alternate patterns of change during pregnancy have also been reported (Evans et al., 1988). Interestingly, oral contraceptives containing oestrogen may also decrease plasma cholinesterase activity by 50% and modify the cholinesterase isoenzymes (Whittaker et al., 1971). This is reversible on withdrawal of the contraceptive. Redderson (1973) has suggested that the effects are caused by a steroid-induced depression of hepatic cholinesterase biosynthesis.

Glucocorticoids and oestrogens also lower the plasma cholinesterase activity. Prednisone treatment may produce a decrease of 50% activity (Foldes et al., 1974), while oestrogen treatment for cancer of the prostate may reduce cholinesterase activity to 20% normal (Archer and Janowsky 1978). Plasmapheresis (Wood and Hall 1978) and haemodilution cardiopulmonary bypass (Viby Mogensen 1982) produces a dramatic fall in cholinesterase.

Many drugs can reduce the cholinesterase activity in vivo. Most

of these are inhibitors, but others may reduce the biosynthesis of the protein in the liver.

Inhibitors are classified as reversible or irreversible. The irreversible group are long lasting and normal enzymic levels are restored a few weeks after withdrawing the drug, when the liver has synthesized enough enzyme to give normal cholinesterase activity. This includes cytotoxic compounds (Wang and Ross, 1963; Zsigmond and Robins, 1972) and cyclophosphamide used in cancer treatment (Walker et al., 1972), echothiopate iodide used in treatment of glaucoma (McGavin, 1965) and the organophosphorous insecticides (Barnes and Davies, 1951).

Reversible anticholinesterases include edrophonium, neostigmine and pyridostigmine (Barrow and Johnson, 1966; Foldes and Smith, 1966). Other drugs are used as muscle relaxants either alone or by potentiating the action of suxamethonium using the inhibitory property of the drug on plasma cholinesterase. Vercuronium (Whittaker and Britten, 1981), propanidid (Doenicke et al., 1968), pancuronium (Katz, 1971; Stovner et al., 1975), local anaesthetics and monoamine inhibitors (Bodley et al., 1969) can potentiate succinylcholine.

The inhibitors which most frequently cause toxicological problems are the nerve gases, such as Tabun and Sarin and the systemic insecticides (eg. parathion and marathion). Many of these potent inhibitors are either organophosphates or carbamates. The pharmacological effects of these agents are due to inhibition of acetylcholinesterase in membrane structures of the nervous system. Most of these substances inhibit both acetylcholinesterase and plasma cholinesterase. In general, the

plasma enzyme is more susceptible to inhibition than the red cell enzyme and this has been used as a biochemical indicator for the presence of some types of inhibitors. A substantial decrease in the activities of both enzymes below normal values is indicative of an environmental effect, such as irreversible organophosphate inhibition.

The rate of binding of the organophosphates to the enzyme varies greatly. It is usually fast following exposure to a high concentration of nerve gas, but slower with the insecticides which often require metabolic activation by the liver before becoming powerful cholinesterase inhibitors.

There is considerable interperson variability in the vulnerability to cholinesterase inhibition. In many glaucoma patients using ecothiopate iodide as eyedrops the cholinesterase activity is not much depressed, whereas in others on exactly the same treatment plasma cholinesterase may be completely inhibited.

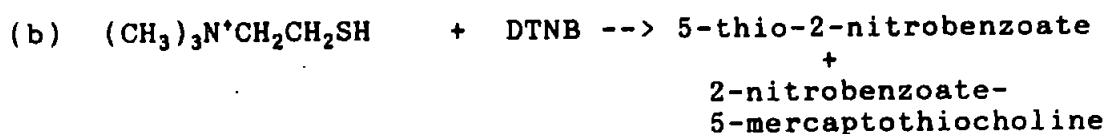
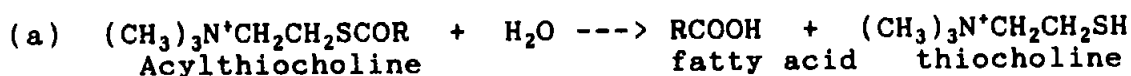
Increased ChE Activity

E2+ variants have on average 30% higher activity than the E2-phenotypes. Other examples of genetically determined high activity have been reported in the Cynthiana variant (Neitlich 1966). In addition, 20% increase in enzymic activity has been reported in thyrotoxicosis (Thompson and Whittaker, 1965; Vincent et al., 1973). Treatment restores the mean esterase level.

Methods of Assay of Acetylcholinesterase and Cholinesterase Activity

Enzyme activity can be measured by a wide variety of techniques (Augustinsson, 1971; Whittaker, 1984), based on the measurement of the rate of hydrolysis of an ester, catalysed by cholinesterase. The enzyme should be demonstrated by low concentrations (10^{-5}M) of specific inhibitors for both AChE and ChE such as eserine, or by specific inhibitors such as E600 (diethyl-4-nitrophenyl phosphate) and quinidine sulphate ($2 \times 10^{-5}\text{M}$) for ChE and 3116CT (bis(3-dimethylamino-5-hydroxyphenoxy) 1,3-propane dimethiodide) for AChE.

Acylthiocholines are hydrolyzed by ChE to the corresponding fatty acid and thiocholine. The rate of formation of the thiocholine can be monitored by the continuous reaction of the thiol group with 5-5'-dithio-2-nitrobenzoate and other products. The rate of formation of the yellow anion can be measured spectrophotometrically at 410nm (Ellman et al., 1961).



When reporting cholinesterase activity it is essential to state the substrate used, since the normal range is characteristic for each substrate. Benzoylcholine and butyrylthiocholine are probably the most commonly used substrates for ChE, with succinylthiocholine and propionylthiocholine having selected use. Currently, activity is recorded in μmol of substrate hydrolysed per minute per ml of serum or plasma at 25°C.

The colorimetric method of Okabe et al. (1977) using a choline oxidase/peroxidase procedure can be used (Panteghini and Bonora, 1984) in conjunction with a centrifugal fast analyser. Chemiluminescent assays using luminol to detect hydrogen peroxide and fluorimetric methods (Pantel, 1981) provide sensitive determination of enzymes at low substrate concentration.

Enzyme Immunoassay

Enzyme immunoassays were introduced about 20 years ago for the identification of precipitin lines obtained by immunodiffusion and electrophoresis (Nakane and Pierce, 1966, 1967; Avrameas and Uriel, 1966). The principles on which they are based are quite similar to those of immunofluorescence, where an antigen is specifically detected due to the discriminatory power of antibodies, and a marker attached to these antibodies indicates that such a reaction takes place. Enzymes are used as markers in enzyme immunoassays since they have, in contrast to fluorescent labels, the capacity of amplification. Peroxidase was used as the enzyme, and thus techniques were known as immunoperoxidase procedures.

An important advance was the use of various solid phases (originally plastics such as polystyrene) that have the capacity of binding antigens or antibodies in high concentrations under certain ionic conditions. These immunosorbants could then select the molecule to be detected from the test fluid which, in turn, could absorb an enzyme-labeled antibody (detector molecule). Application of this approach to the immunoperoxidase method simplified quantitative techniques, eliminating cross reaction in many instances. Automation of these systems also became viable. Development of monoclonal antibody production and auxiliary amplification molecules (avidin/biotin, streptavidin, protein A, lectins) all contributed to the rapidly expanding use of enzyme immunoassays.

Antibody Preparation

It is practically impossible to make reproducible polyclonal reagents. Even antisera from the same animal taken at different times differ in their properties. In contrast, monoclonal antibodies have the same specificity and affinity, can be produced in virtually unlimited quantities and pure immunogen is not required for the immunization. However, a monoclonal antibody is unable to distinguish different antigens if they bear the same epitope, specificity may not be as high as expected, some may cross-react, which, in contrast to polyclonal antisera, cannot be removed with immunosorbents (Brodsky et al., 1979) and production is time consuming.

Monoclonal antibodies may be much more sensitive to inactivation by freezing and thawing, changes in pH or other physical factors (Mosmann et al., 1980) important for their purification. The lower avidity of monoclonal antibodies and the small fraction of high-affinity antibodies (dominant in polyclonal sera) are the probable cause of their frequent lower detectability in enzyme immunoassay (EIA) when compared with polyclonal antisera and makes the selection of high-affinity monoclonal antibodies mandatory. Pooling of different monoclonal antibodies to gain avidity has given conflicting results (Ehrlich et al., 1982). Ultimately however, monoclonal antibodies make new designs of EIA possible. The most important advantage monoclonal antibodies offer for EIA is the possibility to standardize assay methods, the specificities, detectabilities and sensitivities.

Polyclonal Antibody Production

The purpose of immunization is to obtain high-titred antisera with a high avidity. These may be increased by admixture of the immunogen with a suitable adjuvant (Freund's). Alternatively, protein stained with Coomassie blue may be cut as bands from polyacrylamide gels and used directly (Buolard and Lecroisy, 1982). It is also possible to use immune precipitate in agarose gels, eliminating contaminants which copurify with immunogen, but do not coprecipitate. One regime consists of injection of 25-50 µg/kg of immunogen once a month for up to 1 year.

A 10-fold increase in titre may be achieved by immunoglobulin isolation using salt precipitation with saturated ammonium sulphate, ion-exchange chromatography (Levy and Sober, 1960), isolation on Protein A-Sepharose (Langone, 1982), or by immunosorbents (Maze and Gray, 1980).

Monoclonal Antibody Production

Kohler and Milstein (1975) reported the successful fusion of normal mouse antibody-secreting lymphocytes with the cells of a myeloma cell line, thus producing a hybrid-myeloma which combined the properties of cell growth and antibody secretion. The hybrid cell could be grown in tissue culture and yield antibody in the supernatants. The useful antibody secreting cell lines are selected by means of simple screening tests, such as ELISA, on the supernatants. The cell lines are purified by cloning and are stored in liquid nitrogen to guarantee quality and supply.

Enzyme Conjugation

Enzymes most commonly used include horseradish peroxidase and alkaline phosphatase. Three different approaches for conjugation exist. Chemical labeling (Avrameas and Ternynck, 1971), immunological labeling (Mason and Sammons, 1978) and auxiliary nonimmune recognition systems (biotin/avidin, protein A, lectins). Preformed peroxidase-anti-peroxidase (PAP) complexes (Sternberger et al., 1970) may also be used.

Avidin-Biotin Complex Methods

Biotin a small, water soluble vitamin (m.wt. 244) can be easily conjugated to antibodies without damage to the antigen binding ability. Biotinylation does not impair horseradish peroxidase, but alkaline phosphatase activity is severely reduced. Avidin a glycoprotein (m.wt. 67,000) available from egg white has four active binding sites for biotin and since many biotin molecules can be bound to a protein this has a potential for amplification.

Avidin, however binds in a non-specific manner to nuclear material and cell membranes and will react with other biological molecules such as lectins via the carbohydrate moiety. This has resulted in many workers substituting avidin with streptavidin. Streptavidin, a protein (m.wt. 60,000) with four high affinity sites for biotin, is isolated from the bacterium *Streptomyces avidinii* and does not exhibit non-specific binding. It is the strength of the interaction between biotin and streptavidin which enables the development of detection methods with short incubation times, while providing very high sensitivity and specificity. 'Spacer arms' between the biotin and the

macromolecule, allowing the binding between biotin and streptavidin to proceed unhindered are used to increase sensitivity.

The Immobilization of immunoreactants on Solid Phase

Microtitre plates have enjoyed considerable popularity as solid supports for both qualitative and quantitative enzyme immunoassays. It is desirable for the solid phase to have a high capacity for binding immunoreactants, to exhibit minimal dissociation, to incur negligible denaturation of the immobilized molecule and to orientate the immobilized antibody with binding sites towards the solution and the Fc portion towards the solid phase.

Plastic however, being immunoreactant-consumptive, with a low adsorption capacity per unit area, may not be suitable for large antigens due to spacial limitations. The resultant antibody-antigen interactions proceed much slower than in solution. Considerable inter-well variation in protein adsorption may exist, especially noticeable at the edges of the plate where an increased amount of protein attaches to the wells due to pronounced temperature differences across the plate (Burt et al., 1979), wells at the edges being several degrees hotter than those in the interior ('edge effect'). Thus, immunochemical and enzymic reactions in edge wells may proceed at a higher rate than those in cooler interior wells.

Nature of Protein-Plastic Interaction

Non-covalent Attachment

Protein adsorption to plastic is generally attributed to non-specific hydrophobic interactions and is independent of the net charge of the protein, although binding is different and characteristic for each protein (Cantarero et al., 1980). Non-covalently adsorbed antigen may be desorbed during the test (Engvall et al., 1971) and compete for the immunoreactant added, leading to decreased detectability and increased variability. This can be minimized by using the immobilized reactant at the appropriate concentration and washing extensively after immobilization and each subsequent step (Cristensen et al., 1978). With an excess of protein, multiple layers stack on the protein monolayer by protein-protein interactions. Such secondary interactions are not very stable and interfere in the EIA.

The three most important variables for the adsorption of proteins on a solid phase are temperature, time and concentration. The most widely used coating buffer is 0.05M carbonate, pH 9.6. Incubation is usually overnight at 4°C in a humid chamber, but may be shortened by increasing the temperature or the antigen concentration. The optimum coating concentration for purified IgG is 1µg/ml, and for antigens or antibodies between 1 and 10µg/ml (Engvall, 1980), but at least 60min incubation at 37°C is required. The detectability with complete antiserum is about 4 times less than with purified IgG. Free spaces on the solid matrix may be blocked using an albumin solution.

Covalent Attachment

A simple method is pretreatment of polystyrene with glutaraldehyde (GA) or ethylchloroformate (ECF) (Dobbins Place and Schroeder, 1982). Microtitre plates thus treated can be stored for at least 4 weeks. Pretreatment of antibodies with GA prior to coating can decrease their desorption and give a higher detectability in EIA.

Attachment of Antigens or Antibodies to Plastic

Heusser et al. (1981) have described methods to attach cells to the bottom of microtitre plates to form stable cell monolayers suitable to detect antibodies against cell surface antigens. Plates are either pretreated with a heterogenous population of antibodies (IgG fraction) directed against cell surface components or with lectins, such as phytohaemagglutinin (PHA). Attached cells can be subsequently fixed with glutaraldehyde. Fixation of cells with glutaraldehyde in conjunction with poly-L-lysine (Heusser et al., 1981) coating of plates can also be used to prepare stable cell monolayers for solid phase assays. Under these conditions, cells remain firmly attached. Such cell layers can be used for both radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA) to yield specific and reproducible results. Plates containing glutaraldehyde-fixed cells can be stored for several weeks without loss of cell membrane antigens. Nonspecific binding to reactive sites on the plate may be reduced by the use of 1% BSA, 0.1M glycine or 0.05% tween 20.

Design of Enzyme Immunoassays

Two types of EIA can be distinguished, homogeneous (in solution), for the detection of small compounds and heterogeneous (one of the immunoreactants is anchored to a solid phase), for the detection of large compounds. Two differential type of assay have been developed (Kurstak et al., 1984; Tijssen, 1985) namely activity amplification (AA) and activity modulation (AM) assays. In the former the detector immunoreactant is used in large excess to detect the antigen and by the law of mass action, exceedingly small amounts of sample molecules can then be detected. Specificity of such methods may be poor. The contrary applies to AM methods where low concentrations of immunoreactants are used.

Noncompetitive Solid-Phase Enzyme Immunoassays

Both antigens and antibodies may be detected. The immunoreactant, complementary to the molecule to be detected, is immobilized on the solid phase. This sensitized solid phase serves to extract the target molecules from the sample fluid. This is followed by one or more steps with antibodies, which may be labeled with an enzyme.

Competitive Solid-Phase Enzyme Immunoassays

Competition always occurs in the second layer of solid phase reactions. The molecule to be detected competes either with the labeled species for the solid-phase immunoreactant or with the solid-phase immunoreactant for the labeled species. Either antigen or antibody can be labeled.

Homogeneous Noncompetitive Enzyme Immunoassays

Two antibodies are labeled with two different enzymes, the product of one being the substrate of the other (eg. hydrogen peroxide produced by glucose oxidase is consumed by peroxidase).

Homogeneous Competitive Assays

Reaction of the antibody with the hapten attached to enzyme, substrate or cofactor may cause steric inhibition and thus modify the enzymic activity or change the conformation of the enzyme resulting in a modulation of enzymic activity. Haptens present in the sample will bind with the antibody and thus prevent a proportional amount of antibody from reacting with the enzyme. This will decrease the enzyme modulation.

Solid Phase Enzyme Immunoassays

The molecules or complexes to be detected diluted in a suitable buffer are extracted with the appropriate receptor and detected by enzyme-labeled tracers. Nonspecific adsorption to the solid phase is avoided by adding a large excess of blocking agent such as a non-ionic detergent (tween 20, triton X-100) or an inert protein (gelatin or B.S.A.).

The ionic conditions should be such that specific antibody-antigen interactions are not influenced, whereas nonspecific interactions are minimized. For this reason an isotonic buffer is usually chosen. Ionic interactions are the most frequent cause of background staining. The buffer may also influence the enzyme. PBS contains a high concentration of inorganic phosphate which is a strong competitive inhibitor of alkaline phosphatase and

Tris buffer is then advised for use with this enzyme.

The optimal temperature, time and buffer solutions should be established in preliminary tests and checkerboard titrations performed to determine the optimal dilution of the various components of the test. The conjugate dilution and incubation period is chosen giving maximum absorbance with the specific antigen, but below 0.2 for the nonspecific samples. Alternatively, dilution will be suitable where the standard curve shows sufficient linearity of appropriate slope for that particular assay so that the majority of test samples can be evaluated.

Increased sensitivities are possible with bridge methods using avidin-biotin, PAP and lectin. Immune reactions can also be accelerated considerably in the presence of polyethylene glycol (PEG) 6000 (Hellsing and Richter, 1974). Increasing PEG 6000 concentration, both accelerates the reaction and increases detectibility, however the background also increases.

Processing of Data

The standard deviation (SD) and coefficient of variation (CV) should be determined for each system developed and results presented accordingly. Computer programs have been designed to rapidly process EIA data taking these requirements into consideration. Intra and interassay variations of results need to be assessed to evaluate the precision and reproducibility of the system. Reproducibility is generally expressed by testing the samples in duplicate and the SD calculated by the equation

$SD = \sqrt{\sum d^2 / 2N}$ in which d is the difference between duplicate

absorbances and N the number of paired samples.

Results may be presented in a number of ways. In terms of a cut-off value to discriminate presence or absence of test antigen. This level may be 0.15 absorbance units (Halbert et al., 1983), two or three times the mean (Malvano et al., 1982), or the mean plus two or three standard deviations (Richardson et al., 1983). In addition visual inspection has frequently been used for rapid screening of enzyme immunoassays; this is quite subjective however, and difficult to distinguish positive from negative responses at the doubtful interval.

Comparison of absorbance values obtained with that of a standard curve will present results either in arbitrary or relative units or in terms of concentration of the antigen or hapten, often in mg/ml. These dose-response curves which serve to estimate the antigen concentration are usually sigmoidal or hyperbolic. Precision of the standard curve can be considerably improved with suitable plotting systems. Plotting the absorbance data linearly or logarithmically against a logarithmic x-scale often facilitates the construction of a reliable standard curve, but may not always prove satisfactory. Curvilinear fitting may be performed by transforming one or both variables (from linear to square root, logarithm or inversion), by polynomial or other curvilinear regressions, or by transformations for proportions (Tijssen, 1985).

Immunochemistry of the Cholinesterases

Modern immunology has opened new approaches to the identification of subtle structural differences in the apparently homogenous cholinesterases isolated from different sources. Particularly rapid progress has been made in applying immunochemistry, immunocytochemistry and immunoassay to the study of AChE and ChE (Brimijoin and Rakonczay, 1986).

Many polyclonal antibodies have been produced to AChE (Williams, 1969; Rieger et al., 1976; Gurari and Fuchs, 1974; Adamson, 1977; Zanetta et al., 1981) and plasma ChE (Hodkin et al., 1965; Rubinstein et al., 1970, 1976, 1978; Eckerson et al., 1983) providing useful information. Monoclonal antibodies now offer a means of gaining insight into the structure, function and biosynthesis of ChE's (Fambrough et al., 1982; Brimijoin et al., 1983; Mintz and Brimijoin, 1985).

Williams (1969) reported inhibition of non-neuronal AChE by polyclonal antisera. Niday et al. (1977) described an antibody to erythrocyte AChE which gave single precipitin lines with the enzyme on immunodiffusion and rocket, crossed and immunoelectrophoresis. The enzymic activity of purified AChE was partially inhibited by the antiserum, reaching only 35% inhibition even with excess amount of antibody. Results indicated competitive inhibition. Agglutination of human red blood cells by monospecific antiserum to AChE confirmed the localization of the enzyme on the outer surface of the erythrocyte membrane.

Fambrough et al. (1982) reported the production of monoclonal antibodies to human erythrocyte AChE. The specificity of each antibody for human erythrocyte AChE was confirmed when each

antibody interacted with AChE in solution to form discrete complexes sedimenting more rapidly than free AChE. Isoelectric focusing and SDS polyacrylamide gel electrophoresis of the purified immunoglobulin indicated that all the monoclonal antibodies were unique, thus defining at least four antigenic sites on the AChE molecule. The monoclonal antibodies with similar cross-species reactivity (AE-1 and AE-2) were shown to bind to different sites on the ChE molecule by velocity sedimentation techniques. Hybridomas AE-1 and AE-2 were donated to the American Type Culture Collection.

Brimijoin et al. (1983) described the production of several monoclonal antibodies to human AChE and ChE. A solid phase immunoabsorbance assay was used to screen and characterize the antibodies. No cross-reactivity of the AChE antibodies with ChE nor ChE antibodies with AChE was reported. Moreover, none of the antibodies bound to the catalytic active site of AChE or ChE, since neither enzyme was inactivated.

Further studies have been done with the aid of anti-human AChE monoclonal antibodies, fluorescein-isothiocyanate labelled second antibodies with a fluorescence-activated cell sorter (Brimijoin et al., 1986; Dockter and Morrison, 1986). This system has been used to identify separate populations of AChE-positive and AChE-negative red cells in the blood of patients with PNH corresponding to PNH I and PNH II cells respectively (Brimijoin et al., 1986).

Hangaard et al. (1984) reported an immunological method for measuring AChE in different body fluids. A polyclonal antibody was produced in rabbits and bound to the wells of a microtitre

plate. The enzyme was quantitated by its own enzymic activity. This technique forms the basis of an additional test to monitor potential neural tube defects (NTD). In addition to elevated alpha-foetoprotein (AFP) in amniotic fluid and maternal serum, the concentration of AChE is also raised in most cases of NTD.

Sorensen et al. (1986) reported AE-2 inhibited the AChE enzymic activity. The epitope resided on the individual subunits. The rate of phosphorylation of the enzyme by diisopropylfluorophosphate was not affected by the antibody, indicating it does not affect the esteratic site. However, inhibitors directed towards the anionic site(s) competed with antibody binding, suggesting that one of these is the epitope. Further results suggested that interaction between enzyme and antibody took place, not by binding directly to the anionic site itself, but rather inducing an allosteric long distance interaction between the antibody binding site and the anionic site. The titration with antibody is biphasic and yields about 80% inhibition even in the presence of a large excess of antibody. Inhibition is fully reversible upon dilution in a time dependent manner. AE-2 was also found to inhibit human adult and foetal brain AChE. ChE showed no reactivity towards the antibody.

Brimijoin et al. (1987) described a radiometric assay for human AChE using a ^{125}I -labeled antibody against AChE with a detection limit near 100pg. An AChE enzyme-linked immunosorbent assay (ELISA) using a biotinylated second antibody detected by alkaline phosphatase conjugated avidin was also described with a sensitivity similar to that of the radiometric assay (Brimijoin et al., 1987).

The development of an enzyme immunoassay system for erythrocyte AChE has been relatively slow probably due to the number of other methods of assay currently available. These include colorimetric (Whittaker, 1984), fluorometric (Brimijoin et al., 1986) and radiometric (Brimijoin et al., 1987) assays. The colorimetric assay presents results in terms of enzyme activity, while the radiometric and fluorometric procedures, although quantitating amount of enzymic protein, require elaborate and expensive equipment.

A number of methods for the quantitation of proteins on the surface of erythrocytes have emerged over the last few years and these methods have been adapted and compared in an attempt to provide a set of simple yet accurate and reproducible protocols for the characterisation of erythrocyte AChE. These techniques involve the quantitation of AChE in situ on the intact red cell, precluding the laborious extraction of this enzyme molecule using gel filtration (Froman et al., 1980) and centrifugation (Steck et al., 1970) techniques.

It is of interest to know whether the inherited variants of human ChE can be immunologically distinguished. This does not appear to be possible at the moment. Tests with antibodies (Eckerson et al., 1983; Brimijoin et al., 1983) demonstrated immunological similarity between the 'atypical' and 'usual' form of ChE.

Aims of Proposed Work

The established method of immunological quantitation of plasma cholinesterase is by rocket immunoelectrophoresis using polyclonal antisera. The method however is unsuitable for measuring low levels of protein (below 10% of usual). The introduction of enzyme linked immunological techniques in recent years with the increased availability of high quality monoclonal and polyclonal antibodies, make these procedures ideal for the development of a more suitable quantitative assay for the cholinesterases. In addition, the use of a battery of monoclonal antibodies defining different antigenic regions of the molecule will provide a further level of characterization. The genetic variants of plasma cholinesterase will be investigated by ELISA to give greater insight into the relationships which they have with each other and with the usual form of the enzyme. Of particular interest is the group of 'silent' variants where a degree of confusion appears to exist at present. The implementation of immunological measurements on these samples may provide further classification of the group.

The variation seen in plasma cholinesterase in certain conditions will also be investigated using these immunological techniques. An interpretation of the observed decrease in plasma cholinesterase activity which occurs during pregnancy and continues during the early puerperium could be provided by monitoring the changes in molar concentration of the plasma enzyme which occur during this period.

The adoption of microplate techniques has the added advantage of relatively fast mass screening capability with a sensitivity

appropriate to the enzyme under investigation. Several ELISA protocols using different monoclonal and polyclonal antibodies will be used for evaluation and comparison. The best procedure in terms of accuracy, reproducibility, sensitivity and applicability will be presented.

Acetylcholinesterase, also, is well suited for enzyme linked immunological assay since the enzyme is located on the outer surface of the membrane. Both liquid and solid phase techniques will be investigated for the most appropriate assay system. Changes in erythrocyte acetylcholinesterase activity and in the physical properties of the enzyme have been reported in several haemolytic conditions such as paroxysmal nocturnal haemoglobinuria. Enzyme linked techniques will again be used to determine whether the observed catalytic changes are functions of the molar concentrations of the enzyme located in the erythrocyte membrane. Progress in this field will probably be slower than for the plasma enzyme, since acetylcholinesterase is less stable and the relevant pathological and physiological conditions are rare.

Methods

The compositions of buffer, substrate and antibody solutions used are given in Appendix I.

Erythrocyte Acetylcholinesterase

Erythrocyte AChE Activity

The basis of the method is the hydrolysis of acetylthiocholine to acetic acid and thiocholine (Ellman et al., 1961). The catalytic activity is measured by following the increase of the yellow anion, 5-thio-2-nitrobenzoate, produced when thiocholine reacts with 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB). It is necessary to confirm a linear relationship of the assay procedure at the selected temperature (fig 3).

A reagent blank to estimate the non-enzymatic hydrolysis of the substrate was prepared by substituting phosphate buffer solution in place of the sample. 3ml of phosphate buffer were pipetted into a glass tube to which 20µl of washed (*3 with 0.9% NaCl) packed cells were added. 20µl of acetylthiocholine solution (ATI) (Aldrich Chemical Co.) were added, the reagents mixed and incubated at 22°C for 2min. Following centrifugation at 1000*g for 30sec, 1ml of supernatant were removed to which 33µl of DTNB (Aldrich Chemical Co.) were added. 200µl of reaction mixture were transferred to a microplate and the absorbance measured at 405nm. Triplicate testing indicated a CV of 7%.

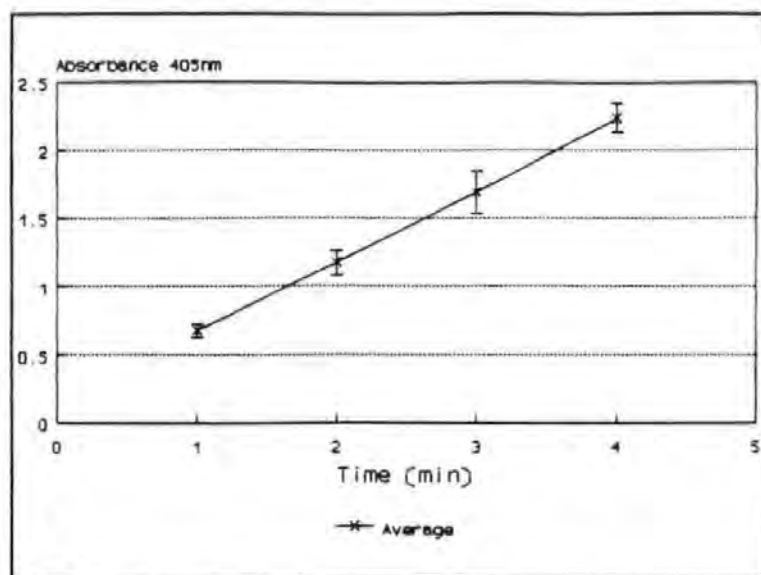


Figure 3
The effect of increasing time of incubation of ATI with adult erythrocytes on absorbance (20 samples tested).

A range of activities were calculated for 30 healthy adult males and test results were presented in terms of arbitrary units (AU) relative to the mean of that range. Alternatively, activity was calculated using equation 1.

Equation 1

$$\text{AChE activity} = \frac{a}{b} * \frac{\Delta A}{\Delta t} * \frac{1000}{13600} * \frac{1}{l} \text{ } \mu\text{mole/min/ml packed cells}$$

where a = total volume; b=sample volume

ΔA = change in absorbance; Δt = change in time

l = depth of light path on the microplate

Molecular extinction coefficient of the

yellow anion = 13600

For 20 μ l of packed cells + 3ml of phosphate buffer +
20 μ l of acetylthiocholine

$$\text{Dilution} = \frac{20}{3040}$$

and for 1ml of supernatant + 33 μ l DTNB

$$\text{Total dilution} = \frac{20}{3040} * \frac{1000}{1033} = \frac{\text{sample volume}}{\text{total volume}}$$

$$\text{Total dilution} = 0.006369$$

$$\frac{a}{b} = \frac{1}{0.006369} = 157$$

For 200 μ l of reagent the depth of the light path = 0.780cm

Enzyme units for the SI system are based on moles/second rather than μ moles/min. The unit of enzyme activity corresponding to the conversion of 1mole/sec of substrate is the katal. To convert U (μ moles/min) to katal (mole/sec) multiply μ moles by 10^{-6} to obtain moles and divide minutes by 60 to obtain seconds.

$$\begin{aligned} 1 \text{ U} &= 1\mu\text{mole/min} = \frac{1 * 10^{-6} \text{ mole/sec}}{60} \\ &= 16.67 * 10^{-9} \text{ katal} \end{aligned}$$

Since 10^{-9} katal = 1 nanokatal

$$1 \text{ U} = 16.67 \text{ nanokatal}$$

Enzyme activity is expressed as μ kat, nkat or pkat per litre to obtain convenient units.

