INVESTIGATIONS ON PLASMA CHOLINESTERASE IN MAN AND ANIMALS USING SUCCINYLCHOLINE AS THE SUBSTRATE.

Sherry Ann Faye

Submitted in accordance with the requirements for the degree of Doctor of Philosophy.

THE UNIVERSITY OF LEEDS.

Department of Chemical Pathology (Clinical Biochemistry, St James's University Hospital).

June 1988.

ABSTRACT

A simple, precise "reaction rate" assay for plasma cholinesterase based on a succinylcholine substrate has been developed.

It's ability to define individuals at risk of succinylcholine sensitivity and identify those who had experienced apnoea was superior to the previously best available assay.

However it was not able to identify abnormal forms of cholinesterase which could hydrolyse conventional assay substrates but not succinylcholine. It was concluded that if these forms exist their numbers are small.

The failure to identify such cholinesterase types may have been because the substrate concentration chosen for the assay was higher than that found pharmacologically. However investigation of the kinetics of the succinylcholine-cholinesterase interaction showed that this was not the case.

The assay was applied to the assessment of liver dysfunction and compared to three established methods was superior. All assays identified patients with severe liver disease but the succinylcholine-based one identified more patients with moderate/mild disease.

The assay was also used to investigate the clinical observation that children require a higher dose of succinylcholine for muscle relaxation than adults. Infants were found to have higher succinylcholine activities than adults which is compatible with their relative resistance

to the drug.

Finally cholinesterase measurements were made, using a range of substrates including succinylcholine, in a variety of animal species. Results show that only when succinylcholine is used as the substrate for the assay of cholinesterase does enzyme activity correlate with tolerance to it's muscle relaxant properties.

The choice of procedure for the analysis of any biochemical variable depends on a number of criteria including ease of assay, precision, accuracy and cost; however the primary consideration should be the ability of the method to provide clinically useful information. Based on all these criteria, in particular the latter, succinylcholine must be considered as the substrate of choice.

CONTENTS.

CHAPTER 1 INTRODUCTION:

Literature Review and Aims.
1.1 History 2-4
1.2 The chemical and physical nature of cholinesterase 4-17
1.2.1 The relative molecular mass 4
1.2.2 The structure and active site 7-9
1.2.3 Multiple forms of cholinesterase
a) Isoenzymes 9-12
b) Genetic variants 12-17
1.3 The biochemical properties of cholinesterase 17-22
1.3.1 Enzyme substrate interaction 17-20
1.3.2 Inhibition and activation 20-22
1.4 The physiological properties of cholinesterase 22-25
1.5 The clinical significance of plasma cholinesterase 26-34
1.5.1 In disease 26-29
1.5.2 In toxicology 29-30
1.5.3 In succinylcholine apnoea 30-34
1.6 Cholinesterase in animals 34-37
1.7 Measurement of plasma cholinesterase 37-43
1.8 Aims 43-44

CHAPTER 2 MATERIALS AND METHODS

2.1 Materials 45-46
2.2 Analytical methods 47-54
2.2.1 Measurement of enzyme activity 47-50
2.2.2 Ascription of cholinesterase genotype 50-52
2.2.3 Preparation of icteric sera 52-53
2.2.4 Ultrafiltration of sera 53
2.2.5 Liver function tests 53-54
2.3 Statistical methods 54-55

CHAPTER 3 DEVELOPMENT OF A SUCCINYLCHOLINE-BASED ASSAY FOR SERUM CHOLINESTERASE

Introduction 56-58
3.1 Preliminary investigations 58
3.2 Determination of the most approprate blank solution for use in the assay 58-59
3.3 Investigation of linearity 59-60
3.4 Calibration 60-61
3.5 Investigation of the most appropriate substrate concentration 61-62
3.6 Investigation of the effect of altering the concentrations of the components of the colour reagent 62-63

3.7 The final format of the method 63-64
3.8 Imprecision 64
3.9 Investigation of interferences 64-66
3.10 Reference range 66
Tables and Figures 66-79

CHAPTER 4 THE EVALUATION OF THE SUCCINYLCHOLINE-BASED CHOLINESTERASE ASSAY FOR THE INVESTIGATION OF SUCCINYLCHOLINE INDUCED APNOEA

Introduction 80
4.1 Comparison of the succinylcholine- and propionylthiocholine activities of healthy phenotypically usual individuals 80
4.2 Comparison of the succinylcholine and propionylthiocholine activities of individuals referred for assessment of cholinesterase status 81-82
4.3 Comparison of succinylcholine and propionylthiocholine activities of samples from individuals with genetic abnormalities of cholinesterase 82-84
4.4 Application of the succinylcholine-based assay to biochemically normal individuals with apnoea following treatment with succinylcholine 84-85
4.5 Discussion 85-88
Tables and Figures 89-104

CHAPTER 5 INVESTIGATION OF THE KINETICS OF THE SUCCINYLCHOLINE-CHOLINESTERASE REACTION USING THE NEWLY DESCRIBED ASSAY

A. INVESTIGATION OF THE TYPE OF KINETICS EXHIBITED BY THE INTERACTION BETWEEN CHOLINESTERASE AND SUCCINYLCHOLINE

Introduction 105
5.1 Kinetic studies using the succinylcholine-based assay for serum cholinesterase 105-106
5.2 Discussion 106-108

B. COMPARISON OF THE Km1 VALUE OBTAINED IN THIS STUDY WITH THOSE REPORTED IN THE LITERATURE

Introduction 108
5.3 Investigation of the assay calibration 108-109
5.4 Studies of potential inhibitors of choliesterase in the assay system 109-110
5.5 Discussion 110-113
Tables and Figures 114-125

CHAPTER 6 THE SUCCINYLCHOLINE-BASED ASSAY IN LIVER DISEASE

Introduction 126
6.1 Patients 126-127
6.2 Cholinesterase activities in patients with liver disease 127-128
6.3 Comparison of albumin and succinylcholine activity in patients with liver disease 128
6.4 Discussion 128-129
Tables and Figures 130-140

CHAPTER 7 THE SUCCINYLCHOLINE-BASED ASSAY IN NEONATES AND CHILDREN

Introduction 141-142
7.1 Subjects 142
7.2 Cholinesterase activities of subjects studied 142-143
7.3 Discussion 143-145
Tables and Figures 146-147

CHAPTER 8 CHOLINESTERASE MEASUREMENTS ON ANIMALS

Introduction 148-149
8.1 Blood samples and methods 149
8.2 Studies on domestic animals 149-151
8.3 Studies on zoo animals 151
8.4 Discussion 151-155
Tables and Figures 156-166

CHAPTER 9 CONCLUSIONS 167-169

REFERENCES.

Table No	Title	Page No
1.1	Nomenclature and organs of origin for cholinesterase and acetyl-cholinesterase	5
1.2	Estimates of the relative molecular mass of the C4 component of human cholinesterase	6
1.3	Approximate frequencies of cholinesterase genotypes in a Caucasian population	17
3.1	Cholinesterase activities in serum samples taken from a volunteer before and after a fatty meal	67
3.2	Cholinesterase activities in a serum sample before and after the addition of bilirubin	68
3.3	Cholinesterase activities in a sample before and after haemolysis	69
3.4	Cholinesterase activities in healthy phenotypically usual individuals	70
4.1	Succinylcholine and propionyl-thiocholine (PTCI) activities in healthy $E1^u$ homozygotes	89-90
4.2	Comparison of the succinylcholine and propionylthiocholine (PTCI) activities of 76 patients referred for routine investigation of cholinesterase status	91-92
4.3	Succinylcholine and propionyl-thiocholine activities in individuals with genetic abnormalities of cholinesterase	93-94
4.4	Cholinesterase activities in biochemically normal patients with apnoea after treatment with succinylcholine	95-96
4.5	Typical velocity versus substrate concentration results for an $E1^u$ homozygote demonstrating Michaelis-Menten kinetics	97
4.6	Km and Vmax values for healthy $E1^u$ homozygotes and for patients with normal cholinesterase status but who	

	developed apnoea following treatment with succinylcholine	98
5.1	Km and Vmax values for the hydrolysis of succinylcholine by cholinesterase at Site 1 and Site 2	114
5.2	Km values for succinylcholine and cholinesterase previously reported	115
5.3	Investigation of the inhibition of cholinesterase by aminophenazone and phenol	116
5.4	Km and Vmax values obtained after reducing the aminophenazone concentration	117
6.1	Full clinical details of patients with liver disease	130-133
6.2	Cholinesterase activities in patients with liver disease	134-135
6.3	Cholinesterase activities expressed as standard deviations from the mean for $E1^u$ homozygotes and modified Childs grading for patients with liver disease	136-137
6.4	Numbers of subjects with low cholinesterase values and their Child's grading	138
7.1	Cholinesterase activities in neonates and infants	146
8.1	Cholinesterase activities in domestic animals	156-159
8.2	Cholinesterase activities in zoo animals	160-162

Figure No.	Title	Page No
3.1	Equivalents of cholinesterase activity shown by spontaneous hydrolysis of a range of succinylcholine concentrations	71
3.2	The relationship between cholinesterase activity and reaction rate	72
3.3	Absorbance produced by the addition of increasing amounts of choline chloride to the reaction mixture	73
3.4	Cholinesterase activities in sera from an individual homozygous for the usual gene and one homozygous for the atypical gene	74
3.5	Succinylcholine activity measured using colour reagent with increasing aminophenazone concentration	75
3.6	Succinylcholine activity measured using colour reagent with increasing peroxidase concentration	76
3.7	Succinylcholine activity measured with colour reagent with increasing choline oxidase concentration	77
3.8	Succinylcholine activity measured using colour reagent with increasing phenol concentration	78
3.9	Succinylcholine activities of healthy individuals homozygous for the usual gene	79
4.1	Comparison of cholinesterase activities of healthy E_1^u homozygotes measured using succinylcholine and propionylcholine substrates	99
4.2	Comparison of enzyme activities of 76 subjects referred for routine investigation of cholinesterase status	100
4.3	Succinylcholine activities of 87 subjects with unusual cholinesterase genotypes	101

4.4	Propionylthiocholine activities of 87 subjects with unusual cholinesterase genotypes	102
4.5	Graphs of substrate concentration against velocity and it's reciprocal for an individual homozygous for the usual gene	103
4.6	Michaelis-Menten constants in healthy $E1^u$ homozygotes and in patients normal by current biochemical criteria but who had experienced apnoea after treatment with succinylcholine	104
5.1	Plot of velocity against log succinylcholine concentration for serum from three individuals homozygous for the usual cholinesterase gene	118
5.2	Lineweaver-Burk plot for serum from one individual homozygous for the usual gene	119
5.3	Plot of velocity against log succinylcholine concentration for serum from an individual homozygous for the atypical cholinesterase	120
5.4	Lineweaver-Burk plot for the serum from an individual homozygous for the atypical gene	121
5.5	Plot of velocity against log succinylcholine concentration for purified cholinesterase	122
5.6	Lineweaver-Burk plot for purified cholinesterase	123
5.7	Absorbance produced by the addition of increasing amounts of choline iodide to the reaction mixture	124
5.8	Lineweaver-Burk plot for serum from one subject before and after reducing the aminophenazone concentration	125
6.1	Cholinesterase activities measured using four substrates in patients with clinically graded liver disease	139

6.2	Albumin concentration and succinylcholine activity in patients with clinically graded liver disease	140
7.1	Cholinesterase activities in neonates and infants measured using succinylcholine and propionylthiocholine as substrates	147
8.1a	Cholinesterase activities in domestic species expressed as a %age of the mean of the appropriate human reference range	163
8.1b	Cholinesterase activities in domestic species expressed as a %age of the mean of the appropriate human reference range	164
8.2	The relationship between cholinesterase activity measured using conventional substrates and tolerance to succinylcholine in a range of species	165
8.3	The relationship between cholinesterase activity measured with succinylcholine as the substrate and tolerance to succinylcholine in a range of species	166

ABBREVIATIONS

All abbreviations are defined in the text. The cumulative list is given below:

DFP	^{32}P di-isopropylfluorophosphate
HPLC	High performance liquid chromatography
SD	Standard deviation
DTNB	5,5´-dithiobis-(2-nitrobenzoic acid)
Tris	[Tris-(hydroxymethyl)-aminomethane] HCL
DMSO	Dimethylsulphoxide
Km	Michaelis-Menten constant
Vmax	Maximum velocity of enzyme substrate reaction
PTCI	Propionylthiocholine
ECT	Electroconvulsive therapy

ACKNOWLEDGEMENTS.

I would like to thank the following:

My mother and father for their support throughout my acedemic career without whom I would never have been in a position to attempt this thesis.

My supervisor Dr Robert Evans for his advice and help throughout the course of this project for which I am extremely grateful.

For their gifts of samples:

Dr Moss — Horse Racing Forensic Laboratories Ltd., Newmarket, Suffolk.

Mr M.S. Bain — Veterinary Investigation Centre, Riseholme, Lincolnshire.

Professor Kerr — Department of Animal Physiology and Nutrition, University of Leeds.

Dr C. Hawkey — Wellcome Research Laboratories, London Zoo, Regents Park, London.

For advice on sections of the thesis:

Dr J. McCormack, Biochemistry Department, University of Leeds and Dr S. Halford, Biochemistry Department, Bristol University for their advice on enzyme kinetics.

Dr A. Hay, Department of Chemical Pathology, University of Leeds for his advice on the animal work.

The biochemists who worked in the Cholinesterase Research Unit, St. James's Hospital during the course of this project:

Dr J McGinlay, Dr C. Rapier, Ms A. Walker, Ms K. Bowden and Ms J. O'Callaghan

for their scientific assistance and companionship.

Dr R. Jones for computer programs which made the writing of this thesis considerably easier.

Finally my husband Paul for his unfailing support and fine scientific advice.

PUBLICATIONS RESULTING FROM WORK CONTAINED WITHIN THIS THESIS.

FAYE S.A. and EVANS R.T. [1986]
Is succinylcholine the substrate of choice in the measurement of cholinesterase activity?
Clinical Chemistry 32, 1477-1480.

FAYE S.A. and EVANS R.T. [1988]
Differences in the response of humans and dogs to succinylcholine.
Anesthesiology 68, 471-472.

FAYE S.A., BANNISTER P. and EVANS R.T. [1988]
Cholinesterase measurement in chronic liver disease. A succinylcholine-based assay is most clinically useful.
Annals of Clinical Biochemistry 25, Supplement, 79-80.

CHAPTER 1 INTRODUCTION

Literature review and aims

1.1 HISTORY.

" The evanescence of the effect, and the regularity of its reproduction with sucessive doses, which, as we shall see, are characteristic of all the actions of acetylcholine, may be connected, with some probability, with the readiness with which the ester is hydrolysed into its relatively inert constituents, choline and acetic acid..... In the blood at body temperature it seems not improbable that an esterase contributes to the removal of the active ester from the circulation, and the restoration of the original condition of sensitiveness. "

DALE 1914

Dale in 1914, with this statement concerning the action of acetylcholine, first predicted the existence of cholinesterase. Experimental support for his view was provided by Loewi and Navratil seven years later when they showed that if eserine was present the effect of acetylcholine on frogs heart was prolonged [Loewi and Navratil 1921]. They attributed this result to the inhibition, by eserine, of the enzyme normally responsible for the degredation of acetylcholine.

It was not until 1932 that the first crude extract of cholinesterase was prepared from horse serum [Stedman, Stedman and Easson 1932]. Between 1940 and 1943 it was established that there were two cholinesterases in the body, one present in erythrocytes, the other in serum, differing in sensitivity, specificity and other properties. This was first shown by Alles and Hawes [1940] who compared their properties. They showed that erythrocyte cholinesterase was several times more active than the serum enzyme at low acetylcholine concentrations.

However, as substrate concentration was increased the erythrocyte enzyme was inhibited whilst serum enzyme activity increased throughout the range of concentrations studied. This work was confirmed and extended by Mendel and his colleagues [Mendel and Rudney 1943; Mendel and Mundell 1943, Mendel, Mundell and Rudney 1943]. They found that a relatively small degree of purification of erythrocyte cholinesterase deprived it of the ability to hydrolyse the aliphatic esters, tributyrin and methylbutyrate, whereas a much higher degree of purification of the enzyme from horse serum and dog pancreas did not. They named the two enzymes "true" and "pseudo" cholinesterase respectively. Soon afterwards they found that benzoylcholine was hydrolysed by "pseudo" cholinesterase but not by "true" cholinesterase. The reverse was true of acetyl-B-methylcholine. Consequently they were able to use this pair of substrates as a means of identifying the two kinds of cholinesterase in blood and tissues.

The work of Mendel and colleagues has not escaped criticism. The use of benzoylcholine and acetyl-B-methylcholine to define pseudo- or serum cholinesterases as distinct from true or acetylcholinesterases has caused confusion as they are not the preferred substrates for cholinesterase and acetylcholinesterase respectively in all species [Myers 1953]. Despite this and controversy concerning the use of the term "pseudo" [Glick 1948], the

work of Mendel and colleagues was important since it emphasised the concept of the existence of two types of cholinesterase, one specific for acetylcholine and a few closely related choline esters, the other capable of hydrolysing both choline esters and those of aliphatic acids.

The terms "true" and "pseudo" cholinesterase have now been replaced by more modern nomenclature. A summary of the terminology relevant to the two enzymes appears in Table 1.1.

1.2 THE CHEMICAL AND PHYSICAL NATURE OF CHOLINESTERASE.

Most of the information concerning the nature of cholinesterase has come from experiments on the enzymes present in horse or human serum. Wide species differences in cholinesterase do occur and will be discussed later.

Human cholinesterase is a glycoprotein. Electrophoretic studies [Surgenor et al. 1949] indicate that it is an α-2 globulin.

1.2.1 The Relative Molecular Mass:

The relative molecular mass of cholinesterase is high, approximately 350,000 daltons, suggesting a complex protein molecule with multiple subunit structure. The molecular weight of the purified enzyme has been estimated by several authors using a variety of techniques (Table 1.2).

TABLE 1.1

NOMENCLATURE AND ORGANS OF ORIGIN FOR CHOLINESTERASE AND ACETYLCHOLINESTERASE.

Systematic name	Enzyme commission number	Accepted trivial name **	Historical trivial name	Principle sites of distribution in mammals
Acetyl-choline acetyl-hydrolase	EC 3.1.1.7	Acetyl-cholin-esterase	Specific cholinesterase true cholin-esterase, E-type cholin-esterase	Grey matter of central nervous system, sympathetic ganglia, motor end plates and erythrocytes
Acylcholine acyl-hydrolase	EC 3.1.1.8	Cholin-esterase	Non specific cholin-esterase, pseudo cholin esterase, butyryl cholin-esterase, S-type cholin-esterase.	Intestinal mucosa, liver, plasma white matter of central nervous system

[Evans 1986]

** Suggested by the Commission on Biochemical Nomenclature of the International Union of Pure and Applied Chemistry and the International Union of Biochemistry [1973] and used throughout the rest of this thesis.

TABLE 1.2

ESTIMATES OF THE RELATIVE MOLECULAR MASS OF THE C4 COMPONENT OF HUMAN CHOLINESTERASE.

VALUE (Daltons)	METHOD	AUTHORS
348,000	Ultracentrifugation: sedimentation equilibrium	Haupt et al. 1966
366,000	Ultracentrifugation in a density gradient and gel filtration	Das and Liddell 1970a
280,000	Sephadex gel filtration	La Du and Snady 1971
345,000	Ultracentrifugation: sedimentation equilibrium	Muensch, Goedde and Yoshida 1976
371,000	Sepharose 6B gel filtration: acrylamide electrophoreisis	Pickard 1976
342,136	Sequencing: HPLC purification Edman degredation and HPLC detection of phenylthiohydantoin derivatives.	Lockridge et al. 1987

1.2.2 The Structure and Active Site:

Muensch, Goedde and Yoshida [1976] first determined the component amino acids of cholinesterase and also estimated the carbohydrate content. They found it to be 24.5% in agreement with the 23.9% reported by Haupt et al. [1966]. Only recently has a complete sequence of the protein portion of the molecule been performed using Edman degredation of purified peptides [Lockridge et al. 1987]. This work showed that the protein contains 574 amino-acids per subunit and confirmed previous reports that human cholinesterase is a tetramer of identical subunits [Lockridge and La Du 1978, Lockridge, Eckerson and La Du 1979]. Each subunit has 9 carbohydrate chains, terminating in sialic acid and attached to the protein through asparagine.

If the sialic acid residues are split off, using bacterial neuraminidase (EC 3.2.1.8), the electrophoretic mobility of cholinesterase is altered, it migrates with the serum β-globulins rather than the α-globulins [Svensmark 1961] and the isoelectric point of the enzyme is changed from less than 3 to 7 [Svensmark and Kristensen 1963]. However despite this, neuraminidase treated human cholinesterase retains it's hydrolytic activity and it's inhibitory characteristics with respect to dibucaine and fluoride [Echobichon and Kalow 1963]. The carbohydrate portion of the molecule therefore cannot be involved in the active site.

The nature of the active site has been extensively studied [Brown et al. 1981]. It possesses anionic and esteratic subsites and hydrophobic areas. The anionic site determines specificity with respect to the choline portion of the substrate while hydrolysis of the ester bond of the substrate occurs at the esteratic site. The role of the hydrophobic areas is unclear but it has been proposed that differences in their length and structure may account for the different kinetic properties of cholinesterase and acetylcholinesterase [Kabachnik et al. 1970]. The nature of the esteratic subsite has been more fully elucidated than the anionic subsite. An organophosphate, [^{32}P] di-isopropylfluorophosphate (DFP), which binds irreversibly at the esteratic site of cholinesterase, has been used to elucidate the structures involved in catalytic activity and has indicated that serine has an active role [Jansz, Brons and Warringa 1959]. However it has been pointed out that the pK of serine is not consistant with the pH optimum of cholinesterase, pH 8 [Bergman et al. 1956]. Only the imidazole ring of histidine, pK 6.95, has a pK in the range required. However the peptide sequence at the active site is similar to other enzymes where serine does have an active role to play in the catalytic process [Blow, Birktoft and Hartley 1969]. In such enzymes both histidine and serine are in close association. In 1986 the active site was sequenced and the presence of both these amino acids was confirmed [Lockridge and La Du 1986].

The number of active sites per enzyme molecule has been variously estimated at either two [Muensch, Goedde and Yoshida 1976] or three [Yoshida and Motulsky 1969] using a DFP titration technique. However using a different method in which a carbamylester was hydrolysed to a fluorescent product, four active sites were demonstrated by Lockridge and La Du [1978].

1.2.3 Multiple forms of cholinesterase:

a) Isoenzymes:

Human serum cholinesterase can be separated into at least four components which appear to be isoenzymes since they have similar substrate specificity and inhibitor susceptibility [La Motta and Woronick 1971].

Harris, Hopkinson and Robson [1962] found four bands (C1-C4) using two-dimensional electrophoresis with paper and starch gel. C1 migrated furthest towards the anode and C4, the major component with approximately 80% of enzyme activity, moved least. Most workers have adopted this numbering system, so that the major isoenzymes of cholinesterase are numbered consecutively, with the form migrating furthest being designated one. Unfortunately there are two sets of symbols for the electrophoretic bands of the enzyme; C1-C4 [Harris, Hopkinson and Robson 1962] and ChE1-ChE5 [La Motta, McComb and Wetstone 1965]. C1-C3 are identical to ChE1-ChE3 but C4 is identified with ChE4 and 5. Using polyacrylamide gel which gives better resolution than starch gel, more cholinesterase bands have

been identified. Juhl [1968] demonstrated 12 bands although other workers using similar techniques found rather less [Gaffney 1970, Das and Liddell 1970].

Staining techniques can influence the number of isoenzymes detected, e.g. more bands will be revealed by the use of nonspecific esterase stains than with butyrylthiocholine which is specific for serum cholinesterase. However Juhl used butyrylthiocholine as the substrate for staining so it is difficult to explain these divergent results.

La Motta, McComb and Wetstone [1965] showed that the isoenzymes of cholinesterase could be interconverted during procedures such as ammonium sulphate precipitation followed by resolubilisation or dialysis against polyvinylpyrollidone. It thus seems that preparative methods can influence the number of bands observed. It can be difficult therefore to establish which bands represent genuine naturally occuring isoenzymes and which are experimental artifacts.

Following storage of the enzyme, extra "storage" bands appear which migrate close to the major component. It is thought that they derive from the breakdown of the C4 component [Harris, Hopkinson and Robson 1962].

Gel filtration on sephadex G-200 showed C4 to have the highest molecular weight and the others to have decreasing molecular weight in the order C3, C2, C1 [Harris and Robson 1963]. La Motta, Woronick and Reinfrank [1970] determined the relative molecular masses of the isoenzymes

by ultracentrifugation in a sucrose gradient. They found values as follows; ChE1 82,000, ChE2 110,000, ChE3 170,000, ChE4 200,000, ChE5 260,000. Other workers have confirmed the values for C1 (ChE1): 86,000 [Boutin and Brodeur 1971] and 85,000 [Muensch, Goedde and Yoshida 1976] but have found higher values for C4: 348,000 [Boutin and Brodeur 1971] and 345,000 [Muensch, Goedde and Yoshida 1976].

Several explanations have been put forward to account for the demonstration of multiple molecular forms of cholinesterase. They include variation in the amounts of sialic acid or partial breakdown of the molecule by proteolysis [Muensch, Goedde and Yoshida 1976]. There is some evidence that the heterogeneity of plasma cholinesterase reflects reversible polymerisation of protein subunits [La Motta, Woronick and Reinfrank 1970]. Boutin and Brodeur were similarly of the opinion that interconversions occur in vitro, but expressed reservations about their occurrence in vivo. Other evidence compatible with La Motta's proposals arises from work in which in vitro treatments, including ultrasonic vibration [Dubbs 1966], the action of urea [Augustinsson 1973], freezing and thawing and treatment with sulphyryl reagents [Muensch, Goedde and Yoshida 1976], have been shown to alter the electrophoretic migration of cholinesterase. By such means the major component of the enzyme can be split into enzymatically active bands with electrophoretic mobilities corresponding to those of other

isoenzymes.

b) Genetic variants of cholinesterase:

These were first recognised following the clinical use of the muscle relaxant, succinylcholine. This drug is a depolarising neuromuscular blocking agent and has a short duration of action due to it's rapid hydrolysis by plasma cholinesterase. Succinylcholine was introduced into clinical practice in 1951 and one year later it was observed that some individuals suffered a prolonged reaction to it. These early cases of sensitivity were all thought to be due to impaired hepatic synthesis of cholinesterase since low levels of the enzyme were often found in patients who recovered slowly from succinylcholine [Bourne, Collier and Somers 1952 and Evans et al. 1952]. They therefore suggested that the drug should not be given in conditions where cholinesterase might be expected to be low, such as liver disease, severe anaemia, malnutrition or following organophosphate poisoning. Subsequently however, several cases were described in which there was no evidence for such conditions [Bourne 1953, Forbat, Lehmann and Silk 1953].

It was Forbat, Lehmann and Silk [1953] who first suggested that there could be a hereditary basis for sensitivity but it was the work of Kalow and his colleagues [1957-1959] which confirmed it.

Kalow and Gunn [1957] showed that cholinesterase from affected patients was abnormal. It did not appear to

hydrolyse succinylcholine in vivo or in vitro and it was unusually resistant to inhibition by the local anaesthetic dibucaine (Nupercaine). Using the percentage inhibition of the enzyme in the presence of a fixed concentration of dibucaine under standard conditions, the "dibucaine number" [Kalow and Genest 1957], Kalow and Staron [1957] divided people into three groups, each possessing two allelic co-dominant genes for cholinesterase. Those homozygous for the usual enzyme form, $E1^{u}E1^{u}$, dibucaine number >70, those heterozygous for the usual and atypical forms, $E1^{u}E1^{a}$, dibucaine number 40-70 and those homozygous for the atypical form, $E1^{a}E1^{a}$, dibucaine number <20. Individuals sensitive to succinylcholine were those homozygous for the atypical form of the enzyme, the product of a gene other than that found in unaffected persons [Kalow 1959].

The results of work with dibucaine stimulated the search for inhibitors of cholinesterase which might help identify other genetic variants of the enzyme. In 1961 inhibition with fluoride led to the description of the fluoride resistant gene ($E1^{f}$) [Harris and Whittaker 1961]. Other inhibitors have been studied including chloride [Harris and Whittaker 1963], Scoline [McComb, La Motta and Wetstone 1965] and butanol [Whittaker 1968] but their use has not led to the discovery of further variants.

Following the investigation of some families of "atypical" individuals, anomalies were found. First degree relatives of "atypical" individuals appeared to have "usual"

cholinesterase [Kalow and Staron 1957]. This suggested that there were a number of allelic genes for "usual" cholinesterase each determining it's production at a different rate, the extreme of which being an allele producing no cholinesterase activity at all. Confirmation of the existence of such a gene came in 1962 when a succinylcholine sensitive individual was described in whom cholinesterase activity was undetectable. It was proposed that the patient was homozygous for the "silent" gene [Liddell, Lehmann and Silk 1962].

In 1976 another allele at the cholinesterase locus was described, the J variant [Garry et al. 1976]. This variant causes reduction of the number of "usual" molecules by approximately 66% so that $El^{u}El^{j}$ heterozygotes have inhibitor numbers indistinguishable from El^{u} homozygotes but reduced enzyme activity. It is unusual since it can only be identified with certainty when it occurs in combination with the atypical or fluoride resistant cholinesterase forms. It then gives rise to inhibitor numbers different from those of other known genotypes. Two years after the desciption of the J variant, the K form was discovered. Similarly it can only be recognised when in conjunction with the atypical gene. In $El^{u}El^{k}$ heterozygotes it causes reduction in the number of usual enzyme molecules by about 33% but, like the J variant, it is not known whether this is due to decreased synthesis or increased degredation [Rubinstein, Dietz and Lubrano

1978].

Recently Whittaker has postulated the existence of another new variant, the H (Hammersmith) form. This variant was associated with a marked reduction in enzyme activity in $E1^uE1^h$ heterozygotes and inhibition characteristics indicative of the $E1^uE1^u$ genotype. $E1^aE1^h$ heterozygotes however were found to have unusual inhibition characteristics with Ro 02-0683, the dimethylcarbamate of (2-hydroxy-5-phenylbenzyl) trimethylammonium bromide. These observations have not yet been confirmed.

The frequencies with which the different cholinesterase genotypes occur varies with the population studied. The frequency of the $E1^a$ gene is highest amongst Caucasian peoples whereas it has not been detected among certain groups of Negroes and East Asians. The $E1^f$ gene, which is rare in most populations, was found with a high frequency among a group of Punjabis and certain Bantu tribes. The silent gene, also uncommon amongst most populations, occurs with a high frequency in some groups of Alaskan Eskimoes [Brown et al. 1981]. Table 1.3 shows the frequency of the different genotypes in a Caucasian population.

All variants previously described derive from allelic genes occuring at a locus designated E1 [Motulsky 1964]. Another variant has been reported characterised by the production of an extra enzyme component, migrating more slowly than the C4 band on starch gel electrophoresis [Harris, Hopkinson and Robson 1962]. It was designated C5.

The gene which determines the production of the C5 component occurs at a locus designated E2 in accordance with the Motulsky system of nomenclature. Approximately 10% of caucasians carry an E2 allelle which specifies the production of the C5 component and these have approximately 30% more plasma cholinesterase activity than those without the C5 band.

Another type of cholinesterase which may be identified by it's electrophoretic characteristics is the Cynthiana variant, first described by Neitlich [1966]. He measured cholinesterase activities in a population of male military personnel and found one individual with an activity consistantly three times the mean. A study of the subject's immediate family indicated other affected relatives, all of whom showed an additional intense band on polyacrylamide gel electrophoresis. The extra band migrated more slowly than the C5 band [Yoshida and Motulsky 1969]. The locus for the Cynthiana gene has not yet been determined. The condition is rare, only two other affected families have been reported [Delbruck and Henkel 1979]. They confirmed the quantitative and structural differences observed previously.

TABLE 1.3

APPROXIMATE FREQUENCIES OF CHOLINESTERASE GENOTYPES IN A CAUCASIAN POPULATION.

Genotype	Frequency
$E1^uE1^u$	95%
$E1^uE1^a$	1 in 30
$E1^uE1^f$	1 in 200
$E1^uE1^s$	1 in 220
$E1^aE1^f$	1 in 19,000
$E1^fE1^f$	1 in 160,000
$E1^fE1^s$	1 in 170,000
$E1^aE1^a$	1 in 2,500
$E1^aE1^s$	1 in 20,000
$E1^sE1^s$	1 in 100,000
$E1^aE1^k$	1 in 250
$E1^aE1^j$	Unknown perhaps 1 in 200,000

[Evans 1986]

1.3 THE BIOCHEMICAL PROPERTIES OF CHOLINESTERASE.

1.3.1 Enzyme-Substrate Interaction.

Plasma cholinesterase is a hydrolase which, under optimal conditions, catalyses the hydrolysis of choline and thiocholine esters at a higher rate than that of other esters. Using a homologous series of esters of choline it has been shown that butyrylcholine is the optimal substrate for usual human plasma cholinesterase and that pentanoylcholine shows the highest Vmax for the atypical enzyme [Davies, Marton and Kalow 1960]. Subsequent studies have shown that the order of the relative rates of hydrolysis by the usual enzyme was butyryl> valyryl> propionyl> acetyl> α-ethylbutyryl> α-methylbutyryl [Sekul, Holland and Breland 1962]. These workers have also shown that the introduction of a double bond at the , position in the alkyl group of choline esters reduces the rate of hydrolysis of the ester compared to that of the corresponding saturated alkyl ester. A double bond at any other position appears to have a less predictable effect. Substitution of an alkyl group at the α-position in the unsaturated acid moiety of a choline ester causes an additional reduction in the rate of hydrolysis.

Indirect evidence suggests that cholinesterase interacts with substrates by first forming an enzyme substrate complex which enables the serine hydroxyl group at the esteratic site to become acylated. The acylated group can then react with any nucleophyllic reagent to regenerate

the free enzyme. This can be represented schematically as follows:

$$EH + XY \underset{k-1}{\overset{k1}{\rightleftharpoons}} EH.XY \xrightarrow[HY]{k2} EX \xrightarrow[k3]{H2O} EH + XOH$$

[Brown et al. 1981]

where EH represents cholinesterase (H belonging to the hydroxyl group of the serine residue present at the esteratic active site) and XY the substrate. The rate constants are k1, k-1, k2 and k3, ($k-1 \lll k2$)

The rate constant k2 determines the rate of acylation and k3 that of deacylation. The rate limiting step is the formation of the acylated enzyme. Deacylation is very fast making it difficult to detect the acyl enzyme by direct observation.