Interference in the assay technique due to uneven sampling of the suspended red cells will result in poor reproducibility in duplicate samples.

Immunological Quantitation of Erythrocyte AChE

Haemagglutination Method

50µl of AE-1 and AE-2 (Bristol Blood Transfusion Centre) were incubated with 50µl of a 2% red cell suspension (in LISS) for 20min in a 37°C water bath. Cells were washed 4 times in isotonic saline and 50µl of rabbit anti-mouse immunoglobulin (DAKO) reagent added. Cells were centrifuged 1000*g for 20sec and examined over a light box for agglutination. Reaction with anti-mouse immunoglobulin produced positivity with slight prozoning, AE-1 showing a titre of 1/128, AE-2 1/512. However, agglutinates were weak and easily disrupted.

Microplate Antiglobulin Test

A rigid polystyrene 'U' well microplate was primed with 100µl of UBS/BSA. 100µl of a 20% red cell suspension were aliquoted into each well and the microplate centrifuged at 1000*g for 2min. The supernatant was discarded and the cells resuspended in 135µl UBS/BSA giving a final concentration of approximately 2%. 25µl aliquots from the master tray were transferred to a dilution microplate ('U' well) and resuspended in 135µl UBS/BSA giving an approximate concentration of 0.2%. 25µl of diluted antiserum (AE-2 diluted 1/100 in UBS/BSA) and 25µl of red cell suspension were added to a 'V' well microplate and incubated for 1hr at 37°C. The plate was washed 4 times with UBS/BSA/tween, the cells being packed hard by centrifugation (1000*g for 2min) after the last wash. 25µl of 1/1000 diluted anti-IgG was added to each well and incubated for at least 15min at room temperature and centrifuged 1000*g for 1min. The microplate was then examined using an angled

light box. Positive reactions result in the red cells remaining at the bottom of the well, negatives stream down the side of the microwell.

The optimum dilution for the rabbit anti-mouse immunoglobulin was 1/1000 for a 1/100 dilution of AE-2 (table 5). This method has the potential for high volume testing of samples and can therefore be used as a convenient screening procedure.

Table 5 Checkerboard titre of AE-2 in the
Microplate Antiglobulin Test

<u>AE-2</u>	<u>Rabbit anti-mouse immunoglobulin dilution</u>							
	<u>1/50</u>	<u>1/100</u>	<u>1/200</u>	<u>1/400</u>	<u>1/800</u>	<u>1/1600</u>	<u>1/3200</u>	<u>1/6400</u>
1/32	+	+	+	+	+	+	+	-
1/64	+	+	+	+	+	+	+	+
1/128	+	+	+	+	+	+	+	-
1/256	+	+	+	+	+	+	-	-
1/512	+	+	+	+	+	-	-	-
1/1024	+	-	-	-	-	-	-	-
1/2048	-	-	-	-	-	-	-	-

Solid Phase Microplate Monolayer Method

(1) Liquid Phase Sensitization

A 2% erythrocyte suspension was sensitized with the antibodies AE-1 and AE-2 by incubation in liquid phase for 20min at 37°C. Microwells were coated with rabbit anti-mouse immunoglobulin by incubating overnight at 4°C at pH9.6 in 0.1M carbonate buffer. 50µl of a 0.1% suspension of sensitized erythrocytes were added to the microwells, incubated for 30min at 22°C and centrifuged at 1000*g for 2min. Microwells were examined for monolayering.

Both monoclonals exhibited prozoning and results are presented on an arbitrary scale of 0-10 (reaction strength:0=negative and 10=maximal positivity) (fig.4). This technique was unable to differentiate levels of AChE from normal adult, cord and PNH erythrocytes.

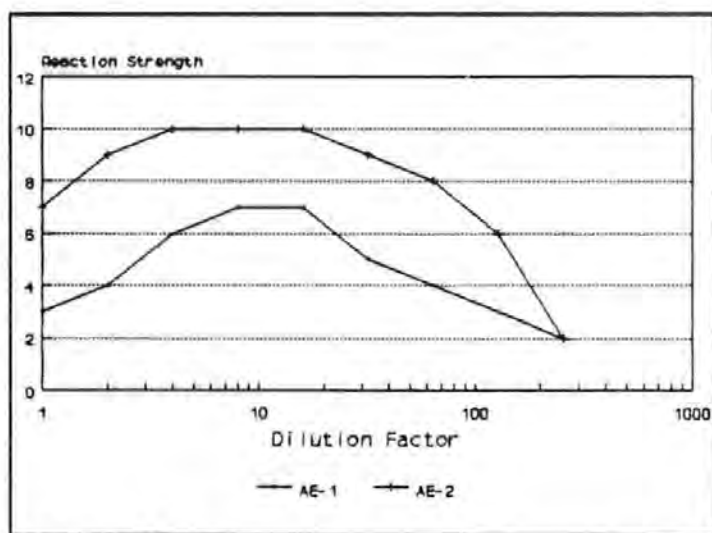


Figure 4

Effect of increasing dilution of AE-1 and AE-2 on the degree of monolayering obtained with normal adult erythrocytes using liquid phase sensitization.

(2) Solid Phase Sensitization

Microplates were coated with rabbit anti-mouse immunoglobulin by incubating overnight at 4°C at pH9.6 in 0.1M carbonate buffer. 100µl aliquots of serially diluted AE-1 and AE-2 in PBS pH7.2 were added to each microwell and incubated at 37°C for 1hr (fig.5). Wells were washed thrice with PBS tween. 50µl of a 0.1% suspension of washed erythrocytes were added to the microwells and incubated for 30min at 22°C and centrifuged at 1000*g for 2min. Microwells were examined for monolayering.

Again, no differentiation was observed between adult and cord samples and no improvement was observed when using ammonium sulphate precipitated AE-1 and AE-2.

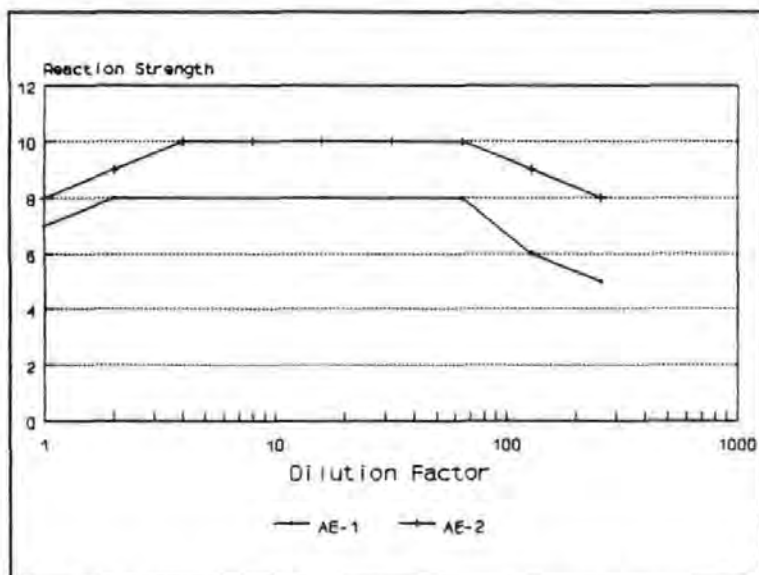


Figure 5
Effect of increasing dilution of AE-1 and AE-2 on the degree of monolayering obtained with normal adult erythrocytes using solid phase sensitization.

(3) Poly-L-lysine and Solid Phase Sensitization

High molecular weight (>400,000) poly-L-lysine is highly positively charged. Incubation of this reagent in the wells of a microplate, which carry a negative charge, results in the wells becoming coated with a positive layer. Incubation of erythrocytes followed by centrifugation results in a fine monolayer of cells coating the well bottom. These cells may then be lysed using distilled water with the 'ghosted' cell membranes remaining on the well surface. Some workers use a glutaraldehyde solution to fix the cells to the well surface, but this may result in an altering of antigenicity.

100µl of poly-L-lysine (BDH) 3µg/ml in PBS (optimum between 3 and 30µg/ml, concentrations higher and lower than these show a marked decline in coating conditions) were added to each well and incubated for 1hr at 37°C. After this time the wells were washed 3 times in PBS/tween 20. 100µl of a 0.25% (in PBS/tween) cell suspension were added to each well and allowed to stand for 5min, after which time the plate was centrifuged for 1000*g for 1min. Again the plate was washed 3 times in PBS tween. The wells were then flooded with distilled water and allowed to stand for 2min at room temperature and washed once in distilled water. 100µl of 0.25% glutaraldehyde in PBS/tween pH 7.2 were added, incubated for 5min at room temperature and washed 3 times in PBS/tween. 100µl of primary antibody for the particular red cell membrane antigen under test was added and incubated for 1hr at room temperature. Plates were again washed 3 times in PBS/tween. 100µl of 1/200 dilution (PBS/tween) of rabbit anti-mouse-HRP conjugate (DAKO) was added and incubated for 2hr at room temperature. After

this time the plate was washed 3 times in PBS/tween. Peroxidase substrate (OPD) was added and the reaction stopped after 15min using 1M H₂SO₄.

Monoclonal anti-A and anti-B produced good positive absorbances. Thirty routine grouping samples were tested in this way and ABO groupings agreed with normal haemagglutination methods. Various subgroups of A were evaluated to see if absorbance values were related to the number of group A antigen sites on the red cell surface (table 6) providing a means of quantitation.

Table 6 Absorbances obtained with A subgroups using solid phase techniques

A subtype	Absorbance	No. of A sites
A ₁	1.565	1 *10 ⁶
A ₂	1.192	2.5*10 ⁵
A ₁ B	1.324	6 *10 ⁵
A ₃ (high grade)	0.845	5 *10 ⁴
A ₃ (low grade)	0.349	3.5*10 ⁴
A _x	0	4.8*10 ³

Thus, an antigen density in excess of 30,000 sites per erythrocyte is required for detection by this method. Since there are approximately 800 AChE dimers per erythrocyte (Ott et al., 1982), this technique is not sensitive enough for this antigen. This was confirmed by the extremely weak reactivity produced with AE-2 while AE-1 produced no reaction.

To quantitate smaller numbers of antigen on the red cell surface biotinylated and avidin/streptavidin conjugated reagents (DAKO) were used at appropriate concentrations and incubation times. This involved the use of the substrate 3,3',5,5'-tetramethylbenzidine (TMB) (Dynatech). Unlike OPD, TMB is non-carcinogenic, non-mutagenic and is not susceptible to light, producing an intense blue colour with a maximum absorption peak at 655nm. Inhibition of reactivity with 1M sulphuric acid changes the colour of the reaction mixture from blue to bright yellow with a two fold increase in colour intensity at 450nm.

Unfortunately, using avidin extremely high absorbances were observed in the negative wells. The cause of this very high background was due to reactivity between the avidin and PLL and reaction was potentiated by biotin. Only gelatin gave any useful blocking of the non-specific reactivity, but was unable to prevent non-specific reaction occurring between avidin and the red cell membrane. Performance of the avidin/biotin assay system with AE-1 and AE-2 has thus been poor. To overcome these problems the use of streptavidin (SA) has been investigated.

Both streptavidin peroxidase conjugate and biotinylated anti-mouse Ig were used from a stock concentration of 500µg/ml. AE-2 at a dilution of 1/4, streptavidin peroxidase 1/1600 and

biotinylated anti-mouse IgG at 1/200 were found to be appropriate (fig.6). Although better results were obtained with the AE-1 and AE-2 monoclonal antibodies using the SA/biotin system there was not a sufficient difference in absorbance between tests to provide a detectable decrease in AChE in the relevant disorders under investigation. Adult and cord cells investigated using this system showed no difference. Solid phase techniques are thus not yet sensitive enough to detect the differences in erythrocyte AChE levels that occur between adult and cord samples.

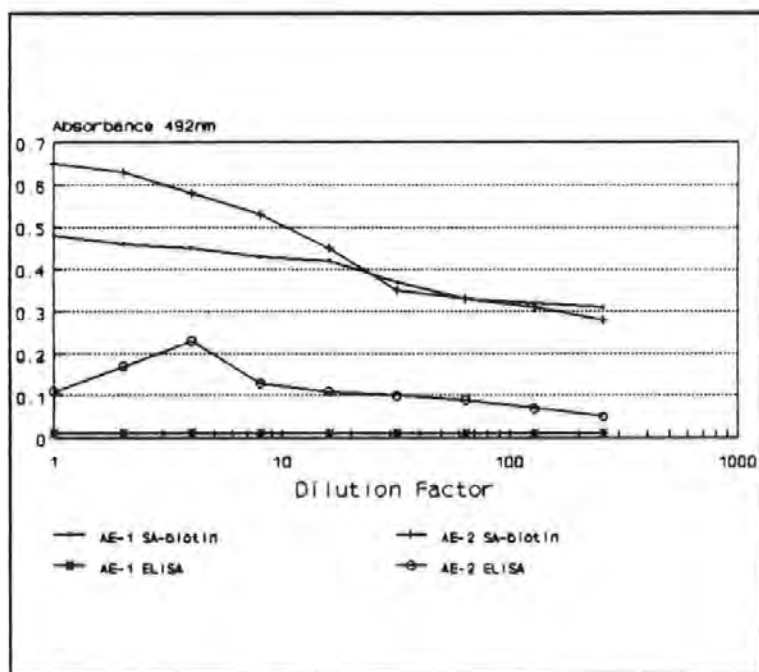


Figure 6
Comparison of AE-1 and AE-2 using HRP conjugated and SA/biotin conjugated antisera using solid phase techniques.

Enzyme Linked Antiglobulin Test (ELAT)

This is a modification of the technique described by Postoway et al. (1985) to quantitate small numbers of human antibody on the surface of red cell membranes. Antibodies attached to specific antigen on the surface of the erythrocyte are detected using an alkaline phosphatase conjugated antibody of high specific activity for the primary antibody. Phosphatase substrate is added and colour development is proportional to the number of antigen sites present on the cell membrane. Alkaline phosphatase is used as the conjugated enzyme since peroxidase activity is endogeneous to the red cell and would give false positive results when HRP is used. Unbuffered saline must be used in the enzyme substrate reaction, since inorganic phosphate is an inhibitor of alkaline phosphatase.

Glass test tubes were filled with PBS/BSA and emptied after a few minutes. 500 μ l aliquots of primary antibody AE-1 and AE-2 diluted to 5mg/l and 1.5mg/l respectively in LISS/BSA, were added to the pre-wetted tubes, followed by 500 μ l of a 2% red cell suspension in LISS/BSA (fig.7). The cells had been previously washed twice with PBS/BSA and once with LISS/BSA. The tubes were mixed, covered with parafilm and incubated for 15min at 37°C prior to centrifugation (1000*g for 30sec) The supernatant containing the antibody was discarded and the compacted red cells carefully washed 3 times in PBS/BSA. 200 μ l of alkaline phosphatase rabbit anti-mouse antibody (1/100 dilution in PBS/BSA) (fig.8) was added. The tubes were again mixed, covered with parafilm and incubated for 1hr at 37°C. After this period the cells were washed 3 times in PBS/BSA and 1ml of UBS/BSA was

added to the red cell button, mixed and the cell suspension transferred to new tubes pre-wetted with UBS/BSA. The tubes were filled with UBS/BSA, centrifuged at 1000*g for 1min and the supernatant discarded. The red cells were mixed with 200 μ l UBS/BSA and 200 μ l of p-nitrophenyl phosphate substrate added. Tubes were incubated at 37°C for 20min (fig.9), centrifuged at 1000*g for 30sec after which the supernatant was transferred to a further tube and neutralized by 25 μ l of 3M NaOH. 100 μ l of coloured product, diluted (1:3) with UBS/BSA, was transferred to a microplate and the absorbance read at 405nm. A control using LISS/BSA in place of AE-2 was included as an indicator of haemolysis.

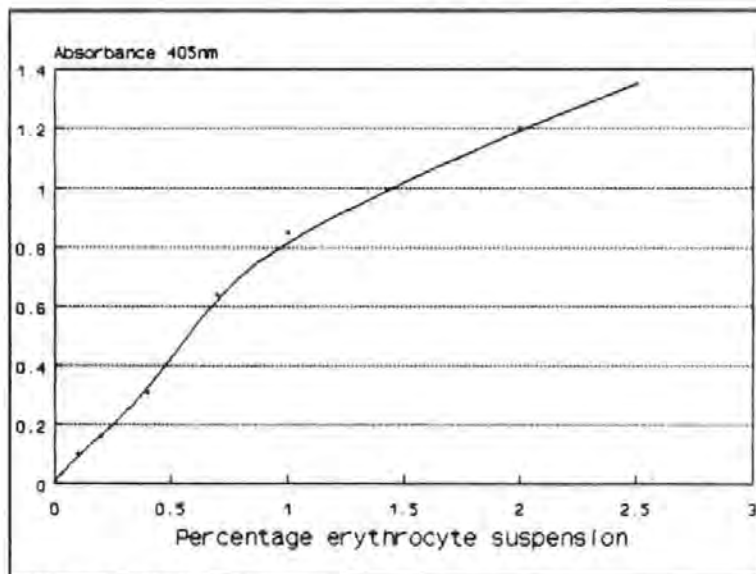


Figure 7

The effect of increasing % erythrocyte suspension on absorbance obtained using AE-2 in the ELAT.

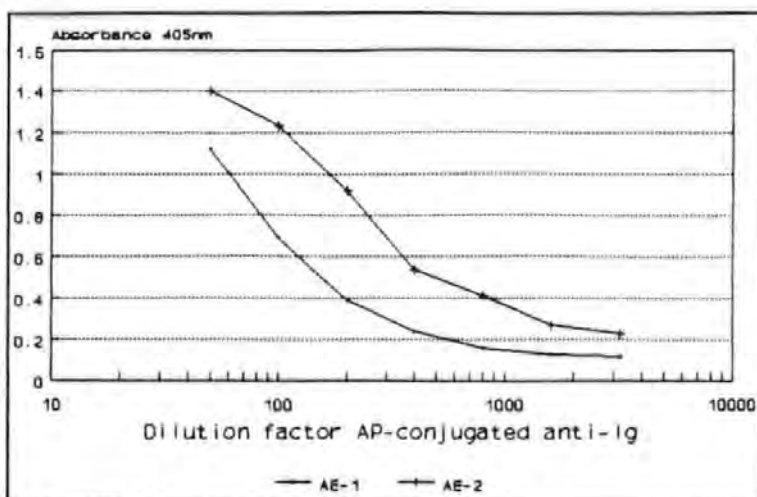


Figure 8

The effect of increasing dilution of alkaline phosphatase (AP) conjugated anti-mouse immunoglobulin on the absorbance obtained using AE-1 and AE-2 in the ELAT.

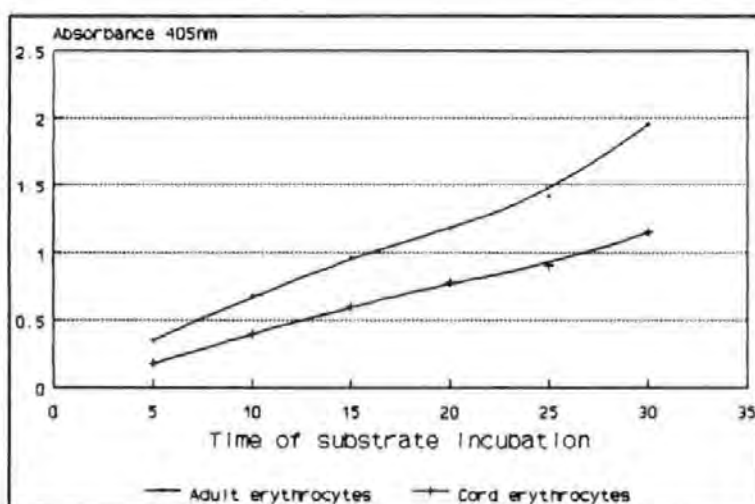


Figure 9

Absorbances obtained at given time intervals after addition of substrate with adult and cord erythrocytes in the ELAT.

Comparison was made between adult and cord cells using AE-1 (fig.10) and AE-2 (fig.11). Conjugate dilution was 1/100.

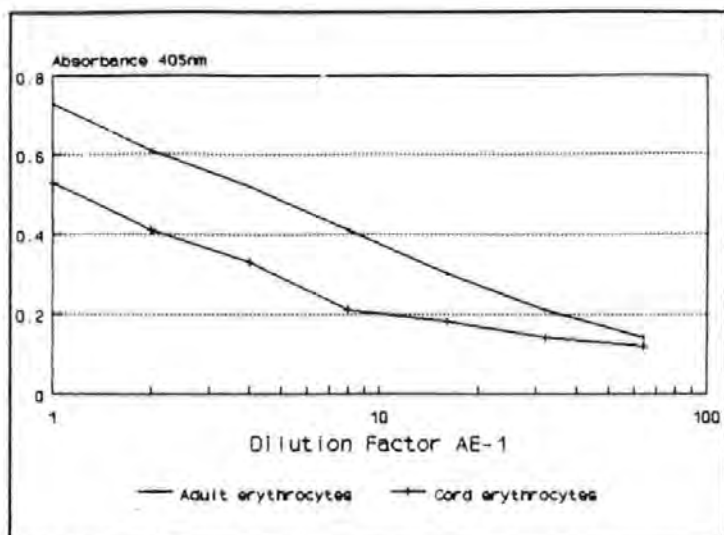


Figure 10
Effect of increasing dilution of AE-1 on absorbance using adult and cord erythrocytes in the ELAT.

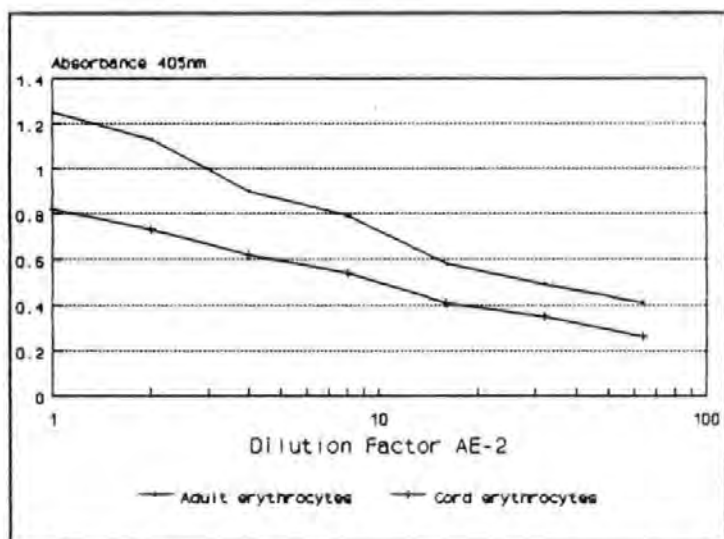


Figure 11
Effect of increasing dilution of AE-2 on the absorbance obtained with adult and cord erythrocytes in the ELAT.

The enzyme linked antiglobulin test has given reliable results. Duplicate testing indicating a CV for this assay system of 7%.

Antibody Consumption Test to Quantitate the Number of IgG Molecules on the Surface of Erythrocytes after Incubation with AE-2

At least 150 molecules of IgG per erythrocyte are required to cause agglutination (Stratton et al., 1983) and between 500 and 2000 molecules of IgG per erythrocyte are required for maximal agglutination (Merry et al., 1984). The number of immunoglobulin molecules (AE-2) attached per erythrocyte during the incubation stage of AE-2 with erythrocytes was estimated using an antibody consumption test to compare the sensitivity of ELAT with the haemagglutination test.

It would be extremely difficult to quantitate the number of immunoglobulin molecules on each erythrocyte directly and so an indirect method of quantitation was devised. An ELISA was used to estimate from a standard curve the amount of immunoglobulin in the antibody solution before and after adsorption. The difference between these two values indicates the amount adsorbed on the erythrocytes. The number of IgG molecules adsorbed per red cell can then be calculated from the equation:

$$\begin{array}{l} \text{Number of IgG} \\ \text{molecules per} \\ \text{red cell} \end{array} = \frac{\text{amt IgG adsorbed(g)}}{\text{m.wt. IgG}} * \frac{\text{Avogadro's constant}}{\text{Number of RBC}}$$

Using an ELISA to quantitate mouse immunoglobulin IgG the absorbances obtained for serial dilutions of AE-2 are indicated (fig.12). Serial dilutions of AE-2 previously incubated with an equivalent volume of 2% erythrocytes were quantitated in parallel. The curves only diverge when the AE-2 dilution is greater than 1/32 indicating the amount adsorbed by the

erythrocytes only becomes detectable at this dilution.

This technique cannot, therefore, be performed at the 1/2 dilution of AE-2 (1.5mg/l) normally used in the ELAT. The dilution of AE-2 would have to be greater than 1/32 and therefore a 1/100 dilution of AE-2 was chosen. Using the above equation 100 molecules of AE-2 were calculated to be adsorbed onto the surface of each erythrocyte after 15min incubation for a 1/100 dilution of AE-2. Using the standard curve of dilution of AE-2 against absorbance obtained during the ELAT (fig.13), if 100 molecules are adsorbed per erythrocyte for a 1/100 dilution of AE-2 then assuming a linear axis, 270 molecules of AE-2 would be adsorbed for a 1/2 dilution of AE-2. The linearity of the axis can be proven by using a second dilution ($>1/32$) of AE-2 suspension. Although, it is accepted that this is an assumption, it does indicate the method to be twice as sensitive as the haemagglutination technique, while providing an objective method of quantitation.

300 μ l of a 1/100 dilution of AE2 was incubated with 300 μ l of a 2% erythrocyte suspension in LISS at 37°C for specific time intervals up to 90min. The cells were centrifuged 1000*g for 30sec and 100 μ l of supernatant added to microtitre wells precoated with rabbit anti-mouse immunoglobulin (RAMI). After 1 hour at room temperature the plate was washed 4 times using PBS/tween and 100 μ l of 1/200 dilution RAMI-HRP conjugate added and incubated for 1 hour at room temperature. The plate was washed 5 times in PBST and 100 μ l of OPD substrate added. After 3min reaction was stopped by adding 100 μ l of 1M H₂SO₄. Absorbance was measured at 492nm.

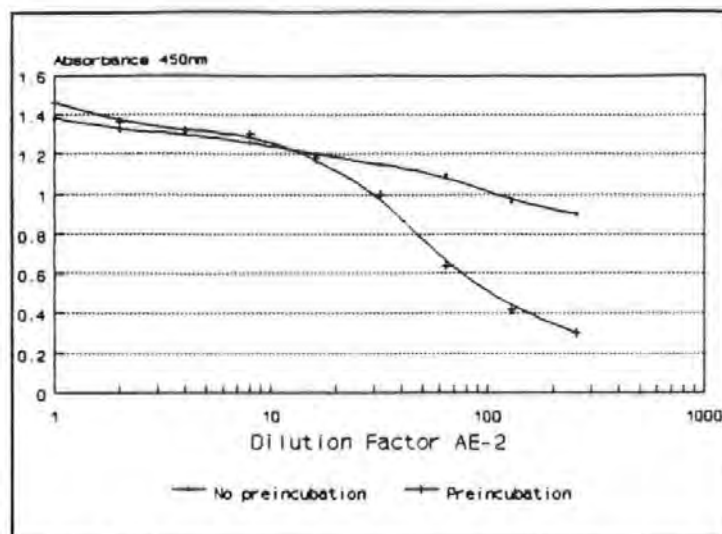


Figure 12
Absorbances obtained with serial dilutions of AE-2 in the ELISA
(1) AE-2 not incubated with erythrocytes.
(2) AE-2 pre-incubated with erythrocytes.

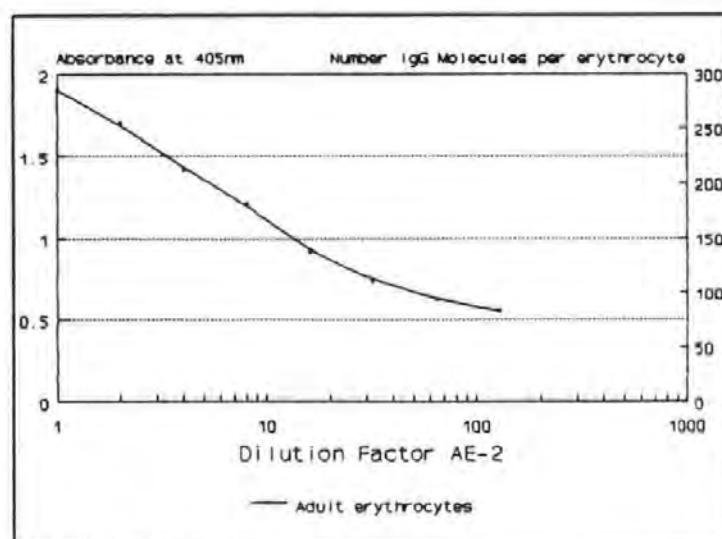


Figure 13
Effect of dilution of AE-2 in the ELAT. The number of IgG molecules adsorbed per red cell are extrapolated from the value obtained with a 1/100 dilution of AE-2. A linear axis is assumed.

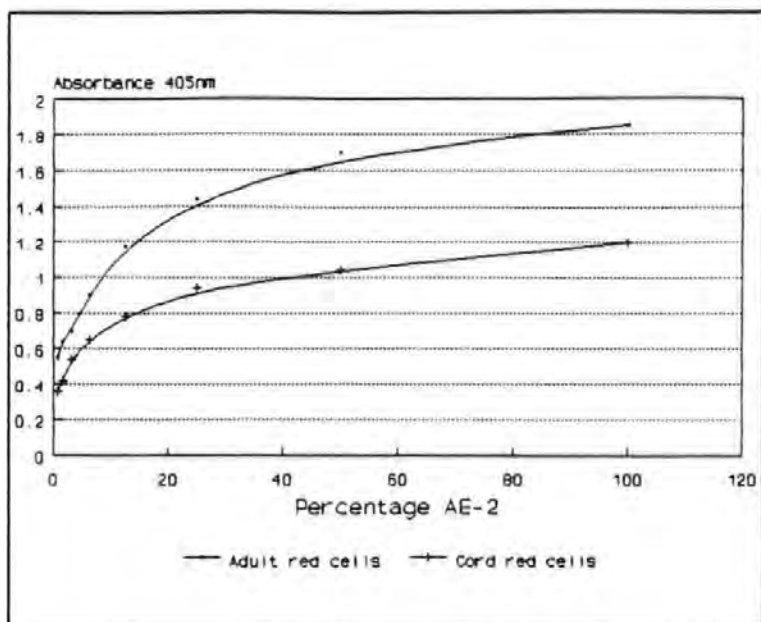


Figure 14 Effect of dilution of AE-2 on absorbance obtained in the ELAT.

If one compares absorbances obtained by the ELAT with doubling dilutions of AE-2, the $1/2$ dilution used in this technique approaches saturating levels (fig.14). The number of immunoglobulin molecules bound per erythrocyte reaches a maximum at 15min incubation with a 2% red cell suspension and then decreases progressively with time (fig.15).

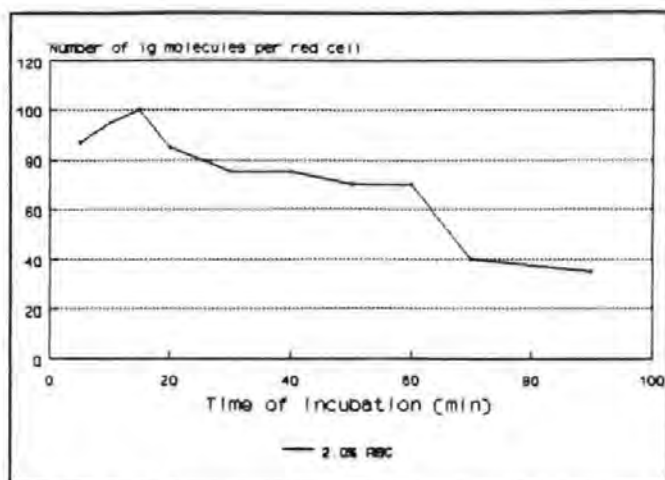


Figure 15
Effect of incubation time of a 1/100 dilution of AE-2 on the number of immunoglobulin molecules adsorbed per red cell using a 2% cell suspension.

The results are presented in terms of activity using the Ellman reaction with acetylthiocholine as substrate and antigen level using the ELAT with p-nitrophenol phosphate as substrate.

Samples were tested over a number of days (table 7). A gradual reduction in AChE was observed. For future testing samples older than 8 days were not used.

Table 7 Effect of Age of Sample on Activity and Immunological Measurements of Erythrocyte AChE

Percentage decrease of original value						
AChE	day0	day2	day5	day7	day8	day10
Activity	0	3.9	4.0	6.0	6.0	7.2
Antigen	0	3.6	5.3	5.5	5.2	6.8

Units for AChE Activity and Antigen Levels

In addition to results given in terms of μmole of substrate hydrolysed per min per ml these are also presented on an Arbitrary Unit (AU) scale. The mean absorbance for the adult male reference range is regarded as 100% and values for the other ranges are presented relative to this. For 30 adult male samples the mean absorbance for activity and antigen levels were 1.18 and 1.20 respectively.

Relationship Between Units of Measurement of AChE

Activity

$$\text{AU} = \frac{\text{test absorbance}}{1.18} * 100$$

Using equation 1 in the methods section:

$$7.39 * \text{test absorbance} = \text{ } \mu\text{mole/min/ml}$$

$$0.0871 * \text{AU} = \text{ } \mu\text{mole/min/ml}$$

Antigen level

$$\text{AU} = \frac{\text{test absorbance}}{1.20} * 100$$

Plasma Cholinesterase

Plasma ChE Activity (Modified Ellman)

A pool of 5 $E_1^uE_1^u$ donors was used as the standard solution of plasma ChE. 100 μ l of diluted test serum and diluted standard (1/8 to 1/128 in 0.01M phosphate buffer pH 7.2) were added to each well of a microplate and 100 μ l of BTI/DTNB (1:9) substrate added using an 8 channel micropipette. A reagent blank was included to monitor non-enzymic hydrolysis of the substrate. For a 1/16 dilution of plasma the change in absorbance at 405nm with time using a substrate concentration of 0.3mg/ml was linear (figs.16 and 17). This dilution of test serum was regarded as the optimum. Activity was calculated as the change in absorbance at 405nm per minute and values read off the standard curve. Results were reported in terms of arbitrary units (AU) (ie. activity relative to the standard pool).

The use of microplates and the linkup of the ELISA reader to a microcomputer enables 48 duplicate tests to be performed in an extremely short space of time. The CV was 5%.

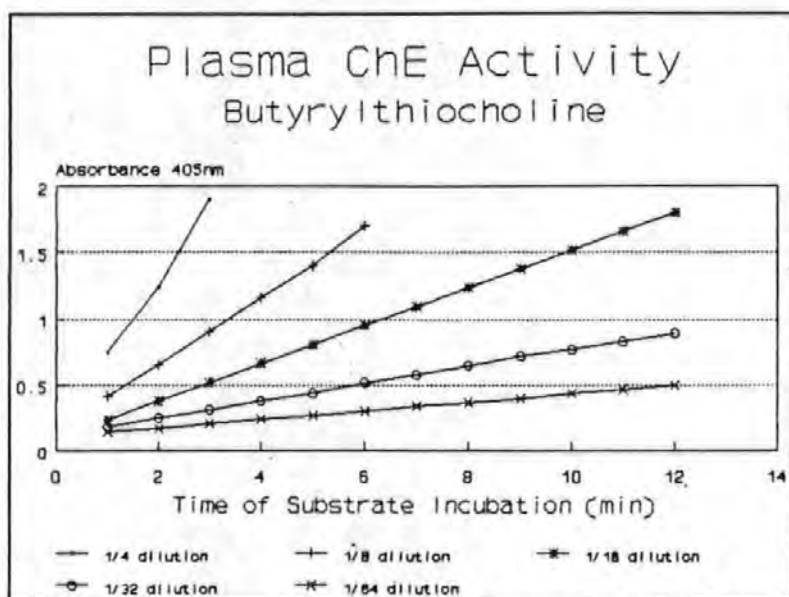


Figure 16 Effect of substrate incubation time on the absorbance obtained with various of dilutions of pooled plasma. Substrate concentration 0.3mg/ml.

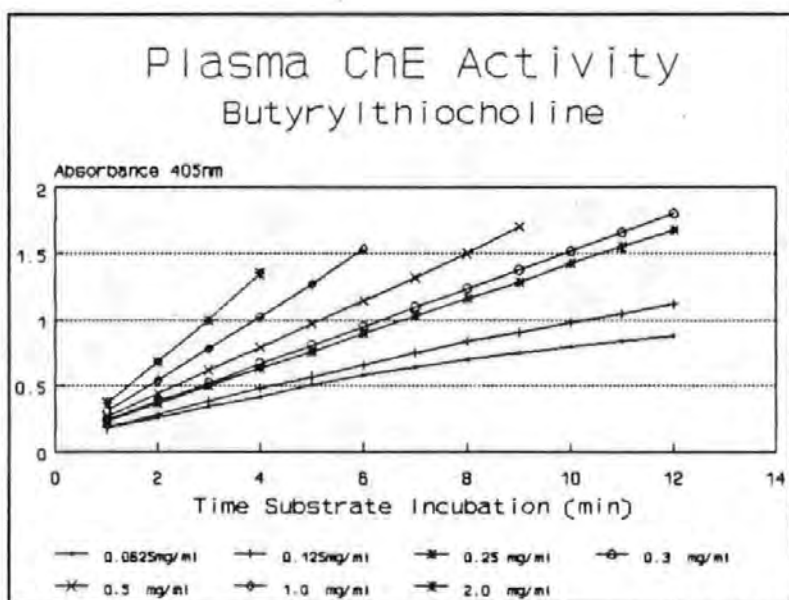


Figure 17 Effect of substrate incubation time on the absorbance obtained with varying substrate concentration. Plasma diluted 1/16.

Immunological Quantitation of Plasma ChE

Rocket Immuno-electrophoresis

Agarose gels were prepared as 1% solutions (0.12g in 12ml) in Tris-veronal (barbitone) buffer pH 8.7 by incubating in a boiling water bath. 10 μ l of anti-cholinesterase (DAKO) were added to the molten agarose prior to pouring onto a glass plate covered with gel bond film. Gels must be cast on the hydrophilic surface of the film. 5 μ l of sera were added to wells (4mm) cut in the solid agarose gel and subjected to immuno-electrophoresis at 2V/cm/plate.

Plates were pressed under filter paper soaked with distilled water for 15min and washed for 15min in 0.1M NaCl and for 2*15min in water, pressed as before and dried in a stream of air at room temperature.

Gels were incubated in the α -naphthyl acetate (Sigma)/fast red (GURR) substrate at 37°C for 10min and observed for staining. α -naphthyl acetate used under these conditions is a specific substrate for serum cholinesterase. 50%, 25% and 10% standards were used and tests were diluted 1 in 4. Cholinesterase can not be seen with protein staining alone.

Superior precipitation rockets were obtained using the Plymouth antibodies, 288 (picture 1) being slightly better than 188 and the DAKO, BCL and SSI antibodies being better than the B'ham anti-ChE. The latter antibody produced diffuse precipitates which were difficult to measure.

Triplicate testing indicated a CV of 12% using this technique.

Electrophoresis using Enzyme Amplification

On completion of electrophoresis as described above the plate was washed 3*30min in 0.1M NaCl. The plate was incubated for 10min in 5% swine serum (DAKO) diluted in PBS pH 7.2 and for 45min in peroxidase labeled swine anti-rabbit immunoglobulin (DAKO) diluted 1/50 in 5% swine serum in PBS. This antibody provides the amplification stage by combining with the rabbit anti-ChE in the precipitation rockets. After pressing and washing 3*30min in 0.1M NaCl the plate was incubated for 15min in substrate, rinsed with water, dried in air and the peak heights measured.

This technique, although well established for quantitating other proteins such as alpha foetal protein (Kjærvig and Ingild, 1983), did not produce reliable results for plasma cholinesterase, probably due to the specific activity of the antibodies used. Other drawbacks of this technique are the length of time of assay and the limited number of samples handled.

Enzyme Immunoassay

The enzyme immunoassay involved immunological quantitation using the enzymic activity of plasma cholinesterase itself. Four antisera DAKO, Plymouth 188, 288, and B'ham anti-ChE were evaluated for reactivity using this technique.

100 μ l of a 1/500 dilution of primary antibody were incubated at 37°C for 1hr, RT for 6hr or 4°C overnight (fig.18). For convenience any of these conditions may be used. Plates were washed thrice with buffer, 100 μ l of diluted plasma (10%-100%) added to each well and the plates incubated for 2hr at 37°C. Plates were washed 4 times with buffer and 100 μ l of BTI/DTNB substrate added. Absorbances were measured at 405nm after 30min. A standard curve can be plotted and results calculated.

0.1M carbonate buffer pH9.6 in the coating stage and phosphate buffer 0.01M pH7.2 in the diluting and washing stages produced no results with any of the antibodies. Increasing substrate concentration did not improve matters. Increasing the pH of the buffers when using the DAKO antibody produced reactivity. Carbonate buffer pH9.6 gave the highest absorbance (fig.19).

However, in spite of concentrated effort using this technique the coefficient of variation was high, in many cases reaching the 40%. This is probably a function of the quality of the antiserum being used.

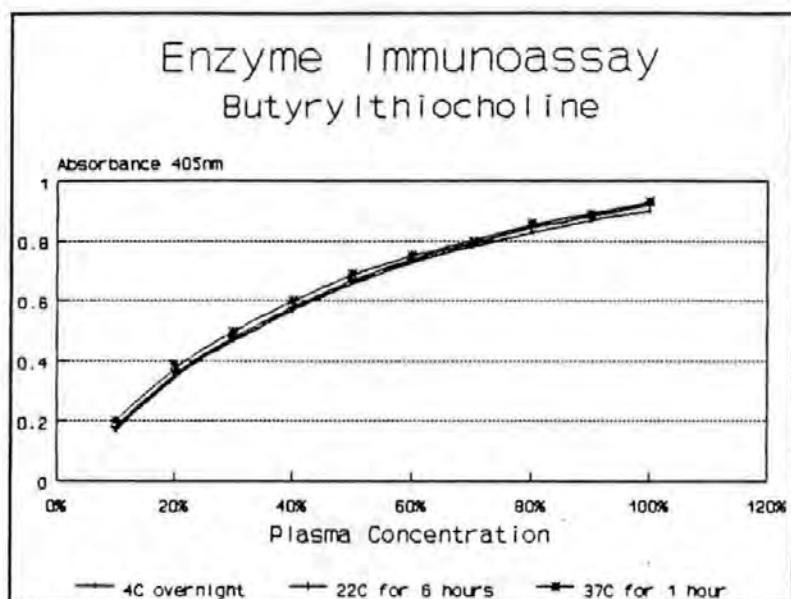


Figure 18. Effect of coating conditions on absorbance in the enzyme immunoassay.

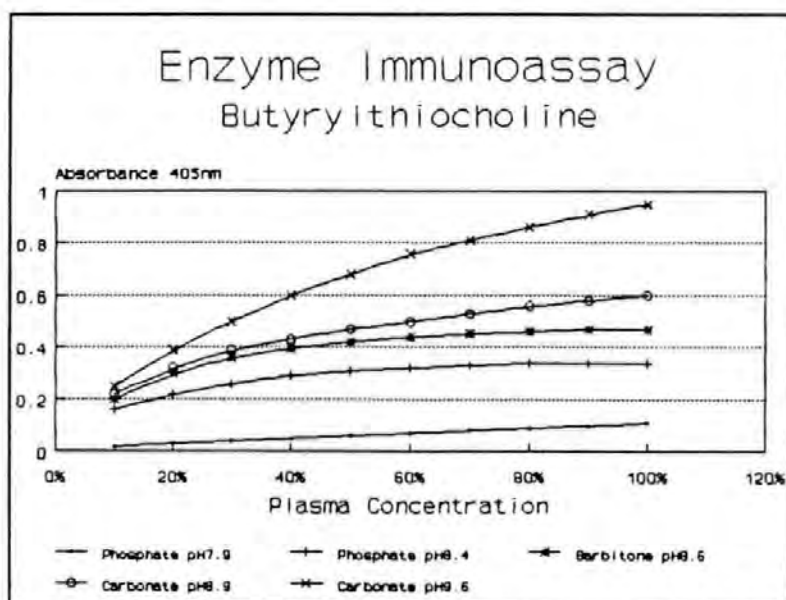


Figure 19. Effect of buffer composition and pH on absorbance in the enzyme immunoassay.

Enzyme Linked Immunosorbent Assay (ELISA)

100 μ l of anti-cholinesterase, diluted 1/1000 in carbonate buffer (0.1M pH9.6), was added to each well of a microtitre plate. The plate was covered with plastic film then incubated at 4°C overnight or at 37°C for 1 hour. The residual liquid was discarded and the plate washed thrice with the phosphate buffered saline. The plate was tapped dry at each stage. 100 μ l aliquots of sera and standards diluted in PBS/tween were added to each well, then incubated at room temperature for 2 hours. The plate was washed as above and 100 μ l of conjugated antibody, diluted in 1/300 in PBS/tween, was added to each well. The covered plate was incubated at room temperature for 1 hour prior to washing 4 times with PBS/tween. 100 μ l of peroxidase substrate, TMB, was added to each well and the initial blue colour development was terminated after 2min by addition of 100 μ l sulphuric acid (1M) when the colour changed to deep yellow.

The standard curve can either be plotted on semilogarithmic paper with Absorbance at 450nm as ordinate and \log_{10} concentration of standard as abscissa, or best of line curve fits may be employed directly to results input into a computer.

Polyclonal Antisera

Although this technique worked well using the established DAKO ELISA antibodies for C-reactive protein (CRP), performance with the DAKO antisera for plasma ChE proved disappointing. Using anti-IgG-HRP conjugate both the anti-ChE and anti-CRP were shown to coat the microwell to the same extent (fig.20), proving the difference in performance was not due to well coating.

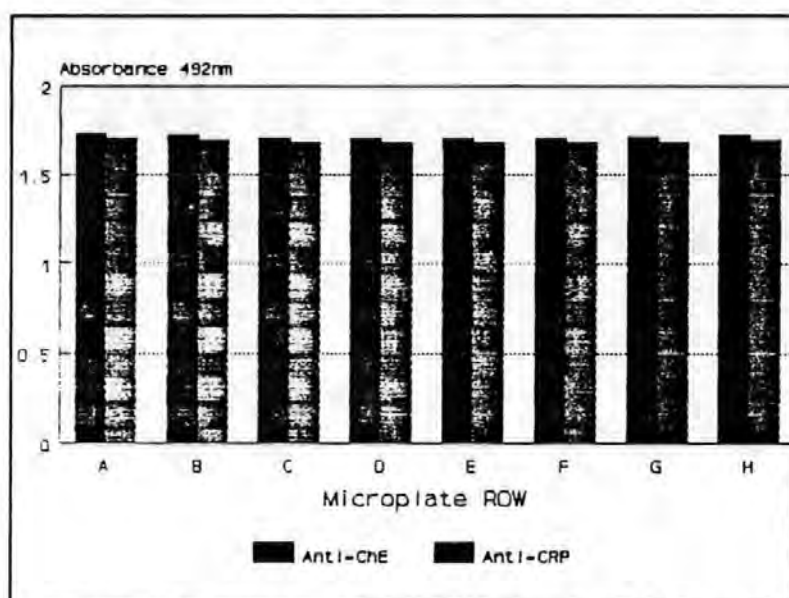


Figure 20. Mean absorbances at different rows along a microplate, using anti-rabbit IgG HRP conjugate to detect anti-ChE and -CRP (mean of 3 assays).

Varying the experimental conditions revealed optimum dilutions of the HRP-conjugated (fig.21) and the primary anti-ChE (fig.22) antibody to be 1/50 and 1/1000 respectively. This dilution of conjugate is unrealistic due to the amount available and financial restrictions.

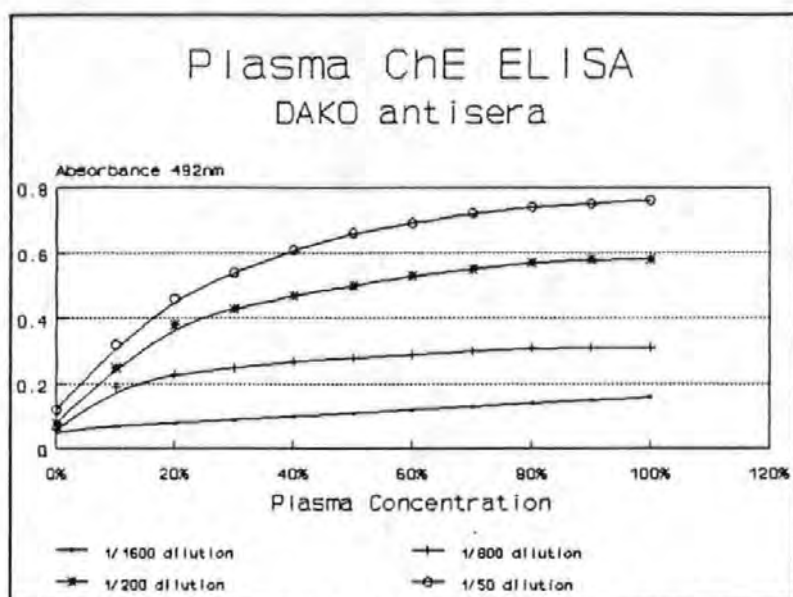


Figure 21. Effect of dilution of plasma on absorbance for a number of dilutions of HRP-conjugated anti-ChE (DAKO).

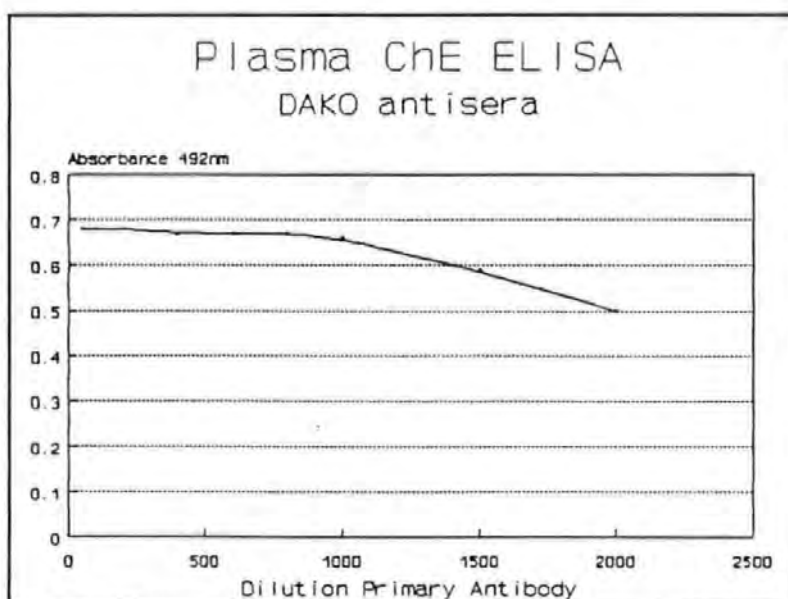


Figure 22. Effect of dilution of the primary antibody (anti-ChE DAKO) on absorbance.

When PEG (polyethyleneglycol) 6000 was used to improve the performance of the system, a large increase in colour development, both background and test was observed when both antigen and antibody were diluted in 3% PEG. Neither 1% nor 3% bovine serum albumin were able to block this high background absorbance. Thus PEG did not produce any significant improvement.

The reducing agents dithiothreitol (DTT), 2-mercaptoethanol (2-ME) and iodoacetamide (IA) were used to investigate whether splitting the cholinesterase tetramer into its component monomers would enable better binding to the coating antibody. Use of the proteolytic enzymes papain and bomelain to break up the ChE molecule and hence expose the epitopes were also investigated. Plasmas treated in this way were subjected to PAGE to examine the ChE banding patterns.

The use of papain in combination with the papain inhibitor E64 after treatment of antigen, prior to addition to the plate produced no improvement over the normal assay method.

Use of DTT produced a large increase in colour production. However, $E_1^sE_1^s$ genotypes, with no immunologically detectable protein by immunoelectrophoresis, still produced high absorbances indicating false positivity with this technique due to the precipitation coating the bottom of the well. Neither 2-ME or IA produced any significant improvement in performance.

A number of test and standard samples were assayed by ELISA using DAKO antisera and quite a high CV% was obtained of the order of 15%. Correspondence with Dr. Agnette Ingild (DAKO) confirmed these findings, high background absorbances again being reported.

A combination of low dilution of the conjugate and relatively high CV% resulted in this antibody being used only as a backup to confirm results obtained with other antibody systems. It would seem that this particular antibody is more suited for immunoelectrophoretic techniques.

When the Behring anti-ChE diluted 1/500 was used as the primary coating antibody a 1/100 dilution of the DAKO anti-ChE HRP conjugate was found to be the optimum. Unfortunately, Behring ceased production of anti-ChE and supplies became restricted so that further use of this antibody was prevented.

The Plymouth anti-ChE, 188 and 288, were presented for use as unpurified rabbit plasma. Microplates were first coated with a swine anti-rabbit-IgG (DAKO 1/400). Checkerboard titres were performed on the anti-ChE diluted 1/20 to 1/200 in PBS/tween. These were complemented by dilutions of 1/50 to 1/800 of the DAKO anti-ChE-HRP conjugate. With antigen dilutions ranging from 50% to 10% no reactivity was observed. The B'ham anti-ChE-HRP contained an extremely potent anti-rabbit immunoglobulin and so its use with the Plymouth rabbit antibodies would result in false positivity. These two rabbit antisera were not suitable for ELISA.

When using the B'ham sheep anti-ChE checkerboard titres were performed between the conjugated and unconjugated antisera to find the optimum dilutions required, a standard 5% antigen concentration being used. Incubation at room temperature for 1h produced positive results even while using very high coating antibody dilutions. Optimum dilution of the conjugated antibody was 1/200. The method was extremely reproducible with low CV

(less than 5%). These are conditions associated with a good ELISA based system. However, when used to analyse test samples unusual results were which did not correlate with activity or quantitation by rocket immunoelectrophoresis. This system was therefore rejected.

Dilutions of antigen were incubated for 2hr at room temperature on microplates previously coated with B'ham anti-ChE (1/1000). Two-fold dilutions of biotinylated monoclonal anti-ChE (MAb 2-1) were added. HRP conjugated streptavidin diluted 1/2000 was used as detector (fig.23). Optimum concentration of the MAb 2-1 biotin conjugate was 1/4000.

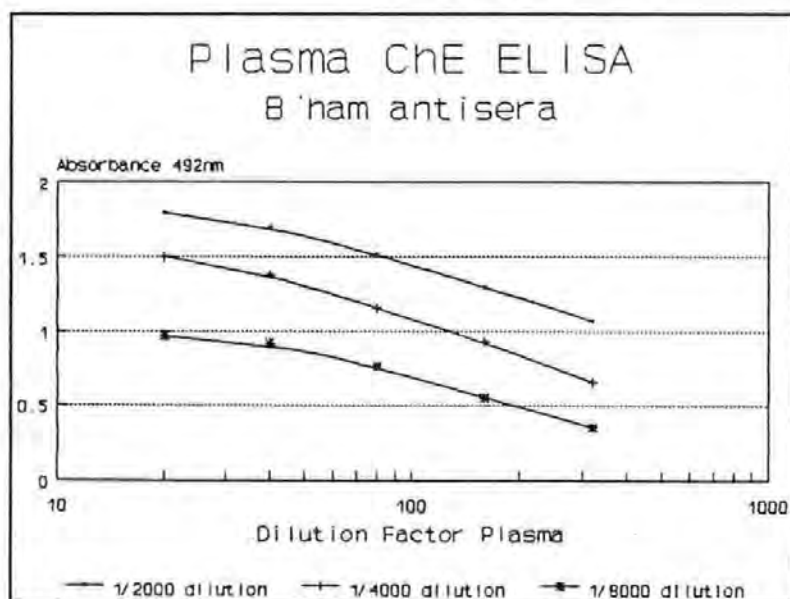


Figure 23 Effect of dilution of plasma on absorbance obtained with B'ham anti-ChE as primary antibody and varying dilution of MAb 2-1 biotin conjugate.

When the SSI rabbit polyclonal anti-ChE was used as the primary coating antibody at a dilution of 1/5000, antigen dilutions from 1/20 to 1/320 were incubated for 2hr at room temperature. Dilutions of monoclonal anti-ChE 2-1 (fig.24) and 2-4 (fig.25) were incubated for 1hr at room temperature and a 1/1000 dilution of DAKO rabbit anti-mouse-IgG HRP conjugate used as detector antibody.

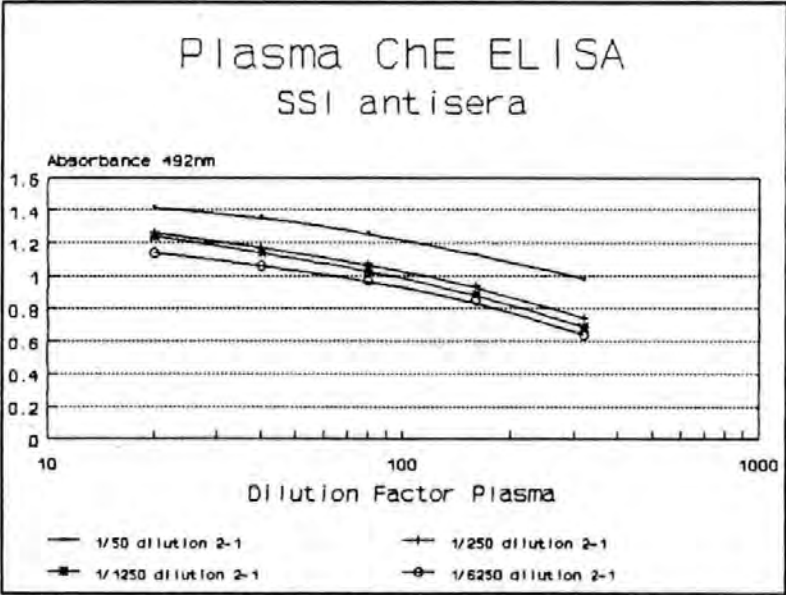


Figure 24 Effect of dilution of plasma on absorbance obtained with SSI as primary antibody and varying dilutions of MAb2-1.

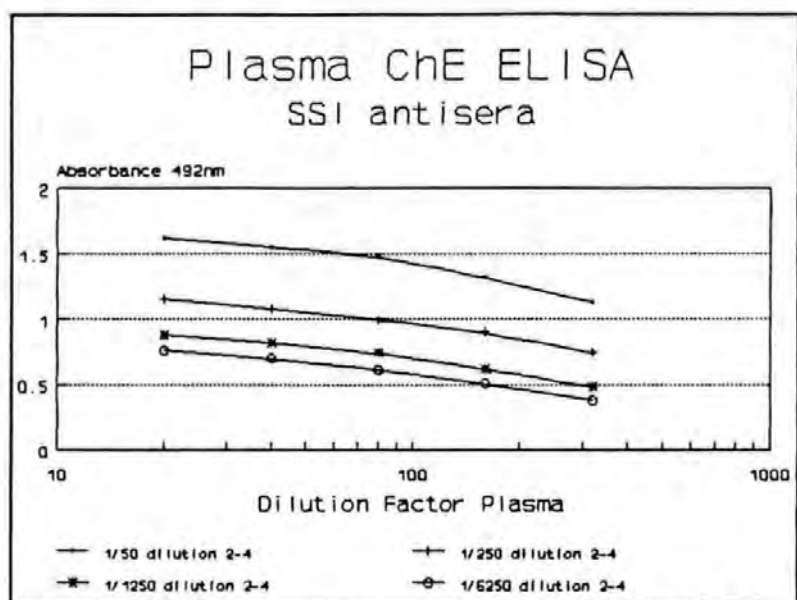


Figure 25 Effect of dilution of plasma on absorbance obtained with SSI as primary antibody and varying dilutions of MAb2-4.

Standard curves for both MAb2-1 and 2-4 using SSI as primary coating antibody were good, enabling very high dilutions of antisera to be used.

Monoclonal Anti-ChE: MAb 2-1; 2-2; 2-3; 2-4

The four monoclonal antibodies to plasma ChE were incubated overnight at varying concentrations to establish the relative amount attached to the microwell using rabbit anti-mouse-IgG HRP conjugate (fig.26).

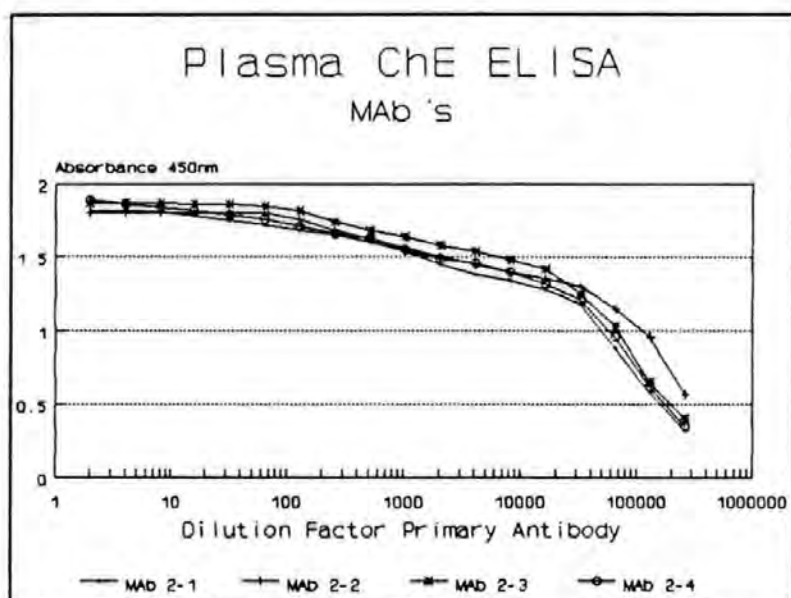


Figure 26 Effect of dilution of MAb's 2-1, 2-2, 2-3, 2-4 on absorbance after incubation overnight at 4°C. Rabbit anti-mouse-IgG HRP-conjugate was diluted 1/500.