Studies on the kinetics of cholinesterase have shown that it does not always obey Michaelis-Menten kinetics. Inhibition is observed at high substrate concentrations with halogenated cholines [Sastry and Chiou 1968], benzoylcholine [Berry 1960], acetylsalicylcholine [Augustinsson 1948] and lactoylcholine [Sastry and White 1968] but not with either acetyl or butyrylcholine [Brown et al. 1981] or with thiocholine esters [Wetherall and French 1986]. The latter workers showed that there is a further increase in enzyme activity at high thiocholine ester concentrations. This has also been shown for butyrylcholine [Hastings 1966]. These observations have

been interpreted in several ways,

a) that there are two enzymes present [Heilbron 1958, Berry 1960]

b) that the enzyme is activated at high substrate concentration [Hastings 1966]

c) that there exists a modifier or an allosteric site [Main 1983].

1.3.2 Inhibition and Activation.

Inhibitors of cholinesterase can be divided into two classes:

a) Those acting reversibly, forming non-covalent bonds.

b) Those acting irreversibly by the formation of covalent bonds.

Many reversible inhibitors contain a quaternary ammonium group, for example neostigmine. The positively charged nitrogen of such compounds is attracted to the negatively charged anionic site of the enzyme. Inhibitors are also bound at this site by coulombic forces which may be augmented by Van der Waals attraction of the alkyl groups to hydrophobic areas near the anionic site [Brown et al. 1981]. Bis quaternary ammonium compounds, for example, pancuronium and succinylcholine are also reversible inhibitors of cholinesterase. On a mole for mole basis they have been found to be twice as potent as the simple quaternary ammonium compounds [Main 1976]. Other clinically interesting reversible inhibitors of cholinesterase are fluoride and dibucaine. Inhibition by

the fluoride ion follows complex kinetics [Heilbron 1965] the mechanism of which is yet to be resolved. Dibucaine has a tertiary amine group and therefore acts in a similar way to quaternary ammonium compounds.

Irreversible inhibitors react with the hydroxyl group of serine at the active site of cholinesterase. They bind at the same site as true substrates but bind covalently to form acyl-enzyme complexes which are resistant to hydrolysis [Brown et al. 1981]. This group of inhibitors includes the organophosphates and carbamates.

Other substances capable of altering cholinesterase activity exist which do not fit into the above catagories, notably n-butanol. Whittaker studied the effects of alkyl-alchohols on human serum [1968a]. Moderate alcohol concentrations activated the usual enzyme and only high concentrations caused irreversible inactivation due to denaturation. The atypical enzyme was more readily inactivated and therefore alkyl-alcohols were advocated as a means of differentiating cholinesterase variants. It was claimed that new phenotypes could be recognised using n-butanol [Whittaker 1968b] but this has not been substantiated.

Several compounds have been shown to enhance the hydrolysis of substrates by human cholinesterase. Erdos and co-workers using benzoylcholine and procaine demonstrated activation by tryptamine and histamine [Erdos et al. 1957], quaternary ammonium compounds [Erdos et

al.1958] and a series of narcotic analgesics [Erdos et al. 1959]. Other compounds reported to activate cholinesterase include divalent cations [Hoftsee 1960], glutathione and pilocarpine [Keeser 1938].

1.4 PHYSIOLOGICAL PROPERTIES OF CHOLINESTERASE.

In a normal healthy population the range of cholinesterase activities is wide but values within an individual show little variation. The main factors affecting cholinesterase activity are age, sex and pregnancy.

There is controversy in the literature concerning cholinesterase levels at birth. Zsigmund and Downs [1971] reported low levels of cholinesterase, approximately 50% those of adults in term neonates and infants up to 6 months. Mirakur, Elliot and Lavery [1984] and Strauss and Modanlou [1986] reported levels equivalent to that of adults in term neonates although they did report a transiently low activity in preterm neonates. Adult levels are achieved at the time of puberty and probably remain constant thereafter, although some studies have noted further slight changes, either an increase [Propert and Brackenridge 1976] or a decrease [Kalow and Gunn 1958]. A number of workers have shown sex differences, with higher activities in men than in women [Sidell and Kaminskis 1975, Propert and Brackenridge 1976]. There have been conflicting reports of the effects of pregnancy on plasma cholinesterase levels. Some workers have found no

difference between pregnant and non pregnant women [Meade and Rosalki 1963, Rimbach and Dacic 1963] whereas others have found lower activities in pregnant women [Tourtellotte and Odell 1950, Robertson 1966]. A large study by Evans and Wroe [1980] has indicated that there is a fall in cholinesterase during the first trimester to levels which remained constant for the rest of pregnancy but with a further fall in the seven days following delivery.

Despite the wide tissue distribution of cholinesterase in man [Silver 1974] there is controversy concerning it's physiological function. Healthy individuals exist who have no detectable cholinesterase activity which suggests either that it's role is not essential or that there is an alternative metabolic pathway which bypasses cholinesterase.

Cholinesterase is found in the white matter of the central and peripheral nervous system in both man and animals, in close association with the acetylcholinesterase of the grey matter [Ord and Thompson 1952]. The function of acetylcholinesterase is to terminate the action of acetylcholine at all sites of cholinergic transmission and cholinesterase has been linked with this. Acetylcholine in high concentrations inhibits acetylcholinesterase but not cholinesterase. Lehmann and Silk [1953] suggested that the function of cholinesterase may be protective, hydrolysing acetylcholine when it reaches concentrations sufficient to

inhibit acetylcholinesterase. In addition theories have been advanced that cholinesterase is a precursor of acetylcholinesterase [Koelle, Koelle and Smyrl 1973] and that it is involved in controlling plasma choline and acetylcholine levels [Funnell and Oliver 1965].

Clitherow, Mitchard and Harper [1963] proposed that cholinesterase may be responsible for the breakdown of toxic choline esters arising from lipid or intestinal metabolism. However no evidence exists for the presence of toxic quantities of choline esters in human plasma or tissues.

More recently there has been increasing interest in the relationship between cholinesterase and lipoproteins. Lawrence and Melnick [1961], after histochemical, immunoelectrophoretic and quantitative studies, showed an association of cholinesterase with β-lipoproteins. They suggested that the cholinesterase β-lipoprotein complex existed for the purpose of transporting cholinesterase in an inactive form and that the union was a physical rather than a chemical one since it could easily be disrupted by physical means. This work was supported by that of Dubbs [1966] who observed increased cholinesterase activity after ultrasonication of human serum. However work by Kutty and colleagues [1973-1980] has indicated that the relationship is an active one and that the association between cholinesterase and β-lipoprotein is a chemical one. Kutty, Rowden and Cox [1973] provided evidence that cholinesterase interacts with the phosphocholine site of

the lecithin of lipoprotein and postulated that it was there for the purpose of stabilising β-lipoprotein. Support for this proposal came from studies using cholinesterase inhibitors which showed that changes in - lipoprotein paralleled those of cholinesterase after the ingestion of an organophosphate [Kutty et al 1975] and that neostigmine decreased the incorporation of tritiated lysine into β-lipoprotein [Kutty, Redheendran and Murphy 1977].

A relationship between cholinesterase and β-lipoprotein undoubtedly exists, whether chemical or physical, since an increase in plasma cholinesterase has been noted in conditions associated with abnormal lipid metabolism, in hyperlipoproteinaemia [Cucuianu et al 1975], obesity [Cucuianu, Popescu and Haragus 1968] and nephrotic syndrome [Way, Hutton and Kutty 1975] and may therefore be of clinical significance.

1.5 THE CLINICAL SIGNIFICANCE OF PLASMA CHOLINESTERASE.

1.5.1 In disease:

Since cholinesterase is synthesised in the liver the amount of enzyme appearing in the plasma is dependant both upon normal liver function and an adequate delivery of amino acids. Measurement of cholinesterase activity may therefore be used as a test of liver function or as an index of protein synthesis.

Many forms of liver disease including cirrhosis [La Motta, Williams and Wetstone 1957], chronic hepatitis [Singh et al. 1976] and malignant disease with secondary liver deposits [Ghooi, Malaviya and Kashyap 1980] have been shown to be accompanied by reduced enzyme activity. There is always reduction of esterase levels following starvation and in particular kwashiorkor [Srikantia, Jacob and Reddy 1964]. Cholinesterase activity is low in other pathological conditions including chronic renal disease [Simon et al. 1969], myocardial infarction [Chawhan et al.1982], burns [Viby-Mogensen et al. 1975], anaemias [Vorhaus and Kark 1957], myxoedema [Thompson and Whittaker 1965], tuberculosis [Vaccarezza, Wilson and Bochi 1966], Crohns disease [Sategna-Guidetta, Bianco and Durazzo 1980] and cancer [Kanaris et al. 1979]. It is probable that this is due to a secondary reduction in protein synthesis. However it is also possible that enzyme synthesis is reduced as a result of derangement of the metabolic processes of the hepatocyte by materials produced in the

diseased tissues or that enzyme activity is inhibited by toxic materials. The reduction seen in cancer is greater than that of albumin or total protein and has led to the suggestion that there is a humoral effect of cancer cells on cholinesterase production or activity [McComb, LaMotta and Wetstone 1964].

There have been many reports of alterations in cholinesterase gene frequency with disease. The majority of these have involved the observation of an increased incidence of the fluoride resistant gene. Whittaker, Spencer and Searle [1977] noted three of four patients, who had survived malignant hyperpyrexia who were heterozygous for the usual and fluoride resistant gene and that this gene was functioning in two additional families who had lost blood relatives as a consequence of the disease. This finding was supported by Ellis and colleagues [1978] but not by other workers [Evans et al. 1981]. An increased incidence of the $E1^f$ gene has also been reported in patients with Down's Syndrome [Drew and Rundle 1977], mental illness (psychosis and Huntingdon's chorea) [Whittaker and Berry, 1977] and in patients undergoing lithium therapy [Whittaker and Spencer 1977]. Studies on gene frequencies in leprosy have also yielded conflicting results some workers finding a high incidence of the atypical gene [Navarrete, Lisker and Perez-Briceno 1979] and others not [Rea and Ng 1978]. Such controversy highlights the difficulties of precise cholinesterase

phenotyping and especially in the identification of the fluoride resistant gene. Fluoride inhibition of cholinesterase is not straightforward [Heilbron 1965] and probably involves prior formation of a fluorophosphate complex which acts as the actual inhibitory agent. In addition it is a difficult inhibitor to handle in the laboratory. Some workers have suggested using only freshly prepared stock solution or adding the inhibitor immediately before analysis. Even so the degree of inhibition of the hydrolysis of benzoylcholine appears to fall during the reaction and consequently it has been advocated that measurements be taken only during the first 5-7 minutes of the assay [Evans 1986].

In routine clinical chemistry the measurement of cholinesterase has been most widely used in the investigation of liver disease. In the early stages of acute hepatitis the plasma enzyme level is slightly reduced but a substantial fall indicates the development of complications. In both chronic hepatitis and cirrhosis low to normal cholinesterase activities are found. Generally there is good correlation between the functional state of the liver and cholinesterase activity. A sudden fall in activity may indicate developing hepatic failure but if acute illness is sufficiently severe, death may occur before any change in cholinesterase is seen. This is probably due to the short half life of the enzyme, estimated between 3.4 and 16 days [Brown et al. 1981]. Cholinesterase is used as a routine liver function test in

many countries of Europe but not the United Kingdom. The wide range of cholinesterase values seen in the healthy population makes the interpretation of isolated values seen during an illness difficult. Interpretation is further hindered by the occurrence of the genetically determined abnormalities which give rise to reduced cholinesterase levels unassociated with disease. Evidence has also been presented which suggests that cholinesterase measurements do not contribute significantly more to the diagnosis and management of liver disease than other tests of liver function [Gross, Audetat and Bircher 1978]. However cholinesterase measurements have been found useful when dealing with specific liver problems such as the surgical treatment of the cirrhotic patient [Hunt and Lehmann 1960] and the monitoring of liver transplants [Evans and Lehmann 1971].

In other disease states the measurement of cholinesterase is of little importance unless administration of succinylcholine is considered. Even then reductions in enzyme activity are rarely sufficient to put patients at risk of sensitivity to the drug.

5.2 In toxicology:

Irreversible inhibition of plasma cholinesterase occurs as a consequence of poisoning with anticholinesterase insecticides, principally carbamates and organophosphates. These compounds are extremely toxic and their characteristic effects are due to inhibition of

acetylcholinesterase at the neuromuscular junction. Both erythrocyte acetylcholinesterase and serum cholinesterase can be estimated as indices of poisoning. When exposure ceases cholinesterase activity returns to normal after five to six weeks as the enzyme is resynthesised by the liver. It takes approximately four months for the red cells to be replaced and acetylcholinesterase activity to achieve normal levels. The measurement of serum cholinesterase is preferred for the screening and monitoring of this type of insecticide poisoning as it is easier to determine accurately and precisely. However it's use in this situation is not without problems, especially if baseline activities for comparison are unavailable. The wide range of cholinesterase levels seen within the population make it difficult to be sure whether poisoning has occurred particularly if the level of intoxication is low. On those occasions when cholinesterase is congenitally lowered or absent acetylcholinesterase will need to be measured as an index of poisoning.

5.3 In succinylcholine apnoea:

Succinylcholine, a depolarising neuromuscular blocking agent, was first introduced into clinical practice in 1951 and is now widely used, facilitating such procedures as intubation and electric convulsive therapy (ECT). When the drug is injected intravenously in the usual dosage, 1mg/kg, most is hydrolysed by plasma cholinesterase within the first minute [Kalow 1962]. A small fraction of the

drug reaches the post-junctional membrane at the neuromuscular junction and competes with acetylcholine for cholinergic receptors. It produces an acetylcholine like but prolonged depolarisation of the end-plate. This depolarisation spreads to the surrounding muscle fibres making them unexcitable. The duration of action of the drug is approximately 2-5 minutes. During this time, after an initial contraction, the muscle fibres passively elongate to give the relaxation required by the anaesthetist. All the skeletal muscles including the respiratory muscles are affected. Prolonged paralysis occurs whenever the breakdown of succinylcholine is reduced. This sensitivity to succinylcholine may arise in several situations; pregnancy [Evans and Wroe 1980] especially when coupled with plasmapheresis for rhesus haemolytic disease [Evans, Macdonald and Robinson 1980], liver disease [Singh et al. 1976], certain disease states especially chronic debilitating diseases, poisoning from organophosphorus insecticides [Mackey 1982], and in patients with genetically determined enzyme abnormalities [Viby-Mogensen 1983]. In all affected patients the inability to use the muscles of the chest in order to breathe requires them to be artificially ventilated until the succinylcholine is eliminated, either by spontaneous hydrolysis or excretion through the kidney.

Apnoea is most prolonged in patients with genetically determined abnormalities of cholinesterase when it may

last for several hours [Viby-Mogensen 1983]. Goedde, Held and Atland [1968] demonstrated that the atypical enzyme has a much lower affinity for succinylcholine and succinylmonocholine than the usual enzyme and thus is unable to hydrolyse the drug at pharmacological concentrations. This was later confirmed by Hobbiger and Peck [1971]. Altered affinity has not been shown for other enzyme variants but this is likely to to be the case. The genotypes most susceptible are $E1^aE1^a$, $E1^aE1^f$, $E1^aE1^s$, $E1^fE1^f$, $E1^fE1^s$ and $E1^sE1^s$. In general individuals who are heterozygotes for the usual enzyme are not sensitive to succinylcholine in the absence of complicating factors.

In cases of succinylcholine sensitivity where the enzyme is not abnormal apnoea is not so prolonged [Anon. 1973]. Viby-Mogensen [1980], in a study correllating the duration of action of succinylcholine with plasma cholinesterase activity in subjects with phenotypically usual enzyme, demonstrated that even cholinesterase activities 30% of the lower limit of normal resulted in an apnoea of only approximately 20 minutes. He suggested that the prolonged apnoeas seen in similar patients [Hunter 1966] were due to factors other than low plasma cholinesterase activity. Additional reasons for the prolonged apnoea have been postulated.

a) Enzyme inhibition [Anon. 1973]

b) Factors affecting the termination of succinylcholine action such as electrolyte concentrations [Laxenaire and Sigiel 1976].

c) A change in the character of the succinylcholine block [Lee 1975].

d) Abnormalities in the response of the muscle end plate [Brown et al. 1981].

e) Other factors such as hyperventilation and/or residual narcotic effect [Viby-Mogensen 1980].

Nonetheless from a practical point of view when enzyme activities fall 2.5 SD below the mean for Elu homozygotes there is a greatly increased risk of some degree of sensitivity.

In all analyses of patients presenting with prolonged apnoea following succinylcholine administration there is a high incidence of patients (28-50%) where both genotype and plasma cholinesterase activity are normal [Kalow 1965, Lehmann and Liddell 1969, Whittaker and Vickers 1970, Bauld et al. 1974, and Viby-Mogensen and Hanel 1978]. Bauld et al. [1974] first postulated that this might be due to the existence of abnormal genotypes not demonstrable by the biochemical methods at present employed. Variants of cholinesterase might exist which whilst able to hydrolyse substrates used in their assay cannot hydrolyse succinylcholine. To explore such a possibility succinylcholine should be used as the substrate for the measurement of plasma cholinesterase. Bauld and coworkers pointed out some of the problems this would entail and advocated the use of succinyldithiocholine, a close analogue. Agarwal et al.

[1975] developed an assay based upon succinylcholine and using this method Goedde and Agarwal [1978] claimed they were able to explain up to 90% of previously unexplained cases of abnormal reaction to succinylcholine. They maintained that there was a further gene $E1^{su}$ detectable only using this substrate. This work has not yet been substantiated.

1.6 CHOLINESTERASE IN ANIMALS.

Cholinesterase was first recognised in horse serum [Stedman, Stedman and Easson 1932] and has since been found to occur widely in the animal kingdom. Its distribution is diverse being found in mammals, reptiles [Lam 1977], birds [Earl and Thompson 1952], fish and amphibians [Augustinsson 1959].

Specificity of cholinesterase towards choline and non-choline esters, sensitivity to inhibitors, activity-substrate concentration relationships and electophoretic patterns are all species dependant [Augustinsson 1948 and Augustinsson 1959a, b, c]. The source of the enzyme must therefore be stated in any work with cholinesterase since it is not possible to apply results obtained with specimens from one species to that of another.

Plasma from rats, rabbits, chickens and frogs have propionylcholinesterases, that is have maximum activity when cholinesterase is measured using a propionylthiocholine substrate. Their properties however are different with respect to inhibitor-sensitivity,

kinetic and electrophoretic behaviour. In contrast plasma specimens from higher order vertebrates, man, horses and dogs, contains cholinesterase with a maximum activity against butyrylthiocholine. The enzymes show similar biochemical properties but have different molecular forms as shown by electrophoresis. Augustinsson [1959c and 1968] further considered the evolutionary implications of the patterns of substrate specificity. He proposed that cholinesterases and carboxylesterases had evolved from a common "serine enzyme" precursor. He suggested that during evolution cholinesterases acquired an anionic site which conferred a special ability to bind cationic substrates. Further mutational changes resulted in the development of differing specificity, butyrylcholinesterases evolving from the propionyl- type.

Although cholinesterase is present throughout the animal kingdom and, according to Augustinsson's theory, actively retained as part of an evolutionary process separate from acetylcholinesterase, it's function and natural substrates are not known. Nonetheless the measurement of animal cholinesterase does have some practical importance. It is of value in determining the degree of exposure of animals to anticholinesterase compounds [Silvestri 1977 and Crookshank and Palmer 1978] and has been used to provide information concerning the pharmacological effect of succinylcholine in different species [Wright et al. 1981].

Succinylcholine was first used as a muscle relaxant in veterinary practice in the early 1950's. It has also been

used for the translocation or culling of wild animals when, depending on the dose, it acts either to immobilise or to cause death by paralysis of the respiratory muscles [Berger et al.1983, Wright et al. 1981]. Considerable controversy surrounds such use since drug action occurs with complete retention of conciousness which aggravates the state of anxiety of the animals. Continued use of the drug in these situations cannot be condoned with the advent of powerful opioid sedative drugs which can be reversed, if necessary, by specific antagonists [Weaver, Dept. of Veterinary Medicine, Bristol University, personal comunication]. However despite this, use of succinylcholine has been advocated in translocation of horses in preference to other drugs [Berger et al. 1983] and it is still used in culling operations "for want of a better method" [Anon. 1984]. In veterinary medicine the drug is reserved for intubation of cats and for use in horses as an adjunct to thiopentone, so that if thiopentone has been slightly underdosed the handlers of the horse are not injured by any subsequent excitement reaction.

A problem complicating the use of succinylcholine is the wide species variation in sensitivity. Hansson [1957] showed considerable differences in sensitivity in four domestic animal species and others demonstrated that these also exist in the wild [Buecher, Harthoorn and Lock 1960].

Viby-Mogensen 1980 showed that plasma cholinesterase activity, in man, was related to the effect of succinylcholine and he was able to predict the duration of apnoea following a single dose of the drug. It seems likely therefore that cholinesterase activity may provide a guide to the susceptibility of different species to succinylcholine. This is important for the assessment of effective dose levels. Wright et al. [1981] investigated 26 species but concluded that plasma cholinesterase activity was unlikely to give more than a very approximate guide to their susceptibility to paralysis by the drug. However the substrate which he used for measuring cholinesterase activity was butyrylthiocholine. It may be that the ability of animal plasma to hydrolyse butyrylthiocholine in vitro is not directly related to the ability to hydrolyse and thus terminate the action of succinylcholine in vivo. A more appropriate assay system would be one based upon succinylcholine.

1.7 MEASUREMENT OF PLASMA CHOLINESTERASE.

Many techniques have been described for the measurement of plasma cholinesterase ranging from simple colorimetric procedures to assays which rely on the gas chromatographic detection of reaction products [Somorin 1978] or the use of a radiolabelled substrate [Hoover et al. 1976]. However few are amenable to routine use. Methods which have been used routinely can be divided into three groups.

a) Those which measure the liberation of acid from a cholinester.

Acid formation has been monitored as follows:

i) by measuring the carbon dioxide released from a bicarbonate-containing medium in the Warburg manometric apparatus [McArdle 1940].

ii) by the time taken to achieve a fixed pH electrometrically [Michel 1949] or colorimetrically using indicators such as phenol red [Reinhold, Tourigny and Yonan 1953] or bromothymol blue [Biggs Carey and Morrison 1958].

iii) by measuring the rate of titration required to maintain a constant pH [Alles and Hawes 1940].

iv) by monitoring the change in pH in the Astrup micro equipment [Johnson and Whitehead 1965].

b) Those which make use of chromogenic substrates.

Hydrolysis of such substrates by cholinesterase produces a compound which is coupled with an indicator to yield a product which can be measured colorimetrically.

The most widely used assays of this type are those in which a sulphydryl group is released from a thiocholine ester. This can be followed by using the nitroprusside colour reaction [McOsker and Daniel 1959], by iodometric titration [Augustinsson 1955], by coupling to the reduction of 2:6 dichlorophenolindophenol [Gal and Roth 1957], by complexing with 2,2′ or 4,4′-dithiodipyridine [Uete et al. 1972] or with dithiobis-

(2-nitrobenzoic acid) [Ellman et al. 1961].

c) Those in which the unreacted ester is measured.

This has been done in fixed-time assays using an acetylcholine substrate by means of the ferric-hydroxamate reaction [De La Huerga, Yesinick and Popper 1952] or with benzoylcholine by following the decrease in absorbance at 240 nm [Kalow and Lindsay 1955]. This last assay is probably the most widely used of all cholinesterase assays since the routine ascription of cholinesterase genotype relies predominantly on the inhibition of benzoylcholine hydrolysis.

Substrates which are the most widely used at present for measurement of cholinesterase activity are those amenable to UV/visible spectrophotometry. They include benzoylcholine and the thiocholine esters. In most laboratories the benzoylcholine assay is used to establish cholinesterase genotype and therefore, if used for activity measurements, has the advantage that results can be obtained simultaneously. It is also specific. It's disadvantages are that the λ max for benzoylcholine is 235 nm a wavelength at which protein absorbs strongly. Because of this problem the reaction is monitored at 240 nm, a wavelength not optimal for the substrate and at which some protein absorbance still occurs. Furthermore the optical density change is small which increases analytical imprecision.

Assays using thiocholine esters as substrates are very

sensitive and so measurements may be made using small volumes of plasma. The first assay of this type used acetylthiocholine as the substrate but was established to measure cholinesterase in tissue extracts and cell suspensions [Ellman et al. 1961]. It was later applied to blood by Garry and Routh [1961]. Disadvantages arise from the instability of acetylthiocholine in solution and it's nonspecificity. It is susceptible to hydrolysis by acetylcholinesterase and therefore if applied to plasma care must be taken to avoid even the slightest haemolysis. These problems have been overcome by the use of other thiocholinesters, notably butyrylthiocholine [Das and Liddell 1970] and propionylthiocholine [Dietz, Rubinstein and Lubrano 1973]. Both these substrates are more stable than acetylthiocholine in solution, are more specific to cholinesterase and are more rapidly hydrolysed. Both have been recommended as the substrate of choice for the measurement of plasma cholinesterase [Silk, King and Whittaker 1979 and Dietz, Rubinstein and Lubrano 1973].

The choice of technique for the measurement of cholinesterase activity, like any other parameter in clinical chemistry, depends upon many factors including speed and ease of assay, precision, accuracy and cost. However the main factor which should govern the choice of any assay procedure and particularly an enzyme, should be it's ability to answer the clinical question. The main clinical requirement for measurements of cholinesterase activity concerns the investigation of suspected

succinylcholine sensitivity. Only to a lesser extent is it used as a liver function test or in the investigation of poisoning by organophosphates and related compounds.

The recommendation that butyrylthiocholine be the substrate of choice for the measurement of plasma cholinesterase [Silk, King and Whittaker 1979] did not take into consideration it's ability to provide useful clinical information. A study by Evans and Wroe [1979] assessed this. They investigated a large group of patients, defined as being sensitive to succinylcholine or not according to clinical criteria or cholinesterase genotype. Cholinesterase activity was measured using benzoylcholine, acetylcholine, butyrylthiocholine and propionylthiocholine as the substrates. It was shown that, on the basis of enzyme activity alone, whilst benzoylcholine identified only 50% of affected patients, propionylthiocholine increased this to over 90%. Acetylcholine and butyrylthiocholine gave intermediate figures [Evans and Wroe 1979]. The authors therefore advocated the use of propionylthiocholine for the measurement of cholinesterase arguing that it was the substrate that maximised clinically useful information. Despite this propionylthiocholine only identified 91-96% of sensitive patients, depending on the cut-off value chosen. In theory an assay using a succinylcholine substrate should enable the identification of an increased proportion of sensitive patients since there may be, as

postulated by Bauld et al. [1971], enzyme variants which hydrolyse non-pharmacological substrates but not succinylcholine. Succinylcholine-based assays have been described but since neither the drug itself nor it's hydrolysis products have, until recently, been amenable to spectrophotometric analysis such methods have not been suitable for routine use. Previously described techniques have involved:

a) Electrophoretic separation of ^{14}C labelled succinylcholine from its products of hydrolysis by cholinesterase [Schmidinger, Held and Goedde 1966] and a similar technique using thin layer chromatographic separation [Agarwal and Goedde 1976].

b) Monitoring the rate of fall of pH, resulting from the release of the carboxyl group from succinylcholine, in the Astrup micro pH apparatus [Fishtal, Evans and Chapman 1972].

c) The use of a bioassay technique for the detection of unchanged succinylcholine [Hobbiger and Peck 1969]. d) The use of choline oxidase (EC 1.1.3.17) to measure the choline released after hydrolysis of succinylcholine by cholinesterase [Agarwal et al. 1975]. In this procedure choline oxidase was used to convert choline to betaine aldehyde. This reaction was followed by coupling it to the reduction of cytochrome c which can be monitored at 550 nm. A rat liver mitochondrial preparation was used as the source of the choline oxidase.

The recent commercial availability of choline oxidase has,

for the first time, given rise to the prospect of developing a simple analytical procedure for a succinylcholine-based cholinesterase assay which can be applied to the routine identification of succinylcholine sensitive individuals.

1.8 AIMS

1. To develop an assay for plasma cholinesterase using succinylcholine as the substrate which is applicable to use in routine clinical chemistry laboratories.

2. To evaluate the ability of the assay to define individuals at risk of sensitivity to succinylcholine and compare it to a propionylthiocholine-based assay, previously shown to be the best available for this purpose.

3. To establish whether there exist abnormal forms of cholinesterase which can hydrolyse conventional assay substrates but not succinylcholine.

4. To investigate the kinetics of the succinylcholine-cholinesterase reaction using the newly described assay.

5. To evaluate the usefulness of the succinylcholine-based assay in the assessment of liver dysfunction and compare it to established cholinesterase assays.

6. To use the succinylcholine-based assay to investigate whether the increased dose of succinylcholine required to

muscle relax neonates and infants compared to adults is due to a difference in the ability of their respective cholinesterases to hydrolyse the drug.

7. To apply the succinylcholine-based assay and three conventional assays to a variety of animal species to

i) document differences in their ability to hydrolyse a range of substrates.

ii) to establish whether they can be used to predict species sensitivity to succinylcholine.

CHAPTER 2:

MATERIALS AND METHODS.

2. 1. MATERIALS:

Adenosine tri-phosphate (ATP)	Boehringer Corp. (London) Ltd., Lewes, U.K.
Amicon Centriflo Membrane cones.Type CF25.	Amicon Corporation Lexington, Mass., U.S.A.
Aminophenazone	B.D.H Chemicals Ltd., Poole, U.K.
Benzoylcholine chloride	B.D.H Chemicals Ltd., Poole, U.K.
B-Nicotinamide adenine dinucleotide (B-NAD)	Boehringer Corp., (London) Ltd., Lewes, U.K.
Butyrylthiocholine iodide	Sigma Chemical Co., Poole, U.K.
Calcium chloride	Sigma Chemical Co., Poole, U.K.
Choline chloride	Sigma Chemical Co., Poole, U.K.
Choline iodide	Sigma Chemical Co., Poole, U.K.
Choline kinase	Boehringer Corp., (London) Ltd., Lewes, U.K.
Choline oxidase	Boehriner Corp., (London Ltd., Lewes, U.K. and Sigma Chemical Co., Poole, U.K.
Cholinesterase (purified human)	Sigma Chemical Co., Poole, U.K. and Behringwerks AG, Marburg, Hoechst Pharma AG, Zurich, Switzerland.

Dibucaine hydrochloride	Ciba Laboratories, Horsham, West Sussex.
Dimethylsulphoxide (DMSO)	B.D.H Chemicals Ltd., Poole, U.K.
Disodium hydrogen phosphate	Sigma Chemical Co., Poole, U.K.
5,5´-dithiobis- (2-nitrobenzoic acid) DTNB	Sigma Chemical Co., Poole, U.K.
Intralipid	Kabivitrum U.K. Ltd., Uxbridge, Middlesex.
Lactate dehydrogenase	Sigma Chemical Co., Poole, U.K.
Peroxidase	Hughes and Hughes, Romford, U.K.
Phenol	B.D.H Chemicals Ltd., Poole, U.K.
Potassium dihydrogen phosphate	Sigma Chemical Co., Poole, U.K.
Propionylthiocholine iodide	Sigma Chemical Co., Poole, U.K.
Pyruvate kinase	Boehringer Corp. (London) Ltd., Lewes, U.K.
Qualify II, Freeze dried control material.	American Monitor U.K. Ltd., Burgess Hill, West Sussex.
Ro 02-0683 dimethylcarbamate of (2-hydroxy-5-phenylbenzyl) trimethylammonium bromide	Roche Pharmaceuticals Ltd., Welwyn Garden City, U.K.
Sodium fluoride	Sigma Chemical Co., Poole, U.K.
Succinylcholine chloride	Sigma Chemical Co., Poole, U.K.
Tris [tris-(hydroxymethyl) -aminomethane]HCL	Sigma Chemical Co., Poole, U.K.
Triton X-100	B.D.H Chemicals Ltd., Poole, U.K.

2.2 ANALYTICAL METHODS:

2.2.1 MEASUREMENT OF ENZYME ACTIVITY.

All measurements of enzyme activity were performed using either a Pye Unicam SP1800 spectrophotometer or on a Pye Unicam PU8800 UV/VIS spectrophotometer incorporating a BBC microcomputer (Pye Unicam Instruments Ltd., Cambridge, U.K.).

a) Cholinesterase activity measured using benzoylcholine as the substrate.

This assay was performed as described by Kalow and Lindsay [1955]. It is a reaction rate assay in which cholinesterase activity is calculated by monitoring the decrease in absorbance of unreacted benzoylcholine at 240 nm. At this wavelength benzoylcholine has a much greater absorbance than it's products of hydrolysis, benzoic acid and choline.

i) Reagents:

Phosphate buffer: 0.067 mol/L, pH 7.4. 47.98g $Na_2HPO_4.2H_2O$ and 8.665g KH_2PO_4 in five litres of water.

Substrate: Benzoylcholine iodide.

Stock solution: $2x10^{-3}$ mol/L. 48.75mg in 100mL phosphate buffer. The solution was stored at $4^{o}C$ for a maximum of 7 days.

Working solution: $2x10^{-4}$ mol/L. Stock solution was diluted 1 in 10 in phosphate buffer and prepared freshly.

ii) Procedure:

Serum was diluted 1 in 100 in phosphate buffer. 2mL was added to 1mL phosphate buffer and 1mL substrate. The reaction was monitored at 240 nm and 25^{o}C against a blank solution with substrate replaced by buffer.

Enzyme activity (IU/mL) is calculated by multiplying the change in absorbance per minute by 30.3.

Precision was assessed using Qualify II, a commercially prepared freeze-dried quality control material. The assay had a between batch coefficient of variation of 6.4% (n=12), at 0.45 IU/mL.

(ii) Cholinesterase activity measured using butyrylthiocholine as the substrate:

The method of Das and Liddell [1970b]was used in which cholinesterase hydrolyses butyrylthiocholine to give butyric acid and thiocholine, the free sulphydryl of which reacts with 5,5´-dithiobis-(2-nitrobenzoic acid)(DTNB). This yields the 5-thio-2-nitrobenzoate ion which has an absorbance maximum at 410 nm. DTNB absorbs maximally at 320nm.

i) Reagents:

Phosphate buffer: 0.067 mol/L., pH 7.4.47.98g $Na_2HPO_4.2H_2O$ and 8.665g KH_2PO_4 in 5L of water.