ELISA's were performed using serial dilutions of MAb's to establish the optimum concentration of each as primary antibody (fig. 27).

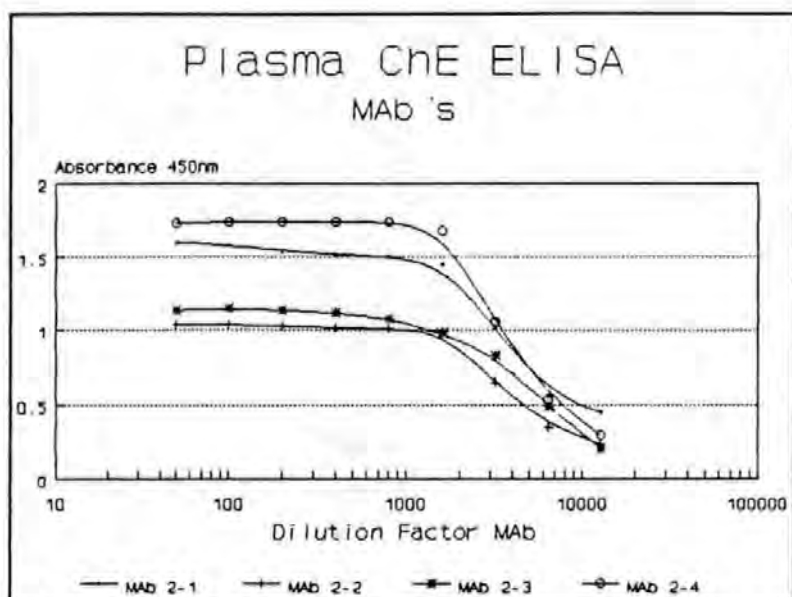


Figure 27 Effect of dilution of primary MAb's 2-1, 2-2, 2-3 and 2-4 on absorbance.

A 1/1000 dilution of MAb's 2-1, 2-2 and 2-4 and 1/400 dilution of MAb 2-3 were chosen for the primary coating antibodies. Using a 1/40 dilution of plasma the optimum dilution of B'ham anti-ChE HRP conjugate was 1/300 (fig.28). Serial dilutions of plasma were used to plot standard curves (fig.29) with these antisera. MAb's 2-1 and 2-4 were superior to MAb2-3 and MAb2-2. The latter proving unsuitable.

Results using MAb's with the DAKO anti-ChE HRP conjugate detector antibody were inferior to the B'ham conjugate, but could be used as a backup antibody for any confirmatory testing.

Thus for the ELISA for plasma ChE, MAb's 2-1 and 2-4 were used in conjunction with sheep anti-ChE HRP conjugate as the detector antibody. This system involved the minimum number of steps and produced good standard curves with a CV of 5%.

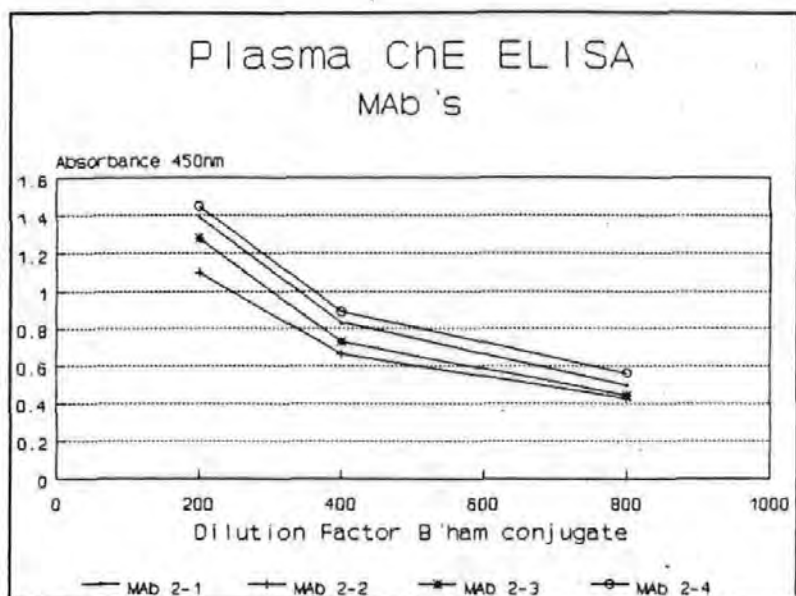


Figure 28 Effect of dilution of B'ham anti-ChE HRP conjugate on absorbance with each of the primary MAB's.

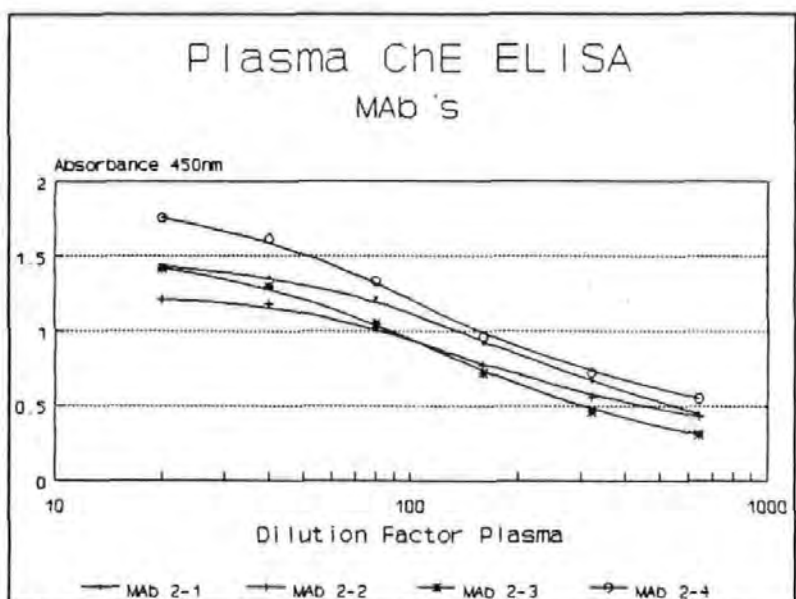


Figure 29 Effect of dilution of plasma on absorbance using ELISA for ChE with MAB's as primary antisera and B'ham anti-ChE HRP conjugate diluted 1/300.

Results

Erythrocyte Acetylcholinesterase

Reference Ranges for AChE Activity using acetylthiocholine as substrate and Antigen Levels by the ELAT technique

Thirty normal adult male and female and normal cord samples were tested to establish reference ranges for each (table 8). Ranges were calculated for activity and antigen levels for 30 adult males. Results are presented relative to the mean of these ranges on an arbitrary unit (AU) scale. In addition, results are given in terms of μ mole of substrate hydrolysed per min per ml of packed cells. Data collected in this way allowed frequency distributions for healthy male, female and cord samples to be plotted (fig. 30 - 32).

Table 8 Reference ranges of Enzymatic and Immunological Activity for Erythrocyte AChE (mean \pm SD) for populations of healthy males, females and cord bloods

Category	Number tested	Activity μ mole/min/ml ¹	AU ²	Antigen AU ²
Adult male	30	8.71 \pm 1.39	100 \pm 16	100 \pm 14
Adult female	30	8.54 \pm 1.22	98 \pm 14	100 \pm 15
Cord	30	4.97 \pm 0.87	57 \pm 10	62 \pm 10

¹ packed cells
² AU relative to the mean for healthy adult males

No significant difference was found between male and female values for either activity or antigen levels. Cord cells, however, averaged 60% of the healthy adult male value.

Table 9 Statistical comparison of AChE activity and antigen levels for adult male and cord erythrocytes

Category	Mean	SD ¹	S.E. ² mean	T	p
<u>Activity</u>					
Adult male	100	16	2.92	12.48	<0.001
Cord	57	10	1.83		

<u>Antigen</u>					
Adult male	100	14	2.56	12.10	<0.001
Cord	62	10	1.83		

<u>Cord Cells</u>					
Activity	57	10	1.83	1.94	0.116
Anitgen	62	10	1.83		

¹ Standard Deviation
² Standard Error of Mean

¹ Standard Deviation

² Standard Error of Mean

AChE activity and antigen levels for adult male and cord samples were compared statistically to establish whether each are separate populations (table 9).

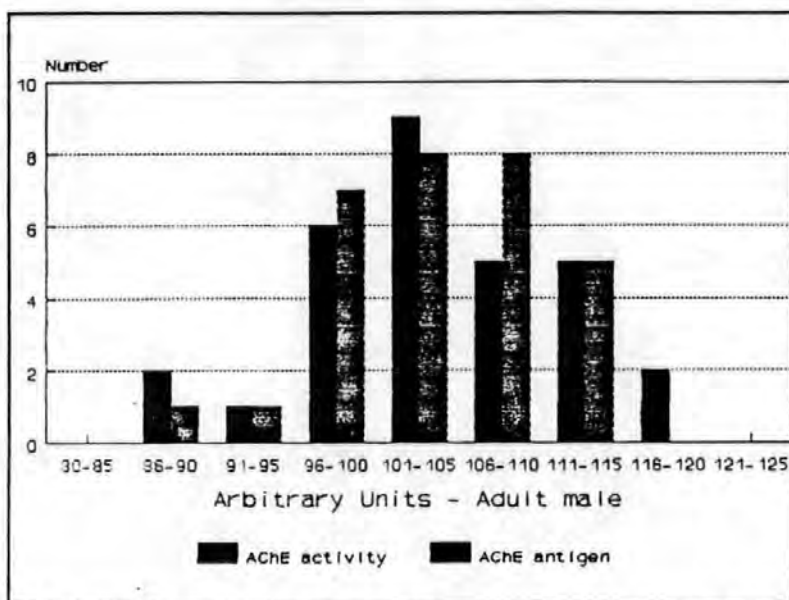


Figure 30 Frequency distribution of AChE activity and antigen levels for healthy adult males.

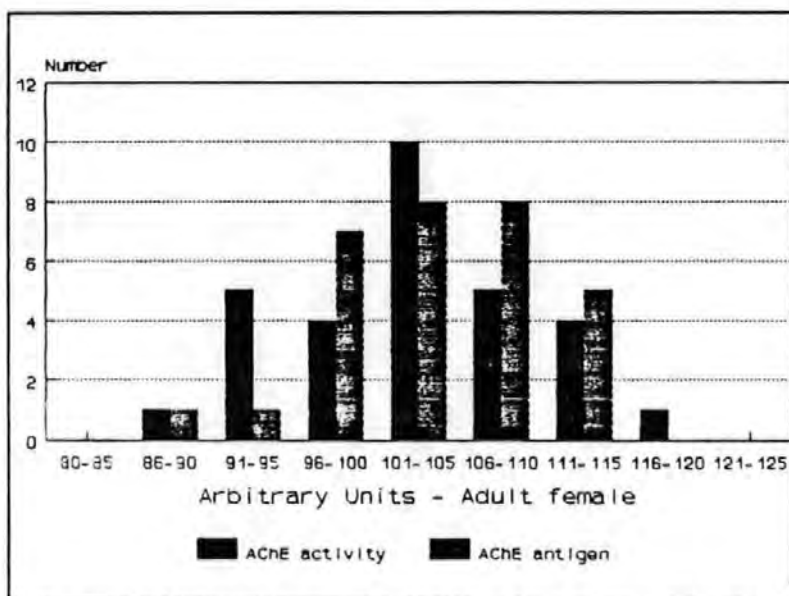


Figure 31 Frequency distribution of AChE activity and antigen levels for healthy adult females.

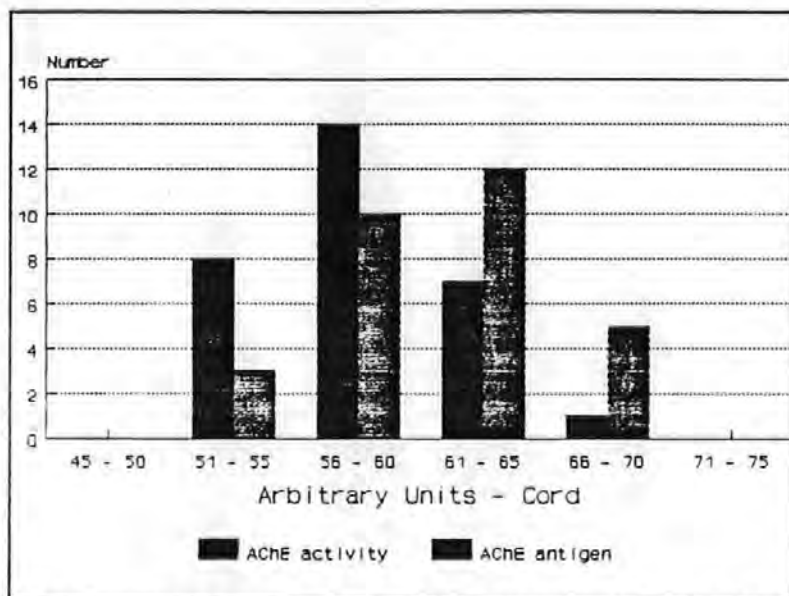


Figure 32 Frequency distribution of AChE activity and antigen levels for healthy cord samples.

Relationship of Erythrocyte AChE with Age of Patient

Erythrocyte AChE activity and antigen levels were evaluated for patients in a number of age groups, from gestational age (fig.33) through postnatal (fig.34) and adolescence (fig.35) to adulthood (fig.36).

AChE activity and antigen levels rise sharply after birth reaching adult values within 5 months. Levels remain remarkably constant throughout the teenage and adult life, with a possible slight downward trend towards later life.

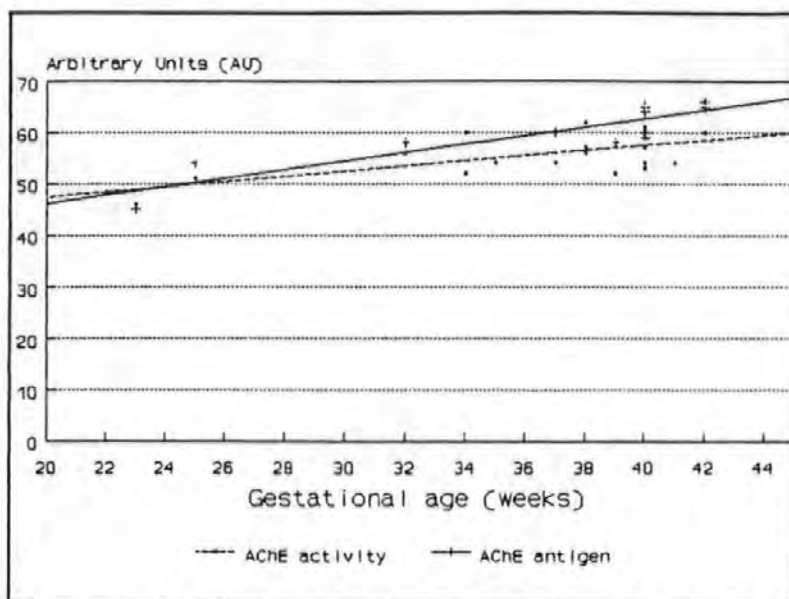


Figure 33 Effect of gestational age on foetal erythrocyte AChE activity and antigen levels.

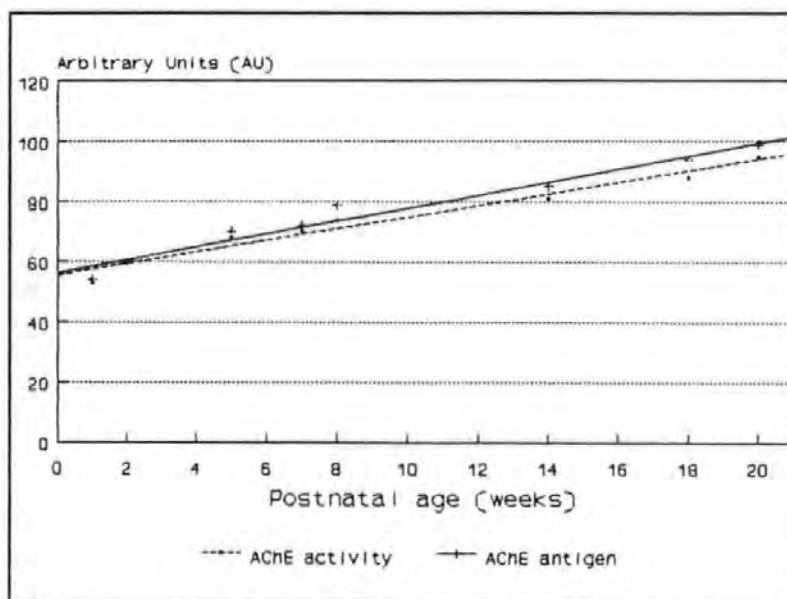


Figure 34 Effect of postnatal age (0 to 20 weeks) on erythrocyte AChE activity and antigen levels.

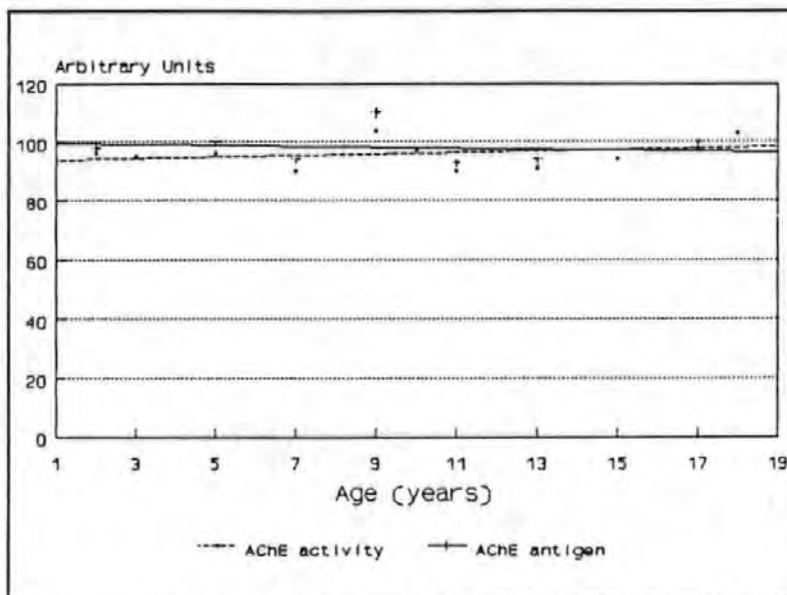


Figure 35 Effect of age throughout adolescence on erythrocyte AChE activity and antigen levels.

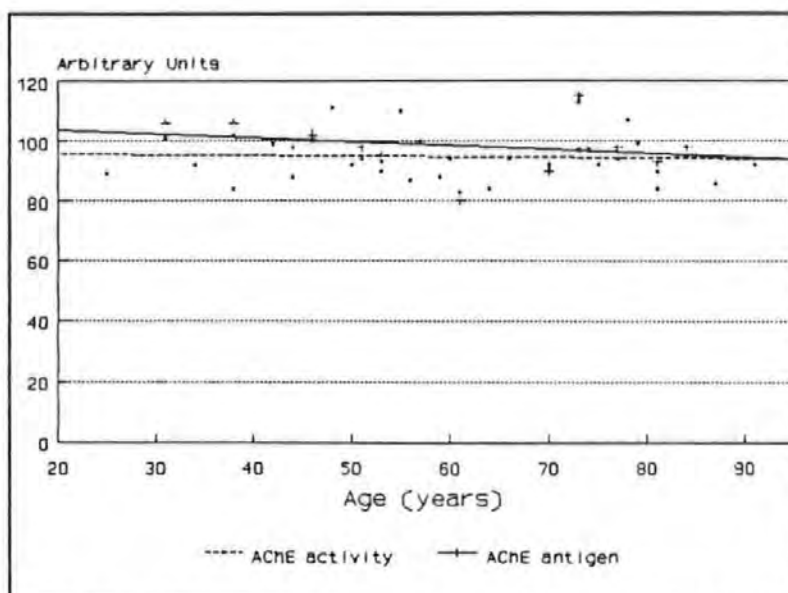


Figure 36 Effect of age throughout adulthood on erythrocyte AChE activity and antigen levels.

Relationship of Erythrocyte AChE with Mean Cell Volume (MCV)

A number of samples were tested to establish whether AChE on the erythrocyte surface is related to the actual size (MCV) of the red cell. Samples of erythrocytes varying in MCV from 65 to 100 were tested for AChE activity and antigen level (fig.37).

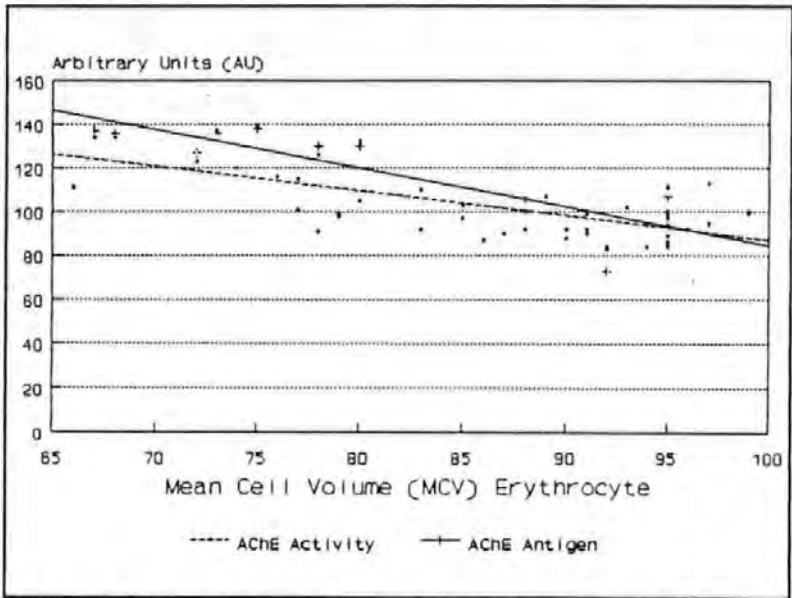


Figure 37 The effect of decreasing mean cell volume on erythrocyte AChE activity and antigen levels in microcytic cells.

It appears that there is an overall trend of increasing erythrocyte AChE activity and antigen level with decreasing cell size for erythrocytes of MCV in the range 65 to 85.

The levels of AChE on the surface of macrocytic erythrocytes (MCV 99 to 113) were also investigated (fig.38). These samples were selected from a number of patients showing varying degrees of vitamin B₁₂ and folate deficiency. There appears to be a wide distribution of results with no apparent trend.

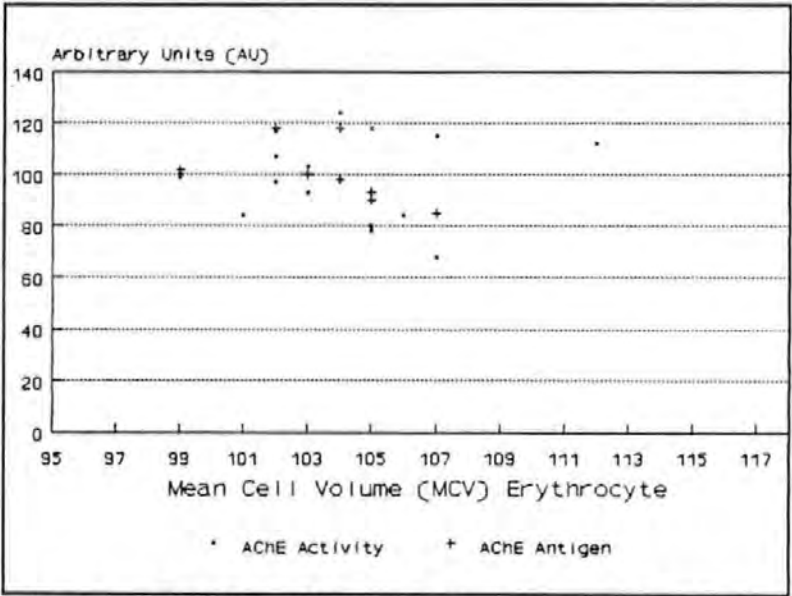


Figure 38 The effect of increasing mean cell volume on erythrocyte AChE activity and antigen levels in macrocytic cells.

Erythrocyte AChE activity and antigen levels during pregnancy

Erythrocyte AChE was monitored at various stages of pregnancy in 93 women (fig.39).

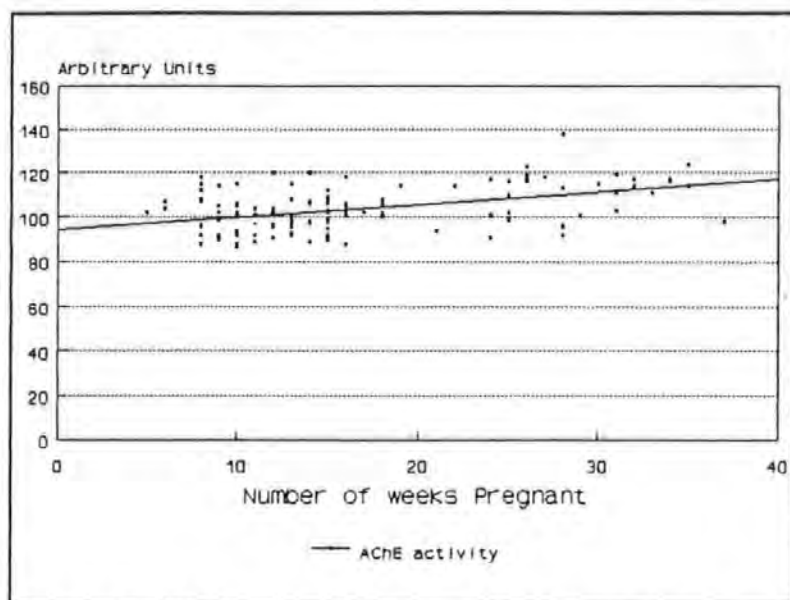


Figure 39 The effect of gestational age on erythrocyte AChE activity during pregnancy.

There appears to be an overall increase in AChE activity as pregnancy progresses. Although AChE antigen levels were not monitored on all these patients, those with high activity also presented with high antigen levels. Postnatal samples between one and three days were investigated to ascertain whether the level of this enzyme activity at delivery was affected (fig.40).

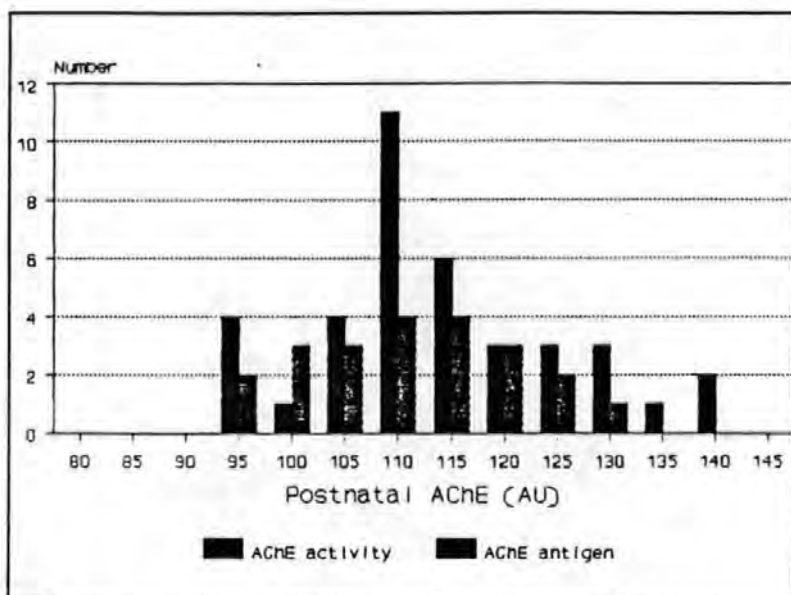


Figure 40 Frequency distribution of erythrocyte AChE activity and antigen levels measured 1 to 3 days postnatal.

A wide distribution of both activity and antigen levels was observed postnatally (fig.40), reflected in the mean and standard deviation (table 10 - 11). On average AChE was found to be increased with several samples presenting with very high activity. Samples later than 3 days postnatal were not available for testing. Statistical comparison of activity and immunological data between females 1 to 3 days postnatal and non-pregnant females was made (table 10 - 11) to establish whether the difference between the two was significant.

Table 10 Erythrocyte AChE activity in a number of patients 1 to 3 days postnatal (mean±SD)

Category	Number	AChE Activity		S.E.	T	p
		AU ¹	μmole/min/ml ²	mean ³		
Postnatal (1 to 3 days)	38	113±24	9.84±2.09	3.89		
					3.22	0.004
Female non-pregnant	30	98±14	8.54±1.22	2.56		

¹ relative to the mean value for healthy adult males
² packed cells
³ Standard error of the mean

Table 11 Erythrocyte AChE antigen level in a number of patients 1 to 3 days postnatal (mean±SD)

Category	Number	AChE antigen	S.E.	T	p
		AU ¹	mean ²		
Postnatal (1 to 3 days)	22	111±22	4.69		
				2.03	0.102
Female non-pregnant	30	100±15	2.74		

¹ relative to the mean value for healthy adult males
² Standard error of the mean

Erythrocyte AChE in Haematological Disorders

A number of haematological disorders were investigated for AChE activity and antigen levels (table 12).

Table 12 Enzymic and antigenic activities of erythrocyte acetylcholinesterase in patients having haematological disorders.

Condition	AChE Arbitrary Units		Comment
	Activity ¹	Antigen ¹	
PNH	73	76	low
PNH	79	81	low
PNH	62	72	low
ALL	57	51	very low
AML	79	84	low
AML	83	82	low
AML (M5)	87	89	low end norm
AML (M5)	88	89	low end norm
CLL	96	104	normal
CLL	79	71	low
CLL	102	100	normal
CMML	112	115	high end
NHL	88	95	normal
Cancer breast	120	117	high
malaria	62	72	low
Auto-immune IgG	62	70	low
Auto-immune IgG+C3d	86	88	low end norm
ABO HDN day 0	47	41	low
ABO HDN day 3	50	47	low to norm
MDPS	94	98	normal
PRV	97	93	normal
alcoholic cirrhosis	104	96	normal
Diabetes	92	92	normal
Iron def. anaemia	110	115	high end norm
Reticulocytosis 27%	115		high end norm
Reticulocytosis 42%	119	91	high/normal
HE	105		normal
HS	130	98	high/norm

¹ AU relative to mean value for healthy adult males

PNH = Paroxysmal Nocturnal Haemoglobinuria
 ALL = Acute Lymphoblastic Leukaemia
 AML = Acute Myeloid Leukaemia
 CLL = Chronic Lymphoblastic Leukaemia
 CMML = Chronic Myelo-Monocytic Leukaemia
 NHL = Non-Hodgkins Lymphoma
 HDN = Haemolytic Disease of the Newborn
 MDPS = Myelodysplastic Syndrome
 PRV = Polycythaemia Rubra Vera
 HE = Hereditary Elyptocytosis
 HS = Hereditary Spherocytosis

Plasma Cholinesterase

The plasma ChE standard consisted of a pool of 5 $E_1^uE_1^u$ blood bank donor plasmas. These plasmas were frozen within 6 hours of collection and kept at -30°C . The ChE activity and antigen levels for this standard were assigned 100 arbitrary units (AU). Values for the samples tested are presented relative to this standard.

Statistical Comparison of Methodology

(1) Plasma ChE Activity

All activities using benzoylcholine as substrate were measured by the Cholinesterase Research Unit, Hammersmith Hospital, London W12 0HS, who also supplied DN, FN and RO2 numbers to identify the genotypes. 170 $E_1^uE_1^u$ samples were assayed for plasma ChE activity using BzCh and BTI as substrate and statistical comparison was made between the results obtained for each (table 13-14). A graph showing linear regression was plotted (fig. 41).

Table 13 Statistical analysis of Plasma ChE Activity using BzCh and BTI as substrates for 170 $E_1^u E_1^u$ samples

	BzCh ¹	BTI ²
Minimum value	0.79	100
Maximum value	1.66	222
Mean	1.02	138
Standard Deviation (SD)	0.16	23
Coefficient of Variation (CV%)	16	17

¹ $\mu\text{mols/min/ml plasma}$
² AU relative to a standard of 5 $E_1^u E_1^u$ donors

Table 14 Regression Analysis of Results Obtained for Plasma ChE Activity for 170 $E_1^u E_1^u$ samples using BzCh and BTI as Substrates

	<u>Regression Analysis</u>	
	Linear	Polynomial
Correlation Coefficient	0.889	0.889
Standard Error	10.86	10.86
Slope	1.266	---
Y Intercept	8.217	---

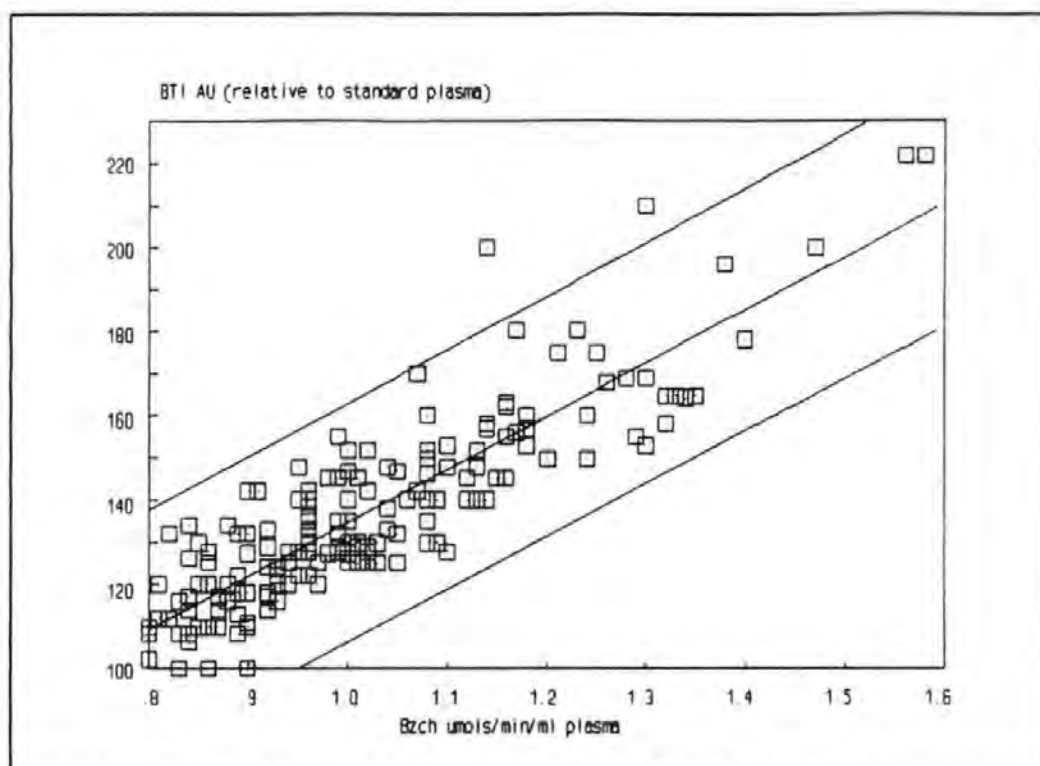


Figure 41 Linear regression analysis showing relationship between the two substrates Bzch and BTI in the assay of plasma ChE activity.

(2) Plasma ChE Immunological Quantitation

(i) Rocket Immunolectrophoresis (288) and ELISA (Mab2-1)

50 $E_1^uE_1^u$ samples were quantitated for plasma ChE levels by R.I. and ELISA. Statistical comparison was made between the results obtained by both techniques (table 15-16) and a graph of linear regression plotted (fig.42).

Table 15 Statistical Analysis of Plasma ChE Quantitation by Rocket Immunolectrophoresis and ELISA for 50 $E_1^uE_1^u$ samples

	R.I. ¹	ELISA ²
Minimum value	85	100
Maximum value	190	190
Mean	137	139
Standard Deviation (SD)	35	26
Coefficient of Variation (CV%)	25	19
¹ AU relative to 5 $E_1^uE_1^u$ donor standard (Plymouth 288)		
² AU relative to 5 $E_1^uE_1^u$ donor standard (Mab2-1)		

Table 16 Regression Analysis of Results Obtained for Plasma ChE Quantitation by Rocket Immunolectrophoresis and ELISA for 50 $E_1^uE_1^u$ samples

	Regression Analysis	
	Linear	Polynomial
Correlation Coefficient	0.740	0.741
Standard Error	18.05	18.10
Slope	0.574	---
Y Intercept	59.98	---

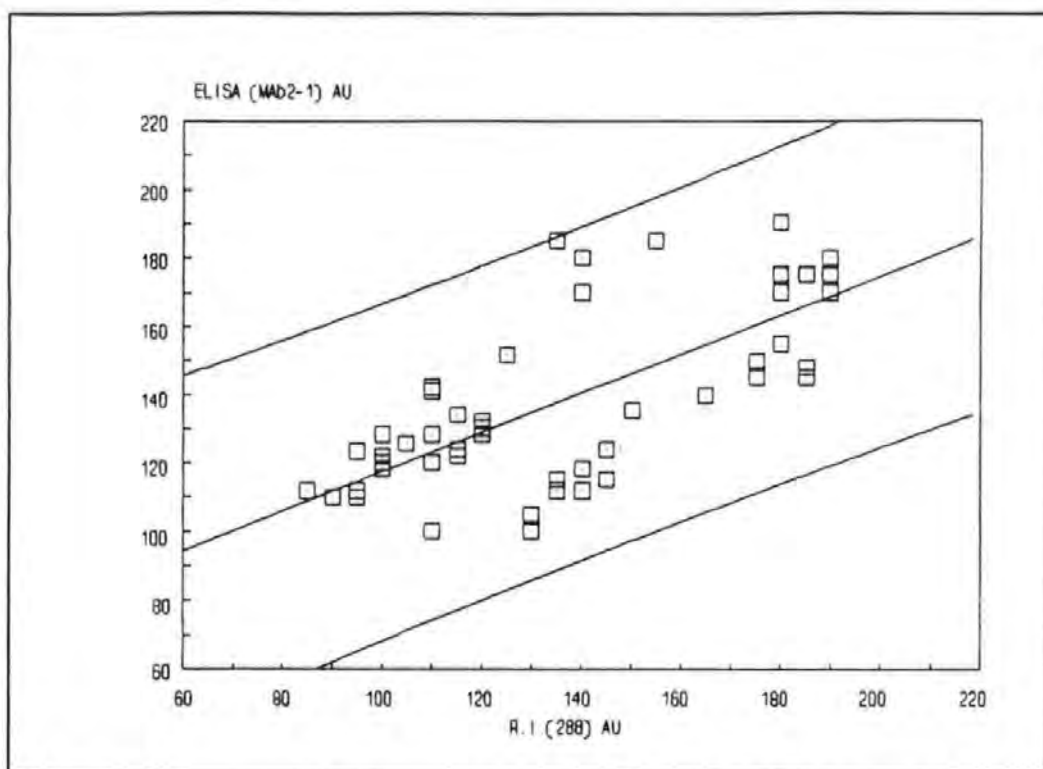


Figure 42 Linear regression analysis showing relationship between rocket immunoelectrophoresis and ELISA in the quantitation of plasma ChE.

(ii) ELISA Using MAb2-1 and MAb2-4 as primary antibodies

170 $E_1^uE_1^u$ samples were quantitated for plasma ChE levels by ELISA using MAb2-1 and MAb2-4 and statistical comparison was made between the results obtained with each antibody (table 17-18) and a graph of linear regression was plotted (fig. 43).

Table 17 Statistical Analysis of Plasma ChE Quantitation by ELISA using MAb2-1 and MAb2-4 for 170 $E_1^uE_1^u$ samples

	MAb2-1 ¹	MAb2-4 ¹
Minimum value	100	98
Maximum value	230	230
Mean	136	144
Standard Deviation (SD)	25	26
Coefficient of Variation (CV%)	18	18
¹ AU relative to a standard of 5 $E_1^uE_1^u$ donors		

Table 18 Regression Analysis of Results Obtained for Plasma ChE Quantitation by ELISA using MAb2-1 and MAb2-4 for 170 $E_1^uE_1^u$ samples

	<u>Regression Analysis</u>	
	Linear	Polynomial
Correlation Coefficient	0.815	0.815
Standard Error	15.36	15.39
Slope	0.868	---
Y Intercept	26.06	---

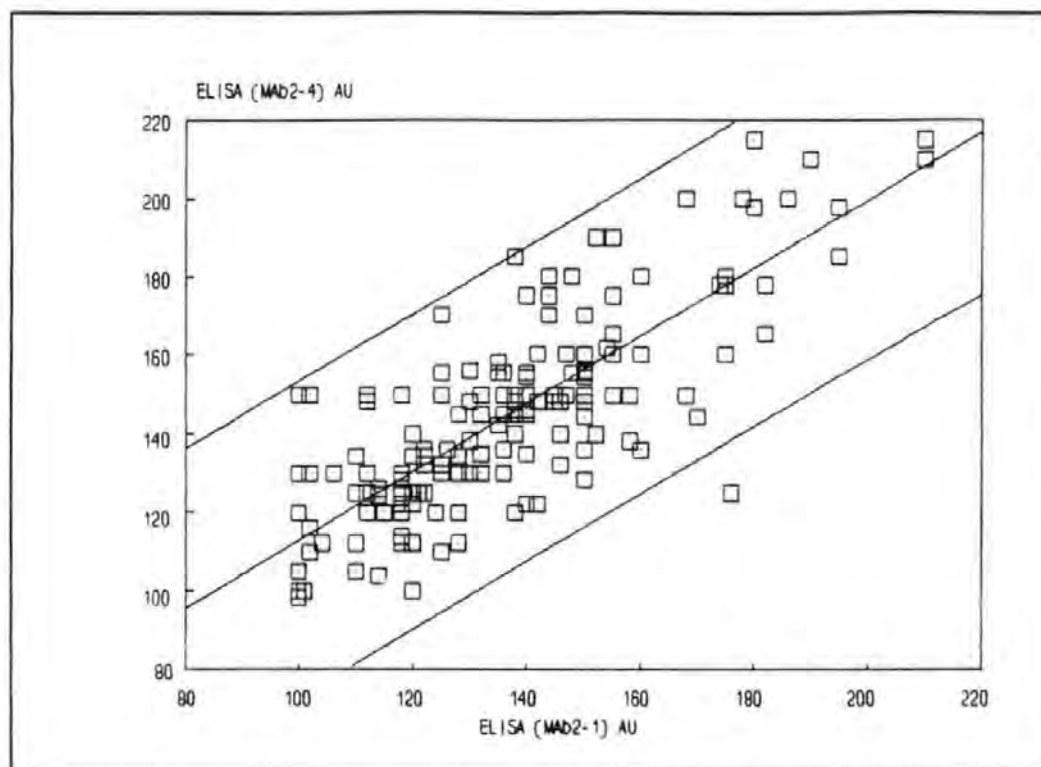


Figure 43 Linear regression analysis showing the relationship between the monoclonal antibodies MAb2-1 and 2-4 used in ELISA for the quantitation of plasma ChE.

Analysis of Genotypic Variants for Plasma Cholinesterase

Data obtained for enzymic activity with butyrylthiocholine and benzoylcholine as substrate and for antigen levels as measured by ELISA, using MAb2-1 and MAb2-4 for a number of genetic variants of plasma cholinesterase (table 19) has enabled frequency distributions for the genotypes $E_1^uE_1^u$, $E_1^uE_1^a$ and $E_1^aE_1^a$ to be plotted (fig. 44-46). Calculations of the ratios of activity and immunological protein for $E_1^uE_1^u$, $E_1^uE_1^a$ and $E_1^aE_1^a$ (table 20) indicates a statistical distinction between these genotypes.

Table 19 Cumulative activity and immunological data for genotypic variants of plasma ChE.
Results are presented as mean \pm SD

Genotype	Number	Activity		R.I. ²	MAb	
		BzCh ¹	BTI ²		2-1 ²	2-4 ²
$E_1^uE_1^u$	170	1.02 \pm .16	138 \pm 23	137 \pm 35	136 \pm 25	144 \pm 26
$E_1^uE_1^a$	60	0.77 \pm .15	104 \pm 23	122 \pm 33	118 \pm 27	122 \pm 25
$E_1^aE_1^a$	56	0.46 \pm .09	57 \pm 13	86 \pm 30	90 \pm 28	90 \pm 27
$E_1^uE_1^s$	7	0.57 \pm .10	81 \pm 13	90 \pm 25	84 \pm 15	79 \pm 12
$E_1^aE_1^s$	11	0.25 \pm .10	33 \pm 14	55 \pm 24	48 \pm 14	45 \pm 12
$E_1^aE_1^k$	20	0.56 \pm .08	74 \pm 12	82 \pm 25	88 \pm 22	89 \pm 21

¹ μ mol/min/ml plasma
² AU relative to a standard of 5 $E_1^uE_1^u$ donors

Table 20 Comparison of activity and immunological data
for the usual and atypical variants of plasma ChE.
Results are presented as mean \pm SD

Genotype	Number	Activity		MAb		Ratio	
		BTI ¹	2-1 ¹	2-4 ¹	BTI/2-1	BTI/2-4	
E ₁ ^u E ₁ ^u	170	138 \pm 23	136 \pm 25	144 \pm 26	1.02 \pm .08	0.96 \pm .08	
E ₁ ^u E ₁ ^a	60	104 \pm 23	118 \pm 27	122 \pm 25	0.88 \pm .08	0.85 \pm .08	
E ₁ ^a E ₁ ^a	56	57 \pm 13	90 \pm 28	90 \pm 27	0.63 \pm .07	0.63 \pm .07	

¹ AU relative to a standard of 5 E₁^uE₁^u donors

Table 21

Statistical comparison of the ratios of activity to immunological protein for the usual and atypical genotypes using the students 't' test.

Genotypes	mean	SD	S.E. ¹ mean	T	p
<u>BTI/MAb2-1</u>					
E ₁ ^u E ₁ ^u	1.02	0.08	0.0061	11.65	<0.001
E ₁ ^u E ₁ ^a	0.88	0.08	0.01		

E ₁ ^u E ₁ ^a	0.88	0.08	0.01	17.86	<0.001
E ₁ ^a E ₁ ^a	0.63	0.07	0.0094		

E ₁ ^u E ₁ ^u	1.02	0.08	0.0061	32.59	<0.001
E ₁ ^a E ₁ ^a	0.63	0.07	0.0094		
<u>BTI/MAb2-4</u>					
E ₁ ^u E ₁ ^u	0.96	0.08	0.0061	9.16	<0.001
E ₁ ^u E ₁ ^a	0.85	0.08	0.01		

E ₁ ^u E ₁ ^a	0.85	0.08	0.01	15.72	<0.001
E ₁ ^a E ₁ ^a	0.63	0.07	0.0094		

E ₁ ^u E ₁ ^u	0.96	0.08	0.0061	27.58	<0.001
E ₁ ^a E ₁ ^a	0.63	0.07	0.0094		

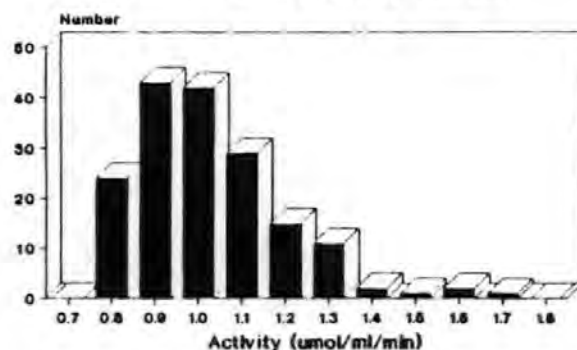
¹ Standard Error of Mean

¹ Standard Error of Mean

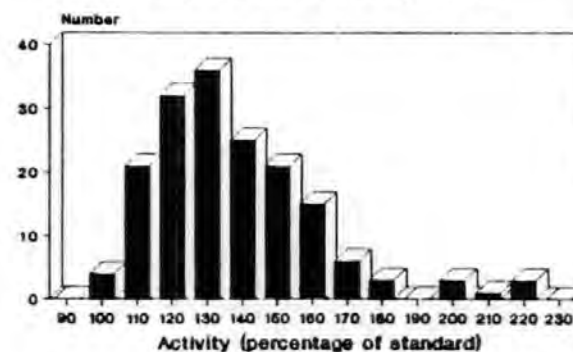
Figure 44

Frequency distribution of $E_1^U E_1^U$ individuals for enzymic activity using (a) benzoylcholine and (b) butyrylthiocholine and immunological quantitation using (c) MAb2-1 and (d) MAb2-4.

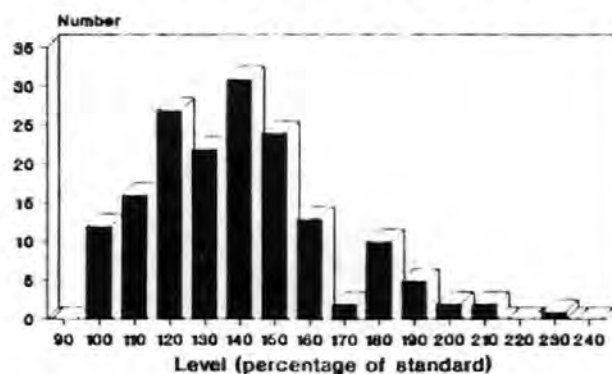
Plasma ChE Activity (BzCh)



Plasma ChE activity (BTI)



Plasma ChE level (MAb2-1)



Plasma ChE level (MAb2-4)

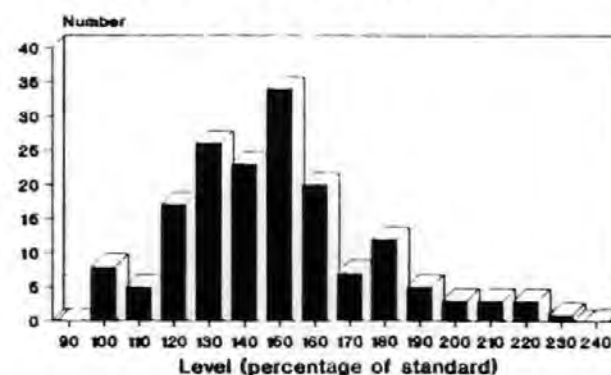
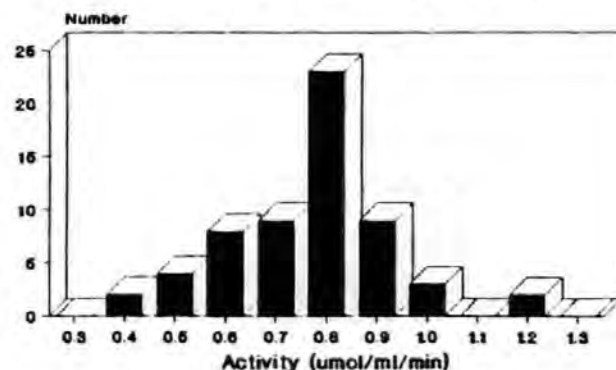


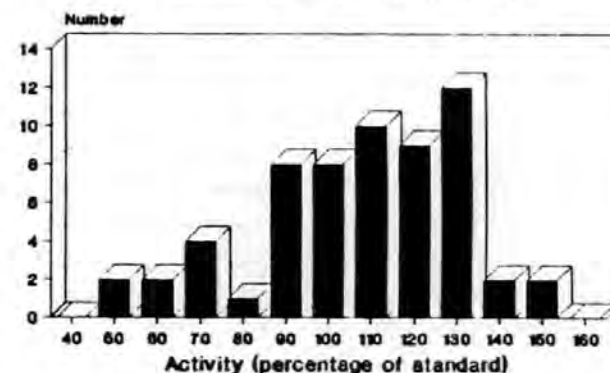
Figure 45

Frequency distribution of $E_1^u E_1^a$ individuals for enzymic activity using (a)benzoylcholine and (b)butyrylthiocholine and immunological quantitation using (c)MAb2-1 and (d)MAb2-4.

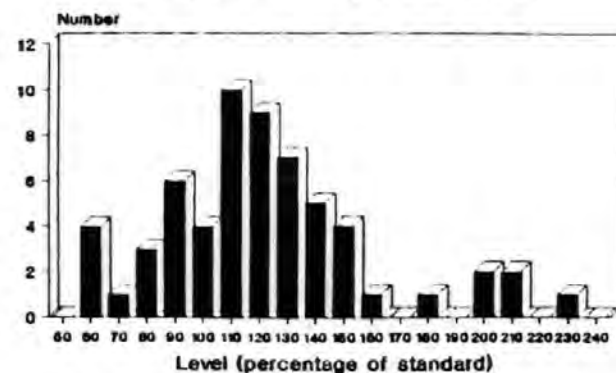
Plasma ChE activity (Bzch)



Plasma ChE activity (BTI)



Plasma ChE level (MAb2-1)



Plasma ChE level (MAb2-4)

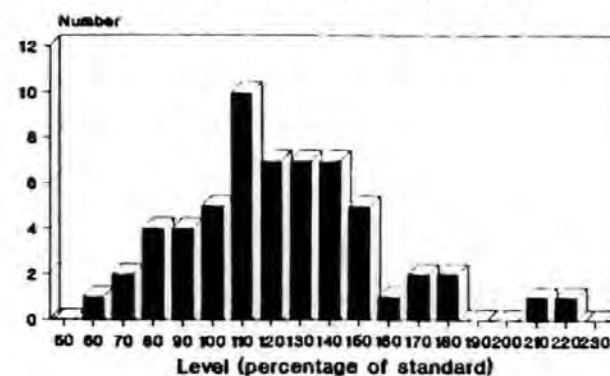
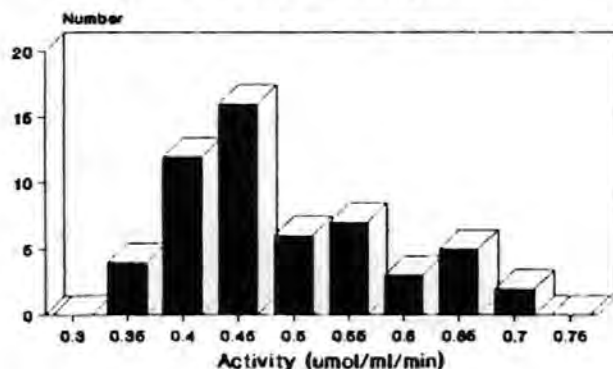


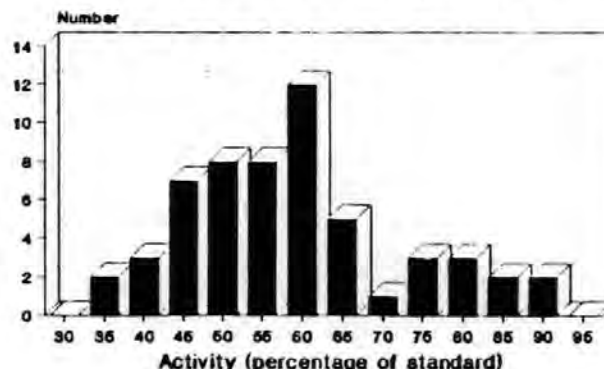
Figure 46

Frequency distribution of $E_1^a E_1^a$ individuals for enzymic activity using (a)benzoylcholine and (b)butyrylthiocholine and immunological quantitation using (c)MAb2-1 and (d)MAb2-4.

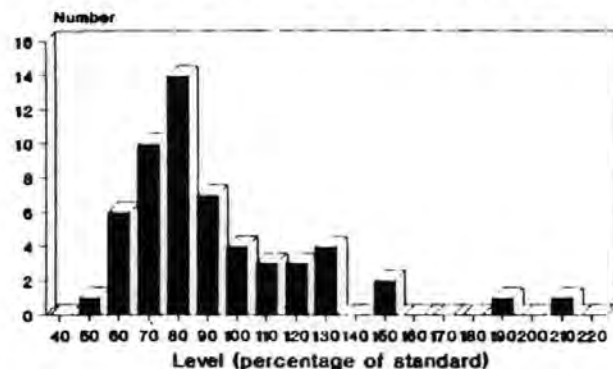
Plasma ChE activity (Bzch)



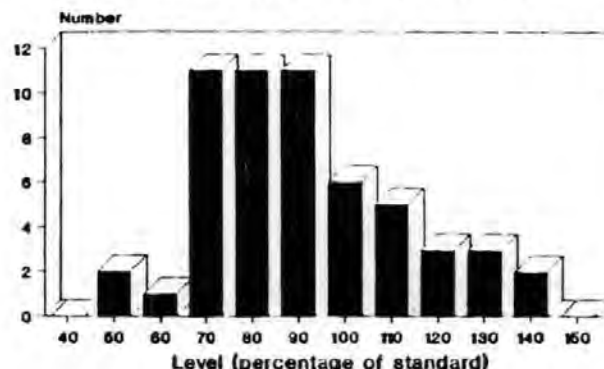
Plasma ChE activity (BTI)



Plasma ChE level (MAb2-1)



Plasma ChE level (MAb2-4)



Investigation of heterogeneity of the silent gene for plasma ChE

ELISA using MAb's 2-1 and 2-4 was used to quantitate the enzyme protein in 43 apparently 'silent' homozygotes with little or no catalytic activity (table 22). The ELISA estimates are expressed as the percentage ratio of the binding of each sample compared with the binding observed for a pool of 5 E₁^uE₁^u plasmas.

Rocket immunoelectrophoresis (R.I.) was performed using the 'piggy-back' method. Rockets of the same height and staining intensity as the 25% standard do not contain any immunologically detectable cholinesterase protein. Longer and weaker staining peaks are indicative of functionally inactive, but immunologically reactive cholinesterases, the amount being proportional to the difference in height of the rocket obtained for the test and the height of the 25% standard (picture 2). Results for R.I. (table 19 and 22) are expressed as a percentage of the 25% standard. All assays were at least duplicated. Cumulative data is given in table 23.

Table 22

Plasma cholinesterase activities and levels
in 43 apparent silent gene homozygotes.

Group	Individual	Activity		R.I. ²	ELISA MAb	
		BzCh ¹	BTI ²		2-1 ²	2-4 ²
1	I LM	0	0	0	0	0
2	I IG	0	0	0	0	0
3	I RR	0	0	0	0	0
4	I TT	0.01	0	0	0	0
5	I SS	0.02	0	0	0	0
6	I LC	0.02	1	0	0	0
7	I YW	0.02	1	0	0	0
8	I AD	0.03	0	0	0	0
9	I DF	0.03	0	0	0	0
10	II AG	0	14	20	17	19
11	II KJ	0	7	--	18	20
12	II DP	0.06	3	20	11	24
13	III CL	0	0	--	12	12
14	III DD	0	0	0	2	2
15	III MP	0	0	0	3	3
16	III JV	0	0	0	2	0
17	III MM	0	0	0	3	0
18	III EE	0.01	4	15	4	5
19	III YW	0.02	0	0	2	0
20	III AB	0.02	1	--	3	1
21	III SK	0.02	1	0	9	6
22	III FK	0.02	0	10	3	0
23	III KQ	0.02	2	10	7	4
24	III AG	0.02	2	0	2	3
25	III MH	0.03	11	18	7	8
26	III SQ	0.04	0	--	2	4
27	III MH	0.04	2	0	2	3
28	III RC	0.06	6	--	7	7
29	III CB	0.12	1	--	0	0
30	IV YL	0	0	---	120	125
31	IV MS	0	0	---	200	210
32	IV GD	0	0	---	150	145
33	IV AF	0	4	---	130	125
34	IV AW	0	0	100	180	200
35	IV JM	0	0	---	210	205
36	IV VH	0	0	160	130	200
37	IV KM	0	0	---	116	112
38	IV CH	0.01	0	110	120	200
39	IV PH	0.02	1	---	180	145
40	IV DS	0.04	0	100	150	150
41	PP	0.13	12	0	0	0
42	VT	0.08	7	35	120	66
43	JK	0.04	18	52	44	48

¹ $\mu\text{mols/min/ml plasma}$

² AU relative to a standard of 5 $E_1^uE_1^u$ donors

Table 23 Cumulative data for apparent silent gene homozygotes.

Group	Patient	Activity		R.I. ²	MAb	
		BzCh ¹	BTI ²		2-1 ²	2-4 ²
I	1 - 9	0.01	0	0	0	0
II	10 - 12	0.02	8	20	15±4	21±3
III	13 - 29	0.03	2	4	4	4
IV	30 - 40	0.01	0	118±29	153±34	165±38
	41	0.13	12	0	0	0
	42	0.08	7	35	120	66
	43	results discarded - probable pathological low activity				

¹ μmols/min/ml plasma
² AU relative to a standard of 5 E₁^uE₁^u donors

Immunological studies of families segregating the silent gene for plasma cholinesterase

Seven family groups segregating the silent gene for plasma ChE were investigated for enzymic activities using the two substrates BTI and Bzch with dibucaine and fluoride inhibition and for antigen levels using MAb2-1 and 2-4 (table 24). The pedigrees of these families are shown (fig. 47).

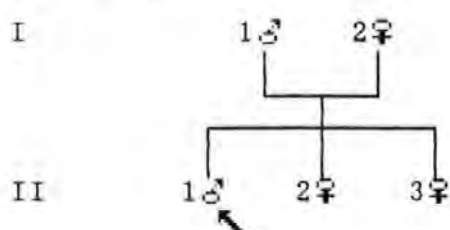
Table 24 Biochemical data for individuals from seven families each segregating an apparently 'silent' homozygote (P = Propositus).