Substrate: Butyrylthiocholine iodide 225 mmol/L.

7.137g in 100mL phosphate buffer.

DTNB: 0.27 mmol/L.

214mg in 2L phosphate buffer. The solution was stored at 4^{o}C in a dark glass bottle.

ii) Procedure:

10 or 20 ul plasma and 100 uL butyrylthiocholine iodide were added to 2.9 mL aliquots of DTNB, mixed and the reaction monitored at 408 nm and 25°C against a blank solution in which the serum had been replaced by buffer. Enzyme activity (IU/mL) was calculated by multiplying the change in absorbance per minute by 221/uL of sample.

A commercially prepared freeze-dried quality control material, Qualify II, was used to assess precision. The assay had a between batch coefficient of variation 5.1% (n=12), at 2.67 IU/mL.

(iii) Cholinesterase activity measured using propionylthiocholine as substrate:

The assay principle is the same as described for butyrylthiocholine. The method of Dietz, Rubinstein and Lubrano [1973] was used. However two modifications were made as their assay showed an inconveniently high sensitivity. The temperature was reduced to 25°C and the volume of diluted serum also reduced, from 1000 to 500 uL. The final volume, 5 mL, was achieved by increasing the volume of DTNB solution from 3 to 3.5 mL. These modifications diminished the rate of increase in absorbance for $E1^u$ homozygotes from a mean value of 0.25 units per minute to 0.07 units per minute. This enabled 10 measurements to be made at 1-minute intervals, thus increasing the accuracy of calculation of the rate of absorbance change without exceeding the capacity of the

spectrophotometer [Evans and Wroe 1978].

i) Reagents:

Phosphate buffer: pH 7.6.

Separate solutions of 13.61g KH_2PO_4 per litre and 7.413g $Na_2HPO_4.2H_2O$ per litre were mixed to give a final pH of 7.6.

DTNB: 0.423 mmol/L.

334mg in 2L of phosphate buffer. This was stored at 4°C in a dark glass bottle.

Substrate: Propionylthiocholine iodide 20mmol/L.

6.064g in 1L phosphate buffer. This was stored at -20°C in aliquots of various volumes and thawed as required.

ii) Procedure:

Serum was diluted 1 in 100 in phosphate buffer. 0.5mL was added to 3.5mL DTNB and mixed. 1mL substrate was added, the reagents mixed again and the reaction monitored at 408nm and 25°C against a blank solution in which diluted serum was replaced by buffer.

Enzyme activity (IU/mL) was calculated by multiplying the optical density change per minute by 73.67.

Precision was assessed using Qualify II, a freeze-dried quality control material.

The assay had a precision of 3.0% (n=12), at 2.82 IU/mL.

2.2.2 ASCRIPTION OF CHOLINESTERASE GENOTYPE.

This was made on the basis of inhibition of benzoylcholine hydrolysis by dibucaine [Kalow and Genest 1957], fluoride [Harris and Whittaker 1961] and the dimethylcarbamate of

(2-hydroxy-5-phenylbenzyl) trimethylammonium bromide (Ro 02-0683) [Liddell, Newman and Brown 1963] and where possible, family studies.

i) Reagents;

Phosphate buffer: 0.067 mmol/L pH 7.4.

47.98g $Na_2HPO_4.2H_2O$ and 8.665g KH_2PO_4 in 5L of water.

Substrate: Benzoylcholine iodide.

Stock solution: $2x10^{-3}$ mol/L. 48.75mg in 100mL phosphate buffer. This was stored at $4^{o}C$ for a maximum of 7 days.

Working solution: $2x10^{-4}$ mol/L. Stock solution was diluted 1 in 10 with phosphate buffer and prepared freshly.

Inhibitors;

Dibucaine hydrochloride.

Stock solution: $4x10^{-4}$ mol/L.

15.2mg in 100mL phosphate buffer. This was stored frozen in 10-20mL aliquots and thawed as required.

Working solution: $4x10^{-5}$ mol/L.

Stock was diluted 1 in 10 with phosphate buffer and prepared freshly on day of test.

Ro 02-0683.

Stock solution: $1x10^{-4}$ mol/L and $1x10^{-6}$ mol/L.

39.6mg in 1L phosphate buffer and 1 in 100 dilution with phosphate buffer. These were stored at $4^{o}C$ at which temperature they are stable indefinitely.

Working solution: $4x10^{-8}$ mol/L.

4mL $1x10^{-6}$ mol/L is made up to 100mL with phosphate buffer and stored at $4^{o}C$.

Sodium fluoride,

Stock solution: $2x10^{-3}$ mol/L.

84mg in 1L phosphate buffer. This was stored at room temperature.

Working solution: $2x10^{-4}$ mol/L. A 1 in 10 dilution of stock in buffer was made on the day of test.

b) Procedure:

Serum was diluted 1 in 100 using phosphate buffer. 2 mL was added to each of 4 tubes. 1mL buffer was added to tube 1 and 1mL of an inhibitor added to the remainder. Inhibition of enzyme activity by Ro 02-0683 requires a minimum of two hours preincubation at room temperature before the addition of 1mL of substrate. Fluoride and dibucaine are added prior to the addition of substrate. The reaction was monitored at 26°C and 240nm. The inhibitor numbers (percentage inhibition of benzoylcholine activity by each of the inhibitors) were calculated as follows:

$$\text{Inhibitor Number} = \frac{100(v-v')}{v}$$

where v is the uninhibited velocity and v′ is the velocity in the presence of inhibitor.

2.2.3 PREPARATION OF ICTERIC SERA.

The method of Billing, Haslam and Wald [1971] was used.

a) Reagents:

Dimethylsulphoxide (DMSO).

Bilirubin: 98 mg in 20 mL dimethylsulphoxide.

Serum: A serum pool was made by mixing the residue of samples sent to the laboratory for routine investigations.

b) Procedure:

Bilirubin was dissolved in DMSO. Warming at 37°C for 1 hour ensured complete solution. 400, 200 and 100 uL aliquots of the bilirubin solution were each made up to 10 mL with serum to give samples with approximate bilirubin values of 340, 170 and 85 umol/L respectively. Assayed values were 365, 195 and 105 umol/L respectively measured on a Unistat bilirubinometer (A.O Scientific Instruments). The higher observed values were due to the bilirubin present in the pooled sera used to make the dilutions.

2.2.4 ULTRAFILTRATION OF SERUM.

Amicon Centriflo membrane cones of type CF25 were used for ultrafiltration. Before use the cones were soaked in distilled water for at least one hour then blotted and centrifuged to remove excess water. Serum was added to the cones and centrifuged at 850 g for 20 minutes. This concentrated high molecular weight molecules by the removal of an ultrafiltrate consisting of serum water and dissolved crystalloids.

2.2.5 LIVER FUNCTION TESTS.

These were performed on automated equipment either an RA1000 analyser (Technicon Ltd., Basingstoke) in the Department of Chemical Pathology, Leeds General Infirmary or on a Parallel analyser (American Monitor U.K. Ltd., Burgess Hill, West Sussex) in the Department of Chemical

Pathology, St. James's University Hospital, Leeds.

a) RA1000:

Bilirubin, aspartate transaminase (EC 2.6.1.1) and alkaline phosphatase (EC 3.1.3.1) were measured using manufacturers reagents by assays based on the following methods, bilirubin [Jendrassik and Grof 1938], aspartate transaminase [Kessler et al. 1971] and alkaline phosphatase [Bessey, Lowry and Brock 1957].

b) Parallel:

Total protein, albumin, bilirubin, alanine transaminase and alkaline phosphatase were measured using manufacturers reagents assays based on the following methods, total protein by a method employing the biuret reaction, albumin by a dye binding assay using bromocresol green [Rodkey 1965], bilirubin by a method based on that of Jendrassik and Grof [1938], alanine transaminase (EC 2.6.1.2) [Karmen, Wroblewski and La Due 1955] and alkaline phosphatase [Bowers and McComb 1975].

2.3 STATISTICAL METHODS.

Statistical analyses were performed on either a Digico Consort 20 Computer (Digico Ltd., Hemel Hempstead, U.K.) using "in-house" software (St. James's University Hospital) or on an Amstrad PC1512 computer (Amatrad Electronics plc, Brentwood, U.K.) using Oxstat 2 (Medstat Ltd., Nottingham, U.K.).

Enzyme kinetic data was analysed on a BBC Microcomputer [Acorn Computers, Cambridge, U.K.] using a program

obtained from the Biochemistry Department, University of Leeds [England 1984]. The program is based on one for non-linear regression and allows data to be fitted to a series of predefined equations representing the more commonly occuring mechanisms. The equation:

$$v = Vmax /(1 + Km/[S])$$

representing Michaelis-Menten kinetics was used.

CHAPTER 3:

THE DEVELOPMENT OF A SUCCINYLCHOLINE-BASED ASSAY FOR SERUM CHOLINESTERASE.

Assays for cholinesterase which use succinylcholine as a substrate have been described before. However, because of their complexity, none have easily been applied to routine use.

The aim of this section of the project was to develop a simple assay which could be used in any clinical chemistry laboratory.

The approach taken was to base an assay on the measurement of the choline released following succinylcholine hydrolysis.

In 1975 Agarwal and coworkers described an assay for cholinesterase based on a succinylcholine substrate in which the choline generated was measured enzymatically as follows:

$$\text{Succinyldicholine} + H_2O \xrightarrow{\text{cholinesterase}} \text{succinylmonocholine} + \text{choline}$$

$$\text{Choline} \xrightarrow{\text{choline oxidase}} \text{betaine aldehyde} + 2H$$

$$\text{cytochrome C} + 2H \xrightarrow{\text{cytochrome C oxidase}} \text{reduced cytochrome C}$$

A rat liver mitochondrial preparation was used as the source of choline oxidase (EC 1.1.3.17) and cytochrome C oxidase. Reduced cytochrome C was measured at 550 nm after the addition of potassium cyanide to prevent reoxidation. There are two drawbacks to the routine use of such a

method; the extraction of rat liver mitochondria and the use of toxic potassium cyanide.

Two developments allowed me to overcome these drawbacks.

a) The commercial availability of choline oxidase from Arthrobacter globiformis (Boehringer Corp. (London) Ltd., Lewes, U.K.).

b) The description of an alternative indicator reaction [Takayama et al. 1977]. In this reaction all the reagents are in common use in clinical chemistry laboratories and are less toxic than potassium cyanide.

The assay described by Takayama and coworkers measured phospholipids. Phospholipase D splits lecithin, lysolecithin and sphingomyelin to yield choline. Using choline oxidase this is converted to betaine with the simultaneous formation of hydrogen peroxide. Hydrogen peroxide, in the presence of peroxidase (EC 1.11.1.7), converts phenol and 4-aminophenazone to a red quinone dye in proportion to the amount of choline liberated. The change in optical density at 500 nm (absorbance maximum for the red quinone dye), following incubation at $37^{o}C$ for 20 minutes, is compared to that of a standard lecithin solution and the phospholipid concentration calculated.

To produce a succinylcholine-based assay for cholinesterase it was necessary to replace the step involving the splitting of lecithin by one involving the hydrolysis of succinylcholine by cholinesterase. The basis of this assay is as follows:

$$\text{Succinyldicholine} + H_2O \xrightarrow{\text{cholinesterase}} \text{succinylmonocholine} + \text{choline}$$

$$\text{Choline} + O_2 + 2H_2O \xrightarrow{\text{choline oxidase}} \text{betaine} + 2\,H_2O_2$$

$$2\,H_2O_2 + \text{phenol} + \text{4-aminophenazone} \xrightarrow{\text{peroxidase}} \text{quinone dye} + 2H_2O$$

3.1 Preliminary Investigations.

In initial experiments concentrations of reagents were as recommended by Takayama and colleagues; 100 IU choline oxidase, 220 IU peroxidase, 12 mg 4-aminophenazone, 20 mg phenol and 8mg calcium chloride dihydrate in 100 mL 50 mmol/L Tris/HCl buffer (pH 7.8 and containing 2g Triton X-100 per litre). Hereafter referred to as colour reagent. For the assay of cholinesterase it was necessary to reduce the pH of the solution to 7.4 to avoid spontaneous hydrolysis of succinylcholine.

50 uL of 10 mmol/L succinylcholine and 50 uL of serum with high cholinesterase activity were incubated at 37^{o}C with 3 mL of colour reagent. The reaction was monitored at 500 nm for 40 minutes against a colour reagent blank. The optical density change was linear over this period. As commercially prepared choline oxidase is expensive the colour reagent volume was reduced to 1 mL and the experiment repeated. The reaction remained linear.

3.2 Determination of the most appropriate blank solution for use in the assay.

a) Five different sera were each added to colour reagent

and monitored for 10 minutes against colour reagent alone. No optical density increase was observed using any of the sera and it was concluded that endogenous choline did not interfere with the assay.

b) Colour reagent alone was observed against a water blank for 10 minutes and no optical density increase was observed.

c) Four stock succinylcholine concentrations of 10, 25, 50 and 100 mmol/L (equivalent to "in assay" concentrations of 0.454, 1.14, 2.27 and 4.54 mmol/L repectively) were added to colour reagent and each measured five times against colour reagent alone over 10 minutes. An increase in optical density was observed, proportional to the concentration of substrate. Results are shown in Fig. 3.1.

It was concluded that a blank containing substrate would be essential to offset the non-enzymatic hydrolysis of succinylcholine.

3.3 Investigation of linearity.

A linear relationship between cholinesterase activity and reaction rate was established by assaying the following sera undiluted and diluted in buffer to provide sample pools with 75, 50, 25 and 10% of the original serum.

a) a serum with an average cholinesterase activity.

b) serum which had been concentrated by ultrafiltration using Amicon Membrane Cones.

Results are shown in Fig. 3.2. The assay is linear up to 145 IU/L (r= 0.995).

3.4 Calibration.

The assay was standardised using choline as the quantity of dye produced is stoichiometrically equivalent to the amount of choline oxidised. Three quantities of choline chloride 25, 50 and 100 nmoles were added to colour reagent, in quadruplicate, and the maximum amount of colour produced noted. Results are shown in Fig. 3.3. The relationship between choline added and final absorbance recorded is linear (r= 0.9985). The y on x regression equation derived from the data is y= 0.0108x + 0.0125. From this a factor can be derived to convert optical density change per minute to enzyme activity.

Cholinesterase catalyses the hydrolysis of succinylcholine as follows:

1. Succinyldicholine $\xrightarrow{\text{cholinesterase}}$ succinylmonocoline + choline

2. Succinylmonocholine $\xrightarrow{\text{cholinesterase}}$ succinic acid + choline

As reaction 2 proceeds much more slowly than reaction 1 [Goedde, Held and Atland 1968], one mole of choline can be considered to be derived from one mole of succinylcholine.

1 unit of enzyme activity is defined as the amount which will catalyse the conversion of 1 umole of substrate per minute.

The optical density change (Δ OD) produced by 100 nmoles choline is 1.09 absorbance units (AU).

Therefore the Δ OD produced by 1 umole choline is 10.9 AU.

However in the experiment 50 uL of choline was substituted for 50 uL of substrate but no replacement was made for the 50 uL serum present in an assay. Therefore the Δ OD produced by 1 umole choline must be corrected to:

$$10.9 \times \frac{1.05}{1.1} = 10.4 \text{ AU}$$

1 IU enzyme activity = 10.4 AU

$$\text{An enzyme activity of x IU} = \frac{\Delta \text{OD per minute}}{10.4}$$

but under the conditions of the assay this enzyme activity (x) is in 50 uL serum. Therefore in 1 L serum:

$$\text{Enzyme Activity (IU/L)} = \frac{\Delta \text{OD per minute}}{10.4} \times 20{,}000$$

The factor to convert ΔOD per minute to enzyme activity in IU/L becomes 1923.

3.5 Investigation of the most appropriate substrate concentration.

Sera from one individual homozygous for the usual form of cholinesterase and one homozygous for the atypical form were assayed five times at each of six substrate concentrations, 0.0454, 0.227, 0.454, 1.14, 2.27 and 4.54 mmol/L. The results are shown in Fig. 3.4. The usual enzyme form exhibited substrate inhibition at

assay concentrations of succinylcholine in excess of 1.14 mmol/L whereas the atypical enzyme had a cholinesterase activity which increased with substrate concentration throughout the range of succinylcholine concentrations tested. Reaction rate was maximal for the usual enzyme at a concentration of 1.14 mmol/L. Since this concentration differentiated well the between the two enzyme forms it was selected for use in subsequent assays.

3.6 Investigation of the effect of altering the concentrations of the components of the colour reagent.

Concentrations of reagents in the indicator reaction; aminophenazone, peroxidase, choline oxidase and phenol, were checked to ensure they were in excess and not inhibiting other stages of the reaction.

In this series of experiments the concentrations of reagents were those shown in Section 3.1 except for that of the reagent under test. This was altered to give values of 25, 50, 100, 150 and 200% of the original. A serum sample from one of two individual homozygous for the usual cholinesterase variant was assayed five times at each concentration. Results are shown in Figs. 3.5-3.8. Cholinesterase activities were tested, using an unpaired T-test, against those obtained with the original reagent concentration to see if they had changed significantly. The only reagent which produced significantly different results with a change in

concentration was choline oxidase. A reduction to 50 or 25% of the original concentration gave significantly lower values for serum cholinesterase ($p<0.001$). As increasing the concentration of choline oxidase to 150 or 200% did not further alter the results obtained the concentration of choline oxidase used was maintained at 1 IU/mL colour reagent.

3.7 The final format of the method.

Reagents:

Calcium chloride: 7.8 g in 100 mL water.

Aminophenazone: 6.0 g in 100 mL water.

Buffer: Tris HCL, 50 mmol/L, pH 7.4, containing 2 g Triton X-100 detergent per litre.

Colour reagent: 50 IU choline oxidase, 110 IU peroxidase and 10 mg phenol in 50 mL buffer to which is added 100 uL each of calcium chloride and 4-aminophenazone solutions. The solution is stored at $4^{o}C$ and made freshly each week.

Substrate: Succinylcholine chloride 25 mmol/L in water. This reagent is stable for up to 4 weeks when stored at $4^{o}C$.

Procedure;

50 uL of substrate and 1 mL of colour reagent are mixed in a cuvette and allowed to equilibrate at $37^{o}C$ for 3 minutes, 50uL serum is added, mixed, and the rate of change in absorbance measured against a blank solution in which the serum has been replaced by buffer.

Enzyme activity, defined as micromoles of choline liberated per minute per litre of serum is determined by use of a factor:

Enzyme activity (IU/L) = ΔOD per minute x 1923

3.8 Imprecision:

The within and between-run coefficients of variation for the assay were determined by replicate analysis of serum samples drawn from volunteer $E1^u$ homozygotes with normal cholinesterase activity. Serum for the assessment of the within batch coefficient of variation was analysed on the day of sampling. Serum for the assessment of between batch coefficient of variation was aliquoted and stored at -20^{o}C until analysis.

The within batch coefficient of variation was 4.5% (n=20) for a sample with a mean cholinesterase activity of 78 IU/L. The between batch coefficient of variation, assessed over a 2 week period, was 5.6% (n=11) for a sample with a mean cholinesterase activity of 55.8 IU/L.

3.9 Investigation of interferences.

a) Lipaemia.

A serum pool was made lipaemic by the addition of Intralipid such that lipid concentrations of approximately 2g/dL (twice the upper normal value for total serum lipids) and 4g/dL were obtained. However these samples could not be analysed spectrophotometrically due to their high initial

absorbance. In an attempt to produce physiological turbidity a volunteer ate a large fatty meal and samples were drawn for cholinesterase analysis before and one and a half hours post ingestion. The resulting samples were clear and turbid respectively. No significant difference in cholinesterase activity was observed between them using an unpaired T-test ($p=0.165$, $n=5$). Results are shown in Table 3.1.

b) Icterus.

Pooled serum (bilirubin 21 umol/L) was made icteric according to the method of Billing, Haslam and Wald [1971]. Samples were prepared with bilirubin values of 105, 195 and 365 umol/L. Their cholinesterase activities were compared with the cholinesterase activity of the pooled starting serum using an unpaired T-test. Results are shown in Table 3.2. The samples with bilirubin values of 105 umol/L were not significantly different ($p=0.364$, $n=6$). However at a bilirubin levels greater than 195 umol/L there was a reduction in cholinesterase activity of 9.3% ($p<0.05$, $n=6$). At 365 umol/L there was a 23% reduction in cholinesterase activity.

c) Haemolysis.

The effect of haemolysis was investigated by comparing cholinesterase values in a serum sample from an Elu homozygote some of which had been separated conventionally and the rest separated after the sample had been frozen and then thawed giving a serum sample which was moderately haemolysed (pink/red to the eye).

Replicate analysis of the two samples showed no significant difference in the cholinesterase activities using an unpaired T-test ($p=0.638$, $n=4$). Results are shown in Table 3.3.

3.10 Reference Range.

Cholinesterase reference ranges were established by assaying serum obtained from male laboratory staff (ages 23-45 years, n=39) and from nonpregnant women of reproductive age who were not taking oral contraceptive medication (n=50). No subjects were included who suffered from any condition likely to affect cholinesterase activity or who possessed genetic abnormalities identifiable by measurement of dibucaine and fluoride inhibition. Two females were excluded from the original group, having been typed $E1^uE1^a$. Succinylcholine activities are shown in Table 3.4 and Fig. 3.9.

The reference ranges (mean = 2 SD) for serum from $E1^u$ homozygotes (phenotypically usual since the presence of J/K variants cannot be excluded by biochemical tests) individuals are 45-86 IU/L for males and 34-72 IU/L for females. These are significantly different ($p < 0.005$), in agreement with reports for assays using other substrates [Siddell and Kaminskis 1975]. The combined reference range for both sexes is 35-82 IU/L.

TABLE 3.1.

CHOLINESTERASE ACTIVITIES IN SERUM SAMPLES TAKEN FROM A VOLUNTEER BEFORE AND AFTER A FATTY MEAL.

	CHOLINESTERASE ACTIVITY (IU/L)	
	PRE	POST PRANDIAL (90 minutes)
	80.8	88.5
	92.3	84.6
	84.6	88.5
	84.6	92.3
	92.3	88.5
mean	86.9	88.5

No significant difference p=0.165.

TABLE 3.2.

CHOLINESTERASE ACTIVITIES IN A SERUM SAMPLE BEFORE AND AFTER THE ADDITION OF BILIRUBIN:

	BASAL	AFTER THE ADDITION OF BILIRUBIN		
Bilirubin: (umol/L)	21	105	195	365
Cholinesterase activity (IU/L)	71.2	71.2	71.2	57.7
	73.1	73.1	73.1	50.0
	76.9	71.2	71.2	53.8
	73.1	76.9	71.2	63.5
	73.1	69.2	65.4	55.8
	73.1	79.6	65.4	57.7
mean	73.4	73.5	69.6	56.4
		p=0.364	p=0.039	p<0.001

There was no significant difference in the results after the addition of bilirubin up to 105 umol/L. At a bilirubin level of 195 umol/L and greater cholinesterase activity was significantly reduced.

TABLE 3.3.

CHOLINESTERASE ACTIVITIES IN A SERUM SAMPLE BEFORE AND AFTER HAEMOLYSIS:

CHOLINESTERASE ACTIVITY (IU/L)	
PRE	POST HAEMOLYSIS
53.8	55.8
53.8	53.8
53.8	51.9
53.8	51.9
mean 53.8	53.4

No significant difference (p=0.638).

TABLE 3.4

SUCCINYLCHOLINE ACTIVITIES IN HEALTHY, PHENOTYPICALLY USUAL INDIVIDUALS.

FEMALE ACTIVITIES (IU/L)		MALE ACTIVITIES (IU/L)	
79.8	40.4	62.5	60.6
65.4	61.5	43.3	75.0
67.3	63.5	66.4	76.0
50.9	46.2	65.4	80.8
71.2	65.4	51.9	63.5
55.8	50.0	63.4	75.0
66.4	59.6	63.4	80.8
49.0	57.7	67.3	72.2
48.1	44.2	80.8	60.0
44.2	50.0	59.6	80.8
55.8	44.2	46.2	85.6
44.3	40.4	57.7	74.0
66.4	57.7	67.3	63.5
53.8	53.8	59.6	50.2
55.8	48.0	48.1	
62.5	44.2	75.0	
53.8	38.5	59.6	
45.2	61.5	80.8	
40.4	55.8	69.2	
50.2	44.2	69.2	
67.4	44.2	62.3	
57.8	46.2	55.8	
42.4	61.5	61.6	
42.4	50.0	62.5	
42.2	51.9	63.5	
mean	53.2	65.7	
lSD	9.6	10.4	

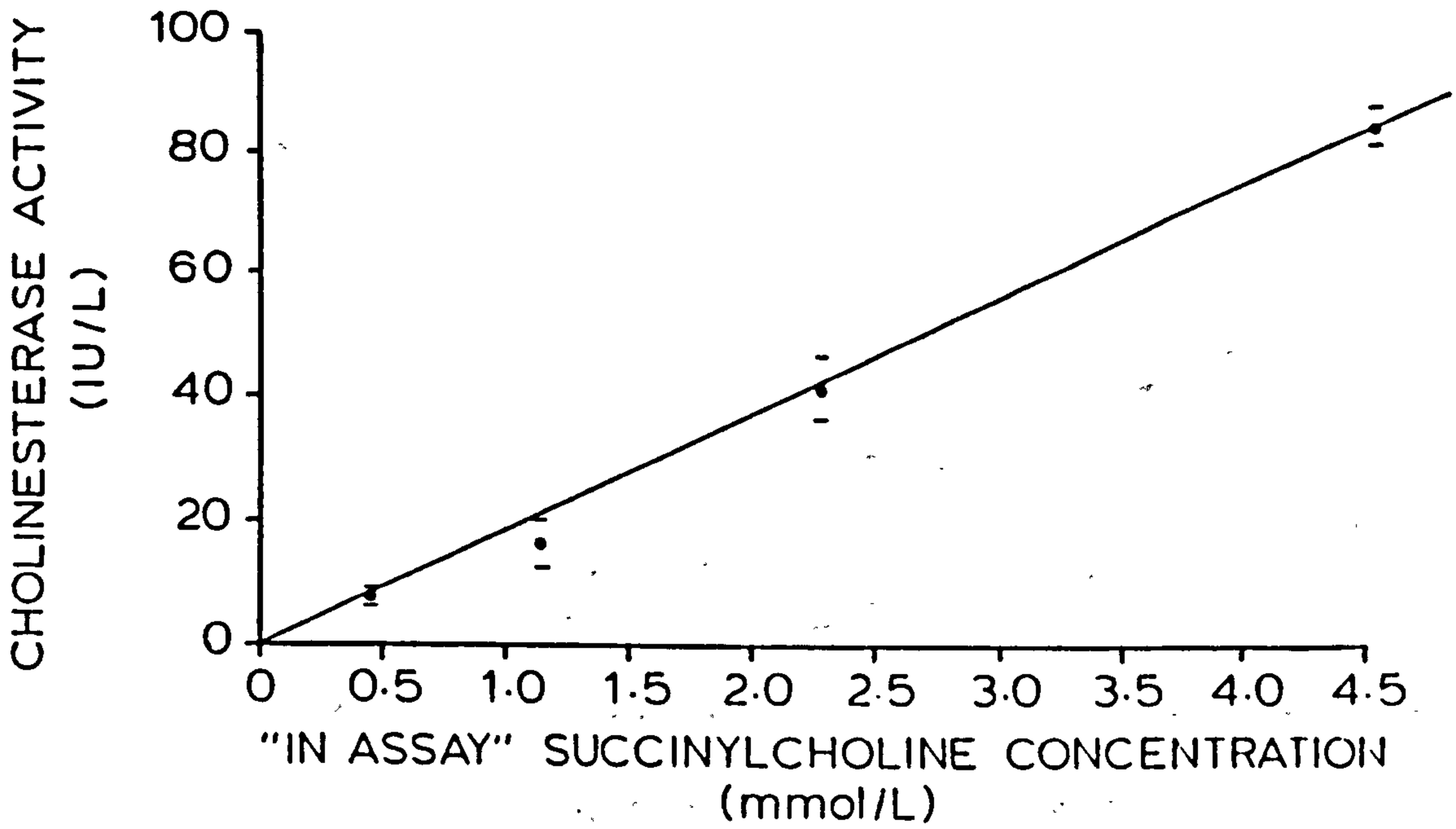

Figure 3.1

Equivalents of cholinesterase activity shown by spontaneous hydrolysis of a range of succinylcholine concentrations.

Symbols denotate mean ± 1SD.

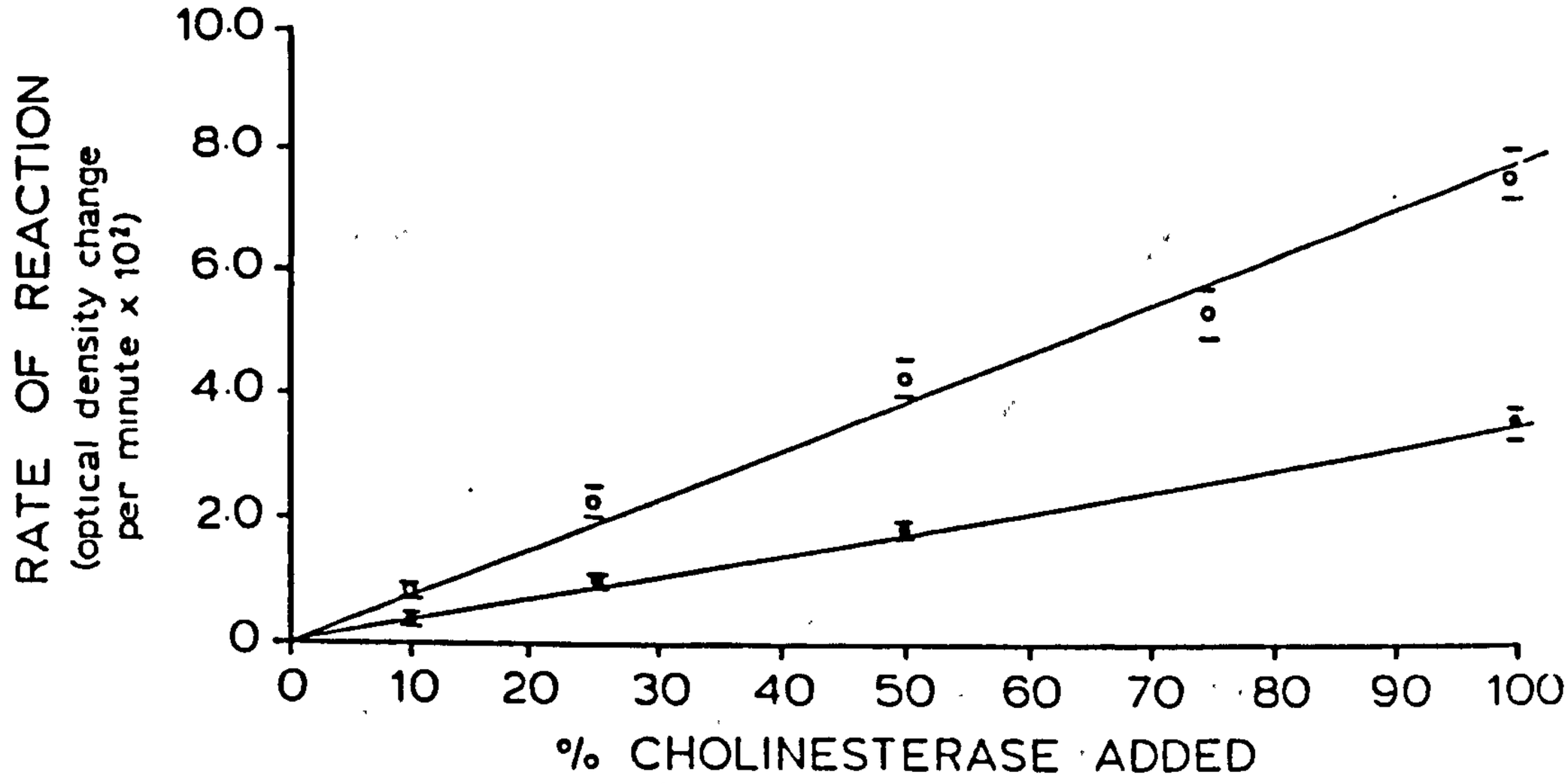

Figure 3.2

The relationship between cholinesterase activity & reaction rate.

Open circles represent ultrafiltrated serum; r=0.995

Closed circles represent serum of average cholinesterase activity; r=0.993

Mean ± 1SD. n=5

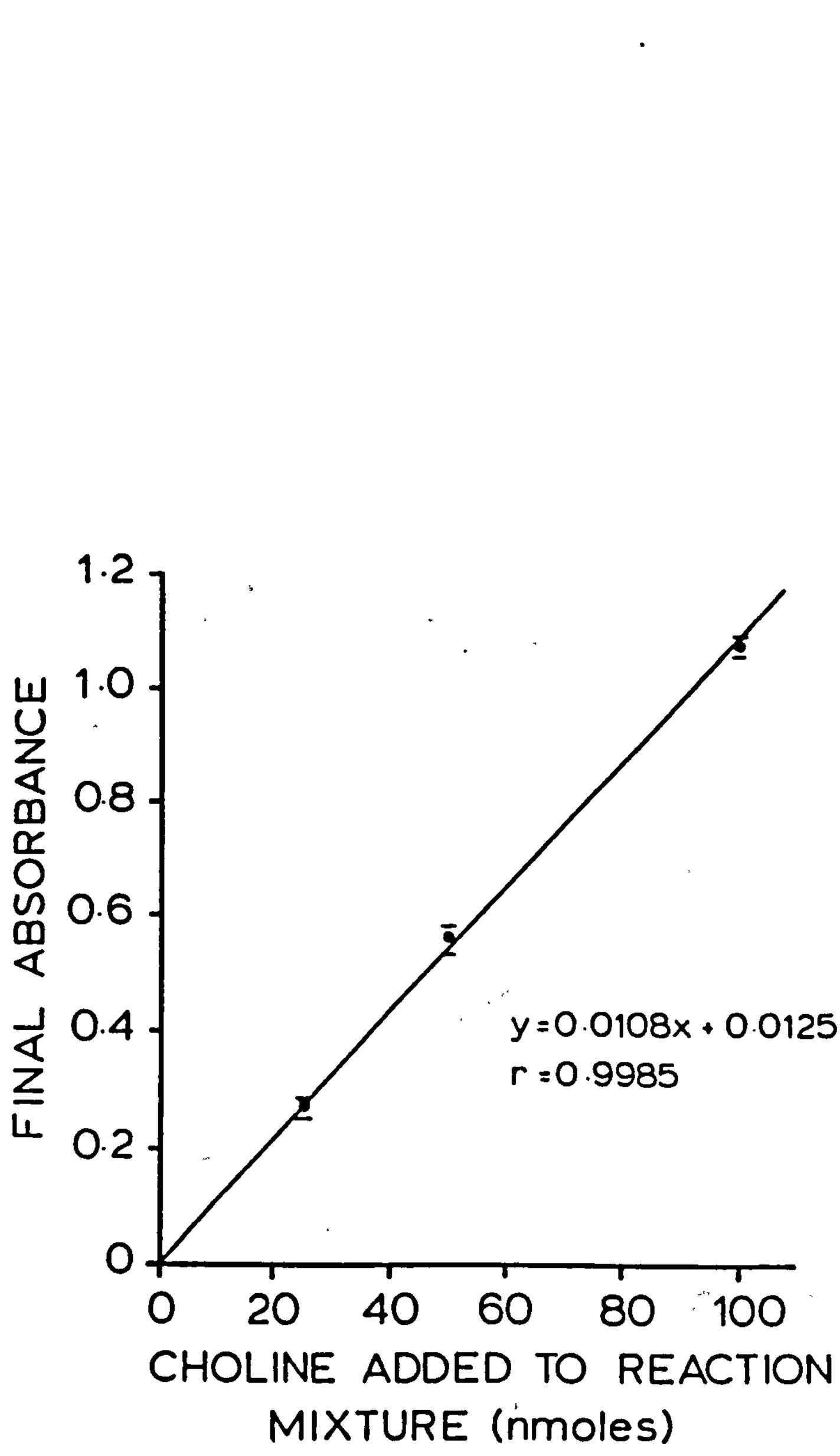

Figure 3.3

Absorbance produced by the addition of increasing amounts of choline chloride to the reaction mixture.

Symbols denote mean ± 1 SD. n=4

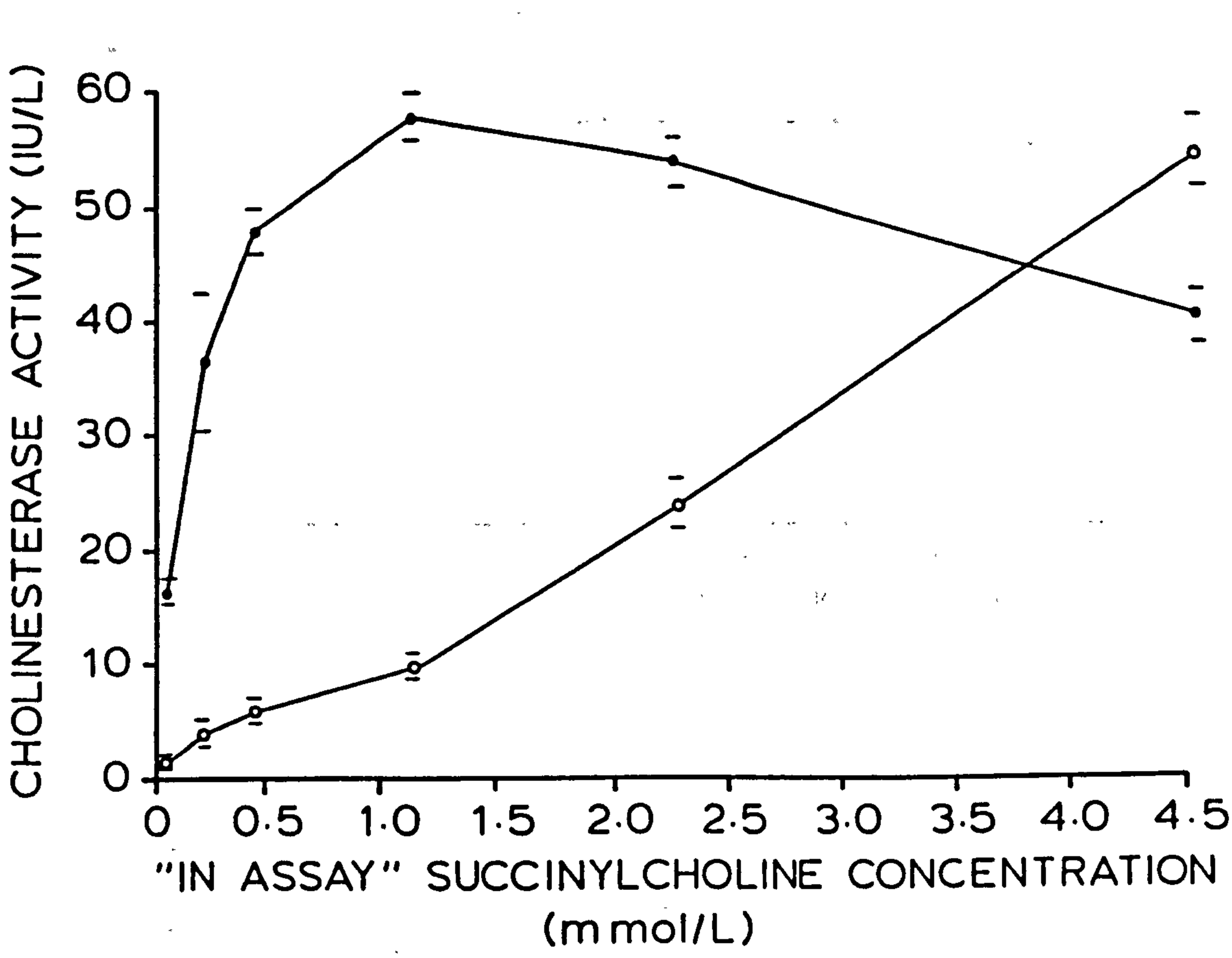

Figure 3.4

Cholinesterase activities in sera from individual homozygous for the usual gene & one homozygous for the atypical gene measured at different succinylcholine concentrations.

Closed circles represent serum from the $E1^uE1^u$ individual.

Open circles represent serum from the $E1^aE1^a$ individual.

Mean $\pm$ 2 SD for 5 determinations.

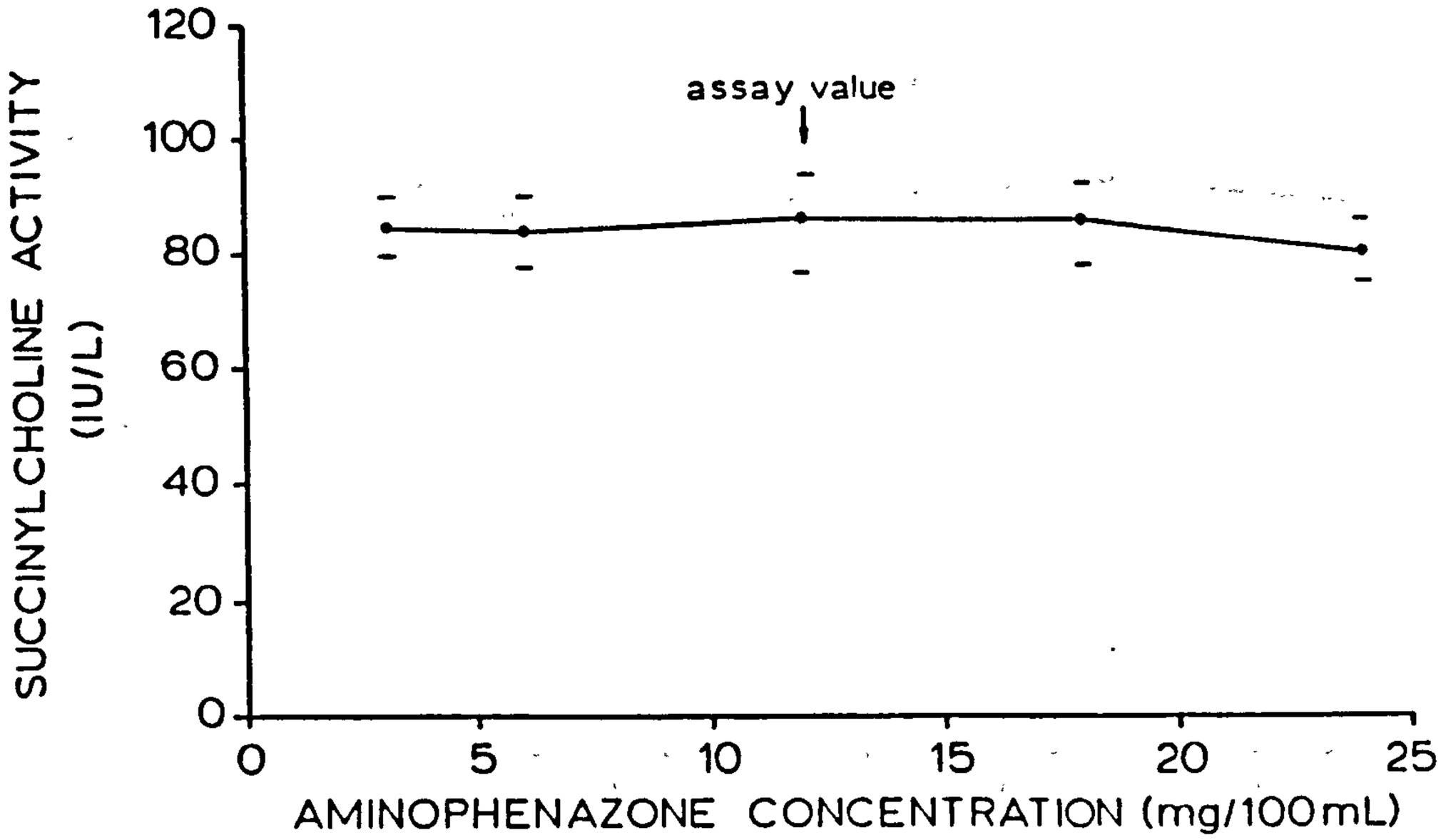

Figure 3.5

Succinylcholine activity measured using colour reagent with increasing aminophenazone concentration.

Mean ± 1 SD; n=5.

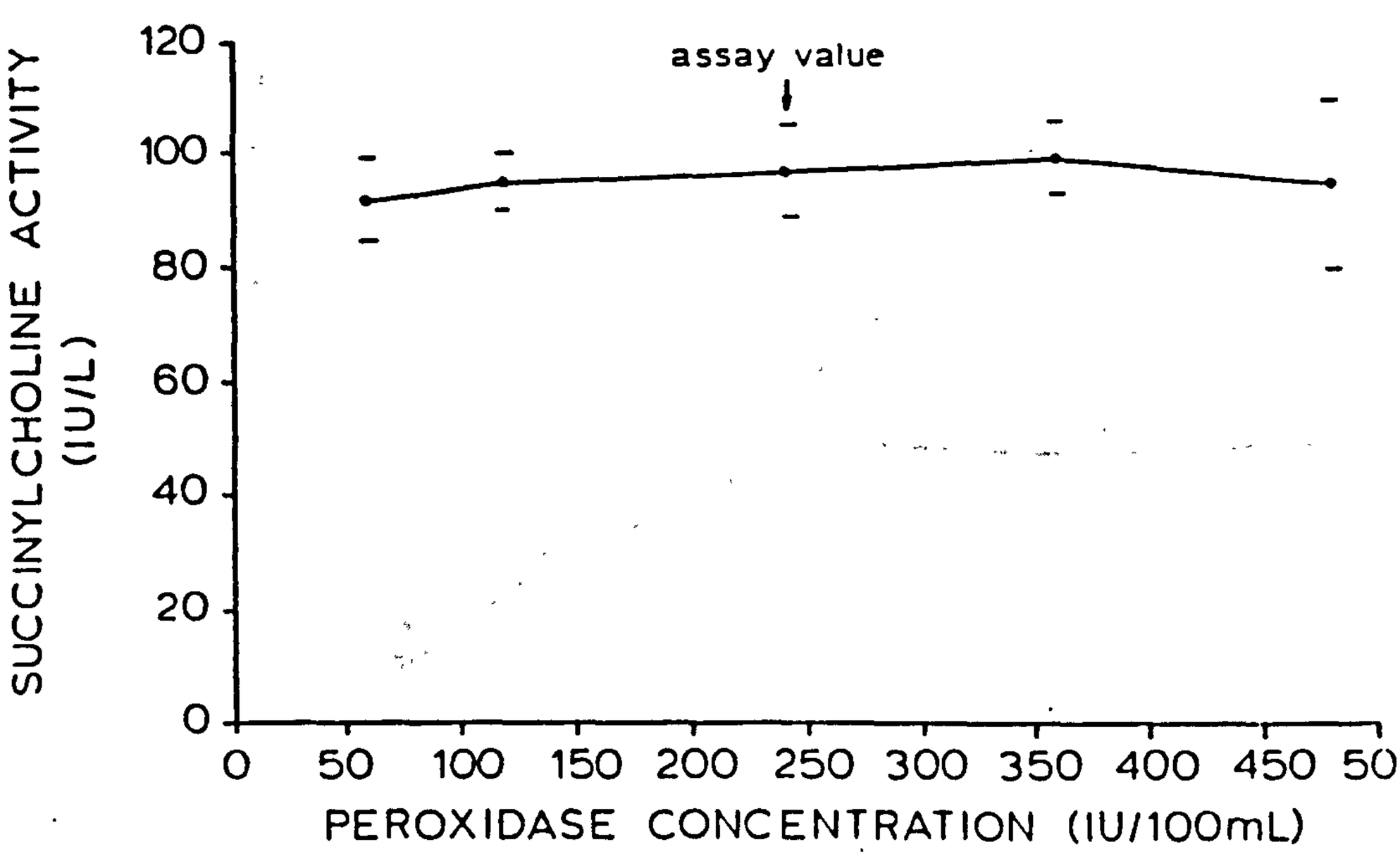

Figure 3.6

Succinylcholine activity measured using colour reagent with increasing peroxidase concentration.

Mean ± 1 SD.

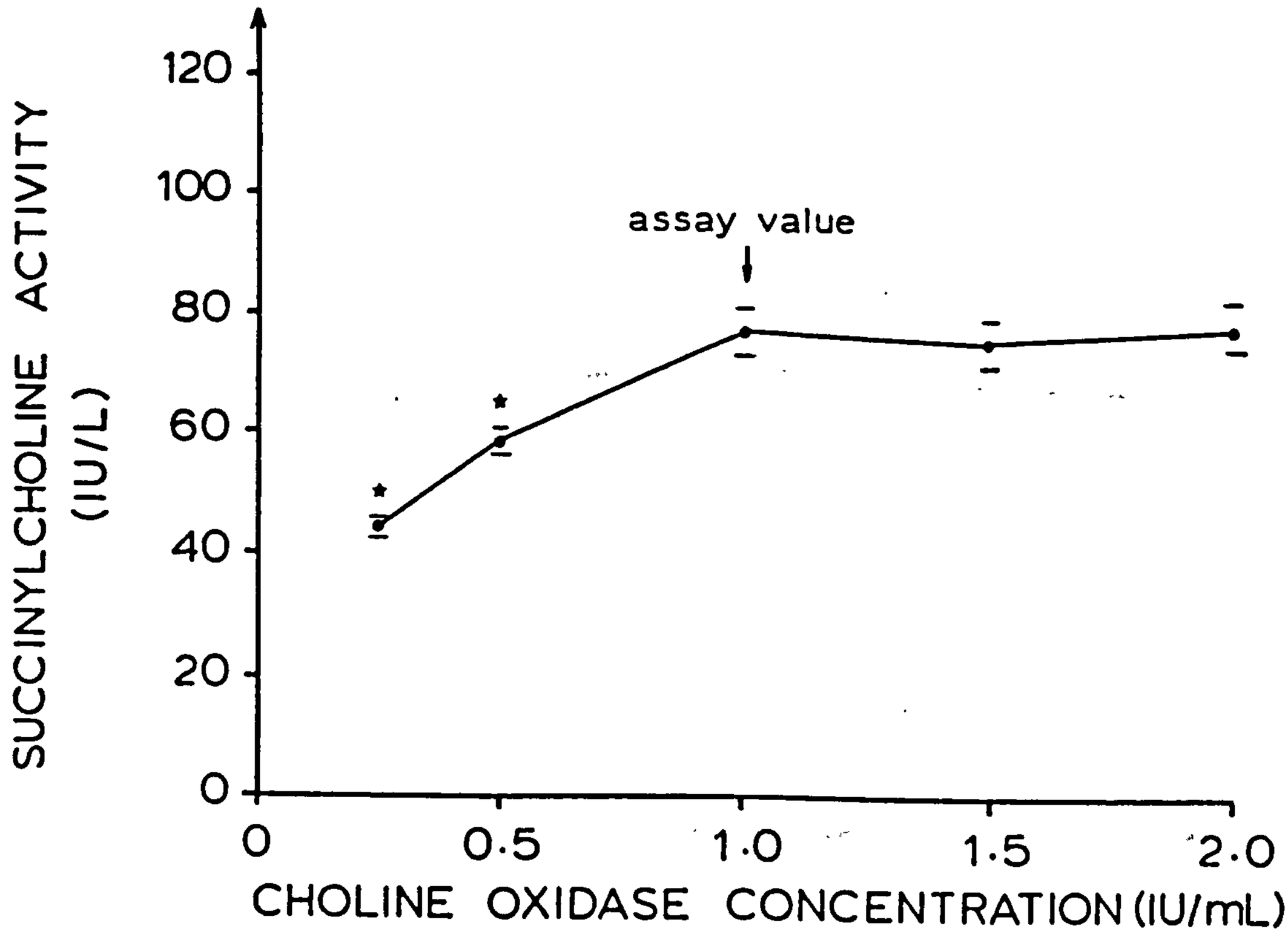

Figure 3.7

Succinylcholine activity measured with colour reagent with increasing choline oxidase concentration.

Mean ±1 SD.

* = significantly different from assay value P< 0.001.

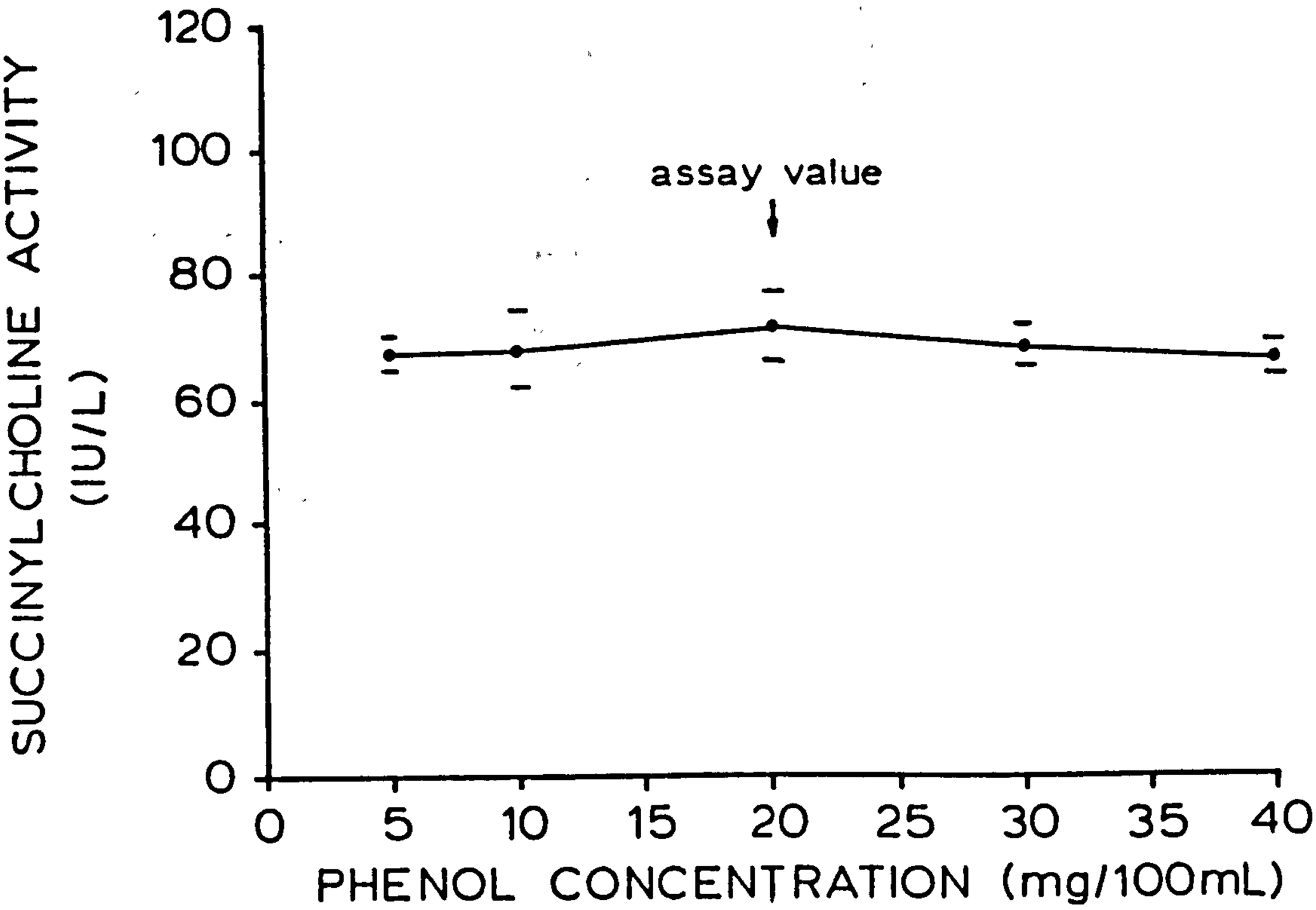

Figure 3.8

Succinylcholine activity measured using colour reagent with increasing phenol concentration.

Mean ± 1 SD; n=5

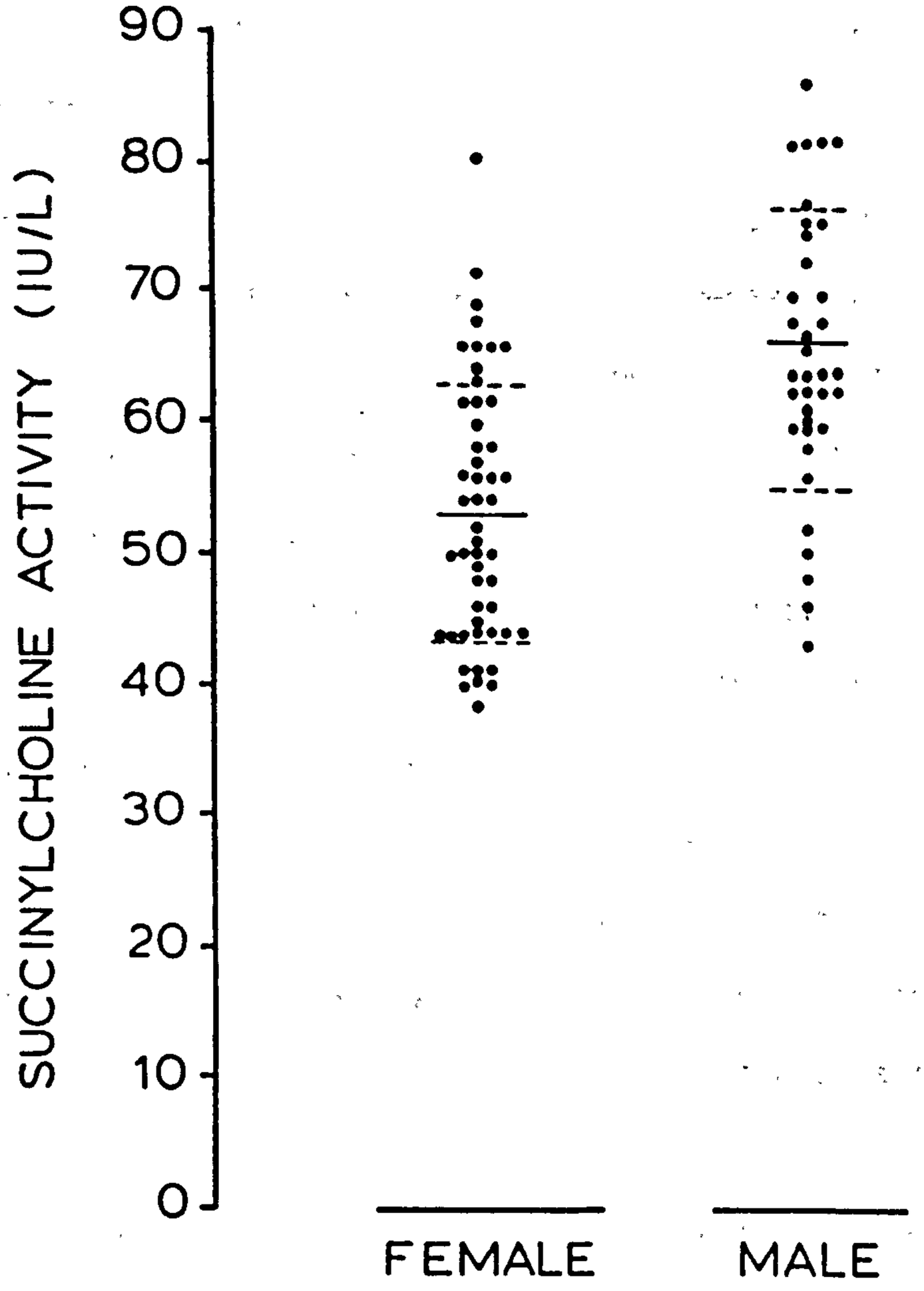

Figure 3.9

Succinylcholine activities of healthy individuals homozygous for the usual gene.

Broken horizontal lines = mean $\pm$ 1 SD.

CHAPTER 4.

THE EVALUATION OF THE SUCCINYLCHOLINE-BASED CHOLINESTERASE ASSAY FOR THE INVESTIGATION OF SUCCINYLCHOLINE-INDUCED APNOEA.

The aims of this section were to evaluate the clinical usefulness of the succinylcholine-based assay and to establish whether forms of cholinesterase exist which can hydrolyse substrates used in their assay but not succinylcholine.

The succinylcholine-based assay for serum cholinesterase was compared with one using propionylthiocholine as the substrate. The latter method has previously been shown to be the best available for the identification of succinylcholine sensitive patients [Evans and Wroe 1978].

In addition the assay was applied to the investigation of a group of individuals who had experienced apnoea after the administration of succinylcholine but whose cholinesterase status was normal according to currently accepted biochemical criteria.

4.1 Comparison of the succinylcholine and propionylthiocholine activities of healthy, phenotypically usual individuals.

Comparison of the succinylcholine and propionylcholine activities was performed using sera from the group of individuals described in Chapter 3, Section 3.10. Results are shown in Table 4.1 and Fig. 4.1.

4.2 Comparison of the succinylcholine and propionylthiocholine activities of individuals referred for assessment of cholinesterase status.

The succinylcholine assay was applied to serum from 76 patients and their relatives referred to the Cholinesterase Investigation Unit of this hospital during a three-month period. In the unit, routine investigation of cholinesterase status involves the measurement of propionylthiocholine activity at 25°C and ascription of cholinesterase genotype on the basis of inhibition of benzoylcholine hydrolysis by dibucaine, sodium fluoride and Ro-0683. Samples are stored at -20 °C, which ensures long-term stability [Turner et al. 1984]. Results are shown in Fig. 4.2 and Table 4.2.

The optimum dividing line for differentiation between succinylcholine sensitive and non sensitive patients using a propionylthiocholine assay has been reported as 2.5 standard deviations (SD) below the mean for $E1^u$ homozygotes [Dietz, Rubinstein and Lubrano 1973]. For succinylcholine this is 29 IU/L and, using this cut-off, the assay identified 19 of 22 patients who had either proven apnoea or a genetic variant associated with it. Three patients were missed. Two had an $E1^aE1^k$ genotype which is not invariably associated with sensitivity to succinylcholine and one was a patient who had developed apnoea after being given succinylcholine but who subsequently proved to be normal by all routine biochemical criteria. He is included in the patients

considered later. One patient with a non-senstive genotype also had an activity of less than 29 IU/L but had not been exposed to succinylcholine so that no definite conclusions concerning sensitivity could be drawn.

For the propionylthiocholine assay the -2.5 SD cut-off value is 1.64 IU/L and using this, it identified 14 of the 22 patients. Of the eight who were missed, four had an $E1^aE1^k$ genotype and one was the patient with usual enzyme referred to above. The remaining three patients missed were all homozygous for the atypical form of cholinesterase, a genotype which is always associated with prolonged succinylcholine induced apnoea. In addition two had clinically proven sensitivity. No apparently non-sensitive patients were included in the "sensitive" group.

4.3 Comparison of succinylcholine and propionylthiocholine activities of samples from individuals with genetic abnormalities of cholinesterase.

Eighty seven samples obtained from individuals with a wide range of genetic abnormalities were taken from a bank of material held by the Cholinesterase Investigation Unit. Samples were assayed using the succinylcholine-based assay and results compared with those obtained using propionylcholine as the substrate. Results are shown in Figs. 4.3 and 4.4 respectively and combined in Table 4.3.

For the succinylcholine-based assay all individuals with genotypes invariably associated with sensitivity had activities of less than 29 IU/L. Of the 87 individuals investigated 22 had experienced an apnoea of 20 minutes or more. The succinylcholine-based assay identified 21 of the 22. The patient missed had an $El^{a}El^{k}$ genotype. One individual heterozygous for the usual and the atypical form of cholinesterase was included in the "sensitive" group, that is with a succinylcholine activity of <29 IU/L. This genotype is not usually associated with succinylcholine sensitivity unless activity has been lowered by disease or pregnancy. However since the patient had been investigated only as part of a family study no clinical assessment was available. Similarly of the 11 $El^{a}El^{k}$ individuals included in this group only one had been exposed to succinylcholine and been found to be sensitive.

Figure 4.4 shows the propionylthiocholine activities on the 87 individuals. Using a cut-off of -2.5 SD (1.64 IU/L) the assay failed to identify three individuals typed $El^{a}El^{a}$, four typed $El^{a}El^{f}$ and one typed $El^{f}El^{s}$. These genotypes are all associated with succinylcholine sensitivity. Of 22 patients who developed apnoea eight had activities greater than the 1.64 IU/mL cut-off. One individual heterozygous for the usual and the silent gene fell into the "sensitive" area (1.64 IU/mL). This is a genotype not usually associated with sensitivity in the absence of complicating factors. As he had not been

exposed to succinylcholine, again no clinical interpretation was possible. Similarly no conclusions can be drawn concerning the 2 individuals of the El^aEl^k genotype who were included in the sensitive group.

4.4 Application of the succinylcholine-based assay to biochemically normal individuals with apnoea following treatment with succinylcholine.

Samples were obtained from 50 patients who had suffered an apnoea of 20 minutes or more after the administration of succinylcholine but on whom routine investigation of cholinesterase status gave normal results. The cholinesterase activities measured using the succinylcholine-based assay are shown in Table 4.4.

Only one had an activity of less than 2.5 SD below the mean for El^u homozygotes (29 IU/L).

The system described here uses a substrate concentration of 1.14 mmol/L which is much greater than that found pharmacologically. Schmidinger, Held and Goedde [1966] have calculated that the peak concentration likely to exist in the plasma after the administration of succinylcholine must be less than 50 umol/L. It is feasible therefore that within the group of 50 patients investigated here, there may exist individuals whose cholinesterase is able to hydrolyse succinylcholine at the relatively high assay substrate concentration but not at pharmacological concentrations. They may possess an enzyme form with low affinity for succinylcholine

which cannot be detected either by this drug-based cholinesterase assay or by those using non-pharmacological substrates.

To investigate this possibility enzyme kinetic studies were performed on this group of patients and compared with those performed on healthy $E1^u$ homozygotes. Individual samples were analysed in duplicate, where possible, using substrate concentrations of 0.045, 0.091, 0.136, 0.227, 0.454 and 1.136 mmol/L. Km and Vmax were calculated using a program based on one for non-linear regression (see Chapter 2, Section 2.3). Using this program a range of equations, representing the commonly occurring kinetic mechanisms, are available to apply to the data. Table 4.5 and Fig. 4.5 show typical results from a healthy $E1^u$ homozygote. These indicate that the enzyme obeys Michaelis-Menten kinetics and therefore this equation was used to analyse the results. Table 4.6 shows the Km and Vmax values for 23 healthy Elu homozygotes and for 30 patients suspected of an abnormality and upon whom sufficient serum was available for analysis. Fig. 4.6 shows the Km values for the two groups.

4.5 Discussion.

In theory a method for the measurement of cholinesterase activity based upon succinylcholine hydrolysis will reflect the in vivo metabolism of the drug and therefore the duration of action, more

precisely than one using a non pharmacological substrate. This assumption appeared to be substantiated following the work of Agarwal, Srivistava and Goedde [1975]. They examined serum from 21 patients with clinical sensitivity but who had no cholinesterase abnormality. In six of these they were able to observe reduced catalytic activity when measured against succinylcholine. This they supposed indicated the existence of cholinesterase variants which were not apparent from the techniques in routine use.

The succinylcholine-based assay described here is better than the one employing propionylthiocholine for the identification of sensitivity due either to genetic abnormalities or pathological reductions in cholinesterase activity. However no evidence has been found for the existence of new genetic variants. Only one of 50 cases biochemically normal but with clinically suspected sensitivity had an activity of less than 29 IU/L (2.5 SD Below the the mean for $E1^u$ homozygotes). This patient was included in the 30 cases on whom there was also sufficient serum to assess the Km values. These were significantly different from the Km values obtained for 23 $E1^u$ homogotes, 107.8 (2 SD range 57.2-158.4) compared with 92.4 (2 SD range 38.8- 146.0). The Km values obtained for the group of biochemically normal patients who had experienced apnoea although higher than values for $E1^u$ do not approach the Km values previously

estimated for the atypical cholinesterase (6 mmol/L [Brown et al 1981]) and therefore are unlikely to be those of a genotype unable to hydrolse succinylcholine. However it is possible that the slightly lowered affinity of their cholinesterase for succinylcholine may predispose them to the effects of other factors which may prolong apnoea.

Nonetheless these observations contrast with those of Agarwal and his colleagues. Although there are significant differences in the two analytical systems it is unlikely that these are sufficient to explain the conflict. It is more probable that much of the explanation lies in their use of benzoylcholine as the routine substrate. Activities measured by this procedure have been shown to have minimal interpretive value [Evans and Wroe 1978].