Family	Individual	Activity		DN	FN	ELISA MAb	
		BzCh ¹	BTI ²			2-1 ²	2-4 ²
1	I-1	0.72	120	80	58	116	115
	I-2	0.63	104	80	60	85	87
	II-1	0	0	--	--	0	0
	II-2	0.99	140	81	59	118	118
	II-3	0.69	102	80	58	96	88
2	I-1	0.55	76	82	62	80	80
	I-2	0.34	53	78	62	48	52
	II-1	0.03	2	--	--	3	3
	II-2	0.31	44	83	60	60	58
	II-2	0.34	54	83	60	60	52
	II-3	0.31	50	79	61	58	60
	II-3	0.18	25	79	61	25	22
	II-4	0.04	0	--	--	2	4
	II-5	1.25	160	79	62	180	180
	III-1	0.53	65	80	57	78	82
3	III-2	0.64	105	81	58	100	110
	I-1	0.21	32	79	65	37	41
	I-2	0.59	60	80	59	70	52
	II-1	0.71	82	80	58	84	70
	II-2	0.02	1	--	--	0	0
	II-3	0.02	1	--	--	3	1
	II-4	0.61	78	82	60	86	70
4	II-5	0.28	40	80	57	35	52
	I-1	0.49	76	82	58	104	90
	II-1	0	0	--	--	12	12
	III-1	0.53	90	80	60	104	102
	III-2	0.29	50	79	62	60	50
	III-3	0.35	60	79	59	66	60
	III-4	0.70	110	82	59	108	110
	IV-1	0.23	40	80	61	52	48
5	IV-2	0.22	42	81	62	65	60
	IV-3	0.27	48	79	58	61	54
	I-1	0.50	80	82	61	69	64
	II-1	0	0	--	--	116	112
6	II-2	0	0	--	--	210	205
	I-1	0.57	67	81	63	205	152
	I-2	0.28	22	81	63	37	35
	II-1	0.02	1	--	--	180	145
7	II-2	0.02	0	--	--	200	150
	I-1	0	0	--	--	200	210
	I-2	0	0	--	--	120	125
	I-3	0	0	--	--	150	150
	II-1	0.33	45	81	60	45	45
	II-2	0.43	65	82	61	58	58
	II-3	0.32	48	81	63	140	140
	II-4	0.46	70	81	61	140	150

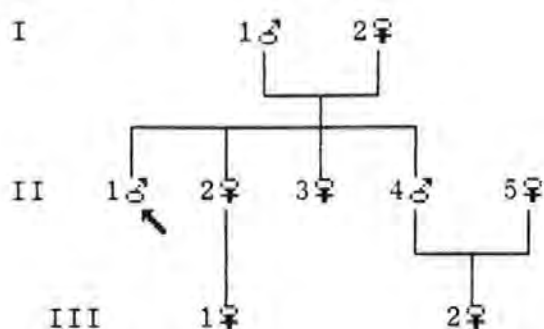
¹ μ moles/min/ml plasma ; ² AU relative to standard

Figure 47 Pedigrees of seven families segregating apparently 'silent' homozygotes

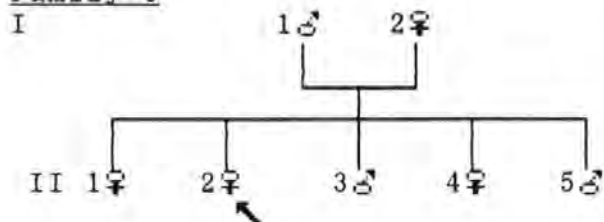
Family 1



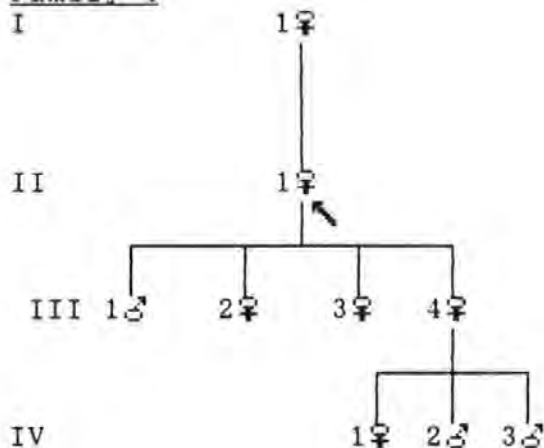
Family 2



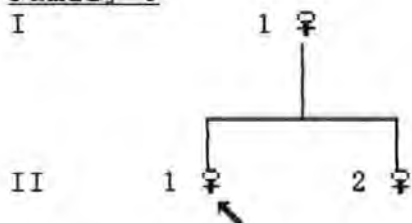
Family 3



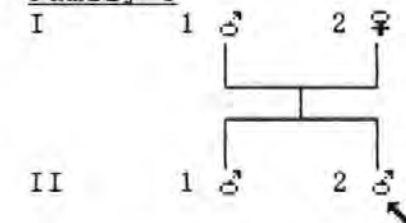
Family 4



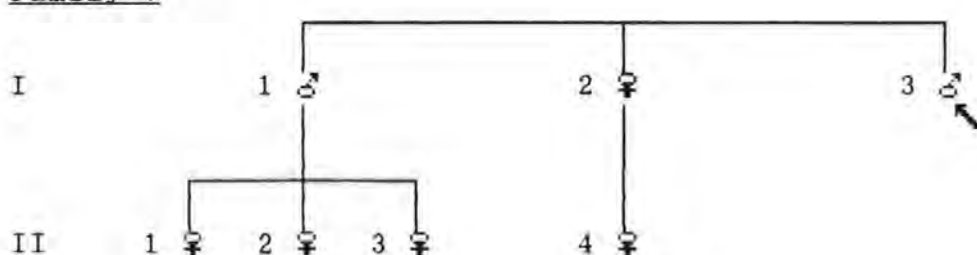
Family 5



Family 6



Family 7



Key
 propositus

Immunological Studies of families segregating the E_1^h gene for Plasma Cholinesterase

Plasma ChE activity and antigen levels were examined for 2 families in which a new allele E_1^h was found to be segregating with the E_1^a gene (Whittaker and Britten, 1987) (table 25). This data enabled cumulative ranges for the heterozygous E_1^h variants within these families to be calculated (table 26). The family pedigrees are shown (fig.48).

Table 25 Plasma cholinesterase for the E_1^h families

Family Individual	Activity BzCh ¹	BTI ²	DN	FN	RO2N	ELISA 2-1 ²	MAb 2-4 ²	Probable Genotype
1 I-1	0.77	108	64	48	73	108	88	$E_1^u E_1^a$
I-2	0.57	100	78	56	100	106	105	$E_1^k E_1^h$
II-1	0.58	90	80	60	100	81	79	$E_1^u E_1^h$
II-2	0.26	36	28	28	33	50	46	$E_1^a E_1^h$
II-3	0.55	85	82	59	100	78	82	$E_1^u E_1^h$
II-4 P	0.24	30	27	26	30	40	48	$E_1^a E_1^h$
II-5	0.59	96	80	58	100	94	88	$E_1^u E_1^h$
II-6	0.46	61	58	44	58	66	62	$E_1^a E_1^k$
II-7	0.58	84	57	45	62	90	80	$E_1^a E_1^k$
II-8	0.96	130	82	59	100	112	130	$E_1^u E_1^k$
II-9	0.26	34	29	28	34	60	54	$E_1^a E_1^h$
II-10	0.25	34	28	27	35	44	45	$E_1^a E_1^h$
II-11	0.63	108	78	57	100	110	104	$E_1^u E_1^h$
III-1	0.53	82	79	57	100	80	62	$E_1^u E_1^h$
III-2	0.67	--	79	58	100	--	--	$E_1^u E_1^h$
III-3	0.95	152	82	58	97	130	122	$E_1^u E_1^u$
III-4	0.98	140	79	59	94	134	148	$E_1^u E_1^u$
III-5	0.94	140	80	58	96	134	140	$E_1^u E_1^u$
III-6	0.87	130	81	59	97	125	105	$E_1^u E_1^u$
2 I-1	0.67	98	65	50	75	110	102	$E_1^u E_1^a$
I-2	0.60	96	78	56	94	105	95	$E_1^u E_1^h$
II-1 P	0.30	--	28	25	32	---	--	$E_1^a E_1^h$
II-2	0.32	55	29	27	28	78	72	$E_1^a E_1^h$
III-1	0.76	120	78	56	97	118	118	$E_1^u E_1^h$

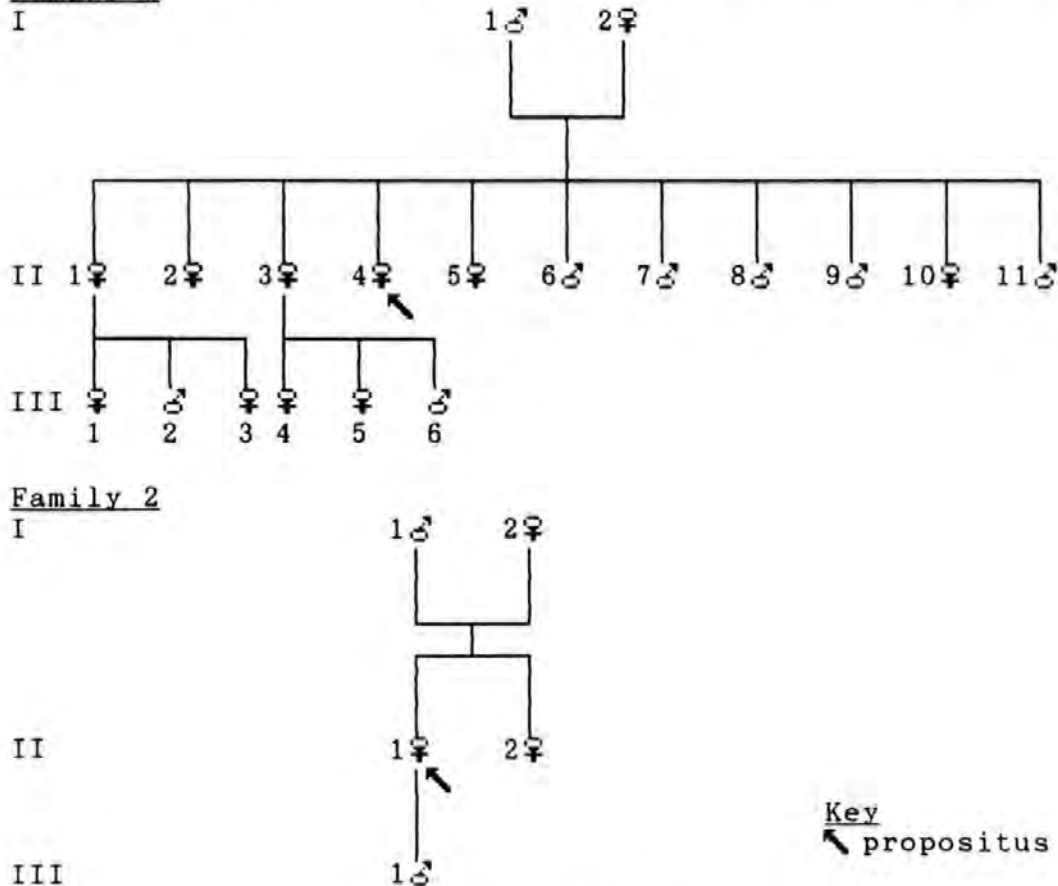
¹ μ mol/min/ml plasma
² AU relative to a standard of 5 $E_1^u E_1^u$ donors
P = Propositus

Table 26 Cumulative data for the heterozygotes of the genotypic variant E_1^h of plasma ChE compared with $E_1^uE_1^u$ and $E_1^aE_1^a$ individuals

Genotype	Number	Activity		ELISA MAb	
		Bzch ¹	BTI ²	2-1 ²	2-4 ²
$E_1^uE_1^h$	7	0.62±.07	97±12	95±15	90±17
$E_1^aE_1^h$	5	0.27±.03	39±10	54±14	53±10
$E_1^kE_1^h$	1	0.57	100	106	105
$E_1^uE_1^u$	170	1.02±.16	138±23	139±23	147±32
$E_1^aE_1^a$	56	0.46±.09	57±13	90±31	90±28

¹ μmols/min/ml plasma
² AU relative to a standard of 5 $E_1^uE_1^u$ donors

Figure 48 Pedigrees of two families segregating the E_1^h gene



Plasma Cholinesterase Levels throughout Pregnancy

Two groups of women were studied. Group A consisted of 16 healthy pregnant women who, at term, underwent spontaneous or induced labour with epidural analgesia followed by vaginal delivery. Patients with any complication of pregnancy or mothers of babies with any neonatal abnormality were not studied. None of these patients had abnormal liver function or any major illness and none received general anaesthesia. Venous blood samples were taken during pregnancy, prior to the administration of epidural analgesia and subsequently during the puerperium (fig.49). The second group of six women (Group B) was studied to clarify the changes in cholinesterase which occurs during the first post partum week (fig.50). All Group B patients were healthy and underwent obstetrically indicated elective caesarian section under epidural analgesia. Venous blood samples were obtained from them prior to delivery and on 1, 2, 3, 4, 5 and 42 days postpartum.

Figure 49 Plasma ChE levels throughout pregnancy

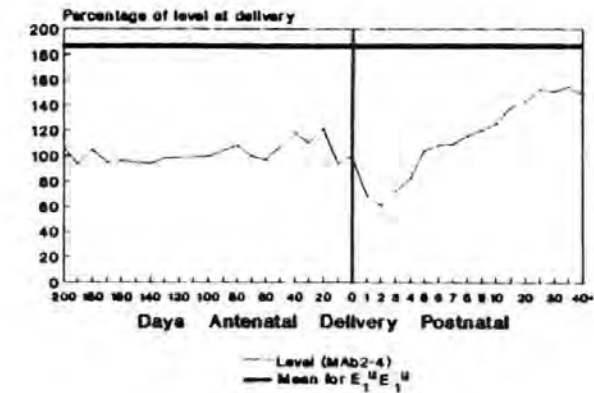
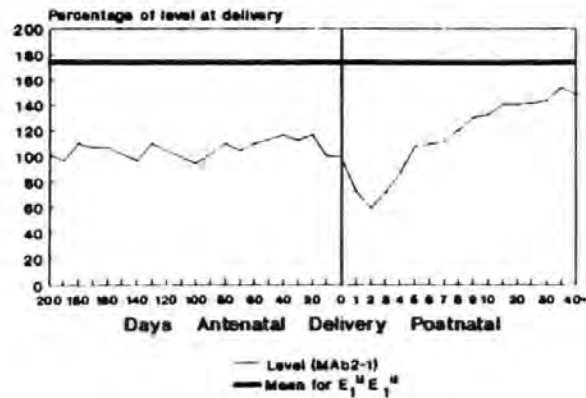
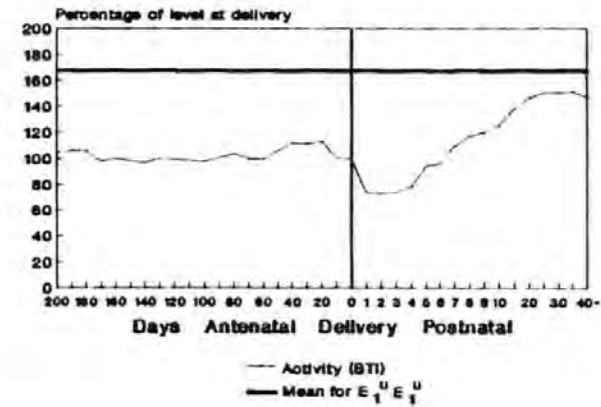
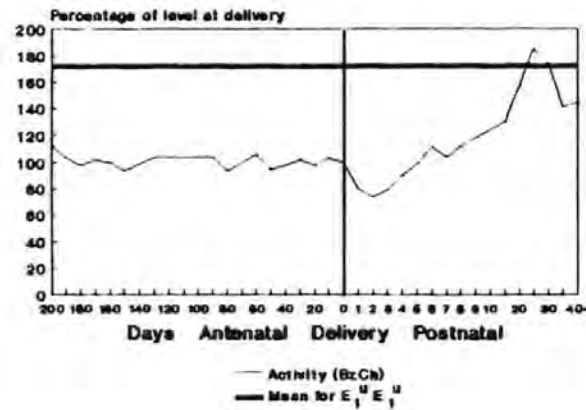
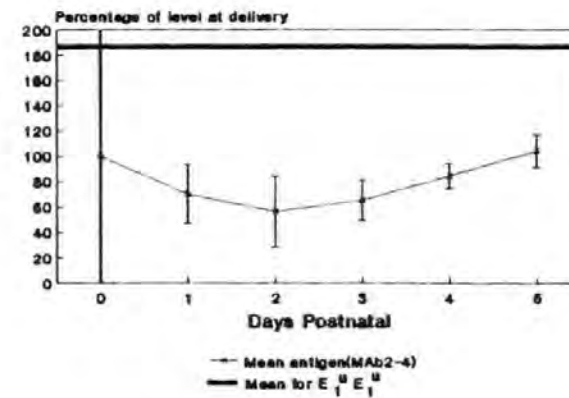
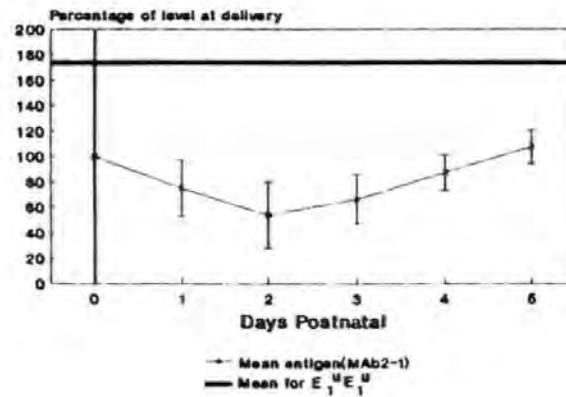
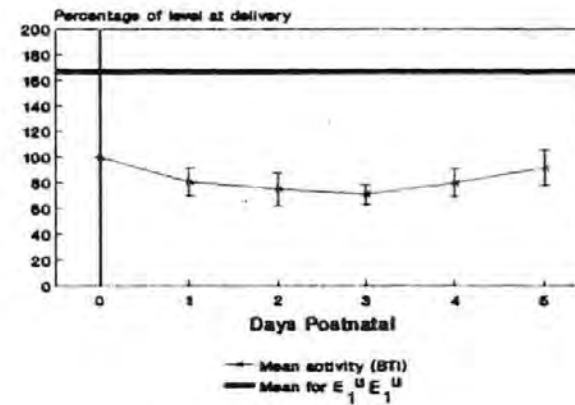
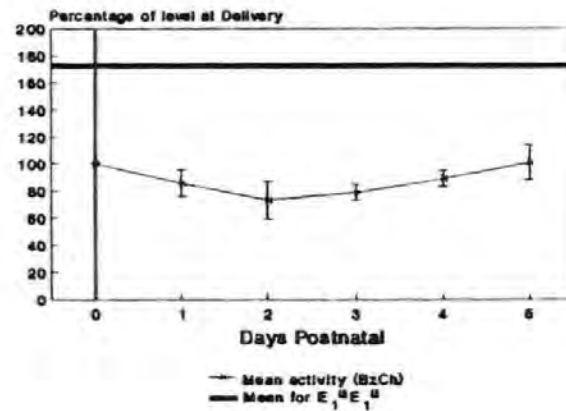


Figure 50 Plasma ChE levels up to 5 days post-delivery



Discussion

Acetylcholinesterase

Traditional methods of assay of erythrocyte AChE involve electrometric (Michel 1949), radiometric (Augustinsson 1971) and colorimetric techniques (Whittaker 1984). Separate validation of the colorimetric method described is not required since it is not significantly changed from the protocol already reported (Whittaker 1984). The method based on the Ellman reaction, provides a reliable method of measuring AChE activity. The 7% coefficient of variation for this technique (CV) is similar to that previously reported (Ellman et al., 1961).

An AChE standard for both activity and antigen levels was unavailable due to the limited storage period of red cells. Activity values are therefore presented in arbitrary units (AU) relative to the mean of the normal range for healthy adult males and also as μ moles of substrate (acetylthiocholine iodide) hydrolysed per min per ml of packed cells. A healthy male sample was always included in the batch as the control sample for the procedure.

The substrate was always present in excess and the temperature was maintained at 22°C. A reaction blank prepared by substituting phosphate buffer solution in place of the sample is mandatory to measure the spontaneous hydrolysis of substrate. AChE activity is maximum between pH 7.5 and 9. At high pH there is considerable non-enzymatic hydrolysis of the substrate which would yield a high blank value. Phosphate buffer pH 7.2 was used in this assay to reduce the blank to negligible proportions.

Erythrocytes are thoroughly washed before assay and

contamination due to plasma ChE is negligible and ChE inhibitors were not considered necessary in this instance. As indicated (fig.3), there is a linear relationship between length of time of incubation of substrate and absorbance at 405nm using the assay conditions described.

The development of monoclonal antibodies for erythrocyte AChE (Fambrough et al, 1982) has enabled alternative methods of quantitation of this enzyme. The enzyme is located on the outer surface of the erythrocyte membrane and, in consequence, the epitope to which these antibodies are directed are available for reaction. This allows the adaptation of a number of serological techniques for the quantitation of erythrocyte AChE.

Haemagglutination using the two monoclonal antibodies AE-1 or AE-2, directed against different epitopes on erythrocyte AChE, gave slightly weaker reactivity with red cells from a cord and PNH sample than with a sample from a normal healthy adult. Agglutinates, however, were weak and easily disrupted. This method does not allow objective quantitation of AChE, since the number of AChE sites are insufficient for this procedure to be sensitive to large percentage changes in the overall number of AChE molecules. It merely acts as a crude test to detect the presence of AChE on the surface of erythrocytes.

The microplate antiglobulin test using AE-2, provides a method of large scale screening with relative ease. The use of weaker red cell suspensions in this method compared to the haemagglutination method requires greater dilution of AE-2, allowing increased sensitivity of reaction. This method merely discriminates and does not quantitate AChE. Results are either

positive or negative (table 5).

Solid phase microplate methodology was investigated to ally the inherent handling advantages of microplates with a quantitative procedure. However, quantitation of AChE, even using the sensitive ELISA based reagents biotin and streptavidin in conjunction with erythrocytes coated with either AE-1 or AE-2 monolayered onto microplate well surfaces, did not result in a method sensitive enough to detect the differences in the number of AChE sites on the surface of adult and cord erythrocytes. This is unfortunate since this method has a number of advantages to recommend it; many samples can be tested with ease, antisera may be used with suitable economy and no transfer of reagents is required. With the advent of even more sensitive reagents, for example alkaline phosphatase/anti-alkaline phosphatase (APPAP) complexes, this method has real potential. The development of a fluid phase homogeneous enzyme linked antiglobulin test (ELAT) for erythrocyte AChE proved extremely successful, constantly providing reproducible results for both adult and cord samples (CV=7%). Samples may be reliably quantitated for as long as 8 days after venesection.

An antibody consumption test indicated a maximum of 270 molecules of AE-2 bound per erythrocyte when using AE-2 at a concentration of 1.5mg/l with a 2% cell suspension following 15min incubation at 37°C. These conditions corresponded to a coloured product having an absorbance of approximately 1.2 units at 405nm (fig.11), which is a convenient location on the absorbance scale. Decreases in the number of AChE binding sites (which directly affects the number of AE-2 molecules bound) can

therefore be easily monitored. This can be illustrated in cord erythrocytes when an absorbance of about 0.7 is usually obtained (fig.11). Incubation of erythrocytes with AE-2 must not be longer than 15min as a decrease in the number of AE-2 molecules per erythrocyte (desorption) was observed after this time (fig.15).

A minimum of 150 molecules of IgG per red cell are required for detection by the haemagglutination method (Stratton et al., 1983). This together with the requirement of between 500 to 2000 molecules of IgG per erythrocyte for maximal agglutination (Merry et al., 1984) suggests that the ELAT is at least twice as sensitive as the haemagglutination method, while also providing an objective means of quantitation. Unfortunately the method is labour intensive and only six duplicate tests can be reliably performed per run. It would be of advantage if an automated or semi-automated procedure could be developed to overcome these difficulties.

AChE results are presented in terms of activity for the Ellman method and antigen quantitation using the fluid phase ELAT. The mean value for the adult male is assigned 100 arbitrary units (AU) for both activity and antigen level, while values for the other groups of samples tested are presented relative to this. No difference was found between adult male and adult female values with both showing normal Gaussian frequency distributions (fig.30-31).

Relationship of Erythrocyte AChE with Age of Patient

Cord blood erythrocytes were found to have a mean activity of 57% of healthy adults, in general agreement with other workers (Sabine 1955; Burman 1960) and mean antigen level of 62% of healthy adults. The frequency distribution of AChE for cord red cells (fig.32) and statistical comparison (table 9) indicate the higher range of values for the antigen levels compared to the activity is fairly significant ($p = 0.116$). This suggests that although there is a smaller number of AChE molecules on the erythrocyte surface as defined by AE-2 these molecules may be of a slightly different conformation to the usual healthy adult AChE molecule resulting in an enzyme which is less efficient catalytically. This difference between activity and antigen levels becomes smaller when samples from babies 0 to 20 weeks old were tested (fig.34). Insufficient numbers of samples were available to perform statistical comparison.

Samples taken from pre-term fetuses and neonates indicate a steady rise in both erythrocyte AChE activity and antigen levels from 47% at 20 weeks to 60% of adult values at term, with the antigen levels rising slightly faster than activity (fig.33). This upward trend is continued post-delivery, adult levels being reached within 5 months (fig.34). Levels remain remarkably constant throughout the teenage and adult period, while a slight downward trend occurs in later life (figs.35-36).

Relationship of Erythrocyte AChE with Mean Cell Volume

The normal range for adult erythrocyte volume (mean cell volume MCV) is quoted as $86 \pm 10 \text{ fl}$ (Dacie and Lewis, 1984). Erythrocytes decrease in cell volume in a number of haematological disorders such as iron deficiency anaemia and increase in conditions such as megaloblastic anaemia. Erythrocytes of reduced MCV have an overall trend of increasing AChE activity and antigen level with decrease in cell size.

The 2% erythrocyte concentration used in the ELAT was determined using the haematocrit, measured with an automated Coulter cell counter and so more cells of smaller volume would be required for a 2% cell suspension. Results of the ELAT are in consequence presented per unit surface area rather than per erythrocyte. Three explanations may be given for erythrocytes of lower MCV resulting in higher antigen values: (i) increased numbers of AChE molecules on the microcytic cell surface; (ii) the same number of AChE molecules on the cell surface as on normal cells, which on a cell of smaller volume would result in a higher site density; (iii) an alteration in shape and/or location of the molecule in the cell membrane with easier access of the monoclonal antibodies to their epitopes. An additional survey of microcytic samples using red cell count (RBC) as the determinant of cell concentration could be used to exclude the first two possibilities.

Results with macrocytic erythrocytes were confusing, activity and antigen levels are apparently unrelated to cell size (fig.38). This may be due to a number of factors. Two causes of macrocytosis are vitamin B₁₂ and folate deficiency; both

substances are required for erythrocyte nuclear development. Reduced amounts of these will therefore impair protein and hence AChE synthesis. In addition, the circulating erythrocytes present in macrocytosis are immature compared to normal cells. The resultant AChE activity and antigen levels observed may be a combination of these factors.

Relationship of Erythrocyte AChE with Pregnancy

An overall increase in AChE activity was observed as pregnancy progresses, patients with high AChE activity presenting with high antigen levels (fig.39; table 10). Samples from patients 1 to 3 days post delivery gave significantly elevated levels of activity (fig.40) when compared to healthy non-pregnant females (table 10 - 11; $p=0.004$). Antigen level comparisons were less significant ($p=0.102$). None of the patients tested presented with microcytic values. This is in agreement with Pritchard et al. (1956) who reported a steady rise in erythrocyte AChE during the first, second and third trimesters, remaining at a constant level at delivery and decreasing after 6 weeks post partum. These authors explained the increase by a progressive rise in the concentration of younger red cells (which contain greater amounts of AChE than older cells) circulating in the blood. This is due to the maintenance of the pre-pregnant haemoglobin levels with a 25 to 35 per cent increase in plasma volume. The rate of erythrocyte production must exceed erythrocyte destruction and thus a greater proportion of the circulating red cells must have been released more recently from the bone marrow than in blood from the normal non-pregnant individual. In part agreement with this hypothesis

the statistical values above indicate that while the numbers of AChE molecules are increased when compared to healthy non-pregnant females ($p=0.102$) there is a more significant difference in activity between these two groups ($p=0.004$) suggesting in addition to an increase in the number of AChE molecules there may be a conformational difference in the AChE molecule allowing greater contact of the catalytic site with the substrate. As discussed later (reticulocytosis) this is indicative of the presence of younger red cells. It is also feasible that this increase in AChE in the cells could be caused by some hormonal effect on erythrocyte production or membrane structure.

These observations are in contrast to those with plasma cholinesterase where a significant decrease in activity occurs during pregnancy (Robertson, 1966), with even lower levels of activity reported during the first 2 to 3 days postpartum (Hazel and Monier, 1971; Robson et al., 1986).

Erythrocyte AChE Activity and Antigen Levels in Various Haematological Disorders

Additional haematological disorders were investigated and categorised into three groups, determined by normal, lower or higher levels of AChE (table 12). No abnormality in AChE levels were observed in polycythaemia, myelodysplastic syndrome, non-hodgkins lymphoma, and 2 out of three cases of chronic lymphatic leukaemia indicating this enzyme can not be used as a marker for these conditions. These results are surprising especially in the cases of polycythaemia and myelodysplasia, since malignant conditions affecting erythrocyte cell lines would be expected to

affect their surface components. As has been previously reported (Abernathy et al., 1988) normal levels were found in a case of alcoholic cirrhosis with decreased plasma cholinesterase activity, in a case of insulin controlled diabetes mellitus (Suhail and Rizvi, 1989) and in hereditary elliptocytosis. In the latter condition the patient was diagnosed as a heterozygous trait with haemolysis being well compensated. Altered erythrocyte AChE activity may well be observed in the homozygous condition.

Reduced levels of AChE activity and antigen level were observed in three cases of PNH not recently transfused. Brimijoin et al. (1986) using fluorescence activated cell sorting (FACS), reported a similar finding and concluded that the cholinesterase defect was dominated by enzyme loss rather than by structural abnormalities affecting enzyme function. Using this technique a fluorescent conjugate is used in place of an enzymic conjugate, but the principle remains the same as for the ELAT. The deficiency of this enzyme is a marker for PNH I cells (complement sensitive) and in consequence this particular method could be important when monitoring the prognosis of patients with PNH, since the relative proportions of AChE deficient and AChE containing cells could be established.