However the selection of patients who are genotypically normal with apparently abnormal reactions to succinylcholine is not without problems since clinical evaluation of the reaction to succinylcholine is uncertain. Two major problems beset the interpretation of cholinesterase findings on patients suspected of having experienced a prolonged succinylcholine induced apnoea. Anaesthetised patients often receive large numbers of drugs any of which may affect the ability to breathe. Secondly it is impossible to assess the cause of an apnoea without the patients myoneuronal conduction having been tested with a suitable stimulator. This can

help eliminate central depression of respiration as a possible cause. It must also be recognised that it is impossible for laboratory conditions ever to simulate entirely those prevailing at the time of the anaesthetic. Substrate concentration, the patients ventilatory state and the presence of other drugs which might influence cholinesterase will differ between the laboratory and the operating theatre. Nonetheless I feel that this work indicates that the number of patients sensitive to succinylcholine due to cholinesterase abnormalities which are failing to be recognised is small. In addition the routine use of a similar succinylcholine-based assay [Abernathy, George and Melton 1984] has not yet led to the recognition of any new genotypes. These observations are in agreement with conclusions reached by Viby-Mogensen who, after careful review and follow up of 225 patients previously reported [Viby-Mogensen and Hanel 1978] with prolonged apnoea following succinylcholine, concluded that the number likely to possess hitherto unknown genotypes is probably very small.

TABLE 4.1

SUCCINYLCHOLINE AND PROPIONYLTHIOCHOLINE(PTCI) ACTIVITIES IN HEALTHY $E1^u$ HOMOZYGOTES.

PTCI activity IU/mL	Succinylcholine activity IU/L	PTCI activity IU/mL	Succinylcholine activity IU/L
7.0	79.0	5.35	57.7
5.6	65.4	4.4	53.8
5.1	67.3	3.4	48.0
4.0	50.9	4.4	44.2
6.5	71.2	3.2	38.5
4.0	55.8	5.8	61.5
5.1	66.4	4.7	55.8
3.4	49.0	3.4	44.2
4.3	48.1	4.6	44.2
2.6	44.2	3.7	46.2
4.2	55.8	5.5	61.5
4.9	62.5	4.2	50.0
3.8	53.8	4.8	51.9
3.4	45.2	2.4	44.3
3.75	40.0	5.1	66.4
4.95	50.2	3.9	53.8
6.35	67.4	4.2	55.8
4.7	57.8	5.3	62.5
4.1	42.4	5.2	43.3
4.0	42.4	4.3	66.4
3.6	42.2	5.6	65.4
3.2	40.4	3.5	51.9
5.5	61.5	5.3	63.4
5.3	63.5	5.2	63.4
3.85	46.2	4.4	67.3
5.85	65.4	7.2	80.8
4.15	50.0	4.3	59.6
3.85	59.6	4.5	46.2
3.8	57.7	4.3	57.7
5.1	44.2	6.0	67.3
4.0	50.0	4.6	59.6
3.7	44.2	3.6	48.1
3.4	40.4	6.5	75.0
3.7	59.6	6.0	80.8
5.5	69.2	5.2	69.2
4.7	62.3	4.9	55.8
4.5	61.6	5.3	62.5
4.9	63.5	4.1	60.6
6.0	75.0	7.0	76.0
6.6	80.6	5.2	63.5
5.4	75.0	5.5	80.8
5.6	72.2	3.6	60.0
7.2	80.8	7.1	85.6

TABLE 4.1 contd.

SUCCINYLCHOLINE AND PROPIONYLTHIOCHOLINE(PTCI) ACTIVITIES IN HEALTHY $E1^u$ HOMOZYGOTES.

PTCI activity IU/mL	Succinylcholine activity IU/L	PTCI activity IU/mL	Succinylcholine activity IU/L
5.3	74.0	5.3	63.5
4.9	50.2		

TABLE 4.2.

COMPARISON OF THE SUCCINYLCHOLINE AND PROPIONYLTHIOCHOLINE (PTCI) ACTIVITIES OF 76 PATIENTS REFERRED FOR THE ROUTINE INVESTIGATION OF CHOLINESTERASE STATUS.

Genotype	PTCI activity IU/mL	Succinylcholine activity IU/L	Apnoea
$E1^uE1^u$	6.0	85.2	-
$E1^uE1^u$	3.3	57.3	-
$E1^aE1^a$	1.05	6.8	YES
$E1^uE1^a$	3.65	52.1	-
$E1^aE1^a$	1.25	7.7	-
$E1^uE1^a$	4.25	71.2	-
$E1^uE1^a$	4.45	62.5	-
$E1^aE1^a$	2.15	12.5	YES
$E1^uE1^u$	4.1	58.8	-
$E1^uE1^a$	5.0	51.5	-
$E1^uE1^a$	3.0	35.7	-
$E1^uE1^u$	3.3	65.3	-
$E1^uE1^u$	6.2	87.9	-
$E1^aE1^a$	1.2	6.1	YES
$E1^uE1^u$	4.0	54.9	-
$E1^uE1^u$	3.45	51.0	-
$E1^uE1^u$	1.0	19.8	YES
$E1^aE1^a$	1.45	3.6	-
$E1^uE1^a$	3.85	50.1	-
$E1^aE1^a$	1.55	9.3	YES
$E1^aE1^k$	3.1	39.1	-
$E1^aE1^k$	3.65	45.5	-
$E1^aE1^a$	1.5	15.1	YES
$E1^uE1^u$	4.3	61.8	-
$E1^uE1^a$	2.55	33.4	-
$E1^uE1^a$	3.95	39.1	-
$E1^uE1^a$	2.85	48.3	-
$E1^uE1^u$	5.1	74.4	-
$E1^aE1^a$	1.15	5.4	YES
$E1^uE1^u$	5.75	87.4	YES
$E1^uE1^u$	5.74	69.6	-
$E1^uE1^u$	6.2	83.7	-
$E1^uE1^a$	2.9	35.2	-
$E1^aE1^k$	2.35	27.9	-
$E1^uE1^a$	4.3	54.0	-
$E1^aE1^a$	1.2	6.4	-
$E1^uE1^a$	5.15	59.3	-
$E1^uE1^u$	5.7	75.8	-
$E1^uE1^a$	4.85	59.9	-
$E1^uE1^a$	3.5	61.8	-
$E1^uE1^u$	5.25	69.3	-
$E1^uE1^a$	6.9	71.3	-
$E1^uE1^u$	5.65	77.8	-

TABLE 4.2 contd.

COMPARISON OF THE SUCCINYLCHOLINE AND PROPIONYLTHIOCHOLINE (PTCI) ACTIVITIES OF 76 PATIENTS REFERRED FOR ROUTINE INVESTIGATION OF CHOLINESTERASE STATUS.

Genotype	PTCI activity IU/mL	Succinylcholine activity IU/L	Apnoea
$E1^uE1^u$	4.7	66.5	-
$E1^uE1^a$	2.9	37.4	-
$E1^uE1^a$	4.2	50.5	-
$E1^uE1^a$	3.1	36.2	-
$E1^uE1^a$	2.9	36.7	-
$E1^uE1^a$	4.7	49.7	-
$E1^aE1^a$	2.05	9.6	YES
$E1^uE1^s$	3.1	43.8	-
$E1^uE1^u$	4.1	53.9	-
$E1^uE1^a$	3.6	39.9	-
$E1^uE1^s$	2.8	37.0	-
$E1^uE1^s$	3.45	40.8	-
$E1^aE1^a$	1.0	14.0	-
$E1^uE1^u$	3.05	42.3	-
$E1^uE1^a$	3.8	46.5	-
$E1^uE1^f$	4.95	73.7	-
$E1^aE1^a$	2.0	16.6	-
$E1^uE1^a$	3.3	43.6	-
$E1^uE1^u$	5.35	76.6	-
$E1^uE1^u$	5.05	65.1	-
$E1^uE1^u$	6.85	91.0	-
$E1^uE1^a$	3.45	58.0	-
$E1^uE1^a$	4.85	54.2	-
$E1^uE1^u$	3.15	40.7	-
$E1^uE1^u$	3.9	47.4	-
$E1^aE1^k$	2.6	25.5	-
$E1^uE1^u$	3.85	56.5	-
$E1^uE1^a$	4.1	51.3	-
$E1^uE1^u$	0.9	14.5	YES
$E1^aE1^a$	0.85	12.0	-
$E1^aE1^a$	1.05	7.5	YES
$E1^uE1^u$	3.65	46.4	-
$E1^aE1^a$	0.95	6.0	-

Apnoea noted was greater than 20 minutes.

TABLE 4.3.

SUCCINYLCHOLINE AND PROPIONYLTHIOCHOLINE ACTIVITIES IN INDIVIDUALS WITH GENETIC ABNORMALITIES OF CHOLINESTERASE.

Genotype	Succinyl-choline activity IU/L	PTCI activity IU/mL
$E1^aE1^k$	15.4	1.64
	25.1	2.3
	34.7	3.7
	24.6	2.7
	34.5	3.3
	31.7	3.2
	19.2	2.2
	32.5	3.3
	44.1	4.0
	19.2	1.8
	18.8	2.1
	18.3	1.3
	29.2	2.8
	39.1	3.1
	45.5	3.7
	27.9	2.4
	14.0	1.0
	25.5	2.6
mean:	27.74	2.62
SD:	9.45	0.85
$E1^sE1^s$	<2.0	<0.1
$E1^aE1^f$	23.1	1.8
	15.4	1.4
	15.4	1.4
	19.2	1.9
	5.8	1.1
	23.7	3.1
	10.5	1.1
	25.5	2.8
mean:	17.33	1.83
SD:	6.88	0.76

Genotype	Succinyl-choline activity IU/L	PTCI activity IU/mL
$E1^aE1^a$	3.9	0.91
	5.8	1.17
	2.9	1.48
	5.8	2.0
	7.7	2.04
	4.8	1.38
	2.9	0.88
	4.8	1.47
	2.9	1.09
	13.5	1.82
	4.8	1.29
	6.8	1.05
	7.7	1.25
	6.1	1.2
	13.0	2.15
	3.6	1.45
	9.3	1.4
	8.2	1.5
	5.3	1.15
	6.4	1.2
mean:	6.31	1.39
SD:	2.99	0.36
$E1^uE1^s$	36.7	3.1
	43.3	3.7
	30.5	1.2
	38.3	2.7
	46.2	3.4
	42.8	3.5
	48.1	3.9
	43.8	3.1
mean:	41.21	3.08
SD:	5.73	0.85

TABLE 4.3 contd.

SUCCINYLCHOLINE AND PROPIONYLTHIOCHOLINE ACTIVITIES IN INDIVIDUALS WITH GENETIC ABNORMALITIES OF CHOLINESTERASE.

Genotype	Succinyl-choline activity IU/L	PTCI activity IU/mL
$E1^uE1^a$	19.2	2.0
	27.0	2.5
	28.9	2.25
	50.1	5.1
	40.4	4.05
	34.7	3.7
	52.1	3.65
	71.2	4.25
	62.5	4.45
	51.5	5.0
	35.7	3.0
	50.1	3.85
	33.4	2.55
	39.1	3.95
	48.3	2.85
	35.2	2.9
	59.3	5.15
	59.9	4.85
	62.8	3.50
	71.3	6.9
mean:	46.62	3.82
SD:	14.96	1.21
$E1^uE1^f$	53.4	3.9
	56.5	6.4
	52.2	5.0
	36.4	3.5
	73.7	5.0
mean:	54.44	4.76
SD:	13.29	1.13
$E1^aE1^s$	6.0	1.0
	4.0	0.5
	5.8	1.2
	5.9	0.85
	<2.0	0.85
	3.9	0.95
mean:	4.27	0.91
SD:	2.30	0.24
$E1^fE1^s$	19.2	1.7

TABLE 4.4.

CHOLINESTERASE ACTIVITIES IN BIOCHEMICALLY NORMAL PATIENTS WITH APNOEA AFTER TREATMENT WITH SUCCINYLCHOLINE.

Duration of apnoea minutes	Propionylthiocholine activity IU/mL	Succinylcholine activity IU/L
*	2.9	50.6
75	3.6	45.9
120	6.5	75.3
*	3.7	66.6
30	4.5	67.3
60	4.6	59.6
40	5.1	54.5
*	6.0	58.8
*	4.6	64.1
35	5.7	65.7
25	3.6	56.1
60	4.0	40.6
*	4.0	78.2
*	3.8	45.3
30	5.2	60.4
30	2.5	27.8
*	3.0	32.8
90	4.5	59.6
25	4.7	47.6
50	3.7	52.9
*	3.1	40.6
30	4.5	83.8
*	3.5	45.9
30	5.1	69.5
*	4.5	73.3
*	4.1	55.5
20	3.4	44.0
60	5.7	64.6
30	4.7	53.4
60	4.2	55.3
*	6.2	70.1
*	3.2	55.6
*	5.8	87.4
45	6.1	80.1
*	4.0	57.8
90	5.7	68.9
45	6.3	74.2
55	5.4	52.9
*	4.0	49.4
*	3.7	55.2
*	5.7	72.9
*	3.2	65.9
*	4.0	59.3
*	5.6	70.2

TABLE 4.4 contd.

CHOLINESTERASE ACTIVITIES IN BIOCHEMICALLY NORMAL PATIENTS WITH APNOEA AFTER TREATMENT WITH SUCCINYLCHOLINE.

Duration of apnoea minutes	Propionylthiocholine activity IU/mL	Succinylcholine activity IU/L
180	4.0	51.5
20	2.1	38.5
45	4.5	68.0
120	8.6	75.2
*	4.1	42.4
*	2.6	30.9

* Length of apnoea unstated but greater than 20 minutes.
a Activity more than 2.5 SD below the mean for $E1^u$ homozygotes.
All patients had cholinesterase inhibitor numbers characteristic of the usual phenotype as determined by inhibition of benzoylcholine hydrolysis by dibucaine, fluoride and Ro 02-0683 (see text).

TABLE 4.5.

TYPICAL VELOCITY VERSUS SUBSTRATE CONCENTRATION RESULTS FOR AN $E1^u$ HOMOZYGOTE: DEMONSTRATING MICHAELIS-MENTEN KINETICS.

Succinylcholine concentration mmol/L	Enzyme activity IU/L
1.136	60.4, 64.6
0.454	51.7, 55.3
0.227	45.4, 46.9
0.136	37.9, 37.1
0.091	30.0, 32.1
0.045	23.2, 19.4

TABLE 4.6.

Km AND Vmax VALUES FOR HEALTHY El^u HOMOZYGOTES AND FOR PATIENTS WITH NORMAL CHOLINESTERASE STATUS BUT WHO DEVELOPED APNOEA FOLLOWING TREATMENT WITH SUCCINYLCHOLINE.

Elu Homozygotes		Biochemically normal patients with apnoea	
Km umol/L	Vmax IU/L	Km umol/L	Vmax IU/L
142	74	129	70
96	79	116	71
87	73	62	128
82	88	110	41
87	71	122	56
78	63	82	133
82	61	129	74
89	57	97	52
73	49	99	43
96	97	118	76
62	72	104	42
70	99	104	44
86	50	107	56
87	76	62	58
63	81	71	49
74	92	83	53
84	45	82	54
55	49	106	56
83	71	95	61
144	101	128	66
136	53	120	71
137	73	102	73
133	55	97	74
		195	67
92.4	70.8(mean)	113	95
26.8	17 (SD)	111	74
		98	72
		117	71
		115	85
		107.8	67.8(mean)
		25.3	21.8(SD)

The Km results for the two groups are significantly different $p < 0.05$.

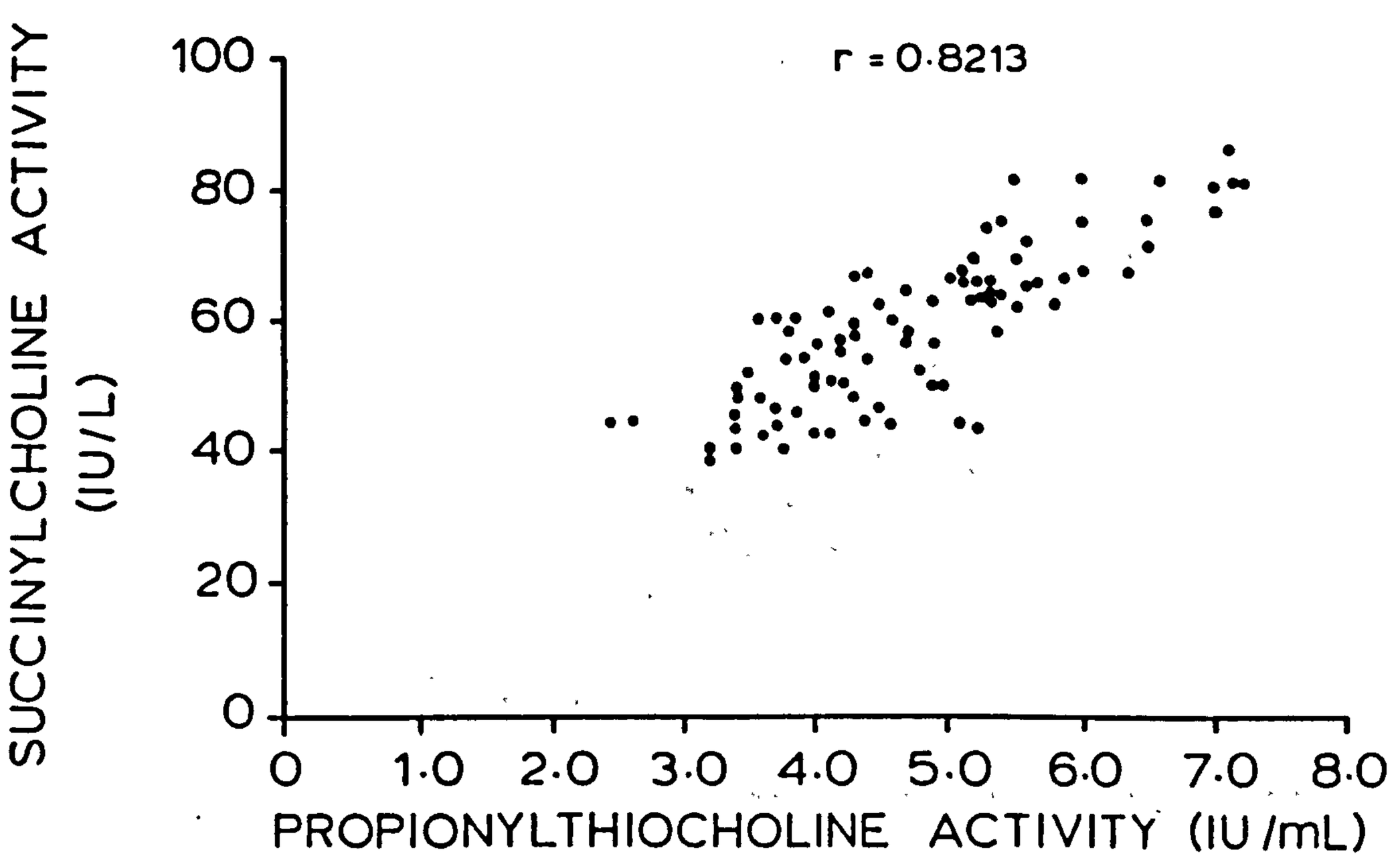

Figure 4.1

Comparison of cholinesterase activities of healthy $E1^u$ homozygotes measured using succinylcholine and propionylthiocholine substrates.

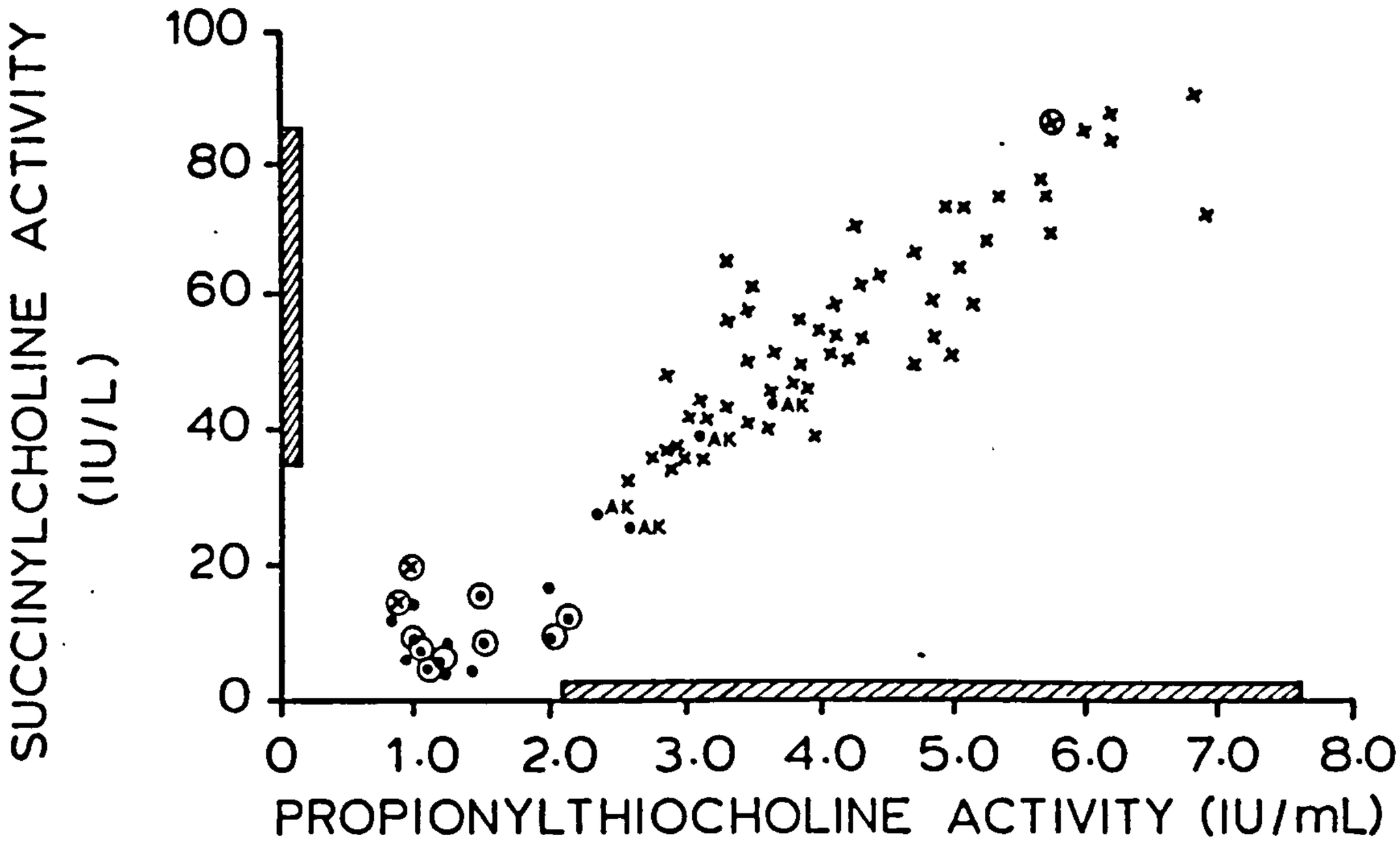

Figure 4.2

Comparison of enzyme activities of 76 subjects referred for routine investigation of Cholinesterase status.

Circles = Individuals with succinylcholine sensitivity.
Crosses = Individuals with at least one usual gene.
Circles annotated AK = $E1^a, E1^k$ homozygotes.
Circled symbols = subjects treated with succinylcholine who developed apnoea.

Shaded bars denote the reference interval for Elu homozygotes for each method.

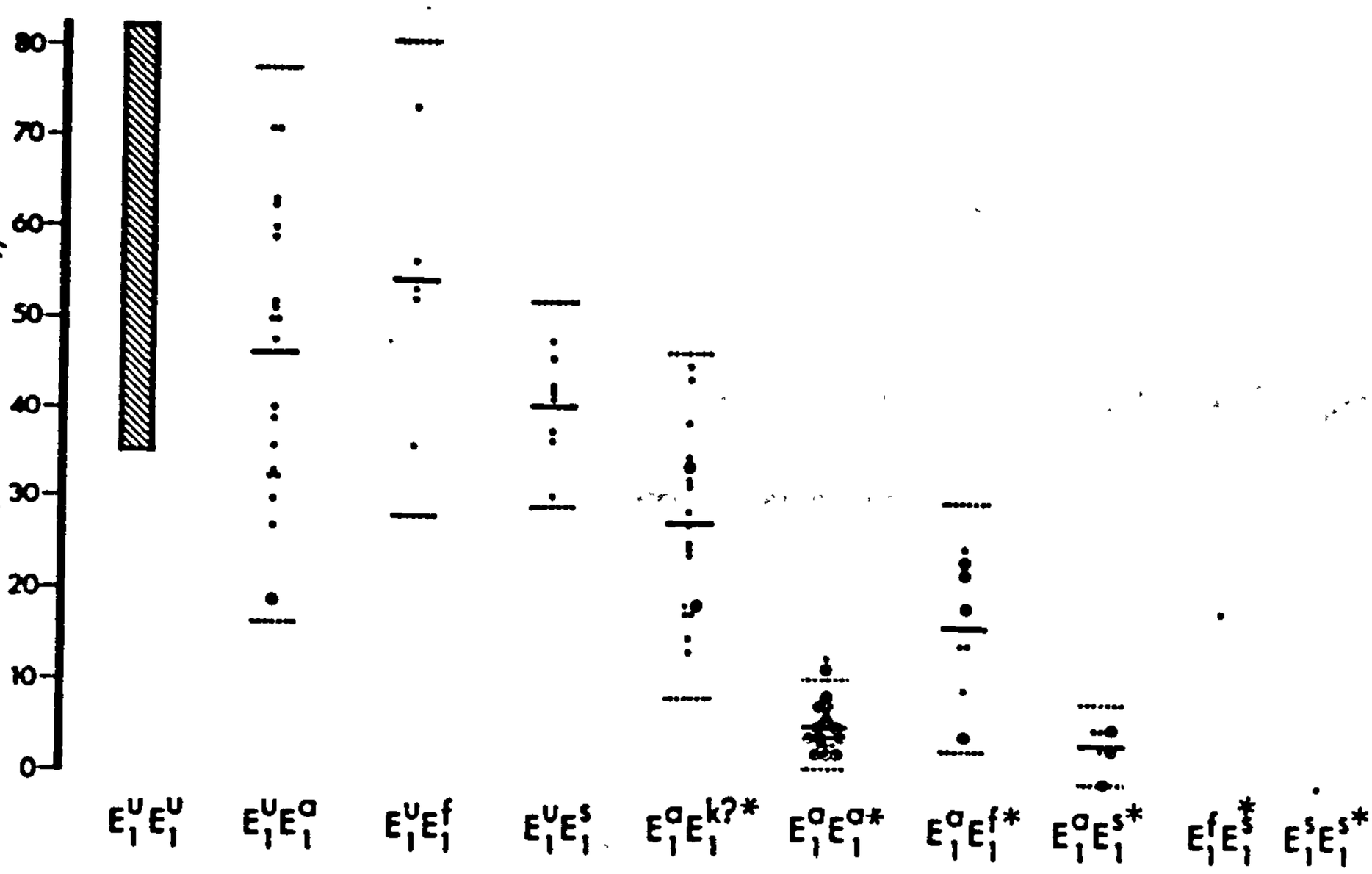

Figure 4.3

Sucinyl choline activities of 87 subjects with unusual cholinesterase genotypes.

Circled symbols indicate patients treated with succinylcholine who developed apnoea.

Shaded bars denote the Elu homozygote refernce range. Heavy and broken horizontal lines denote mean ± 2 SD.

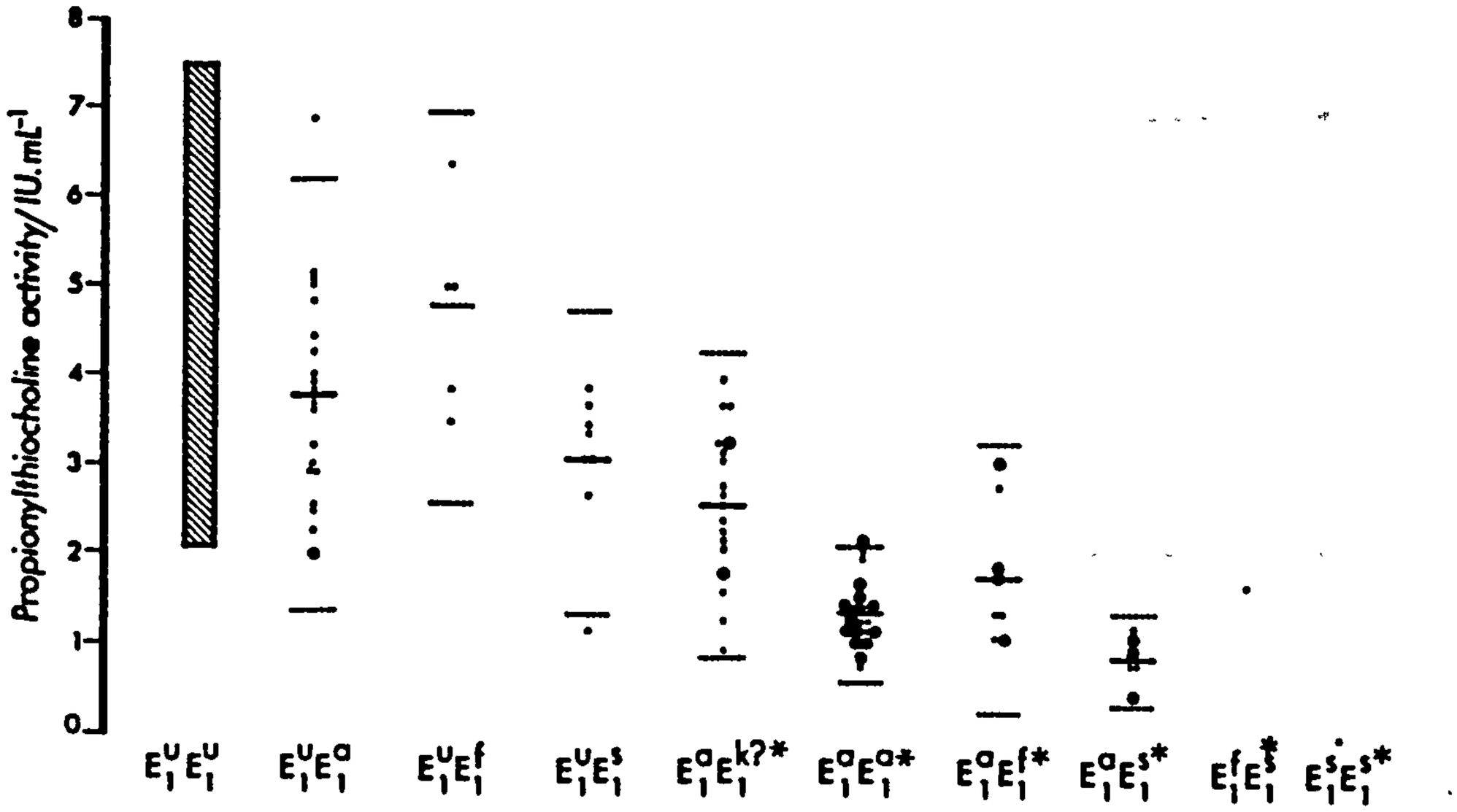

Figure 4.4.

Propionylthiocholine activities of 87 subjects with unusual cholinesterase genotypes.

Circled symbols indicate patients treated with succinylcholine who developed apnoea.

Shaded bars denote the Elu homozygote refernce range. Heavy and broken horizontal lines denote mean $\pm$ 2 SD.

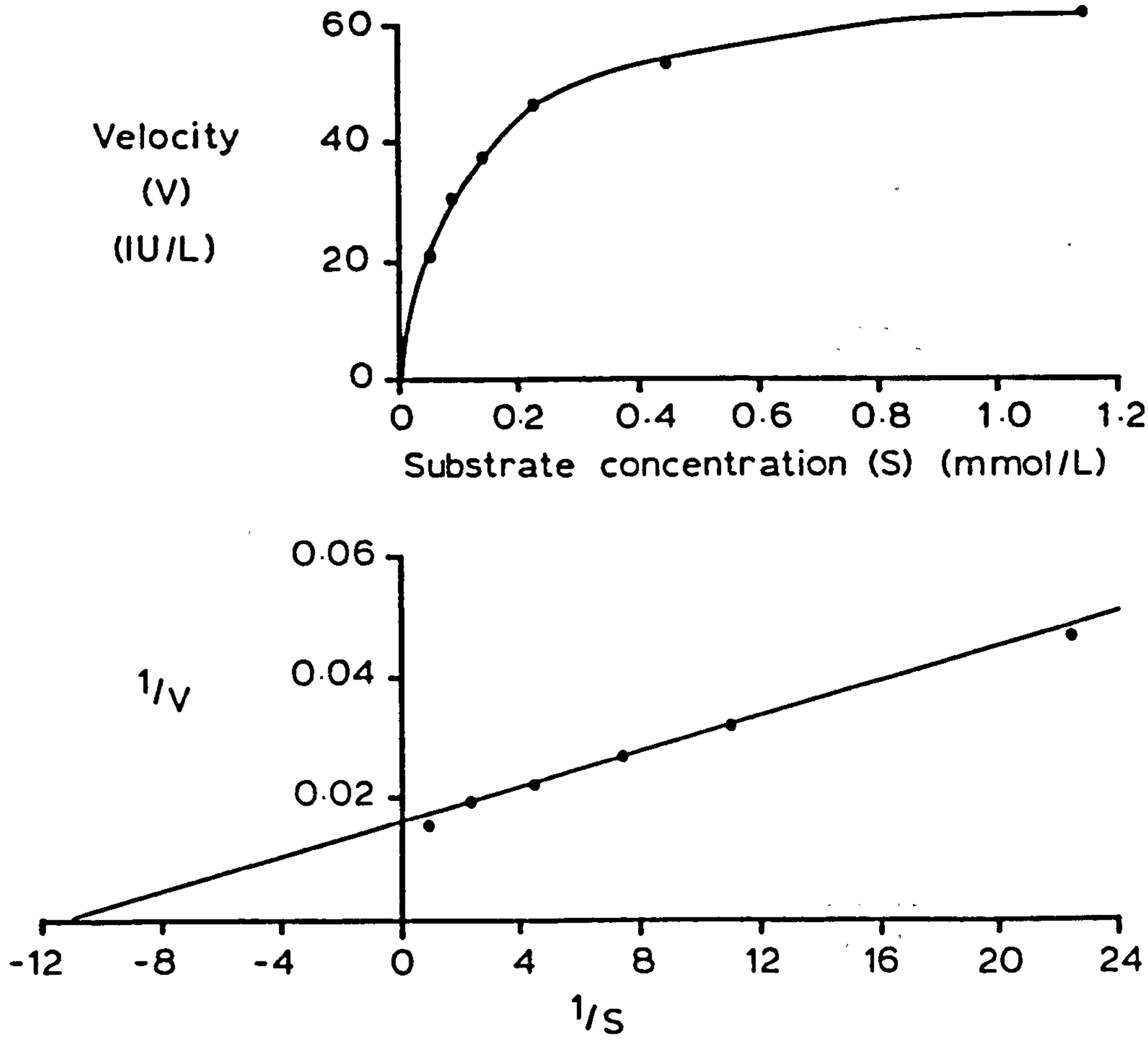

Figure 4.5

Graphs of substrate concentration vs velocity & it's reciprocal for an individual homozygous for the usual gene.