All cases of acute leukaemia examined presented with low values for AChE, with one case of acute lymphoblastic leukaemia having particularly low levels. Two of the cases were newly diagnosed, while the rest were in relapse. This is in agreement with Scott et al. (1973) who reported low red cell AChE levels in patients in relapse and normal values when in remission. If one considers the common origins of blood cells it is not surprising that a

malignancy in the white cell line would present with an abnormally low level of AChE in the erythroid cell line, but it is surprising that low levels should appear in this type of leukaemia and not in the malignant conditions mentioned earlier. The observation of patients with low red cell AChE in patients in relapse becoming normal in remission is of important diagnostic value (Scott et al., 1973). It may well be that the drugs used in relapse, being more powerful than those used in remission, may be directly affecting AChE activity on erythrocytes. This would not appear to be the case, however, since immunological levels of the enzyme were also found to be low in relapse indicating a probable decrease in the numbers of AChE molecules in the erythrocyte membrane. Toxic inhibition would be reflected by decreased enzyme activity, but normal immunological quantitation. The possibility exists that these cytotoxic drugs may be influencing the implanting of AChE in the erythrocyte membrane at the erythropoietic level in the bone marrow. A larger investigation with closer correlation to the patient's clinical condition, such as remission or relapse and emphasis on drug therapy or other treatment would be required to substantiate the use of erythrocyte AChE as a marker for acute leukaemia. The measurement of AChE activity and antigen levels on the white cell surface would also be of value in these conditions. One case of CLL also gave low values of enzymic and antigenic activities, but it is not known whether this patient was in relapse or whether additional complications were involved.

An interesting case of malaria gave very low values of AChE enzymic and antigenic activity despite the patient presenting

with microcytic erythrocytes. The malarial parasite once inside the erythrocyte completely takes over, becoming fully competent for replication, protein synthesis and aerobic energy metabolism. The erythrocyte, however, has lost its nucleus, ribosomes and mitochondria and with them most of its energy supply, except for glycolysis. Maintenance of the integrity of the cell membrane therefore becomes increasingly difficult. However, since the malarial parasite is only detected in about 1% of the red cell population it is rather surprising that this alone would produce the AChE level seen in this patient and it is probable that it is influenced by other factors. Additional patients are required to clarify these findings.

Low AChE levels were observed in two cases of auto-immune haemolytic anaemia (AIHA) where the responsible antibody was IgG. Similar findings were reported by Scott and Rasbridge (1971) who concluded that IgG type antibodies are more likely to cause reduced AChE levels. Sirchia et al (1970) reported that AChE activity was significantly subnormal in the IgG and IgG + complement types of AIHA, while it was not significantly lower than normal where complement alone coated the red cells.

Two cases of ABO haemolytic disease of the newborn (HDN) were found to have low levels of AChE. Similar cases were observed (Ferrone et al., 1968; Herz et al., 1968) in which low levels were reported for ABO HDN, but normal levels for Rhesus haemolytic disease. These results are confusing since both antibodies must be of the IgG type to cross the placental barrier to sensitize the foetal erythrocytes. Erythrocyte AChE is associated with the protein on which the AB(H) antigens are

located (band 3) and the binding of antibodies to these sites may disrupt this association. There are approximately 300,000 A or B sites per erythrocyte in the newborn and antibodies reacting with these structures may have a profound effect on the integrity of the membrane or simply mask the AChE catalytic and immunological binding sites. In contrast there are only 10 - 20,000 Rh(D) sites per erythrocyte and combination of antibodies with these structures probably would not have such an effect.

A raised AChE activity, but with a normal antigen level, was observed in ^{5/11} ~~two~~ cases of reticulocytosis. This seems to indicate a change in the orientation of the molecule in the cell membrane on reticulocytes allowing greater contact of the catalytic site with the substrate, whilst maintaining normal orientation of the epitope towards AE-2.

A case of hereditary spherocytosis (HS) similarly presented with increased AChE activity and normal antigen levels. This appears to be in agreement with Streichman et al (1983) who proposed that the AChE molecules appear more fully exposed at the cell surface in HS cells, possibly due to diminished hydrophobic interactions with neighbouring molecules in the membrane. This suggests a modified orientation of AChE in the erythrocyte membrane allowing greater enzymic activity, whilst the total number of molecules, as indicated by the ELAT, remains within the normal range.

Elevated AChE levels were found in cases of chronic myelomonocytic leukaemia (CMML) and breast cancer, where it is possible that some drug therapy or iatrogenic involvement may affect the erythropoietic system in some way.

The exact function of AChE in the erythrocyte membrane is unknown, but apart from a report of a single family with hereditary deficiency of erythrocyte AChE (Johns, 1962) and also a family with altered erythrocyte AChE (Coates and Simpson, 1972) the enzyme appears to be present on all erythrocyte samples tested. Additional analysis of disorders where AChE levels are altered may provide further insight into the function that this enzyme has in the erythrocyte membrane. The variation observed in certain disorders may be of diagnostic value in a number of conditions such as leukaemia.

Recent reports (Daniels 1989) have linked the erythrocyte membrane protein DAF to the Cromer blood group system. In an effort to link AChE to a blood group system Herz et al (1972^b) investigated AChE in En(a-) cells, but found normal activity. In addition, the blood groups Holley and Gregory have been discounted (B.G.R.L. unpublished data), although links with the Cartwright system cannot be excluded. It is hoped that a link between AChE and a blood group system, possibly through the identification of an AChE null phenotype, will be established in the near future. Monoclonal antisera and techniques similar to the ELAT will aid in this goal.

Plasma Cholinesterase

The examination of cholinesterase activity using a combination of substrates and inhibitors (Whittaker, 1968^a, 1968^b; Whittaker et al., 1981, 1982; Whittaker and Britten, 1980) provide a particularly sound basis for characterization of the various genotypic variants of cholinesterase.

An assay of plasma ChE activity using butyrylthiocholine (BTI) as substrate has been developed using a microplate Ellman technique. Butyrylthiocholine has an affinity for human plasma ChE twice that of acetylthiocholine, is more stable with respect to pH and temperature (Szasz, 1968.) and is therefore the substrate of choice for human ChE using the Ellman method of assay.

Although maximum activity of the enzyme is observed at pH 8.5 - 9.0 (Silk et al., 1979) there is considerable non-enzymatic hydrolysis at this pH and in consequence phosphate buffer pH 7.2 is used for the microplate assay. This reduces the non-enzymatic hydrolysis to negligible proportions and still allows the linear relationship of the assay procedures to be obtained (figs.16-17).

In general, haemolyzed samples and heavily jaundiced samples were avoided since they were found to interfere with absorbances obtained. If plasma ChE activity was required on jaundiced samples, these were further diluted and results adjusted accordingly. A plasma blank was also used in these cases. All assay procedures were performed at 22°C. Das and Liddell (1970) have shown that temperature correction can be made by adding or subtracting 9% for each 1°C variation of the reaction mixture

from 25°C.

The plasma ChE standard consisted of a pool of 5 $E_1^u E_1^u$ blood bank donor plasmas previously stored in plastic packs containing anticoagulant/preservative (citrate, 6g/l; phosphate, 0.5g/l; dextrose, 5.8 g/l) and frozen at -30°C for approximately 8 months before use. The pool was divided into small aliquots and kept frozen until required. Each aliquot was used during a single day and then discarded. This pool provided the large volume of standard required for use throughout the study. Any continuity of standard would not have been possible using plasma from commercial sources. Supply of standard in such volumes would also have been prohibative on a cost basis.

The ChE activity and antigen levels for the standard were assigned 100 arbitrary units (AU). Values for the samples tested are presented relative to this standard. The means for both ChE activity (138AU) and antigen (136AU and 144AU as defined by MAb2-1 and 2-4 respectively) for 170 $E_1^u E_1^u$ individuals are considerably higher than activity (100AU) and antigen levels (100AU) for the standard (table 19). This is surprising as the standard is a pool of $E_1^u E_1^u$ samples. Additional pooled standards were examined and gave similar levels. The low values may, in part, be due to the presence of citrate, which has been shown to be a partial inhibitor of ChE (Whittaker, unpublished work). Alternatively, Ca^{2+} and Mg^{2+} have been reported to activate ChE (Kobayashi et al., 1988) and the chelation of citrate with these ions would prevent this activation. The effect of these ions on the antigen levels is not known. Possibly a conformational change altering the epitope to which the antibody combines may be

involved. Comparison of plasma ChE activity using BzCh as substrate with the microplate Ellman method using BTI for 170 $E_1^uE_1^u$ samples indicate a good linear relationship between the two techniques (correlation coefficient of 0.889 - fig.41). The coefficients of variation for the $E_1^uE_1^u$ population tested for both substrates were similar (16% for BzCh and 17% for BTI; table 13 - 14). The microplate Ellman method using BTI as substrate is therefore a good alternative to BzCh when measuring plasma ChE activity. The use of microplates provides a convenient method of measuring large numbers of samples in a relatively short period of time. The coefficient of variation (CV) for this method (ME) was found to be 5%, which is comparable to that previously reported for the method using a cuvette (Whittaker, 1984).

Quantitation of ChE has traditionally involved immunodiffusion and immunoelectrophoresis using precipitation, usually with polyclonal rabbit antisera. These techniques have been particularly useful in elucidating the heterogeneity of the silent gene phenotypes (Goedde et al., 1965; Rubinstein et al., 1970). Optimum results were obtained with rocket immunoelectrophoresis, both by the normal and the 'piggy back' technique, with the Plymouth rabbit antisera. The method however is time consuming and therefore unsuitable for large numbers of samples. It involves subjective analysis with inherent inaccuracies, is unsuitable for measuring low levels of protein (below 10% of usual), and does not lend itself to direct computer manipulation. A comparison was made with the enzyme linked immunosorbent techniques under development. Unfortunately, the enzyme immunoassay, which utilizes the enzyme activity of the

plasma ChE itself, using DAKO polyclonal antisera was discounted as unsuitable due to the very high CV%. The enzyme linked immunosorbant assay (ELISA) using conjugated and unconjugated DAKO antisera also proved a disappointment, due to high background absorbances and CV (15%). Absorbance differences between buffer (blank) and $E_1^u E_1^u$ sera using these antibodies were small even using enhancers such as PEG. It would seem that the DAKO antibody is more suited to immunoelectrophoretic techniques than ELISA.

Both Plymouth polyclonal antisera (188 and 288) were also unacceptable for ELISA, but produced very good results by immunoelectrophoresis and were especially useful in the 'piggy back' technique to evaluate 'silent' individuals for plasma ChE.

The B'ham sheep polyclonal antisera (used both in primary and secondary conjugated stages) gave unusual results in the ELISA which did not correlate with those obtained from the other techniques. Possible cross-reaction with human IgG was discounted since the sheep anti-rabbit IgG had been absorbed out in one of the stages of preparation of the reagent by the Binding Site.

A comparison of the DAKO (fig.21), SSI (fig.24), and B'ham anti-ChE (fig.29) polyclonal antisera as secondary detector antibodies with the monoclonal antibody MAb2-4 as primary coating antibody indicated optimum performance was obtained using the B'ham anti-ChE, HRP conjugated as the secondary antibody.

The ELISA technique using the monoclonal and B'ham antibodies involves only 3 stages and with the use of pre-coated microplates results were easily obtained within 5 hours. In consequence, although plates may be coated by incubating the monoclonal

antibody in the wells at 37°C for 1 hour, coating overnight at 4°C in a humid chamber proved more convenient. Although the 4 monoclonal antibodies appeared to coat the microwell surface to the same degree (fig.26) when these antibodies were evaluated using standard plasma from an $E_1^uE_1^u$ donor pool with HRP-conjugated B'ham anti-ChE as secondary antibody the performance of the antisera decreased in the order MAb2-4, 2-1, 2-3, 2-2 (fig.27). The concentrations of each antisera as determined by the supplier (State Serum Institute) do not explain this difference (MAb2-1, 2.3mg/ml; MAb2-2, 1.45mg/ml; MAb2-3, 4.36mg/ml; MAb2-4, 2.46mg/ml). It can only be concluded that the relative affinity of the antibodies for plasma ChE also decreases in this order. MAb2-2 also gave high background absorbance. For this reason MAb2-1 and 2-4 were used routinely for the quantitation of plasma ChE and MAb2-3 was used as a backup antibody when required. MAb2-2 with its relative poor performance was not used. ELISA using these antibodies produced good standard curves (fig.29) with a CV of 5%.

Analysis of results obtained for the quantitation of plasma ChE of 50 $E_1^uE_1^u$ samples by rocket immunoelectrophoresis (R.I.) and by ELISA (MAb2-1) showed the mean values for each range to be in close agreement (table 15). The coefficients of variation for the population, however, was greater for R.I. (25%) than for ELISA (19%) (table 15) probably indicating the former to be a less precise technique. A linear relationship exists between the two methods (fig. 42) with a correlation coefficient of 0.740 (table 16). This fairly low correlation reflects the low precision of the immunoelectrophoretic technique. This is supported by the

high coefficient of variation for this technique (CV=12%).

The mean values of the ranges for plasma ChE levels in 170 $E_1^uE_1^u$ samples measured by ELISA was slightly higher using MAb2-4 than 2-1 (table 17) possibly indicating easier access of the MAb2-1 epitope in the $E_1^uE_1^u$ pooled standard. The coefficient of variation for the population was the same for both antibodies (18% - table 17). Results obtained with MAb2-1 and 2-4 are linearly related (fig.43) with a correlation coefficient of 0.815. This correlation is surprisingly lower than expected and one which is difficult to explain because with many of the samples a correlation coefficient of 1 was observed. In spite of repeat assay some samples gave divergent values. Further work may resolve this problem.

Characterization of plasma ChE was thus obtained by measuring activity using benzoylcholine and butyrylthiocholine, inhibition characteristics using DN, FN and RO2N as well as immunological quantitation by rocket immunoelectrophoresis and ELISA using the monoclonal antibodies 2-1 and 2-4 in conjunction with the B'ham anti-ChE-HRP conjugate.

Analysis of Genetic Variants for Plasma Cholinesterase

Normal Gaussian distribution curves were obtained for both activity and antigen level determinations with $E_1^u E_1^u$ (fig.44), $E_1^u E_1^a$ (fig.45) and $E_1^a E_1^a$ (fig.46) genotypes. The ^{ratios} ranges for cholinesterase activity and antigen levels are significantly different for each genotype ($p < 0.001$ in all cases - table 21).

Results presented below are in the order activity with BTI, and immunological levels of ChE as defined by MAb2-1 and 2-4 respectively. All are given in arbitrary units relative to the pool of $E_1^u E_1^u$ donor plasmas.

Assuming each gene contributes equally to the activity and antigenicity in the homozygotes, one can calculate the gene contributions to these values for the E_1^u and E_1^a genes. Using the values so obtained a theoretical value expected for the heterozygote, $E_1^u E_1^a$ can be calculated.

Mean values (table 19) obtained with $E_1^u E_1^u$ individuals (138AU, 136AU and 144AU) indicate the presence of the E_1^u gene results in the production of approximately 69AU of cholinesterase activity and 68AU and 72AU of immunological protein as defined by MAb2-1 and MAb2-4 respectively (ie. each gene contributes half the value of the genotype). The values for the E_1^u gene should tally with those for the $E_1^u E_1^a$ genotype if the E_1^a gene is truly silent. However, results for $E_1^u E_1^a$ individuals (81AU, 84AU and 79AU) were slightly higher than expected, possibly due to the small number of samples tested or to the heterogeneity of the 'silent' gene.

Activity and immunological data (table 19) from $E_1^a E_1^a$ samples (57AU, 90AU and 90AU) indicate the presence of the E_1^a gene cause

values to be decreased on average to 29AU, 45AU and 45AU. This represents a decrease of 58%, 34% and 37% respectively when compared to the E_1^u values. Confirmation of these values for the E_1^a gene were obtained from $E_1^aE_1^a$ samples. Assuming the E_1^a gene is truly silent, contributing neither to activity nor to immunological protein, the E_1^a gene gave values of 33AU, 48AU and 45AU. These figures are in close agreement with the results obtained from the atypical homozygotes.

Values for the E_1^u gene (69AU, 68AU and 72AU) and for the E_1^a gene (29AU, 45AU and 45AU) would give expected theoretical values for the $E_1^uE_1^a$ genotype of 98AU, 113AU and 117AU. These are in close agreement with the actual values obtained with this genotype (104AU, 118AU and 122AU).

Similar activity and immunological data obtained from $E_1^aE_1^k$ individuals (74AU, 88AU and 89AU) and the calculated values for the E_1^a gene (29AU, 45AU and 45AU) indicate the E_1^k gene produces 45AU of activity and immunological protein of 43AU and 44AU as defined by MAb2-1 and 2-4 respectively.

These values are obtained from the mean of ranges for these genotypic variants and any individual may vary from values presented here. The mean contribution each gene makes towards the activity and antigen levels are shown (table 27). The effective efficiency (EE) of the activity of the cholinesterase produced by each genotypic variant was calculated using the immunological data in the equation:

$$EE = \frac{\text{Enzyme Activity (BTI as substrate)}}{\text{Immunological level (mean of MAb2-1 and 2-4)}} * 100\%$$

Table 27 Contribution of the E_1^u , E_1^a and E_1^k genes to enzyme activity and antigen levels.

Gene	Contribution of each gene			Effective Efficiency of gene product using BTI as substrate (%age)
	BTI ¹	2-1 ¹	MAb 2-4 ¹	
E ₁ ^u	69	68	72	99
E ₁ ^a	29	45	45	64
E ₁ ^k	45	43	44	103

¹ AU relative to standard donor plasma

Atypical plasma cholinesterase differs qualitatively from usual cholinesterase due to a point mutation at position 70 on the amino acid chain (McGuire et al., 1989). This could explain the lower enzyme activity that this variant exhibits. However, a quantitative decrease, although much smaller than that observed with activity, also occurs. Consideration of activity values indicates that the E_1^a gene product is only $(29/69 * 100\%)$ 42% as active as the E_1^u gene product when butyrylthiocholine is used as the substrate (table 27). However, this assumes that the same number of molecules occur in each variant. Immunological data indicates that for the E_1^a gene $(45/70 * 100\%)$ 64% of the number of cholinesterase molecules are present compared to the E_1^u individual (table 27). This indicates that the relative efficiency of the atypical cholinesterase molecule is

(42%/64% * 100%) 66% (and not 42%) compared with the usual form of the enzyme. A combination not only of a qualitative, but also a quantitative difference between atypical and usual forms of the enzyme is therefore indicated.

This hypothesis is contrary to the findings of Rubinstein et al (1978) who compared the concentrations of immunologically reactive cholinesterase in a few atypical variants and concluded that the E_1^a allele results in the production of about the same number of immunopotent cholinesterase molecules as the E_1^u allele. The data presented here supports the conclusions of Eckerson et al (1983) who indicated the average concentration of enzyme protein as defined by a rabbit antiserum and enzymic activity at V_{max} , using either benzoylcholine or o-nitrophenylbutyrate as substrate, was about 30% lower in the atypical compared with the usual enzyme. Eckerson et al (1983) therefore suggested that the atypical enzyme not only has altered catalytic properties (K_m), but also may be synthesized more slowly, or alternatively cleared more rapidly in vivo, than the usual enzyme. Our results clearly show that the dynamic ratio of synthesis and degradation of the two variant enzymes differ as well as their catalytic activity.

Analysis of data from $E_1^aE_1^k$ samples shows that the cholinesterase enzyme produced by the E_1^k gene has an efficiency of 103% compared to 99% for E_1^u . However, activity values obtained for this genotype were not consistent with accepted levels reported in the literature (Whittaker, 1986), but it must be emphasised that only a small number of samples were tested. Nevertheless, it appears that the resultant cholinesterase

activity observed in E_1^k individuals is due to a quantitative rather than a qualitative abnormality in agreement with Rubinstein et al (1978) who presented enzymic and immunological data indicating the E_1^k gene causes reduction of usual E_1^u molecules by 33%.

Analysis of the Heterogeneity of the Silent Gene

It has been recognised for many years that the so-called 'silent gene' represents a heterogeneous complex (Goedde and Altland, 1968; Rubinstein et al., 1970). At present, 3 separate genes, E_1^s , E_1^t and E_1^r , have been described (Scott, 1973; Scott and Wright, 1976). The relative affinities for different substrates (Altland and Goedde, 1970), electrophoresis (Rubinstein et al., 1970) as well as immunodiffusion (Goedde and Altland, 1968) have been used to differentiate the variants. Results obtained by ELISA and rocket immunoelectrophoresis are in close agreement (table 22). Moreover, it can be seen from this table that the majority (40) of the 43 samples can be separated into four groups leaving a heterogenous pool.

The first section of patients (1-9) shows that an immunologically reactive cholinesterase cannot be detected with any of the monoclonal or polyclonal antisera by either technique. This group probably represents the $E_1^sE_1^s$ homozygote. In all 9 cases no detectable protein following electrophoresis, nor significant binding to monoclonal antibodies, nor activity with BTI as substrate were observed. Such observations could indicate the absence of protein synthesis by the E_1^s gene for plasma

cholinesterase. However, 0-3% of the normal enzyme activity was found in this group using benzoylcholine as substrate. It therefore seems more likely that a protein is synthesized in which the antigenic sites for the monoclonal antibodies are absent, disorientated or permanently blocked by an inhibitor. In addition, the catalytic site is disorientated or partially blocked. Goedde et al. (1965) suggested a qualitative difference between the silent gene enzyme protein and the normal cholinesterase protein as the likely explanation of the absence of enzyme activity and antigen binding by immuno-electrophoresis or double immunodiffusion tests in two silent homozygotes. A structural alteration of the enzyme protein was assumed to be more likely than a quantitative difference. Results presented in table 22 are not in variance with this hypothesis.

The second group of patients (10-12), although showing diminished immunological reaction relative to the $E_1^u E_1^u$ genotype show increased immunological reaction relative to groups 1 and 3 by the ELISA technique as well as by rocket immunoelectrophoresis. The catalytic activity is also real, although not appreciable. It can be assumed that the structural modification or disorientation of the cholinesterase molecules in these individuals restricts the binding of the monoclonals to their antigenic sites. These findings of weaker than normal affinity of the antibodies support the observations of Rubinstein et al. (1970) and suggest that this group may be type II, which is controlled by the E_1^t gene, and probably represent the homozygotes $E_1^t E_1^t$.

The third group of patients (13-29) show definite, but low

levels of immunoreactive protein - in fact, intermediate between our first and second groups of patients. The enzymic activity, by either substrate, in this third group is less than that found in our second group. Such findings are suggestive of a heterozygote, indicating that this third group could possibly be the heterozygote $E_1^s E_1^t$. This hypothesis requires confirmation by detailed family studies some of which are reported in the next section.

The fourth group (30-40) shows appreciable binding of antibody, both by ELISA and by rocket immunoelectrophoresis, indicating that cholinesterase protein is present in all 11 samples. These values are much greater than those previously reported. There is, however, no enzymic activity using BTI and little activity with benzoylcholine (0-0.04%). This group may, therefore, represent a new gene, E_1^x . Evidence indicates that the antigenic sites at which the monoclonal antibodies react are not only intact, but may be more accessible for binding or alternatively more protein molecules may be synthesized. The low activity indicates that the catalytic site is disorientated or inhibited.

Individual 41, who differs from all the others, may represent the third type of serum cholinesterase deficiency found in Eskimoes (Scott and Wright, 1976). This variant is reported to have about 10% of the normal cholinesterase activity and the gene has been designated E_1^f . No immunoreactive protein has been demonstrated by any of the monoclonals used in the ELISA.

Individual 42 has appreciable immunoreactive protein but, unlike individuals in group 4, this patient has definite catalytic activity with both substrates. This individual may be

an $E_1^r E_1^x$ heterozygote, but this is a very tentative suggestion.

Individual 43 is quite unique amongst the present group under investigation. This lady died shortly after the present sample was taken and it is possible that pathological involvement may be the explanation of the low activity found with benzoylcholine. The immunological studies certainly indicate the presence of immunoreactive protein.

Immunological Studies of Families segregating the Silent Gene

Seven family groups comprising apparently 'silent' homozygotes for plasma ChE and their relatives were screened by ELISA. All individuals in these families have the most common phenotype if one considers only the dibucaine and fluoride numbers. There is only one individual in these family studies who can be classified as a truly silent homozygote having the genotype $E_1^s E_1^s$. All biochemical parameters for individual II-1 in family 1 are zero (table 24) and in consequence it is mandatory that both parents must be segregating the E_1^s gene. Activity and immunological levels indicate that both these parents and the daughter II-3 have the genotype $E_1^u E_1^s$. The remaining daughter II-2 presents with normal activity and immunological levels together with normal inhibition characteristics and hence must be $E_1^u E_1^u$.

Three families 2, 3 and 4 appear to be segregating both the E_1^s and E_1^t genes in the propositus. One of them II-1 (family 2) has been phenotyped as $E_1^s E_1^t$ (table 24) and one of his siblings II-4 has the same phenotype. In this family the father I-1 is segregating a usual gene E_1^u , but it is difficult to assign the E_1^s gene to the father I-1 or to the mother I-2 for preference,

but it is probably the mother I-2 who has the genotype $E_1^k E_1^t$. Individuals II-2 and II-3 have similar biochemical parameters to their mother I-2 and therefore are of the genotype $E_1^k E_1^s$. The apparent diversity of results given in table 15 for individual II-3 in family 2 has interesting implications. The first sample was taken before marriage and the second after marriage when taking oral contraceptive. It has been observed that not only is the cholinesterase activity decreased by oral contraceptives, but that the isoenzyme pattern following electrophoresis is also changed (Whittaker et al., 1971). Both quickly return to normal on cessation of oral contraceptive. This is however, the first indication that the extent of immunobinding protein is also decreased by the use of oral contraceptives. It is therefore desirable that the possible influence of oral contraceptives on any low values of immunobinding protein in healthy young women should be eliminated prior to the assignment of the segregation of a rare gene. The two samples for the sister II-2 were also taken pre- and post- marriage, but this lady does not take oral contraceptives and the biochemical parameters in her case are very similar for both samples. In family 3, although the propositus II-2 has no detectable immunological protein using MAb2-1 and 2-4, he does have slight activity with both BTI and Bzch and therefore has the probable genotype $E_1^s E_1^t$. This is substantiated using data from his brother II-3 who exhibits the same amount of activity, while demonstrating detectable immunological protein. II-3 therefore, also has the genotype $E_1^s E_1^t$. In this family the E_1^k gene appears to be segregating with the father I-1 and is also present in the two sisters II-1 and

II-4 and the brother II-5. It is difficult to establish whether the E_1^s gene is segregating with the father or with the mother, but since both father and sister II-5 have the E_1^k gene and activity for the father is lower than II-5 it is assumed that the father is segregating the E_1^s gene and II-5 the E_1^t gene. The father therefore has the genotype $E_1^kE_1^s$ and II-5 the genotype $E_1^kE_1^t$. The mother of the propositus must therefore have the genotype $E_1^uE_1^t$. II-1 and II-4 having the same values for activity and immunological protein with Mab2-1 and 2-4 have the genotype $E_1^uE_1^k$. Family 4 is again unusual with the probable segregation of the E_1^k gene as $E_1^kE_1^s$ in two of the children III-2 and III-3 of the propositus II-1. It seems probable that the other children of II-1 have genotypes $E_1^uE_1^s$ (III-1) and $E_1^uE_1^t$ (III-4) respectively. There are problems in assigning suitable genotypes to the children IV-1, IV-2 and IV-3 of the sibling III-4. All the children have the same genotype and all must have inherited the E_1^t gene from their mother, but their low activities and low levels of immunological ChE protein is contrary to the inheritance of the E_1^u gene. The three children must have inherited a rare gene from their father. It is not the E_1^a gene, since their phenotype is usual and it seems likely that the E_1^k or even E_1^j may be segregating in the fourth generation of this family. Both genes are associated with diminished enzyme activity - 33% and 66% reduction of the usual E_1^u activity for E_1^k and E_1^j respectively (Rubinstein et al., 1976; 1978). It is remarkable that the E_1^k gene appears to be segregating in the three families each having a propositus with the genotype $E_1^sE_1^t$. Although this is a striking result no significance can be attached to this

segregation within these families until more data is available.

Two of the propoiti in families 5, 6 and 7 appear to be segregating the new 'silent' gene E_1^x . The propositus II-1 and her brother II-2 in family 5 each show high immunological ChE protein with MAb2-1 and MAb2-4 and from this it may be deduced that the E_1^x gene is segregating in this family. However, the ELISA results for their mother I-1 with either MAb2-1 or MAb2-4 are low and so it is extremely improbable that she is segregating the E_1^x gene. Thus, from the biochemical data it may be concluded that this lady has the genotype $E_1^u E_1^s$. In this case, her children cannot be homozygote for the E_1^x gene, but must be heterozygote $E_1^x E_1^s$. In family 6 the propositus II-2 and his brother II-1 must have the same genotype, since their biochemical data are very similar and both are segregating the E_1^x gene. The father I-1 has a high level of immunological ChE protein whereas the mother I-2 is definitely low. It can only be assumed that the father I-1 is segregating the E_1^x gene and his enzymic activities with the two substrates are indicative of an $E_1^u E_1^x$ genotype. The genotype of the mother is suggestive of $E_1^k E_1^s$. This means that their children II-1 and II-2 are heterozygotes with genotype $E_1^s E_1^x$. Although there is considerable variation in the ELISA results obtained for the siblings I-1 and I-2 or the propositus I-3 in family 7, it is nevertheless apparent that the E_1^x gene is segregating throughout the generation. One may query whether the diverse results of I-2 and I-1 represent a heterozygote and the homozygote of E_1^x respectively. The children of I-1 should resolve this problem, since if I-1 is homozygous then all his children II-1, II-2 and II-3 must segregate the E_1^x gene. But the

ELISA values for II-1 and II-2 are low and so we must assume that the E_1^x gene is not segregating in these children. In contrast, the high ELISA values for both monoclonal antibodies for II-3 indicates that the E_1^x gene is segregating. I-1 must therefore be a heterozygote with a probable genotype $E_1^s E_1^x$ with his children II-1, II-2 and II-3 having the genotype $E_1^k E_1^s$, $E_1^u E_1^s$ and $E_1^k E_1^x$ respectively, since the low enzymic activities of II-1 and II-3 probably indicate that the E_1^k gene is segregating within this family. The E_1^x gene is also segregating in individual II-4 and her genotype is probably $E_1^u E_1^x$.

Immunological Studies of families segregating the E_1^h gene for Plasma Cholinesterase

Unusual inhibition characteristics in two unrelated suxamethonium sensitive individuals indicative of a new allele, E_1^h , segregating with the E_1^a gene was reported by Whittaker and Britten (1987). Both families were evaluated for activity with butyrylthiocholine as substrate and immunological quantitation as defined by MAb's 2-1 and 2-4.

In the initial study 3 of the siblings (II-2, II-9 and II-10) in family 1 had identical inhibition characteristics to the propositus I-1. Activity and immunological data confirmed these findings, with similar results in all 4 cases (table 25). Immunological results fell within the ranges characteristic for $E_1^a E_1^s$. Siblings II-1, II-3, II-5, II-11 and III-1 have been genotyped as $E_1^u E_1^h$. Activity and immunological data for these individuals fall within the ranges for both $E_1^u E_1^s$ and $E_1^u E_1^t$, except II-11 who presents with slightly higher values, which fall

just outside the range for $E_1^u E_1^s$ (table 19).

Samples from the propositus in family 2 were not available for testing, but activity (Whittaker and Britten, 1987) is very similar to that of the sibling II-2. Activity and immunological levels for this sibling were slightly higher than expected, falling outside the higher end of the $E_1^a E_1^s$ range. In addition, individual III-1 also exhibits high activity and immunological levels for an $E_1^u E_1^h$. Since the parent I-2 has a level of cholinesterase within the range for an $E_1^u E_1^h$ the E_1^h gene cannot be the cause of this high activity observed in sibling III-1. The high activity must therefore be due to the E_1^u gene. This individual may therefore be C_5^+ .