Mean of duplicate determinations at each substrate concentration are shown.

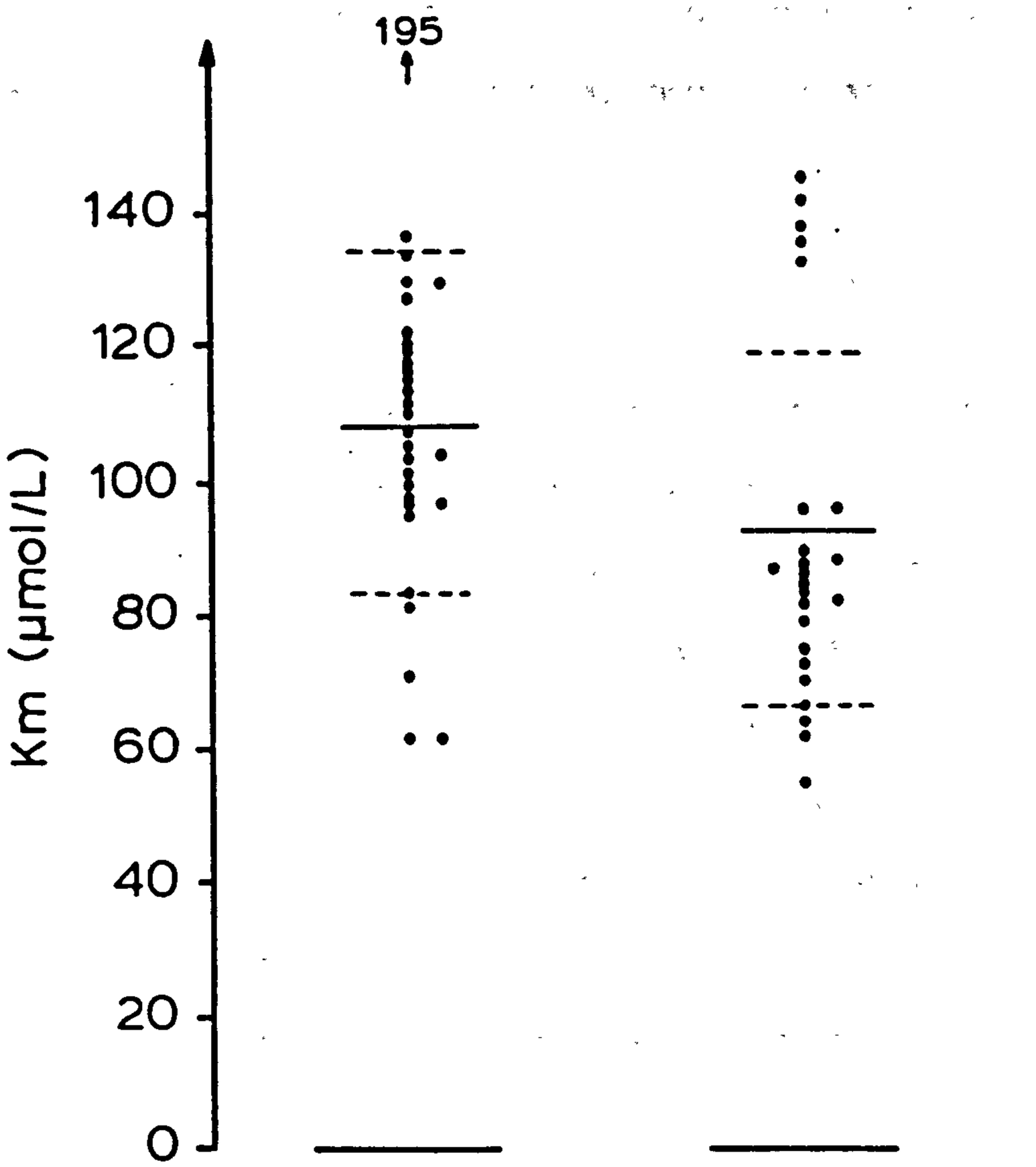

Figure 4.6

Michaelis-Menten constants in healthy El^u homozygotes and patients normal by current biochemical criteria but who had experienced apnoea after treatment with succinylcholine.

Heavy and broken horizontal lines = mean$\pm$ 1 SD.

CHAPTER 5

INVESTIGATION OF THE KINETICS OF THE SUCCINYLCHOLINE-CHOLINESTERASE REACTION USING THE NEWLY DESCRIBED ASSAY.

A. Investigation of the type of kinetics exhibited by the interaction between cholinesterase and succinylcholine.

Kinetic studies on plasma and purified preparations of cholinesterase have demonstrated that the hydrolysis of choline esters does not follow Michaelis-Menten kinetics [Heilbron 1948, Berry 1960, Main 1983 and Wetherell and French 1986]. Preliminary kinetic studies, performed whilst setting up the sucinylcholine-based assay for cholinesterase and during it's application to patients who developed sensitivity to the drug, indicated that when hydrolysing succinylcholine, cholinesterase did obey Michaelis-Menten kinetics. In order to clarify this the work was extended and samples were analysed using a wider range of substrate concentrations.

5.1 Kinetic studies using the succinylcholine-based assay for serum cholinesterase.

The following samples were analysed

a) serum from 3 individuals typed $E1^{u}E1^{u}$ (2 male and 1 female).

b) serum from one individual typed $E1^{a}E1^{a}$.

c) purified cholinesterase from two sources, Sigma Chemical Company, Poole (a relatively crude preparation, method undisclosed) and Calbiochem,

Cambridge (cholinesterase purified 10,000-fold by the method of Haupt et al. [1966]).

Each sample was analysed in quadruplicate over a range of "in assay" substrate concentrations 0.0454, 0.0909, 0.136, 0.227, 0.454, 1.136, 2.272, 3.41, 4.54, 6.81, 9.088, 13.62, 22.7 and 45.5 mmol/L.

Results for all samples are shown in Figs. 5.1-5.6. Km and Vmax values calculated from this data are shown in Table 5.1.

In experiments using the usual enzyme succinylcholine activity starts to increase more rapidly at substrate concentrations greater than 4.54 mmol/L allowing the calculation of two Km and Vmax values, one set with low the other with high values for both parameters. One interpretation of this data is that succinylcholine is hydrolysed by usual cholinesterase at two sites and by the atypical form at only one.

5.2 Discussion.

The hydrolysis of succinylcholine by human plasma or purified human cholinesterase does not follow normal Michaelis-Menten kinetics when substrate concentrations greater than 4.54 mmol/L are also studied.

The observed kinetics are consistant with previous observations using other substrates, that cholinesterase has two active sites [Heilbron 1958, Berry 1960 and Weatherall and French 1986] the presence of which have been interpreted in different ways:

a) that two enzymes exist both capable of hydrolysing the substrate [Heilbron 1958].

b) that enzyme activation occurs at high substrate concentrations [Hastings 1966].

c) that the enzyme molecule contains a modifier or allosteric site [Main 1983].

Since the two sites are present whether plasma, crude or very pure preparations of cholinesterase are used as the source of the enzyme it is unlikely that they arise from the presence of two enzymes.

The clinical dose of succinylcholine for adults is 1 mg/kg and has been estimated to produce plasma levels of approximately 50 umol/L [King, Silk and Whittaker 1978]. This concentration would be hydrolysed by the Km1 site of cholinesterase. The kinetic constant obtained for the atypical cholinesterase approaches those obtained for the Km2 site of usual cholinesterase. The value of 6.922 mmol/L is in agreement with that found previously (6.0 mmol/L) [Brown et al. 1981] and provides confirmation that the long-lasting paralysis in a person with atypical cholinesterase is due to the inability of that cholinesterase to hydrolyse succinylcholine at pharmacological concentrations [Kalow 1959]. Sufficient samples have not been analysed to demonstrate whether this difference between atypical and usual cholinesterase is due the absence of the Km1 site but nonetheless the work is consistant with that of

Lockridge and La Du [1978] who concluded that the atypical enzyme is a structurally modified form of cholinesterase.

B. Comparison of the Kml values obtained in this study with those reported in the literature.

The range of Kml values obtained in this study for usual cholinesterase is 92.4 + 26.8 umol/L (mean + 1SD), n= 23. Other workers have reported lower values (see Table 5.2). Possible explanations for these findings have been examined. The assay calibration was checked using an alternative choline salt bought specifically for the purpose and components of the assay system were investigated to see if they act as cholinesterase inhibitors.

5.3 Investigation of the assay calibration.

The assay calibration (see Chapter 3 section 3.4) was reassessed using choline iodide instead of choline chloride. In addition 50 uL of buffer was added to the reaction mixture to substitute for serum usually present, rather than correct for it in the calculation, as in the original calibration. Five quantities of choline 0.5, 12.5, 25, 50 and 100 nmoles were added to the colour reagent and the maximum amount of colour produced recorded. Results are shown in Fig. 5.7. The absorbance produced by 100 nmoles choline iodide (calculated from the y on x regression equation derived

from the data shown in Fig 5.7) was 0.974. From this value the factor to convert OD per minute to enzyme activity is 2053. This was derived in the same way as the original factor of 1923 but is 7% higher. Thus calibration with choline iodide would give higher Km values.

5.4 Studies of potential inhibitors of cholinesterase in the assay system.

Augustinsson [1948] stated that both aminophenazone and phenol were inhibitors of cholinesterase. To investigate whether inhibition occurred at concentrations present in the succinylcholine assay system, aminophenazone and phenol were added to an assay system for cholinesterase using propionylthiocholine as the substrate. They were added in amounts which kept their ratio to serum equivalent to that in the succinylcholine assay system. Serum samples from 6 $E1^u$ homozygotes with a range of cholinesterase activities were analysed in duplicate before and after the addition of aminophenazone or phenol to the assay system. Results are shown in Table 5.3. Cholinesterase activity was significantly lower after the addition of aminophenazone ($p<0.001$, using a paired t-test) but there was no significant difference in the results following the addition of phenol ($p=0.142$). Aminophenazone, at concentrations used in the succinylcholine-based assay system, therefore inhibited serum cholinesterase whilst phenol did not. The

inhibition was further examined by applying the succinylcholine-based assay to serum from an $E1^u$ homozygote using a range of succinylcholine concentrations, before and after reducing the aminophenazone concentration to 25% of the original value . Results are shown in Fig. 5.8 and Table 5.4. As the concentration of aminophenazone is increased in the assay system both Km and Vmax are reduced, it appears therefore to be an uncompetitive inhibitor of cholinesterase.

5.5 Discussion.

Calibration with choline iodide yielded a factor producing results 7% higher than the original calibration with choline chloride. It is difficult to explain these findings. Such results might occur if choline iodide had absorbed water. However this is unlikely since the choline iodide was bought specifically for the calibration procedure and is less hygroscopic than the chloride [Dittmer and Wells 1969]. Impurities in either salt might account for the discrepancy but both reagents were Analar grade and stored dessicated at 4^oC until use. If the higher calibration value was used for the assay Km values would also be higher.

Aminophenazone is an uncompetitive inhibitor of cholinesterase and as such it's presence would decrease rather than increase the observed Km. Neither

calibration with choline chloride nor the presence of aminophenazone appear therefore to be responsible for the higher Km values obtained using this assay system. Tris [Pavlic 1967] and Triton X-100 [Barenghi et al. 1986] have previously been reported to inhibit cholinesterase. Their omission would decrease the observed Km only if they proved to be competitive inhibitors of cholinesterase. However they are both essential components of the assay system. Triton X-100 is required for the proper development of the coloured dye product [BCL Ltd., choline oxidase product information] and Tris is preferred to phosphate buffer as use of this latter buffer causes precipitation of calcium, another necessary assay component.

The problem of the presence of potential inhibitors in this type of assay system can be avoided by performing the assay in two stages involving:

1. The enzymic hydrolysis of succinylcholine.
2. The inhibition of cholinesterase by physostigmine and the determination of the choline produced.

This approach was taken by Abernathy, George and Melton [1984]. However it is not without problems. Timing of the enzymic hydrolysis is critical as is complete inhibition of cholinesterase activity. Inhibition of cholinesterase activity is achieved using a high concentration of physostigmine which can lead to contamination of other cholinesterase assays even with the use of good laboratory technique.

The Km value for the usual form of cholinesterase using this technique was 53 umol/L [Abernathy, George and Melton 1984] however this estimate was obtained following one experiment on pooled plasma. Other direct estimates [Wakid, Tubbeh and Baraka 1985 and Goedde, Held and Atland 1968] were derived similarly. This study reports the mean Km derived from separate analysis of samples from 23 $E1^u$ homozygotes, a more reliable method of estimating a value representative of the whole population.

In addition the Km derived in the present study is consistant with clinical observation. The plasma concentration of succinylcholine resulting from a typical dose has been reported to be less than 50 umol/L. At this substrate concentration and using the equation:

$$v = \frac{Vmax}{1 + \frac{Km}{[S]}}$$

The velocity of the reaction between cholinesterase and succinylcholine can be calculated. The mean values of Vmax and Km for $E1^u$ homozygotes have been estimated earlier (see Table 4.6). Values were 70.8 IU/L and 92.4 umol/L respectively and thus the velocity of the reaction is 24.8 IU/L (umoles of substrate converted per minute per litre of serum. If pharmacological concentrations of succinylcholine are 50 umol/L of serum they should be hydrolysed in 2.02 minutes. The duration

of action of succinylcholine in vivo is 2-5 mins thus the kinetic values obtained in this study are compatible with clinical observations.

TABLE 5.1

Km AND Vmax VALUES FOR THE HYDROLYSIS OF SUCCINYLCHOLIINE BY CHOLINESTERASE AT SITE1 AND SITE2.

Sample	Km1 mmol/L	Vmax1 IU/L	Km2 mmol/L	Vmax2 IU/L
$E1^uE1^u$	0.144	101	7.92	226.3
$E1^uE1^u$	0.137	72.5	10.80	231.5
$E1^uE1^u$	0.136	52.7	10.77	253.5
$E1^aE1^a$	6.922	171.9	-	-
Sigma purified enzyme (crude preparation)	0.085	58.1	12.886	307.8
Calbiochem purified enzyme (highly purified preparation)	0.121	40.6	19.523	228.9

TABLE 5.2.

Km VALUES FOR SUCCINYLCHOLINE AND CHOLINESTERASE PREVIOUSLY REPORTED.

KM VALUES	AUTHORS
38 umol/L	Goedde, Held and Atland 1968
53 umol/L	Abernathy, George and Melton 1984
30 umol/L	Wakid, Tubbeh and Baraka 1985
22 umol/L	Goedde, Held and Atland 1968 **
6 umol/L	King and Griffin 1973 **

** Km calculated indirectly using the pI50 from succinylcholine inhibition studies.

TABLE 5.3.

INVESTIGATION OF INHIBITION OF CHOLINESTERASE BY AMINOPHENAZONE AND PHENOL.

PTCI activity IU/mL	PTCI activity with added aminophenazone IU/mL	PTCI activity with added phenol IU/mL
1.54	1.40	1.75
4.15	3.79	4.29
5.92	5.46	5.88
1.41	1.16	1.32
7.65	7.24	7.76
2.72	2.32	3.01
	p<0.001 t=6.9042	p=0.142 t=1.7422

TABLE 5.4.

Km AND Vmax VALUES OBTAINED AFTER REDUCING THE AMINOPHENAZONE CONCENTRATION

1. Km Values (mmol/L):

Sample No.	Original colour reagent	Colour reagent with decreased aminophenazone
1	137	190
2	136	159
3	133	170
4	144	193
5	139	192
6	143	185
	t=9.1402 p<0.001 (paired T-Test)	

2. Vmax (IU/L):

Sample	Original colour reagent	Colour reagent with decreased aminophenazone
1	72.5	96.2
2	52.7	55.5
3	73.9	75.9
4	101.1	112.1
5	49.5	61.7
6	95.1	98.7
	t=2.7114 p=0.042 (paired T-Test)	

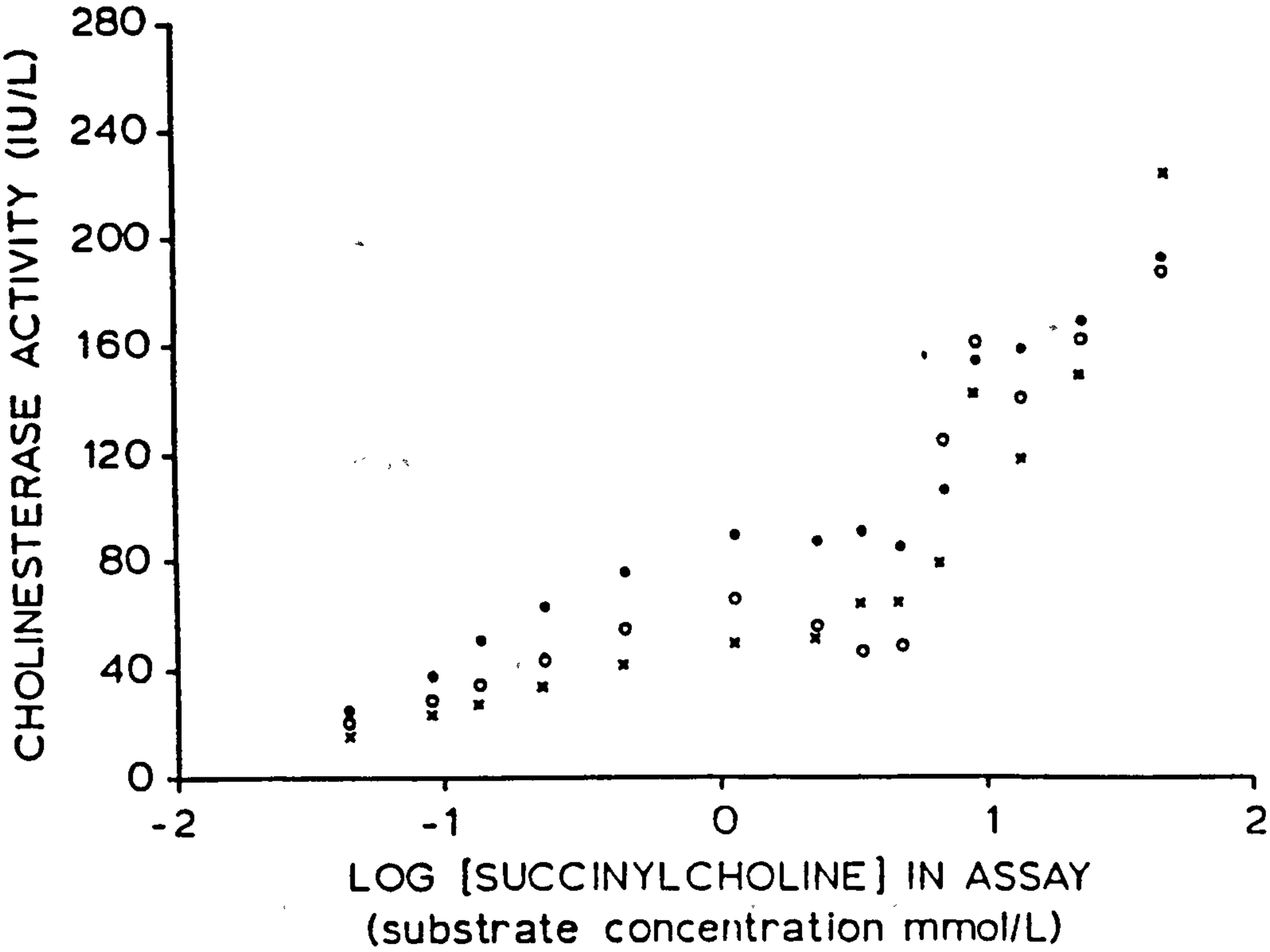

Figure 5.1

Plot of velocity against log succinylcholine concentration for serum from 3 individuals homozygous for the usual cholinesterase gene.

Symbols = mean of 4 replicates for each individual.

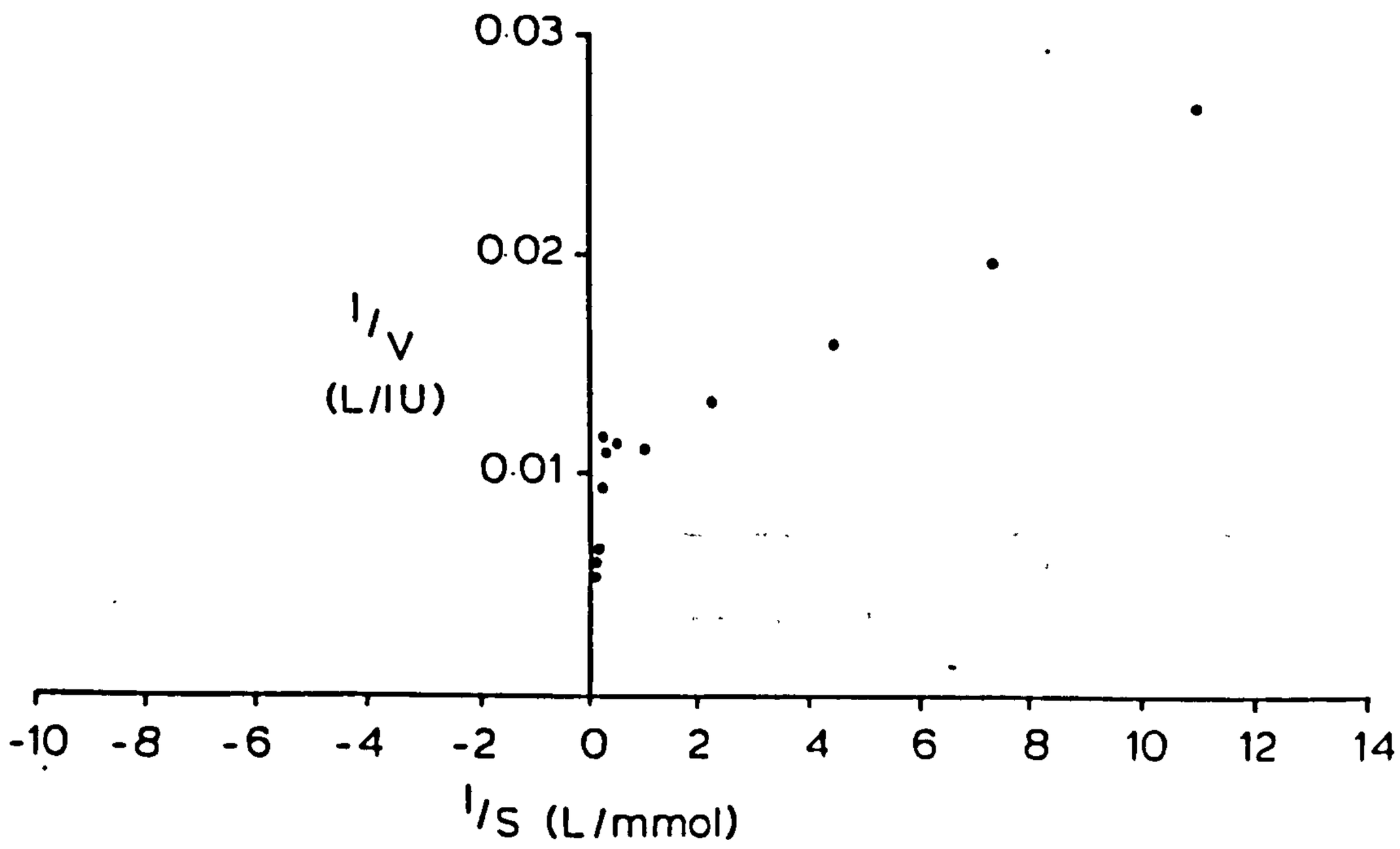

Figure 5.2

Lineweaver-Burk plot for serum from one individual homozygous for the usual gene.

Mean of 6 determinations at each substrate concentration are shown.

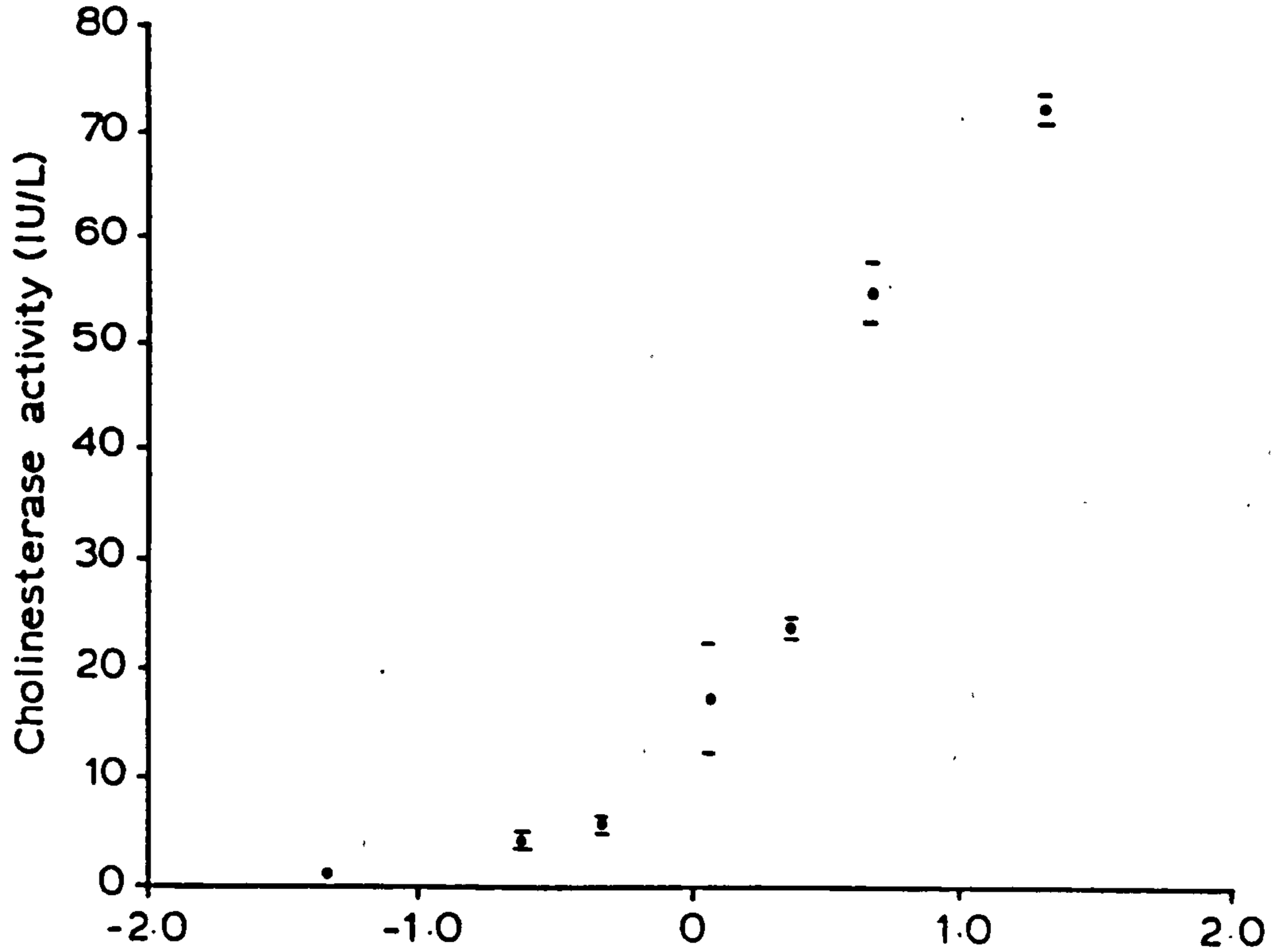

Figure 5.3

Plot of veloioty against log succinylcholine concentration for serum from individual homozygous for the atypical cholinesterase gene.

Mean ± 1 SD; n=5

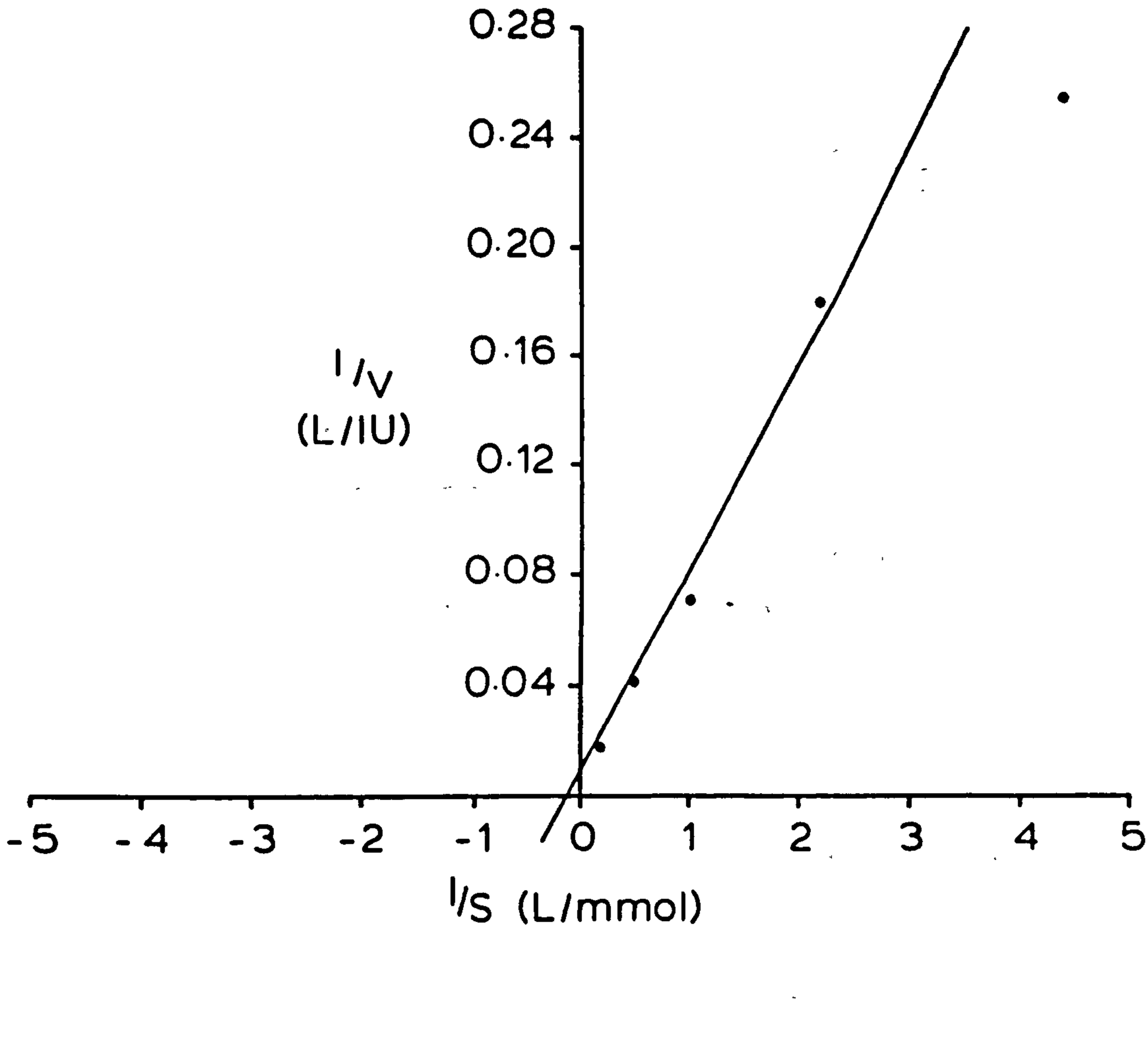

Figure 5.4

Lineweaver-Burk plot for the serum from an individual homozygous for the atypical gene.

Mean of 4 determinations at each substrate concentration.

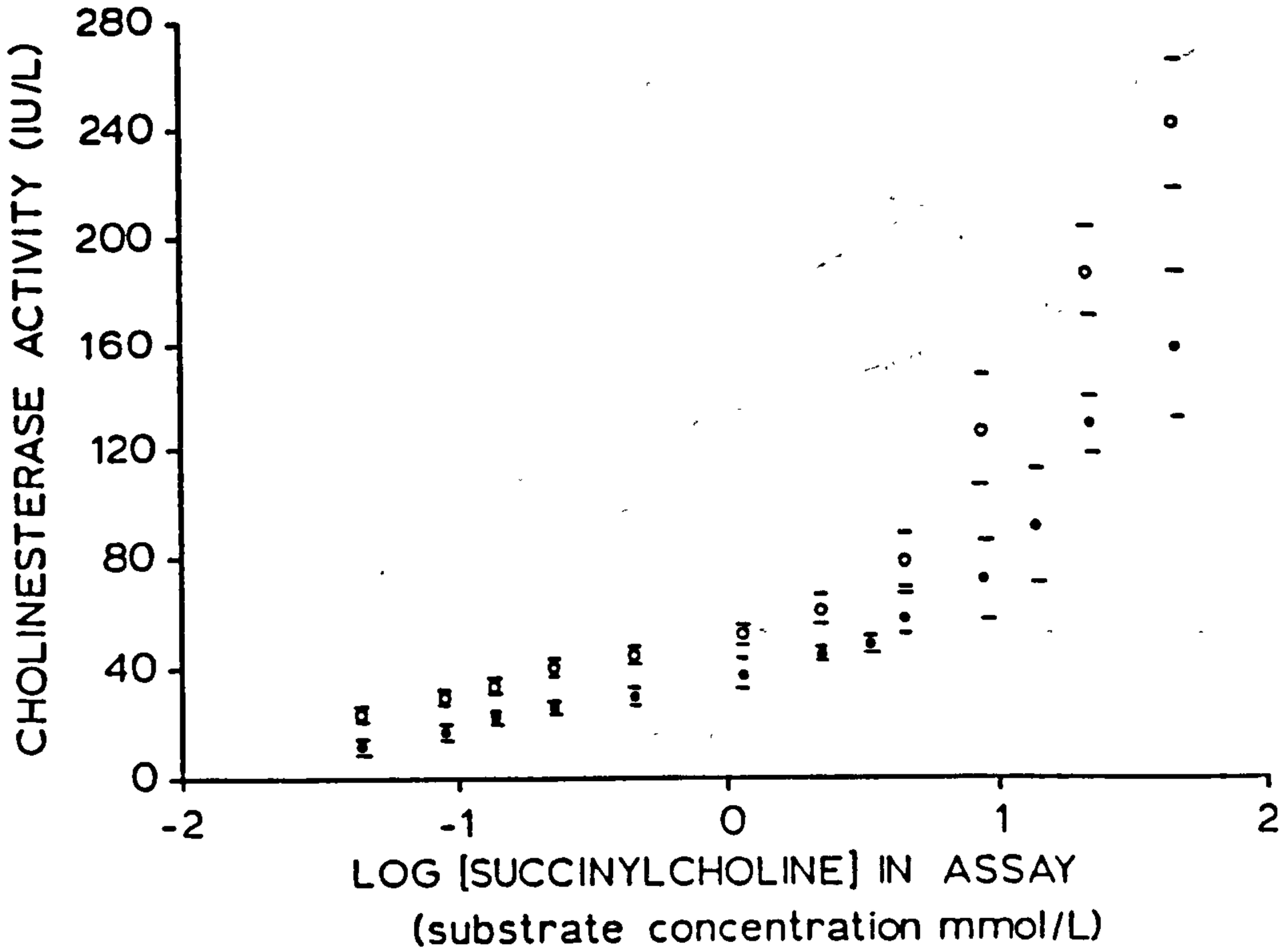

Figure 5.5

Plot of velocity against log succinylchlone concentration for purified cholinestease.

Circles = Sigma cholinesterase (Crude preparation)
Closed circles = Calbiochem Cholinesterase.
Purified 10,000 fold.

Horizontal lines ± 1 SD. n=6

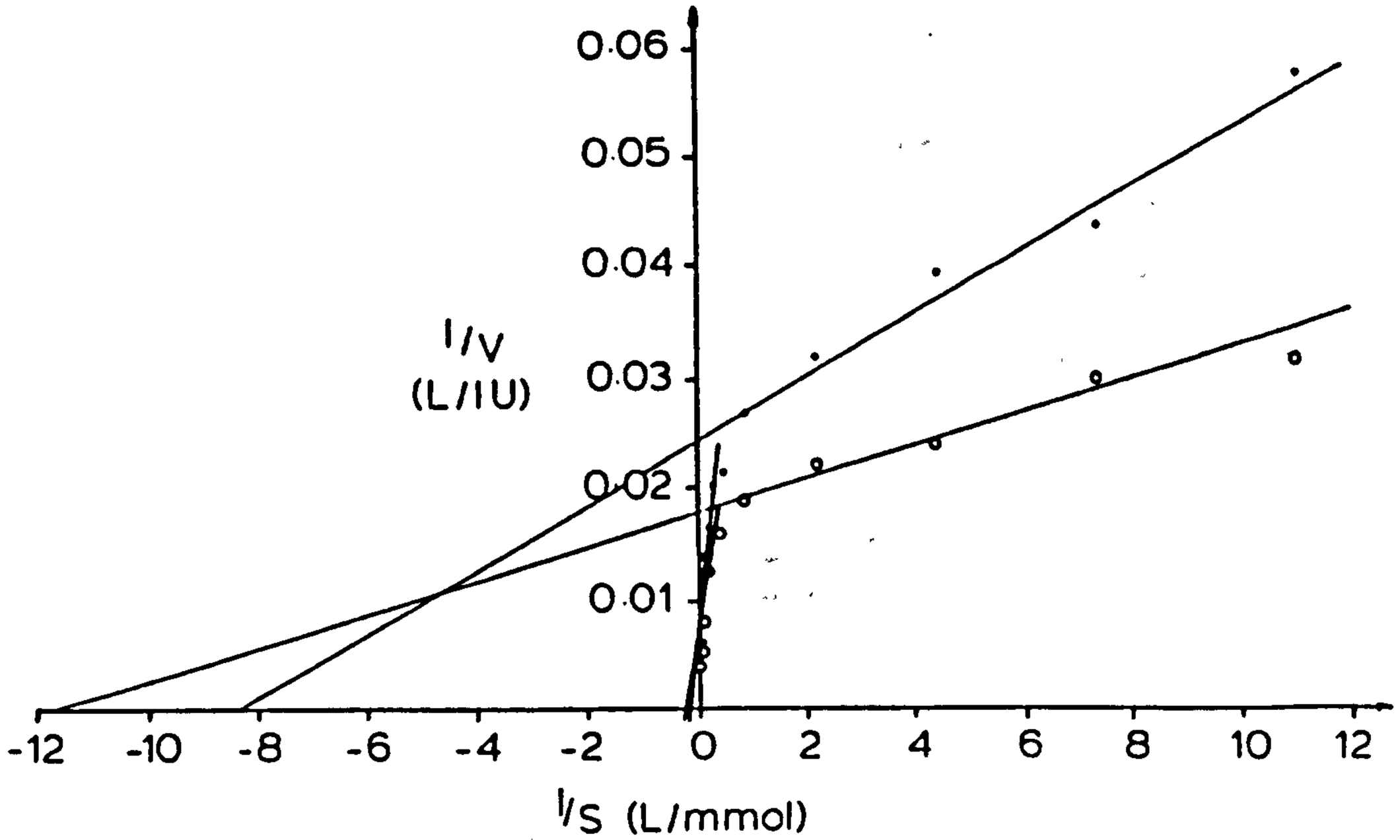

Figure 5.6

Lineweaver-Burk plot for purified cholinesterase.

Open circles = Sigma enzymes (crude preparation)
Closed circles = Calbiochem Enzyme.
Purified 10,000 fold.

Mean of 4 determinations at each substrate concentration.

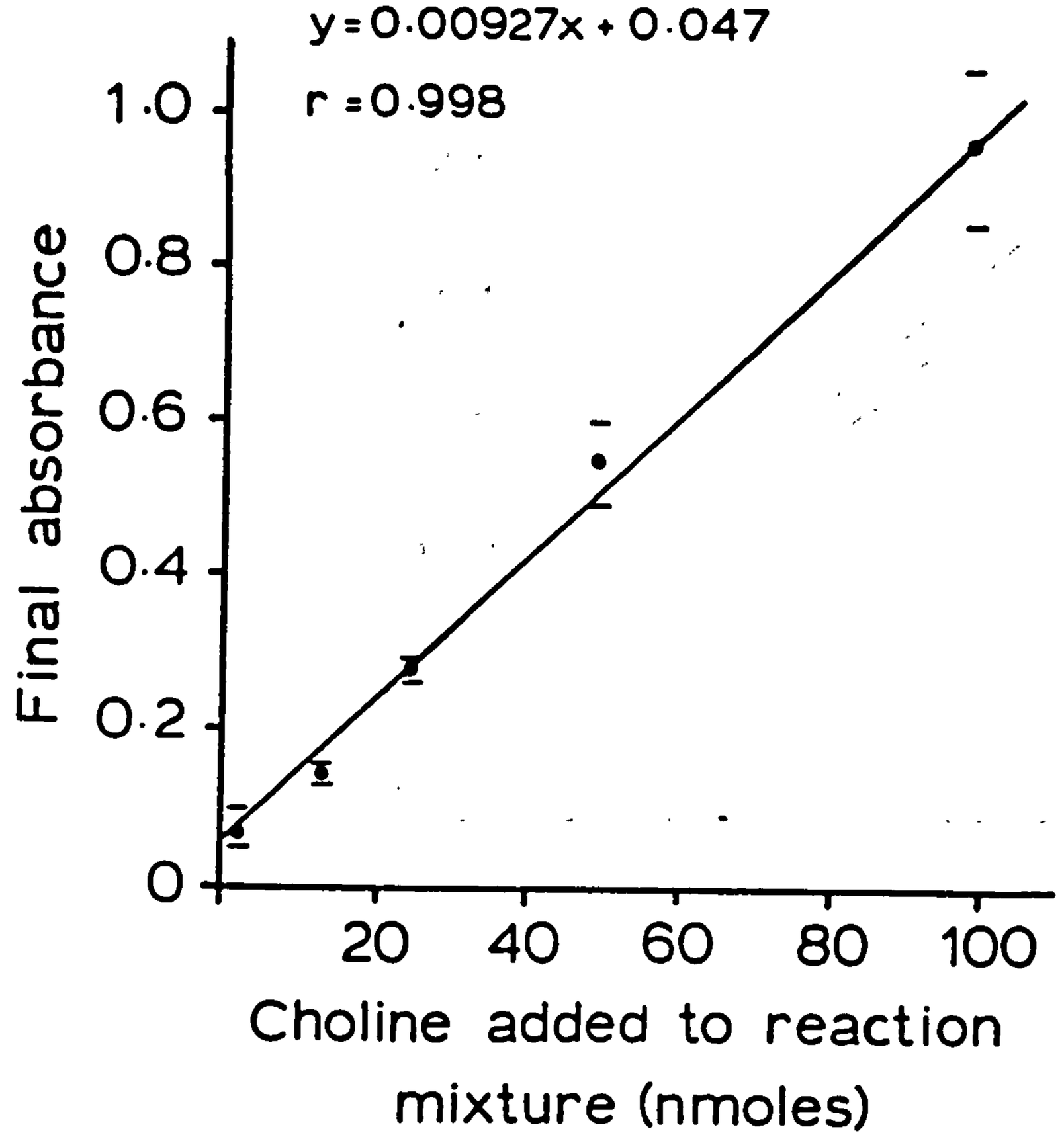

Figure 5.7

Absorbance produced by the addition of increasing amounts of choline iodode to the reaction mixture.

Mean± 1 SD; n=4

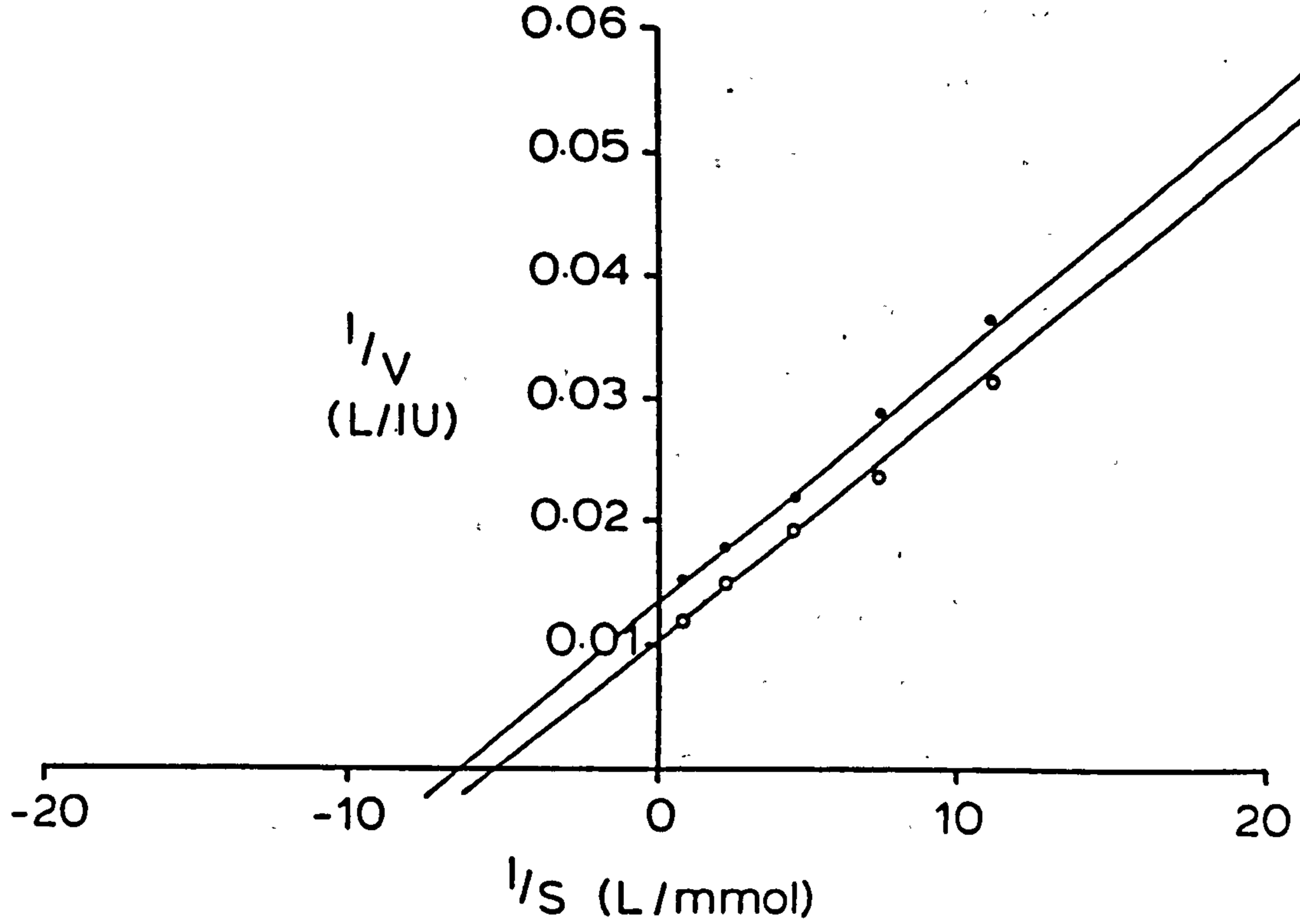

Figure 5.8
Lineweaver-Burk plot for serum from 1 subject before and after reducing the aminophenazone concentration.

Closed circles= original colour reagent.
Open circles = after reduction of aminophenazone concentration.

Mean of determinations at each substrate concentration.

CHAPTER 6

THE SUCCINYLCHOLINE-BASED ASSAY IN LIVER DISEASE.

Cholinesterase is synthesised in the liver and therefore can be used as a test of liver function in the same way as albumin. In Europe it is a frequently used test but in this country it has only been advocated in specific circumstances, for example in the surgical treatment of cirrhosis [Hunt and Lehmann 1960] and the monitoring of liver transplants [Evans and Lehmann 1971].

I have compared cholinesterase activities measured using succinylcholine with those obtained using conventional substrates; propionylthiocholine, benzoylcholine and butyrylthiocholine, in patients with clinically graded liver disease. A further comparison has been made between succinylcholine activity and serum albumin which is the most widely used test of the liver's synthetic ability.

6.1 Patients.

Blood samples were obtained from 54 patients with established liver disease in whom inhibition studies revealed no evidence for the presence of genetic variants of cholinesterase. These comprised 26 women and 28 men age range 19-72 years. The severity of the liver disease was assessed by a modification of the procedure originally described by Child [1964] and modified by Pugh et al. [1973]. It is based upon both clinical signs and laboratory measurements i.e. the presence or absence

of encephalopathy and ascites, serum concentrations of albumin and bilirubin and prothrombin time. The system scores 1, 2 or 3 points for increasing abnormality of each of the five parameters used. Patients whose liver function remains good and where scores total 5 or 6 are designated grade A. Scores of 7, 8 or 9 are equated with grade B disease and patients with 10-15 points, and therefore with poorest hepatic function, are designated grade C. Full patient details are shown in Table 6.1. All diagnoses were histologically proven by needle or open liver biopsy, the biopsies being reported by an independant histopathologist. Confirmative immunology was performed where appropriate.

6.2 Cholinesterase activities in patients with liver disease.

Cholinesterase activities are shown in Table 6.2.

The results expressed as standard deviations from the mean for Elu homozygotes and the patient's corresponding modified Childs grading are shown in Table 6.3 and Fig. 6.1.

Of the 54 patients 17 were found to have low activities (more than 2 standard deviations below the mean for $E1^u$ homozygotes) using propionylthiocholine, 19 using benzoylcholine and 20 using butyrylthiocholine. However using succinylcholine there were 31 low activities. Seventeen patients had low activities by all four procedures including all 10 who were graded C.

The relationship of abnormally low cholinesterase values to clinical grading of liver disease is shown in Table 6.4.

6.3 Comparison of albumin and succinylcholine activity in patients with liver disease.

Serum albumin and succinylcholine activity were measured in all patients. Albumin concentrations are shown in Table 6.1 and succinylcholine activities in Table 6.2. Low albumin values (less than 30g/L, the lower limit of the reference range) were found in 11 of the 54 patients and comprised 7 individuals with grade C and 4 with grade B disease. Low succinylcholine activities (less than 35 IU/L) were found in 31 patients made up from 6 with grade A, 15 with grade B and all 10 individuals with grade C liver disease.

Fig. 6. 2 illustrates the relationship between succinylcholine activity and serum albumin concentration.

6.4 Discussion.

All assays appear to be able to differentiate severe liver disease (Childs grade C) but the succinylcholine-based assay was able to identify more patients with moderate and mild disease.

This study demonstrates that there is little difference between conventional assays in their ability to assess hepatic function in agreement with the observations

of others [Prellwitz, Knapp and Muller 1976]. However a succinylcholine-based assay is superior.

Reasons why succinylcholine activity is a better index of hepatic function are, at present, speculative. Decreased plasma cholinesterase activity may reflect not only impaired synthetic ability by the liver but also possible inhibition by metabolites accumulating secondary to liver disease. Succinylcholine hydrolysis may be more susceptible to such factors.

Cholinesterase activity, irrespective of substrate, is a better index of severity of liver disease than albumin. It may reflect additional aspects of liver function in particular short term changes in synthetic capacity: cholinesterase has a shorter half life than albumin, 3.4 to 16 days [Brown et al. 1981] compared with 17 to 26 days [Eastham 1978].

Currently measurement of serum albumin is offered by most clinical chemistry laboratories in the profile of biochemical tests of liver function which are often requested routinely in the non-specific investigation of illness. Albumin may have advantages over cholinesterase in this situation in that it is an acute phase protein responding to many aspects of disease and is required to correctly interpret total calcium measurements. However if an assay is required specifically for the assessment or monitoring of liver disease a succinylcholine-based assay of plasma cholinesterase could well become a more desirable choice.

TABLE 6.1

FULL CLINICAL DETAILS OF PATIENTS WITH LIVER DISEASE.

Patient No.	Sex	Age yrs	Diagnosis + Childs grading	Bili umol /L	ALT IU /L	Alb g/L	PT sec	Ascites	Ence-phalo-pathy
1	M	48	AC [B]	27	65	29	15/ 12	++	+
2	M	51	CC [A]	12	31	39	12/ 12	-	-
3	F	58	CC [A]	31	58	38	12/ 12	-	-
4	F	63	PBC [A]	27	28	39	12/ 12	-	-
5	M	19	CC [A]	7	19	39	12/ 12	-	-
6	F	61	PBC [A]	10	48	43	12/ 12	-	-
7	M	50	SC [B]	92	46	34	14/ 12	+	-
8	F	59	PBC [B]	68	75	31	15/ 12	+-	+
9	M	37	CAH + C [B]	19	28	32	16/ 12	++	-
10	M	21	CAH + C [A]	9	31	42	12/ 12	-	-
11	M	22	CC [C]	43	38	18	18/ 12	++	+
12	F	38	CAH [A]	12	16	38	12/ 12	-	-
13	M	61	AC [B]	25	31	27	12/ 12	++	-
14	F	50	CPH [A]	13	62	41	12/ 12	-	-
15	F	71	AC [B]	29	48	31	18/ 12	-	-

TABLE 6.1 contd.

Patient No.	Sex	Age yrs	Diagnosis + Childs grading	Bili umol /L	ALT IU /L	Alb g/L	PT sec	Ascites	Encephalopathy
16	F	66	PBC [B]	240	39	22	12/ 12	+	-
17	F	48	PBC [B]	210	71	30	20/ 12	+	-
18	M	63	AC [B]	33	39	32	14/ 12	++	-
19	F	59	CAH [A]	9	11	43	12/ 12	-	-
20	F	71	CAH + C [B]	23	9	31	14/ 12	+	-
21	M	72	CAH [A]	10	19	42	12/ 12	-	-
22	M	61	AC [C]	35	25	33	21/ 12	+++	++
23	M	48	AC [C]	16	61	32	18/ 12	++	-
24	F	66	PIC [A]	8	8	43	12/ 12	+	-
25	M	23	CC [A]	12	110	40	12/ 12	-	-
26	M	48	AC [B]	18	42	28	-	++	-
27	F	56	CC [B]	27	159	34	16/ 12	+	-
28	F	60	CAH + C [A]	21	24	36	12/ 12	-	-
29	F	56	PBC [A]	32	40	38	12/ 12	-	-
30	M	60	AC [C]	25	36	24	17/ 12	+++	++
31	M	65	PBC [A]	17	41	37	12/ 12	-	-

TABLE 6.1 contd.

Patient No.	Sex	Age yrs	Diagnosis + Childs grading	Bili umol /L	ALT IU /L	Alb g/L	PT sec	Ascites	Encephalopathy
32	M	59	CAH + C [B]	29	78	36	16/ 12	+	+
33	M	67	AC [A]	20	75	42	12/ 12	-	-
34	M	39	CC [A]	11	21	44	-	-	-
35	F	44	AC [A]	21	79	46	12/ 12	-	-
36	M	61	CAH + C [B]	28	39	30	12/ 12	+	-
37	F	37	CPH [A]	10	19	39	12/ 12	+	-
38	F	64	CAH + C [A]	16	24	44	12/ 12	-	-
39	F	42	CC [A]	7	17	39	12/ 12	-	-
40	M	59	AC [C]	40	51	29	16/ 12	++	++
41	M	68	CAH + C [B]	33	39	39	18/ 12	+	-
42	F	54	CC [B]	26	110	36	12/ 12	-	-
43	F	62	CPH [A]	16	28	42	12/ 12	-	-
44	M	42	AC [B]	21	35	32	19/ 12	+	-
45	M	69	CAH + C [C]	50	75	19	22/ 12	+++	++
46	M	70	CC [A]	21	23	39	12/ 12	-	-
47	M	52	AC [C]	38	160	30	21/ 12	+++	+

TABLE 6.1 contd.

Patient No.	Sex	Age yrs	Diagnosis [+ Childs grading]	Bili umol /L	ALT IU /L	Alb g/L	PT sec	Ascites	Ence-phalo pathy
48	M	64	CC [C]	30	60	23	18/ 12	++	-
49	M	45	AC [A]	11	57	38	12/ 12	-	-
50	M	49	AC [A]	17	23	41	12/ 12	-	-
51	M	51	AC [B]	19	17	31	15/ 12	++	-
52	F	61	CC [C]	60	190	28	19/ 12	+++	+
53	F	39	CAH + C [C]	53	104	26	26/ 12	+++	++
54	F	62	CAH [A]	11	26	41	12/ 12	-	-

Diagnoses:
AC Alchoholic cirrhosis
CC Cryptogenic cirrhosis
PBC Primary biliary cirrhosis
SC Sclerosingcholangitis
CAH Chronic active hepatitis
CPH Chronic persistant hepatitis
PIH Post infective hepatitis

TABLE 6.2.

CHOLINESTERASE ACTIVITIES IN PATIENTS WITH LIVER DISEASE.

	Propionyl thiocholine activity IU/mL	Benzoyl choline activity IU/mL	Butyryl thiocholine activity IU/mL	Succinyl choline activity IU/L
mean ± 2SD for Elu homozygotes	4.58 ± 2.32	0.88 ± 0.508	5.04 ± 2.54	60.5 ± 25.5
Patient number				
1	1.12	0.18	1.02	16.3
2	9.16	1.40	8.18	97.0
3	2.01	0.32	2.03	24.7
4	3.38	0.55	3.14	32.0
5	4.81	0.75	4.53	59.8
6	3.87	0.61	3.53	39.3
7	4.43	0.44	2.70	35.0
8	3.22	0.53	3.12	46.6
9	3.18	0.46	2.75	30.0
10	3.21	0.42	2.86	35.1
11	1.82	0.28	1.56	24.1
12	4.56	0.65	4.20	49.5
13	1.73	0.27	1.57	23.5
14	7.07	1.032	7.36	70.0
15	3.06	0.41	2.76	34.9
16	2.80	0.39	2.98	31.2
17	1.76	0.31	1.87	19.7
18	1.81	0.32	1.80	23.5
19	5.33	0.84	5.20	62.5
20	3.11	0.60	3.19	36.4
21	4.16	0.68	4.27	49.7
22	0.97	0.16	0.94	16.1
23	1.74	0.21	1.61	22.6
24	3.84	0.68	3.89	37.5
25	2.49	0.39	2.44	36.0
26	1.79	0.27	1.79	24.5
27	4.07	0.65	3.73	39.4
28	3.13	0.50	3.02	36.3
29	2.64	0.37	2.48	30.2
30	1.20	0.29	1.22	14.2
31	4.18	0.64	3.66	41.4
32	2.55	0.45	2.86	26.7
33	4.58	0.75	4.20	42.6
34	6.14	0.96	5.77	56.3
35	5.76	0.77	5.38	37.3
36	3.06	0.45	2.83	34.2
37	4.61	0.87	4.42	29.0

TABLE 6.2 contd.

	Propionyl thiocholine activity IU/mL	Benzoyl choline activity IU/mL	Butyryl thiocholine activity IU/mL	Succinyl choline activity IU/L
mean ± 2SD for Elu homozygotes	4.58 ± 2.32	0.88 ± 0.508	5.04 ± 2.54	60.5 ± 25.5
Patient number				
38	5.28	0.82	5.03	57.3
39	4.61	0.75	4.88	47.8
40	1.88	0.28	1.80	24.2
41	3.40	0.60	3.22	33.9
42	2.50	0.43	2.72	29.8
43	1.99	0.29	1.88	25.1
44	2.59	0.37	2.29	29.7
45	1.23	0.21	1.34	18.4
46	3.99	0.67	3.74	47.8
47	2.04	0.34	1.90	24.8
48	1.18	0.18	1.32	18.7
49	2.90	0.47	2.86	27.1
50	4.96	0.70	4.80	49.3
51	3.90	0.57	3.40	29.0
52	0.85	0.13	0.75	10.9
53	1.57	0.25	1.48	17.8
54	6.08	1.04	5.66	67.6

TABLE 6.3.

CHOLINESTERASE ACTIVITIES EXPRESSED AS STANDARD DEVIATIONS FROM THE MEAN FOR Elu HOMOZYGOTES AND MODIFIED CHILDS GRADING FOR PATIENTS WITH LIVER DISEASE.

Patient No.	Mod. Childs grading	Propionyl thio-choline activity IU/mL	Benzoyl choline activity IU/mL	Butyryl thio-choline activity IU/mL	Succinyl choline activity IU/L
		mean±1SD	mean±1SD	mean±1SD	mean±1SD
		4.58±1.16	0.88±0.25	5.04±1.27	60.5±11.7
1	B	-2.98	-2.75	-3.16	-3.5
2	A	+2.9	+2.66	+2.47	+2.86
3	A	-2.21	-2.2	-2.03	-2.81
4	A	-1.03	-1.29	-1.5	-2.24
5	A	+0.2	-0.51	-0.45	-0.06
6	A	-0.6	-1.06	-1.2	-0.67
7	B	-0.15	-1.73	-1.84	-2.0
8	B	-1.18	-1.37	-1.51	-1.09
9	B	-1.2	-1.6	-1.8	-2.39
10	A	-1.18	-1.5	-1.71	-1.99
11	C	-2.34	-2.36	-2.74	-2.84
12	A	-0.02	-0.9	-0.66	-0.87
13	B	-2.45	-2.4	-2.81	-2.91
14	A	+2.14	+0.6	+1.82	+0.74
15	B	-1.31	-1.85	-1.79	-2.01
16	B	-0.62	-1.93	-1.62	-2.30
17	B	-2.43	-2.24	-2.5	-3.20
18	B	-2.4	-2.2	-2.55	-2.90
19	A	+1.0	-0.3	-0.13	+0.15
20	B	-1.26	-1.1	-0.15	-1.81
21	A	-0.36	-0.8	-0.61	-1.85
22	C	-3.11	-2.83	-3.22	-3.48
23	C	-2.44	-2.63	-2.46	-2.98
24	A	-1.12	-0.3	-1.07	-1.80
25	A	-1.8	-1.93	-2.04	-1.93
26	B	-2.4	-2.4	-2.9	-2.82
27	B	-0.44	-0.9	-1.03	-1.65
28	A	-1.25	-1.5	-1.32	-1.90
29	A	-1.67	-2.0	-2.01	-2.38
30	C	-2.91	-2.32	-3.01	-3.63
31	A	-0.34	-0.94	-1.10	-1.5
32	B	-1.75	-1.53	-1.71	-2.65
33	A	0	-0.52	-1.40	-0.66
34	A	+1.34	+0.30	+0.57	-0.33
35	A	+1.01	-0.43	+0.26	-1.82
36	B	-1.31	-1.69	-1.40	-2.07
37	A	+0.03	0	-0.48	-2.47
38	A	+0.6	-0.02	0	-0.25
39	A	+0.02	-0.44	-0.13	-1.00

TABLE 6.3 contd.

Patient No.	Mod. Childs grading	Propionyl thio-choline activity IU/mL	Benzoyl choline activity IU/mL	Butyryl thio-choline activity IU/mL	Succinyl choline activity IU/L
		mean±1SD	mean±1SD	mean±1SD	mean±1SD
		4.58±2.16	0.88±0.25	5.04±1.27	60.6±11.7
40	C	-2.32	-2.36	-2.55	-2.85
41	B	-1.01	-1.1	-1.29	-2.09
42	B	-1.8	-1.77	-1.82	-2.42
43	A	-2.23	-2.32	-2.49	-2.78
44	B	-1.71	-2.01	-2.16	-2.42
45	C	-2.88	-2.63	-2.91	-3.3
46	A	-0.5	-0.82	-1.02	-1.00
47	C	-2.2	-2.12	-2.47	-2.8
48	C	-2.9	-2.75	-2.93	-3.28
49	A	-1.44	-1.61	-1.71	-2.60
50	A	+0.2	-0.71	-0.18	-0.88
51	B	-0.58	-1.22	-1.29	-2.47
52	C	-3.18	-2.95	-3.37	-3.9
53	C	-3.3	-2.48	-2.8	-3.38
54	A	+1.27	+0.63	+0.48	+0.55

TABLE 6.4.

NUMBERS OF SUBJECTS WITH LOW CHOLINESTERASE VALUES AND THEIR CHILDS GRADING.

Assay Substrate	Low cholinesterase	Childs Grading A	B	C
Propionylthiocholine	17	2	5	10
Benzoylcholine	19	3	6	10
Butyrylthiocholine	20	4	6	10
Succinylcholine	31	6	15	10
Breakdown of all patients		26	18	10

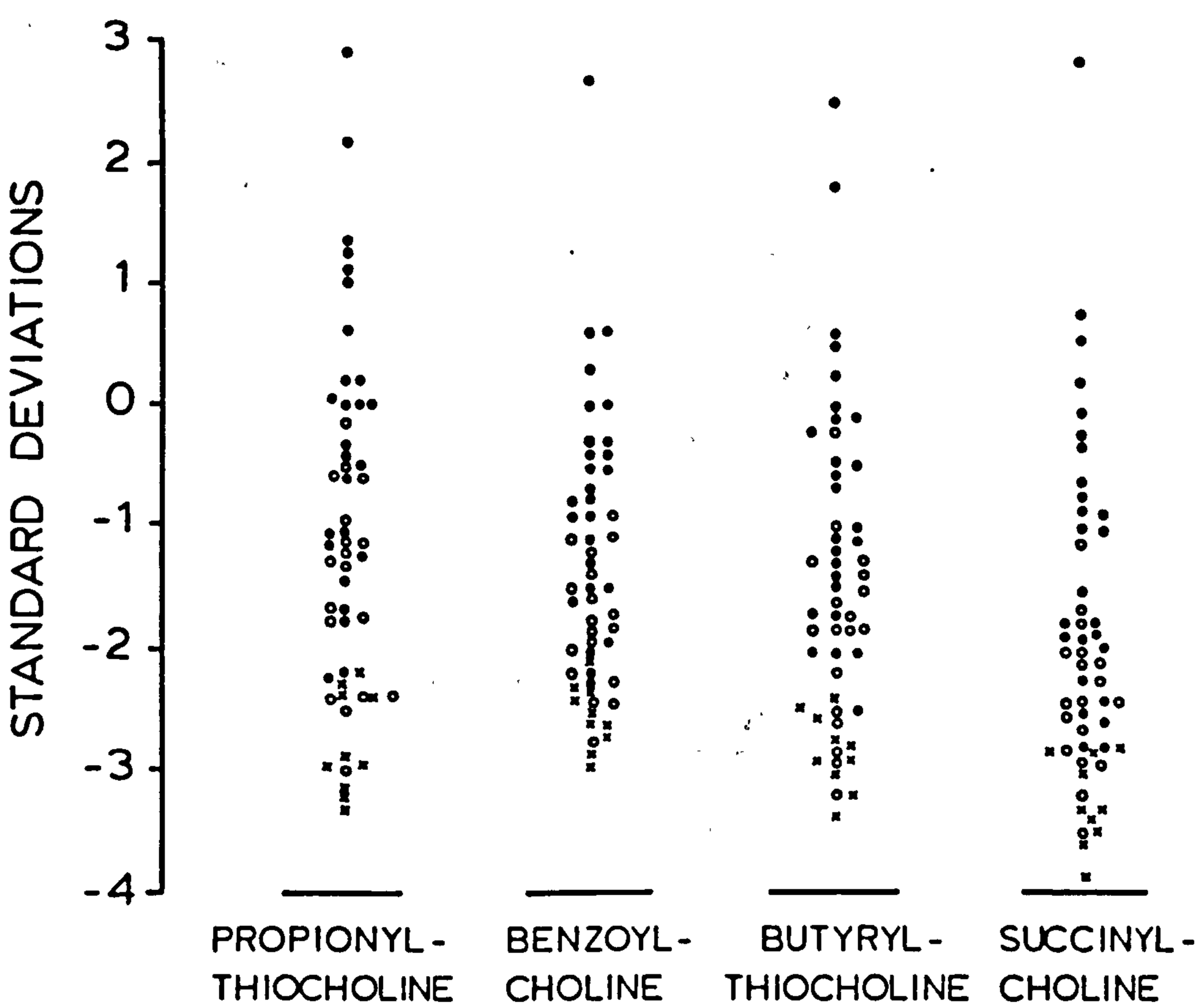

Figure 6.1

Cholinesterase activities measured using 4 substrates in patients with clinically graded liver disease.