The parents I-1 in family 1 and I-1 in family 2 fall within the range for $E_1^u E_1^a$, while I-2 in family 2 has typical values for $E_1^u E_1^h$. However, values for I-2 in family 1 are far higher than would be expected for the genotype $E_1^k E_1^h$ and if it were not for the inhibition characteristics for I-1 and the results obtained with the rest of the family, I-2 could mistakenly have been typed $E_1^u E_1^h$.

Analysis of Cumulative Activity and Immunological Data for the 'Silent' Variants

Using cumulative data for activity and immunological levels obtained for the 'silent' (table 23) and E_1^h (table 26) genes, mean values for the contribution of each of these genes may be calculated (table 28). The mean values for $E_1^s E_1^s$ (0AU, 0AU and 0AU) indicate the E_1^s gene contributes neither to activity nor to immunological protein. Data (table 23) obtained with the

homozygote $E_1^t E_1^t$ (8AU, 15AU and 21AU) gives values for the E_1^t gene of 4AU, 8AU and 11AU. Values for the genotype $E_1^s E_1^x$ (0AU, 153AU and 165AU) are due to the E_1^x gene and for the genotype $E_1^r E_1^s$ (12AU, 0AU and 0AU) are due to the E_1^r gene since the E_1^s gene is truly silent for both activity and immunological protein. Values for activity and immunological protein for the genotype $E_1^a E_1^h$ (39AU, 54AU, 53AU) and the estimated values for the E_1^a gene (29AU, 45AU and 45AU) give values for the E_1^h gene of 10AU, 9AU and 8AU. If the same calculation is performed for the $E_1^u E_1^h$ genotype then values for the E_1^h gene are much higher (28AU, 25AU and 16AU). The standard deviation for the ChE activity using BTI as substrate (table 19) for $E_1^a E_1^a$ individuals (13) is much smaller than for $E_1^u E_1^u$ individuals (23). In spite of the standard deviations for immunological quantitation of ChE protein being the same for both genotypes (table 19) due to the small numbers of samples tested it is assumed that the values for the $E_1^a E_1^h$ samples are more reliable in the assignment of values to the E_1^h gene.

Table 28 Contribution of the 'silent', E_1^h and E_1^a genes to enzymic activity and antigen levels of plasma cholinesterase.

Gene	Contribution of each gene		
	BTI ¹	2-1 ¹	MAb 2-4 ¹
E_1^u	69	68	72
E_1^r	12	0	0
E_1^s	0	0	0
E_1^x	0	153	165
E_1^t	4	8	11
E_1^h	10	9	8
¹ AU relative to standard donor plasma			

Comparison of Plasma Cholinesterase Activity with Immunological Protein Levels in Genotypic Variants

Results presented below are in the order ChE activity with BTI, and immunological levels of ChE as defined by MAb2-1 and 2-4 in arbitrary units.

Cholinesterase activity and immunological protein levels for E_1^s , E_1^t and E_1^h genes combined with E_1^a and E_1^k genes (table 29) were evaluated and compared with results obtained earlier for these genes (table 27). Values for the $E_1^a E_1^s$ genotype (33AU, 49AU and 46AU) are due to the E_1^a gene, since the E_1^s gene does not contribute to any of these and are in close agreement with values calculated from the homozygote (29AU, 45AU, 45AU).

Values for $E_1^k E_1^s$ individuals (46AU, 51AU and 52AU) and the E_1^s gene (0AU, 0AU, 0AU) and $E_1^k E_1^t$ individuals (46AU, 51AU and 53AU) and the E_1^t gene (4AU, 8AU and 11AU) indicate average values for the E_1^k gene of approximately 44AU, 47AU and 47AU. These values are in close agreement with those presented earlier for this gene (45AU, 43AU, 44AU) (table 27).

Table 29 Cholinesterase levels of the 'silent' heterozygotes compared to the usual and atypical homozygotes

Genotype	Number	BTI ¹	MAb	
			2-1 ¹	2-4 ¹
E ₁ ^u E ₁ ^u	170	138	136	144
E ₁ ^u E ₁ ^s fam	7	81	84	79
E ₁ ^u E ₁ ^s	9	86	88	85
E ₁ ^u E ₁ ^t	3	93	93	91
E ₁ ^u E ₁ ^h	7	97	95	90
E ₁ ^u E ₁ ^x	2	69	174	151
E ₁ ^a E ₁ ^a	56	57	90	90
E ₁ ^a E ₁ ^s	11	33	49	46
E ₁ ^a E ₁ ^h	5	39	54	53
E ₁ ^k E ₁ ^s	7	46	51	52
E ₁ ^k E ₁ ^t	5	46	51	53
E ₁ ^k E ₁ ^h	1	100	106	105
E ₁ ^k E ₁ ^x	1	48	140	141

¹ AU relative to standard donor plasma

Comparison of Ratios of Enzyme Activity to Immunological Protein for Genotypic Variants of Plasma ChE

Comparison of the ratios of cholinesterase activity to immunological protein (table 30) indicate that genotypes with ratios around 1.0 all have the E_1^u gene present, agreeing with Altland et al (1971) who reported a high correlation coefficient of enzyme activity with the corresponding amount of antigen.

Evaluation of activity and immunological data of the genotypes presenting with ratios lower than 1.0 reveal that ratios decrease in the order $E_1^k > E_1^a > E_1^x$ when these genes are present (table 30).

The E_1^k gene only results in a slight decrease in the ratio. The E_1^a gene decreases the ratio of enzyme activity to immunological protein in combination with other genes in the order: $E_1^u > E_1^k > E_1^h > E_1^s > E_1^a$. The higher amounts of immunological protein detected in the case of the E_1^a gene may indicate a mechanism whereby more ChE molecules are produced to compensate for the production of a qualitatively abnormal molecule. This hypothesis is supported by lower ratios than expected with the genotypes $E_1^a E_1^h$ and $E_1^a E_1^s$ where only 1 E_1^a gene is present. The E_1^s gene does not contribute and the E_1^h gene only contributes a negligible amount to both activity and immunological protein. Only the atypical gene contributes to the ChE production and an increased number of cholinesterase molecules are synthesized as a compensatory mechanism.

Activity values for $E_1^u E_1^x$ are equivalent to those obtained with $E_1^u E_1^s$ and those for $E_1^k E_1^x$ are equivalent to those for $E_1^k E_1^s$ and $E_1^k E_1^t$ (table 29). The E_1^x gene would therefore appear to produce

greatly increased amounts of immunologically detectable, but enzymically dysfunctionate protein even in the heterozygote. This enables family studies to be performed on these individuals with ease.

Table 30 Comparison of ratios of activity to immunological protein for genotypic variants of plasma ChE

Genotype	Number	Ratio	
		BTI/2-1	BTI/2-4
E ₁ ^u E ₁ ^k	2	0.94	1.15
E ₁ ^u E ₁ ^h	7	1.02	1.08
E ₁ ^u E ₁ ^s	9	0.97	1.03
E ₁ ^u E ₁ ^f	6	1.14	1.02
E ₁ ^u E ₁ ^s	7	0.99	1.02
E ₁ ^u E ₁ ^t	3	0.99	1.02
E ₁ ^u E ₁ ^u	170	1.02	0.96
E ₁ ^k E ₁ ^h	1	0.94	0.95
E ₁ ^k E ₁ ^s	7	0.87	0.87
E ₁ ^a E ₁ ^f	2	0.91	0.87
E ₁ ^k E ₁ ^t	5	0.87	0.85
E ₁ ^u E ₁ ^a	60	0.88	0.85
E ₁ ^a E ₁ ^k	20	0.88	0.83
E ₁ ^a E ₁ ^h	5	0.72	0.74
E ₁ ^a E ₁ ^s	11	0.69	0.74
E ₁ ^a E ₁ ^a	56	0.63	0.63
E ₁ ^u E ₁ ^x	2	0.40	0.46
E ₁ ^k E ₁ ^x	1	0.34	0.34

DNA Analysis of Genetic Variants

Dynamic progress has now been made in the DNA analysis of the genotypic variants of plasma ChE. Genotyping methods using the polymerase chain reaction amplification method have allowed the identification of the mutations responsible for several of the variants of cholinesterase (La Du 1989). The aspartate 70 to glycine point mutation in atypical variants was the first to be identified (McGuire et al., 1989).

Two different point mutations that give rise to the E_1^f variant have been described (Bartels et al., 1990^a). The first mutation, a transition from Thr 243 ACG to ATG (Met) was identified in an $E_1^a E_1^f$ individual. The mutation was called fluoride-1. The change from the threonine residue to another amino acid in this position would result in the enzyme subunit having 8 carbohydrate chains instead of the usual 9, since Asn-X-Thr or Ser is the sequence necessary for glycosylation at the Asn site. A second mutation was found at Gly 390, changing GCT to GTT (Val). This mutation was called fluoride-2. The entire coding region of an $E_1^j E_1^f$ individual was sequenced and no other mutations were found. The 2 types of fluoride variants may explain the 2 sets of immunological data obtained when analysing the E_1^f variant (table 24). Further activity and immunological analysis of a larger number of fluoride-1 and fluoride-2 variants are required to clarify these results.

The E_1^k variant has been shown to be associated with an exon 4 point mutation changing codon 539 from GCA to ACA resulting in Ala being replaced by Thr at this position (Bartels et al., 1990^b). This mutation has also been shown to be in linkage

disequilibrium with the exon 2 mutation responsible for the atypical phenotype. 88% of the atypical alleles tested were linked with Thr⁵³⁹ which may explain why it was previously only possible to diagnose the E₁^k variant when it was seen in combination with the heterozygous atypical (E₁^aE₁^k).

A frameshift mutation at position 117 changing GGT (Gly) to GGAG (Gly + 1 base) was identified in 2 unrelated families segregating the E₁^s gene (Nogueira et al., 1990). The extra base changes the reading frame from Gly 117 to a new stop codon created at position 129, upstream of the active site (Ser 198). Thus a protein only 22% of the size of the usual ChE molecule is produced which does not possess the catalytic serine. This type of mutation probably corresponds to the E₁^sE₁^s variant described (table 23) where no activity or immunological protein could be detected. Examination of the other 'silent' variants for ChE (E₁^t, E₁^r and E₁^x) using these DNA analysis techniques will prove invaluable in elucidating their cause, aiding the characterization of each and possibly enabling the identification of even more 'silent' alleles.

A point mutation at position 142 changing GTG (Val) to ATG (Met) was found during family pedigree analysis of the E₁^h variant (Jensen et al., 1990). The reason why this mutation should result in such a drastic reduction in ChE activity remains unknown. Similarity between activity and immunological data for the E₁^h and E₁^t variants (table 28) suggest a great similarity between the two variants. Use of the polymerase chain reaction technique would prove extremely valuable in proving whether these two variants are one and the same.

At least 7 specific sites within the ChE gene which determine either polymorphisms or rare genetic variants have been identified (La Du, 1990). These intra-genetic loci are very close to each other and one ChE gene may carry more than one ChE mutation. Linkage disequilibrium has been noted between the atypical and E_1^k variant, with one DNA strand carrying both mutations.

Plasma Cholinesterase during Pregnancy and the Puerperium

A significant decrease in cholinesterase activity occurs during pregnancy (Robertson, 1966). The reduction is rapid during the first trimester, but no further decrease occurs during the second and third trimester (Evans and Wroe, 1980). Reports have indicated that even lower levels of activity occur during the first 2 to 3 days postpartum (Hazel and Monier, 1971; Robson et al., 1986). This is followed by an increase in activity to approximately normal nonpregnant values by the end of the puerperium (Robson et al., 1986).

The interpretation of these observations are confused despite several hypotheses being advanced. These include haemodilution, altered hepatic function and steroid inhibition of the enzyme, but none are entirely satisfactory.

For the 21 women investigated in this study there is a very good correlation between the enzymic activities as measured kinetically by the rate of hydrolysis of either benzoylcholine or butyrylthiocholine and the amount of cholinesterase present as indicated by the binding of the monoclonal antibodies MAb2-1 and MAb2-4 to the protein molecule at their respective epitopes. A fairly wide variation between patients was found not only for enzymic activities, but also for the binding of the monoclonal antibodies to the enzyme molecule. The former variation could be predicted from the flat Gaussian distribution curve obtained whenever a large healthy population is screened for cholinesterase activity (Whittaker, 1986). Therefore the measurement of enzymic activity during pregnancy and the puerperium appears to be a function of the number of

cholinesterase molecules present. There is no indication of any inactive molecules, since the binding ratios for the antibodies correlate to the enzymic activity. Other monoclonal antibodies binding different epitopes on the cholinesterase molecule may not show such a correlation.

Results with these women show that the maternal cholinesterase activity falls to about 67% of the non-pregnant level by the end of the first trimester and moreover the binding of either of the monoclonals decreases by the same amount (fig.49). These decreases are maintained during the second and third trimester with a transient increase, probably insignificant, in these parameters at about 20 days prior to delivery. Further decreases are observed during the puerperium which on average reach a minimum at about 2 days for the enzyme activity and for the binding of either monoclonal (fig.50). Both activity and monoclonal antibody binding ratio steadily increase as the puerperium proceed until non-pregnant levels are observed in all cases at 6 weeks post partum, but often at 3 weeks and sometimes earlier during the puerperium.

Haemodilution appears to be an attractive hypothesis to interpret not only the decrease in enzymic activity, but also the lower binding capacity of antibody to the cholinesterase molecule during gestation. There is a smooth and progressive rise (approximately 50%) in total plasma volume during gestation, until a plateau is reached at about the thirty sixth week (Lund and Donovan, 1967). This 50% increase in volume is maintained until delivery. Thus, haemodilution may be the major factor for the observed decrease in cholinesterase activity and monoclonal

binding capacity during gestation. However, half of the total post-partal fall in mean plasma volume, either by diuresis or osmotic processes into the tissues, occurs during the first five days after delivery and normal non-pregnant volume is restored at about 6 weeks. It is therefore impossible, on the basis of haemodilution, to interpret the further decrease in cholinesterase activity or antibody binding of MAb2-1 or MAb2-4 to the enzyme which occurs during the first 2 days post partum.

The hypothesis of impaired hepatic function may account for a decreased protein biosynthesis as indicated by the reduced protein binding of the monoclonals and cholinesterase activity in this obstetric population. In normal pregnancy the total serum protein concentration decreases significantly below the normal non-pregnant values (De Alvarez et al., 1961), but if one takes into account the 50% increase in plasma volume during gestation then it would seem that there is no reduction in total plasma protein at term. Some of this protein is pregnancy associated protein and additionally there is a pregnancy zone protein. The former protein disappears early in the puerperium but the latter is detectable for several weeks post partum (Lin et al., 1976). It is unlikely that these observed changes in cholinesterase should be inversely proportional to the changes in pregnancy zone protein and by such means exert a constant osmotic effect. Serum albumin, like cholinesterase, also decreases during early gestation to a constant level. The hypoalbuminaemia, which in a non-pregnant woman stimulates increased albumin synthesis is probably controlled in pregnancy by progesterone or oestrogen. Oestrogen has been shown to decrease albumin levels in non

pregnant women, presumably by inhibiting its biosynthesis (Laurell et al., 1968). These steroids have a similar effect on cholinesterase (Redderson, 1973). But, after delivery of the placenta, oestrogen is rapidly cleared from the plasma and is not usually detectable after the third day. At this time the observed decreases in cholinesterase are maximum and it must be concluded that oestrogen is not inhibiting cholinesterase and thereby blocking both the catalytic active site and epitope. However, during the first few days of the puerperium levels of other plasma proteins such as coagulation factor VII and X, as well as fibrinogen are elevated presumably in preparation for the haemostatic challenge of placental separation. These proteins, like cholinesterase, are synthesized in the liver. These coagulant proteins are also elevated during oral contraceptive therapy (Amundson and Pilgermann, 1963), while cholinesterase is decreased (Whittaker et al., 1971). It would thus seem plausible that an agent, possibly a steroid such as oestrogen, would exert an effect on the biosynthesis of a number of proteins produced by the liver in response to the demands made on the body during delivery. If this hypothesis is true then proteins important to delivery are elevated and non-essential proteins are decreased in production. The existence of an inhibitor in the plasma is discounted by the finding of elevated levels of erythrocyte AChE during pregnancy and the puerperium (fig.39 - 40).

Nature of future work

Although the ELAT is a reliable method for quantitating erythrocyte AChE, it is cumbersome and only a small number of tests can be performed per run. An alternative method allowing high numbers of samples to be tested reliably and with relative ease is required. At present the solid phase technique for quantitating erythrocyte AChE is not sensitive enough, but with the advent of even more sensitive reagents, for example alkaline phosphatase anti-alkaline phosphatase (APAAP) complexes, the real potential of this method will be realised. Further evaluation of this technique is required.

The wider availability of fluorescence activated cell sorters (FACS) opens up the possibility of quantitation of AChE using a fluorescence conjugated anti-mouse antibody after the initial sensitization of the erythrocytes with AE-2. This method would involve fewer stages than the ELAT since no substrate stage would be required and hence no laborious washing away of inorganic phosphate with unbuffered saline. This together with the facility of direct linkup to a computer makes for an attractive method of quantitation and one which should be explored. Further work using a FACS in the monitoring of patients with PNH will provide essential data concerning prognosis, while also providing information on the average life of the transfused red cells required to maintain these patients. Further analysis of erythrocytes from leukaemic patients may provide a method of diagnosis and monitoring states of remission and relapse.

Further activity and antigen data from cord erythrocytes is necessary to establish whether the low activity is due solely to

decreased numbers of AChE molecules, a foetal form of the enzyme of lower activity than the adult form or a combination of both. A larger survey of erythrocyte AChE from women at delivery and during the puerperium is also required to positively identify the cause of the increased activity in these patients.

Although, very elaborate techniques in DNA analysis have now been developed (Arpargus et al., 1990) which can pinpoint the mutation responsible for the more common genetic variants of plasma cholinesterase, enzyme kinetics and inhibition characteristics will still be required to initially identify these variants. In addition, DNA analysis can not as yet provide direct information concerning the molar concentration of this enzyme, thus immunological quantitation will provide further valuable information in defining the overall characterization of plasma ChE. Further work in enzyme kinetics, immunological characterization and DNA analysis is required with larger numbers of genetic variants to substantiate data already presented here and to investigate new genetic variants. Additional monoclonal antibodies defining different antigenic regions of the molecule will also prove valuable in providing further characterization.

Since steroids are known to be powerful inhibitors of cholinesterase in vitro (Whittaker and Britten, 1980) it would also appear worthwhile to establish a steroid profile present in plasma during the early puerperium and subsequently ascertain whether any of these steroids exert an effect by binding to ChE or inhibiting biosynthesis of the protein.

It is hoped that the future development of techniques will establish the role of the cholinesterases not only on the

erythrocyte surface but also in the plasma.

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Appendix I

Solutions

Phosphate Buffer, 0.01M pH 7.2.

$\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 0.345gm (0.0025M)

$\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 2.680gm (0.0075M)

Distilled water up to 1 litre.

Phosphate Buffered Saline, PBS 0.01M pH 7.2.

Phosphate buffer, 0.01M pH 7.2

NaCl , 8.470gm (0.1450M) per litre

For PBS/tween add 1ml tween/1 PBS.

Unbuffered Saline, UBS, pH 7.0.

NaCl , 8.470gm (0.1450M)

Distilled water up to 1 litre.

Low Ionic Strength Solution (LISS), pH 6.7.

NaCl , 1.79g/l

KH_2PO_4 , 0.257g/l

Na_2HPO_4 , 0.158g/l

Glycine, 18g/l

PBS/BSA, UBS/BSA and LISS/BSA contained 2g/l BSA.

Sodium Carbonate Buffer, 0.1M pH 9.6.

NaHCO_3 , 8.4g/l

Na_2CO_3 , 10.6g/l

9ml NaHCO_3 + 1ml Na_2CO_3 .

Sodium Carbonate buffer, 0.05M pH 9.8

NaHCO_3 , 4.2g/l

Na_2CO_3 , 5.3g/l

6ml NaHCO_3 + 4ml Na_2CO_3 .

contained 0.001 mol/l MgCl_2 .

Citric Acid-Phosphate Buffer, 0.1M pH 5.0.

Citric acid H_2O , 7.30gm (0.034M)

$\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 23.87gm (0.0667M)

Distilled water up to 1 litre

Store at 4°C, stability 2 months.

Tris-veronal Buffer, pH 8.7.

Sodium Barbitone, 4.48gm

Tris (glycine), 8.86gm

Sodium Azide, 0.20gm

Distilled water up to 1 litre

0.05M Acetate/Acetic acid pH 5.0

(a) CH_3COONa (0.05M) 4.1g/l

(b) CH_3COOH (0.05M) 2.85ml glacial acetic

acid made up to 1 litre

100ml of (a) is adjusted to pH5.0 by adding

approximately 50ml of (b).

Substrate Solutions

Acetylcholinesterase Substrate.

39.6mg 5,5' dithio-bis-2 nitrobenzoic acid
(DTNB), in 10ml phosphate buffer (0.01M pH7.2)
15mg sodium bicarbonate.
108.35mg Acetylthiocholine iodide (ATI), in
5ml distilled water.

Cholinesterase Substrate.

20mg DTNB in 100ml of 0.05M (6.8g/l)
KH₂PO₄ pH 7.0.
30mg Butyrylthiocholine iodide, BTI in 10ml
distilled water.
Add 1ml BTI + 9ml DTNB.

Cholinesterase Substrate

0.2mg α -naphthyl acetate (previously
dissolved in acetone as a 1% solution)
per ml 0.01M phosphate buffer pH 7.2
and 0.5mg/ml of Fast Red TR.

Peroxidase Substrate.

Orthophenylenediamine, OPD.
8 mg 1,1-phenylenediamine dihydrochloride in
12 ml 0.1M citric acid-phosphate buffer, pH5.0
Add 5 μ l 30% H₂O₂. Prepare shortly before use.

Peroxidase Substrate.

3,3',5,5'-tetramethylbenzidine (TMB) 0.4g/l
in an organic base and
0.02% H₂O₂ solution in a citric acid buffer
premade (Kirkegaard and Perry Labs Inc.,
2 Cessna Court, Gaithersburg, Maryland).
Add together in ratio 1:1 vol:vol.

Peroxidase Substrate.

20mg of 3-amino-9-ethyl carbazole dissolved
in acetate buffer. To this mixture add 25µl
of 30% H₂O₂. Prepare immediately before use.

Alkaline Phosphatase Substrate.

40mg p-Nitrophenylphosphate capsule dissolved
in 20ml 0.05M carbonate buffer, pH 9.8.

Antibodies Used

Erythrocyte AChE

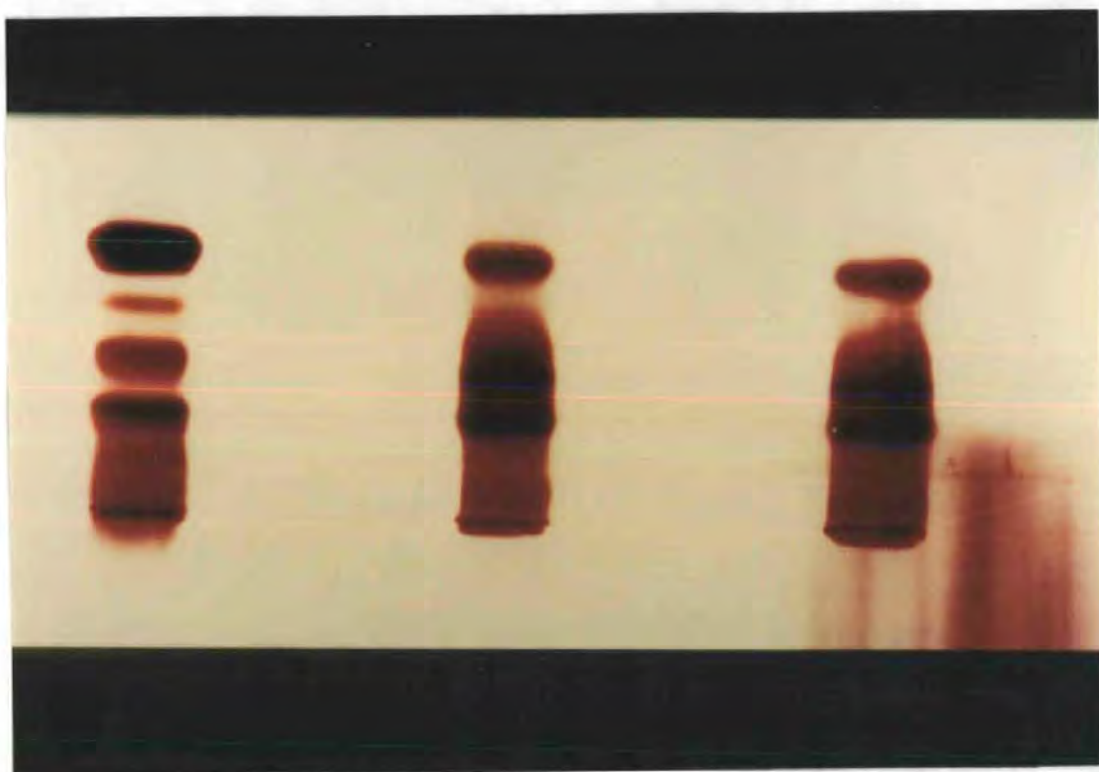
Hybridomas AE-1 and AE-2 were donated to the American Type Culture Collection (ATCC) by Fambrough et al. (1982). These are murine monoclonal antibodies to human erythrocyte AChE.

Plasma ChE

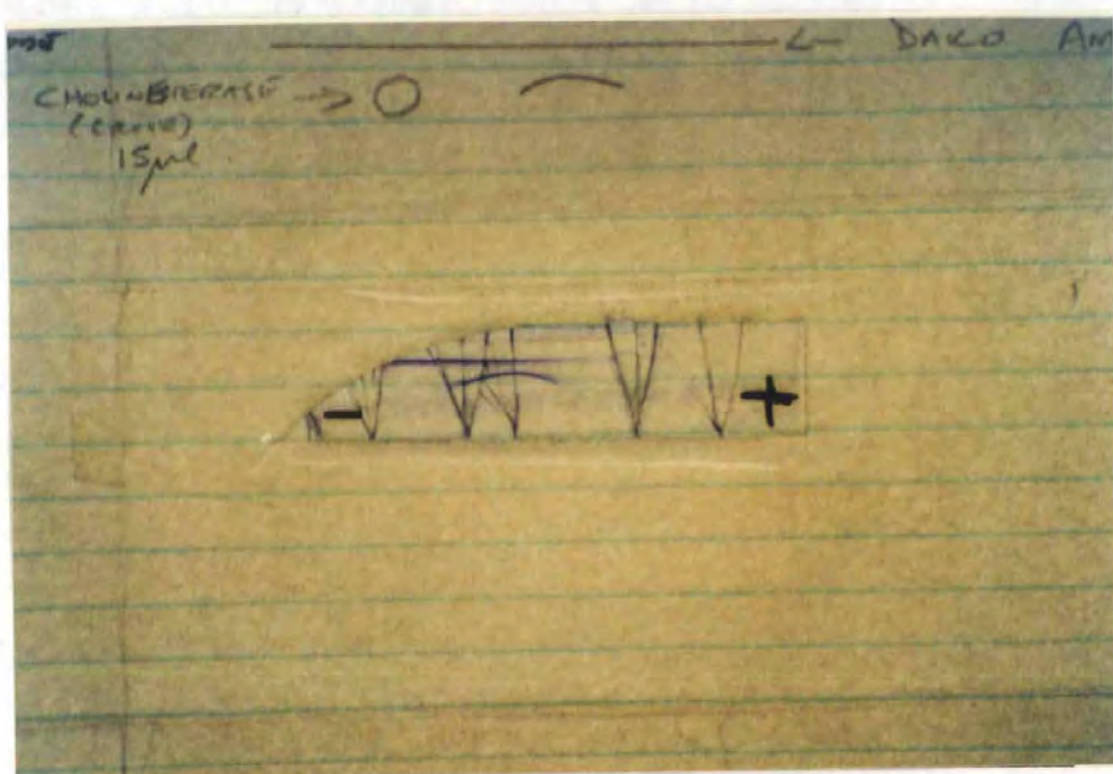
In addition to the commercially available rabbit anti-cholinesterase available from DAKO, a number of other sources of antibody were sought. Polyclonal antisera were produced with the help of the Immunology Department of Plymouth Polytechnic and the 'Binding Site' at Birmingham University.

A pure form of serum cholinesterase was required for production of suitable antisera. The BCL human cholinesterase reagent is not highly purified, being isolated from Cohn Fraction IV from human plasma (Das and Lidell 1970) and is therefore unsuitable for use in preparing antibodies. Electrophoresis confirmed this (picture 3). The 2 banding patterns of the BCL reagent on the right showing β_1 , β_2 and a diffuse gamma band. Only the α band was absent compared to the normal plasma on the left. Thus, precipitation techniques were employed to purify the reagent. The DAKO anti-cholinesterase and BCL cholinesterase reagents were subjected to immunoelectrophoresis (picture 4) on agar gel and the coomassie blue stained precipitin arc (rabbit IgG/cholinesterase complex) was cut out, solubilised and injected into 2 rabbits at Plymouth and a sheep at Birmingham. On day 1 10 μ l of complex (corresponding to less than 5 μ g cholinesterase) and on day 30 50 μ l of complex were injected. On day 37 a trial

bleed was tested using immunoelectrophoresis of human serum and BCL cholinesterase against the antiserum produced. Once the presence of anti-cholinesterase was confirmed the animal was further bled and the antiserum adsorbed with human IgG.



Picture 3 Electrophoresis banding pattern of normal plasma (left) and BCL reagent (middle and right).



Picture 4 Immunoelectrophoresis of BCL cholinesterase reagent with DAKO anti-cholinesterase.

These polyclonals together with a number of monoclonal antibodies (table 31) were evaluated in an enzyme linked quantitation of plasma cholinesterase.

Table 31 Antibodies Used for the Quantitation of the
Cholinesterases

Antibody	Antigen	Source	Animal	Type
AE-1	AChE	BTS	Mouse	Monoclonal
AE-2	AChE	BTS	Mouse	Monoclonal
A-032	ChE	DAKO	Rabbit	Polyclonal
B-1	ChE	Behring	Rabbit	Polyclonal
188	ChE	Plymouth	Rabbit	Polyclonal
288	ChE	Plymouth	Rabbit	Polyclonal
B'ham	ChE	Binding Site	Sheep	Polyclonal
SSI	ChE	SSI	Rabbit	Polyclonal
2-1	ChE	SSI	Mouse	Monoclonal
2-2	ChE	SSI	Mouse	Monoclonal
2-3	ChE	SSI	Mouse	Monoclonal
2-4	ChE	SSI	Mouse	Monoclonal
SSI = State Serum Institute				

Appendix II
PUBLICATIONS