Results expressed as standard deviations from the appropriate mean for $E1^{U}$ homozygotes.

Childs grading.
Closed circles = grade A.
Open circles = grade B.
Crosses = grade C.

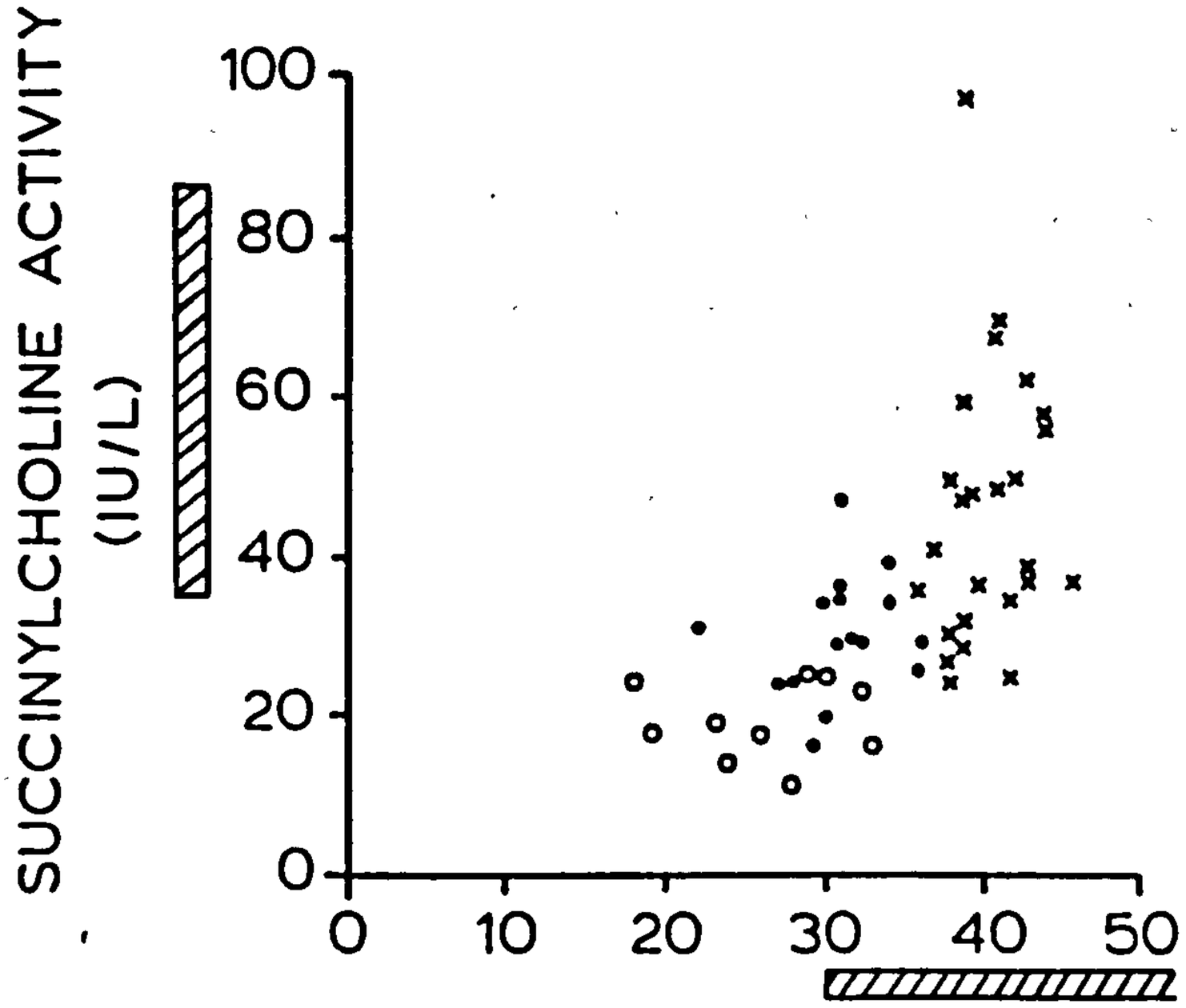

Figure 6.2

Albumin concentration and succinylcholine activity in patients with clinically graded liver disease.

Childs grading.
Closed circles = grade C
Open circles = grade B
Crosses = grade A

Shaded bars denote the respective reference intervals.

CHAPTER 7.

THE SUCCINYLCHOLINE-BASED ASSAY IN CHILDREN.

There is considerable evidence that children require more succinylcholine than adults to produce the same intensity of neuromuscular block. The intravenous dose of succinylcholine required for intubation varies with the patient's age. The recommended dose of succinylcholine for children ranges from 1.5 to 3 mg/kg [Letty et al 1981] whilst adults require a dose of 1 mg/kg. In addition it has been found that complete suppression of twitch after succinylcholine was present in only 19/23 children who received 4 mg/kg [Lui et al. 1981] whereas 15 adults who received the same dose all developed complete inhibition of twitch [Walts and Dillon 1967].

The observation may be related to differences in plasma cholinesterase. The duration of action of succinylcholine has been shown to be associated with plasma cholinesterase activity in adults [Hodges and Harkness 1954], particularly so when the enzyme activity is outside normal limits [Viby-Mogensen 1980]. A strong negative correlation has also been demonstrated between plasma cholinesterase activity and the duration of succinylcholine block in post-puerperal patients [Ganga et al. 1982]. Published work however suggests that plasma cholinesterase is similar [Mirakur, Elliot and Lavery 1984] or even lower [Hutchinson and Widdowson 1952] in children compared with adults and therefore should not account for the shorter

duration of action of succinylcholine in children. These activity measurements were performed using choline esters other than succinylcholine and therefore do not necessarily reflect the ability of plasma cholinesterase to metabolise the drug. The object of this section of the project was to use the succinylcholine-based assay to measure cholinesterase in young children and to compare the results with those obtained in adults.

7.1 Subjects.

Plasma samples were obtained from 32 infants, 17 female and 15 male, age range 1 day to 14 months. No subjects were included having a serum bilirubin of greater than 100 umol/L, liver enzymes (aspartate transaminase and alkaline phosphatase) outside the appropriate age-related reference range, or who possessed a genetic abnormality of cholinesterase. Plasma was collected by pooling what remainded after the requested biochemical tests had been performed. Subjects fell into two groups, neonates (n=17) age range 1-4 days the majority of whom were preterm, and infants (n=15) age range 1-14 months.

7.2 Cholinesterase activity of subjects studied.

Succinylcholine and propionylthiocholine activities were measured. Results are shown in Table 7.1 and Fig. 7.1.

Neonates had lower mean values than adults using both succinylcholine and propionylthiocholine substrates, 48.7 IU/L and 3.09 IU/mL compared with 58.5 IU/L and

4.58 IU/mL respectively. Propionylthiocholine activities were similar in both infants and adults, 4.68 and 4.58 IU/mL respectively but the mean succinylcholine activity of 77.1 IU/L was much higher than that in adults (58.5 IU/L).

6.3 Discussion.

The lower mean cholinesterase activities found in neonates is consistant with the work of Strauss and Modanlou [1986] and Ehrich and Rothganger [1987]. Both groups found reduced cholinesterase activity in preterm infants compared with full term babies or adults which rose to normal within a few weeks of birth.

The infants studied here had propionylthiocholine activities similar to those of adults whereas their succinylcholine activities were higher. The results obtained using the succinylcholine-based assay contast with those obtained elsewhere using different substrates. Zsigmond and Downs [1971] using benzoylcholine found lower activities in infants than adults. Mirakur, Elliot and Lavery [1984] using acetylthiocholine and Strauss and Modanlou [1986] using butyrylthiocholine both found that activities were similar in infants and adults.

The results which I have obtained using the succinylcholine indicate that cholinesterase may have a role to play in the apparent resistance of infants to succinylcholine. However activities were not

sufficiently high to be the sole explanation for this.

It has previously been suggested that a redistribution of succinylcholine from the end plate into the extracellular fluid (ECF) space thus avoiding breakdown by cholinesterase, may be the primary determinant of it's duration of action [Cook 1981]. Since children have a larger ECF volume to muscle mass ratio than adults, this may account for their relative resistance to the drug. Supportive evidence for this is that the effects of succinylcholine are the same in children as in adults when the the drug is administered on the basis of body surface area [Walts and Dillon 1969]. Another explanation for the shorter duration of action of succinylcholine in children is the reported difference in the apparent first order elimination rate constant between infants and adults [Nugent, Laravuso and Rogers 1979].

Therefore these factors in addition to the increased cholinesterase activity in infants may all contribute to the apparent resistance of infants to the action of succinylcholine.

From the above observations it is clear that the substrate used for the measurement of cholinesterase activity will alter the conclusions drawn. For example, on the basis of their results, using a benzoylcholine assay, Zsigmond and Downs [1971] suggested that the dose of succinylcholine could be reduced by one third in children of less than twelve months of age. This is not

supported by use of a succinylcholine-based assay which in contrast indicates that the dose needs to be increased, in line with current clinical practice. Therefore any extrapolation concerning sensitivity to succinylcholine may be misleading unless the substrate used reflects accurately the rate of hydrolysis of the drug in vivo. The succinylcholine-based assay is the one most likely to do this.

TABLE 7.1

CHOLINESTERASE ACTIVITIES IN NEONATES AND INFANTS.

a) Age range 1-4 days.

Age days	Succinyl choline activity IU/L	Propionyl thiocholine activity IU/mL
2	55.4	3.8
4	38.0	2.7
2	48.4	2.3
3	69.4	4.0
1	45.1	3.3
3	63.5	3.6
3	39.4	2.3
1	69.2	4.1
1	56.4	5.1
2	36.6	2.0
3	34.7	1.7
3	29.9	*
1	27.5	*
2	70.6	3.1
1	38.9	2.5
3	59.7	*
1	45.6	2.8
mean	48.74	3.09
SD	14.3	0.95

b) Age range 1-14 months

Age months	Succinyl choline activity IU/L	Propionyl thiocholine activity IU/mL
2	57.4	3.9
4	81.1	4.4
1	70.2	3.4
4	127.9	*
2	85.8	*
5	37.5	*
6	105.2	*
2	89.1	5.0
1	59.2	5.0
1	85.8	5.4
3	63.0	4.7
14	60.7	4.3
4	113.2	7.7
5	51.6	5.4
8	68.7	4.3
mean	77.09	4.86
SD	24.03	1.12

* insufficient sample for analysis.

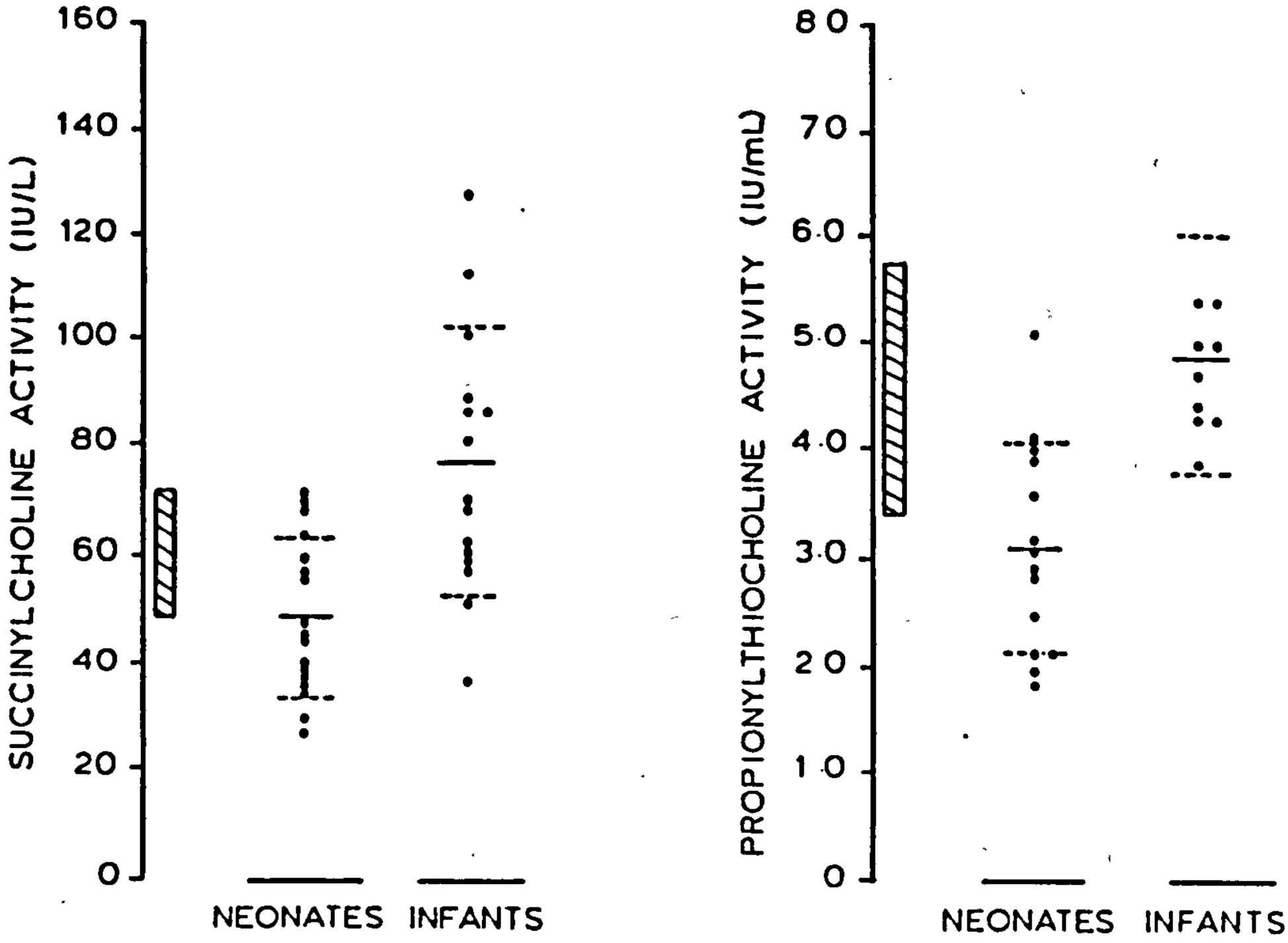

Figure 7.1

Cholinesterase activities in neonates & infants measured using succinylcholine and propionylthiocholine as substrates.

Shaded bars = reference intervals for adult $E1^u$ homozygotes for each method.

Heavy and broken horizontal lines = mean $\pm$1 SD

CHAPTER 8

CHOLINESTERASE MEASUREMENTS IN ANIMALS

Cholinesterase occurs throughout the animal kingdom though with different physicochemical and biochemical properties. Despite this extensive distribution, the natural substrate and function of the enzyme is not known. Nonetheless cholinesterase measurements do have practical significance. They have been used to assess the degree of exposure to organophosphates [Crookshank and Palmer 1978] and in experiments to provide information concerning the effect of succinylcholine on animals [Wright et al. 1981].

There is wide species variation in response to succinylcholine. Domestic animals have been most extensively studied. The horse, pig and cat are relatively resistant whereas dogs, sheep and cattle are sensitive to even small doses [Hall and Clarke 1983].

Since hydrolysis by cholinesterase is the major factor in recovery from the effects of succinylcholine attempts have been made to correlate species sensitivity with plasma cholinesterase activity. The ability of plasma cholinesterase to hydrolyse butyrylthiocholine can be used to predict sensitivity to succinylcholine in man but it has not been of value in animals [Hansson 1957, Wright et al. 1981]. It is possible that in species other than man the rate of hydrolysis of butyrylthiocholine does not parallel that of succinylcholine and if so, a

succinylcholine-based assay could give a better index of the species ability to hydrolyse the drug in vivo. The object of this section of the thesis was to investigate this hypothesis.

8.1 Blood samples and methods.

Blood samples from animals were obtained as follows,

a) Dogs and cats from a local veterinary surgeon.

b) Cattle, pigs and sheep from Mr M.S Bain, Veterinary Investigation Centre, Riseholme, Lincolnshire.

c) Horses from Dr M.S Moss, Horse Racing Forensic Laboratories Ltd., Newmarket, Suffolk.

d) Chickens from Professor Kerr, Department of Animal Physiology and Nutrition, University of Leeds.

e) Zoo animals from Dr C Hawkey, Wellcome Research Laboratories, London Zoo, Regents Park, London.

Samples from dogs and cats were collected and separated daily then stored at -20^{o}C. All other samples were collected, separated and stored (at -20^{o}C) by the laboratories concerned.

Cholinesterase activity was measured using propionylthiocholine, benzoylcholine,butyrylthiocholine and succinylcholine as substrates.

8.2 Studies on domestic animals.

The following domestic species were studied, pigs (two breeds Landrace and Large White), dogs, cats, cattle, sheep, chickens and horses. Cholinesterase activities were measured using all four substrates except with

specimens from cats. These were small and so only propionylthiocholine and succinylcholine activities were performed. Results are shown in Fig. 8.1 and Table 8.1. Dogs and horses demonstrate highest cholinesterase activity with butyrylthiocholine as the substrate whereas chickens show highest activity with propionylthiocholine. The ruminants studied had low cholinesterase activities with all substrates.

In any attempt to correlate cholinesterase activity with response to succinylcholine it is first necessary to find a comparable dose of drug in all species. This is difficult since quoted doses result in different durations of action depending on the requirement in a particular animal. Ideally evidence is needed for the amount of succinylcholine which would paralyse each species for the same time. I did not have the facilities to perform such work so an alternative approach was taken.

Tolerance to succinylcholine has been estimated experimentally for cattle, dogs, pigs (Landrace) and horses [Hansson 1957]. The dose required to produce muscle fasiculation without the loss of the ability to stand was used as a reproducible end-point. In man an infusion of 28-43 ug/kg/min produces muscle relaxation [Laurence and Bennett 1980]. Approximately half this dose produces muscle fasiculation, as seen when succinylcholine is used as an adjunct to ECT therapy [Dept. of Anaesthesia, Leeds General Infirmary, personal

communication]. Cholinesterase activities measured using the four substrates were plotted against the data obtained from Hanssen's study and that for man. Results are shown in Figs. 8.2 and 8.3.

Results demonstrate that cholinesterase activity measured using succinylcholine as the substrate is directly proportional to tolerance to the drug whereas those obtained using any of the other substrates there show no such relationship.

7.3 Studies on zoo animals.

Cholinesterase activities using all four substrates are shown in Table 8.2. In addition they are expressed as a percentage of the mean of the appropriate reference range for human Elu homozygotes, to aid comparison.

Of the Orders studied, Aves, Cetacea and Proboscidea contained only one animal each so no conclusions can be drawn. Of the remaining Orders cholinesterase activities measured using propionylthiocholine, benzoylcholine and butyrylthiocholine are similar, Man> Reptila> Primata> Perissodactyla> Carnivora> Artiodactyla. Whereas using succinylcholine there is a different pattern of hydrolysis, Primata> Man> Carnivora> Perissodactyla> Reptila> Artiodactyla.

8.4 Discussion.

The substrate specificity patterns of plasma cholinesterases from different species are diverse. Some show maximum activity against butyrylthiocholine and

some against propionylthiocholine. Ruminants demonstrated little activity using any of the substrates. These results agree with those of Myers [1948].

Cholinesterase activities plotted against a measure of tolerance to succinylcholine show that only with succinylcholine as the assay substrate does plasma cholinesterase correlate with the species ability to hydrolyse the drug. This is at odds with the results of Hansson [1957] who did not show any relationship between succinylcholine hydrolysis in vitro and the species sensitivity to the drug. However Hansson's succinylcholine-based assay was relatively insensitive and gave undetectable hydrolysis rates for cholinesterase from the plasma of cattle and dogs.

Dogs have a prolonged relaxation when treated with succinylcholine in a dose which would be appropriate for a human [Merin 1986]. It was initially thought this was due to a different plasma cholinesterase in canines but it has not been substantiated [Hall and Lehmann 1953 and Hansson 1957]. Results presented here show that dogs have lower cholinesterase than humans, whichever substrate is used for it's assay. The contrast however is most marked when results obtained using succinylcholine are compared. The mean activity in dogs was found to be 14.8% that of the mean activity in humans when succinylcholine was used as the assay

substrate. With other substrates the mean activities were 40-49% that of humans. Muscle relaxation in dogs is readily achieved following administration of 0.1-0.2 mg succinylcholine /kg of body weight. This is 10-20% of that required in humans. This observation can be reconciled with plasma cholinesterase activity only when the latter is measured using succinylcholine.

The in vitro hydrolysis of succinylcholine by cholinesterase appears to parallel it's breakdown in vivo in different domestic animal species whereas the hydrolysis of other substrates does not. This emphasises the importance of using such a method in the investigation of all animal species, but especially in in wild animals where experience with succinylcholine is limited.

Wright and colleagues [1981], in an attempt to explain the susceptibility of different species to succinylcholine, measured cholinesterase activity against butyrylthiocholine in 26 wild animal species from South Africa. They could not explain differences in susceptibility using butyrylcholine hydrolysis or use it to predict effective doses of the drug.

In the present study 33 species from 9 orders were studied. The hydrolysis of three additional substrates as well as butyrylthiocholine was examined and results confirm findings with respect to wide species variation in cholinesterase activities [Wright et al 1981]. Some species having low or undetectable activities whilst

others have levels comparable with the highest seen in man.

Using propionylthiocholine, benzoylcholine and butyrylthiocholine as assay substrates the pattern of cholinesterase activities of the different Orders studied was similar. Highest activities occur in Man and the lowest in Artiodactyla. Succinylcholine hydrolysis patterns were different. However it is difficult to assess whether this difference means it can be used to predict the susceptibility of different species to it's muscle relaxant properties.

In domestic species the doses of succinylcholine used are well documented both clinically and experimentally [Hall and Clarke 1983]. Such information is not available on wild species. Where dose levels are documented they concern single or low numbers of animals and no durations of paralysis are given [Buechner, Harthoorn and Lock 1960a and 1960b] thus information is anecdotal and not readily comparable.

The only large cohort of animals these workers studied was a group of 50 Adenota kob thomasi (antelope). A dose which effectively immobilised all animals was 0.35 mg/kg approximately one third of the muscle relaxant dose in man. The antelope is of the Order Artiodactyla and in the present study only cholinesterase activities measured using succinylcholine are of a magnitude compatible with this clinical observation, being 10-30%

of that seen in man. With other substrates, activities were undetectable or only up to 5% of values in man. Clearly the sensitivity of the antelope, like that of the domestic species studied, can be predicted solely by use of the succinylcholine-based assay.

The work presented in this chapter confirms the hypothesis that cholinesterase activity measured using succinylcholine rather than conventional assay substrates, gives a better index of the ability of different species to hydrolyse the drug in vivo.

TABLE 8.1. CHOLINESTERASE ACTIVITIES IN DOMESTIC ANIMALS.

1. PIGS (Landrace)

No.	Propionyl thiocholine activity IU/mL	Benzoyl choline activity IU/mL	Butyryl thiocholine activity IU/mL	Succinyl choline activity IU/L
1	0.32	0.01	0.26	30.5
2	0.34	0.01	0.27	29.9
3	0.31	0.01	0.24	25.9
4	0.34	0.02	0.21	34.6
5	0.23	0.02	*	19.9
6	0.28	0.02	0.27	31.5
7	0.29	0.02	0.27	39.8
8	0.32	0.02	0.27	*
9	0.35	0.02	0.30	38.2
10	0.39	0.01	0.34	43.2
11	0.27	0.02	0.27	36.0
12	0.34	0.03	0.26	31.5
13	0.38	0.04	0.33	22.8
14	0.23	0.03	0.24	16.6
15	0.43	0.05	0.36	64.4
16	0.33	0.04	0.35	49.4
mean	0.322	0.023	0.288	34.28
lSD	0.054	0.012	0.047	12.06

2. PIGS (Large White)

No.	Propionyl thiocholine activity IU/mL	Benzoyl choline activity IU/mL	Butyryl thiocholine activity IU/mL	Succinyl choline activity IU/L
1	0.42	0.07	0.51	58.7
2	0.27	0.02	0.26	19.1
3	0.32	0.02	0.32	50.7
4	0.38	0.05	0.36	24.1
5	0.41	0.04	0.43	70.5
6	0.36	0.04	0.32	50.2
7	0.41	0.05	0.33	68.3
8	0.38	0.03	0.39	56.4
9	0.35	0.05	0.30	58.5
10	0.22	0.02	0.25	49.2
11	0.34	0.02	0.31	45.7
12	0.20	0.02	0.15	51.3
13	0.33	0.03	0.30	44.9
14	0.56	0.06	0.60	80.0
mean	0.351	0.037	0.345	51.97
lSD	0.086	0.017	0.112	16.32

3. DOGS

No.	Propionyl thiocholine activity IU/mL	Benzoyl choline activity IU/mL	Butyryl thiocholine activity IU/mL	Succinyl choline activity IU/L
1	1.94	0.35	2.2	3.9
2	1.73	0.30	1.9	3.3
3	1.75	0.34	2.2	6.3
4	2.46	0.44	3.0	8.9
5	2.33	0.39	*	9.1
6	2.1	*	*	8.2
7	7.55	*	*	13.5
8	0.81	*	*	7.2
9	1.8	*	*	8.4
10	2.45	*	*	8.6
11	1.0	*	*	4.0
12	1.6	*	*	11.6
13	1.3	*	*	ND
14	0.97	*	*	19.2
mean	2.127	0.364	2.325	8.01
lSD	1.65	0.053	0.427	4.75

4. CATS

No.	Propionyl thiocholine activity IU/mL	Benzoyl choline activity IU/mL	Butyryl thiocholine activity IU/mL	Succinyl choline activity IU/L
1	0.83	0.11	1.18	ND
2	0.79	*	*	9.0
3	1.7	0.16	*	10.7
4	1.2	*	*	6.4
5	4.95	*	*	8.0
6	0.62	*	*	10.7
7	*	*	*	5.2
8	0.77	*	*	8.2
9	ND	*	*	7.0
mean	1.358			7.24
lSD	1.530			3.28

* insufficient sample for analysis
ND cholinesterase activity not detected

5. CHICKENS

No.	Propionyl thiocholine activity IU/mL	Benzoyl choline activity IU/mL	Butyryl thiocholine activity IU/mL	Succinyl choline activity IU/L
1	1.23	0.038	0.71	11.4
2	1.27	0.046	0.76	6.8
3	1.14	0.039	0.65	10.2
4	1.13	0.050	0.62	13.2
5	1.49	0.048	0.87	10.8
6	1.55	0.053	0.90	16.0
7	1.36	0.046	0.81	13.8
8	1.11	0.041	0.63	9.9
9	1.21	0.034	0.69	12.6
10	1.50	0.037	*	14.4
11	1.52	0.041	0.88	*
mean	1.319	0.043	0.752	11.91
lSD	0.171	0.006	0.107	2.65

6. CATTLE

No.	Propionyl thiocholine activity IU/mL	Benzoyl choline activity IU/mL	Butyryl thiocholine activity IU/ml	Succinyl choline activity IU/L
1	0.11	ND	ND	ND
2	0.074	ND	ND	ND
3	0.068	ND	ND	ND
4	0.063	ND	ND	2.5
5	0.068	ND	ND	ND
6	0.055	ND	ND	2.5
7	0.068	ND	ND	ND
8	0.032	ND	ND	2.9
9	0.039	ND	ND	ND
10	0.037	ND	ND	ND
11	0.033	ND	ND	3.5
12	0.033	ND	ND	ND
mean	0.057			0.95
lSD	0.023			1.42

* insufficient sample for analysis
ND no cholinesterase activity detected

7. SHEEP

No.	Propionyl thiocholine activity IU/mL	Benzoyl choline activity IU/mL	Butyryl thiocholine activity IU/mL	Succinyl choline activity IU/L
1	0.047	ND	ND	3.0
2	0.063	ND	ND	2.2
3	0.061	ND	ND	3.5
4	0.053	ND	ND	ND
5	0.061	ND	ND	2.2
6	0.058	ND	ND	4.8
7	0.028	ND	ND	ND
8	0.043	ND	ND	ND
9	0.011	ND	ND	ND
mean	0.047			1.74
lSD	0.018			1.82

ND no cholinesterase activity detected

8. HORSES

No.	Propionyl thiocholine activity IU/mL	Benzoyl choline activity IU/mL	Butyryl thiocholine activtiy IU/mL	Succinyl choline activity IU/L
1	3.68	0.25	4.15	33.0
2	5.15	0.31	5.43	40.0
3	2.71	0.19	3.37	25.5
4	4.68	0.30	5.03	39.2
5	4.50	0.23	4.95	33.9
6	5.24	0.29	5.73	36.9
7	3.98	0.27	4.49	33.3
8	4.57	0.27	4.61	32.4
9	3.07	0.28	3.64	23.8
mean	4.18	0.266	4.46	33.1
lSD	0.88	0.037	0.78	5.5

TABLE 8.2. CHOLINESTERASE ACTIVITIES IN ZOO ANIMALS.

	Propionyl thiocholine activity IU/mL	Benzoyl choline activity IU/mL	Butyryl thiocholine activity IU/mL	Succinyl choline activity IU/L
	Figure in brackets = result expressed as % of appropriate mean human reference range (see end of table)			
PERISSODACTYLA				
Onager	0.99(21.6)	0.12(13.6)	1.12(22.2)	13.4(22.9)
Donkey	4.55(99.3)	0.55(62.5)	5.70(124.4)	36.1(61.6)
White Rhino	1.36(29.7)	0.10(12.5)	1.73(34.3)	26.4(45.1)
Onager	1.45(31.7)	0.11(12.5)	1.68(33.3)	15.0(25.6)
mean	2.09(45.6)	0.22(25.3)	2.56(53.5)	22.7(38.8)
CARNIVORA				
Kinkajou	12.0(262.9)	*	34.0(677)	76.9(131)£
Serval	2.69(58.7)	*	3.9(76.8)	57.7(98.4)
Jaguar	1.25(27.3)	*	1.5(29.8)	44.9(76.6)
Cheetah	0.34(7.4)	*	0.3(6.3)	34.1(58.2)
Spectacled bear	0.28(6.1)	*	0.2(4.2)	11.5(19.6)
Spectacled bear	0.30(6.6)	*	0.2(4.4)	15.7(26.8)
mean	0.97(21.2)		1.22(24.3)	32.8(55.9)
REPTILES				
Gaboon Viper	0.29(6.3)	*	0.29(5.7)	9.6(16.4)
Aldabra giant Tortoise	0.03(0.7)	*	0.01(0.2)	12.0(20.5)
Iguana	3.65(79.7)	*	2.65(52.6)	9.2(15.7)
Iguana	11.3 (246.7)	*	13.21(262)	35.4(60.4)
Iguana	5.3(115.7)	*	6.32(125.4)	18.1(30.9)
mean	4.11(89.8)		4.50(89.2)	16.9(28.8)
AVES				
Muscovy duck	0.39(8.5)	0.07(7.9)	0.35(6.9)	11.1(18.9)

TABLE 8.2 contd.

	Propionyl thiocholine activity IU/mL	Benzoyl choline activity IU/mL	Butyryl thiocholine activity IU/mL	Succinyl choline activity IU/mL
	Figure in brackets = result expressed as % of mean of appropriate human reference range (see end of table)			
ARTIODACTYLA				
Red Deer	0.06(1.3)	0.04(4.5)	0.02(0.5)	6.8(11.6)
Gaur	0.14(3.1)	ND	0.07(1.3)	15.4(26.3)
Goat	0.14(2.9)	0.04(4.5)	0.02(0.5)	6.7(11.4)
Guanaco	0.13(2.8)	ND	0.05(1.1)	7.7(13.1)
Kudu	0.04(0.9)	ND	0.03(0.5)	6.0(10.2)
Fallow Deer	0.04(1.0)	ND	0.07(1.3)	7.3(12.4)
Fallow deer	0.066(1.4)	ND	0.013(0.3)	17.0(29.0)
Eland	0.085(1.9)	ND	0.107(2.1)	17.0(29.0)
Mouflon	0.10 (2.2)	ND	0.020(0.4)	10.4(17.4)
Scimitar horned onyx	0.05 (1.1)	ND	0.016(0.3)	12.7(21.7)
Yak	0.07(1.5)	ND	0.013(0.3)	13.5(23.0)
mean	0.08(1.8)	0.007(0.8)	0.039(0.8)	11.0(18.6)
CETACEA				
Bottle nosed dolphin	0.04(0.9)	ND	0.02(0.4)	47.9(81.7)
PROBOSCIDEA				
Indian elephant	0.01(0.2)	ND	0.045(0.9)	7.0(11.9)

TABLE 8.2 contd.

	Propionyl thiocholine activity IU/mL	Benzoyl choline activity IU/mL	Butyryl thiocholine activity IU/mL	Succinyl choline activity IU/L
	Figure in brackets = result expressed as % of mean of appropriate human reference range (see end of table).			
PRIMATA				
Baboon	4.2(91.7)	0.85(96.6)	7.01(139.1)	89.7(153.1)
Sacred Baboon	3.79(82.8)	0.88(100)	4.9(97.2)	101.9(173.9)
Vervet	1.02(22.3)	0.16(18.2)	1.53(30.4)	50.0(85.3)
Green Monkey	0.08(1.7)	ND	1.53(30.4)	15.4(26.3)
Chimp	5.65(123.4)	0.95(108)	6.35(126)	78.0(133.1)
Chimp	5.4(117.9)	0.91(103.4)	5.95(118.1)	75.0(128)
Assamese Macaque	2.01(43.9)	0.41(46.6)	2.86(56.7)	80.8(137.9)
Sykes monkey	2.67(58.3)	0.47(53.4)	4.18(82.9)	88.5(151.0)
L'Hoest's monkey	4.42(96.5)	0.64(72.7)	4.41(87.5)	100(170.6)
Roloway monkey	0.28(5.8)	0.05(5.7)	0.45(8.1)	19.2(32.8)
Patas monkey	0.42(9.2)	0.05(5.7)	0.68(13.5)	26.9(45.9)
mean	2.72(59.4)	0.45(55.5)	3.62(71.8)	65.9(112.5)

£ excluded because of marked difference from rest of group
* insufficient specimen for analysis
ND no cholinesterase activity detected

Mean cholinesterase activity for human Elu homozygotes.

Propionylthiocholine activity	4.58	IU/mL
Benzoylcholine activity	0.88	IU/mL
Butyrylthiocholine activity	5.04	IU/mL
		[Evans and Wroe 1978]
Succinylcholine activity	58.6	IU/L

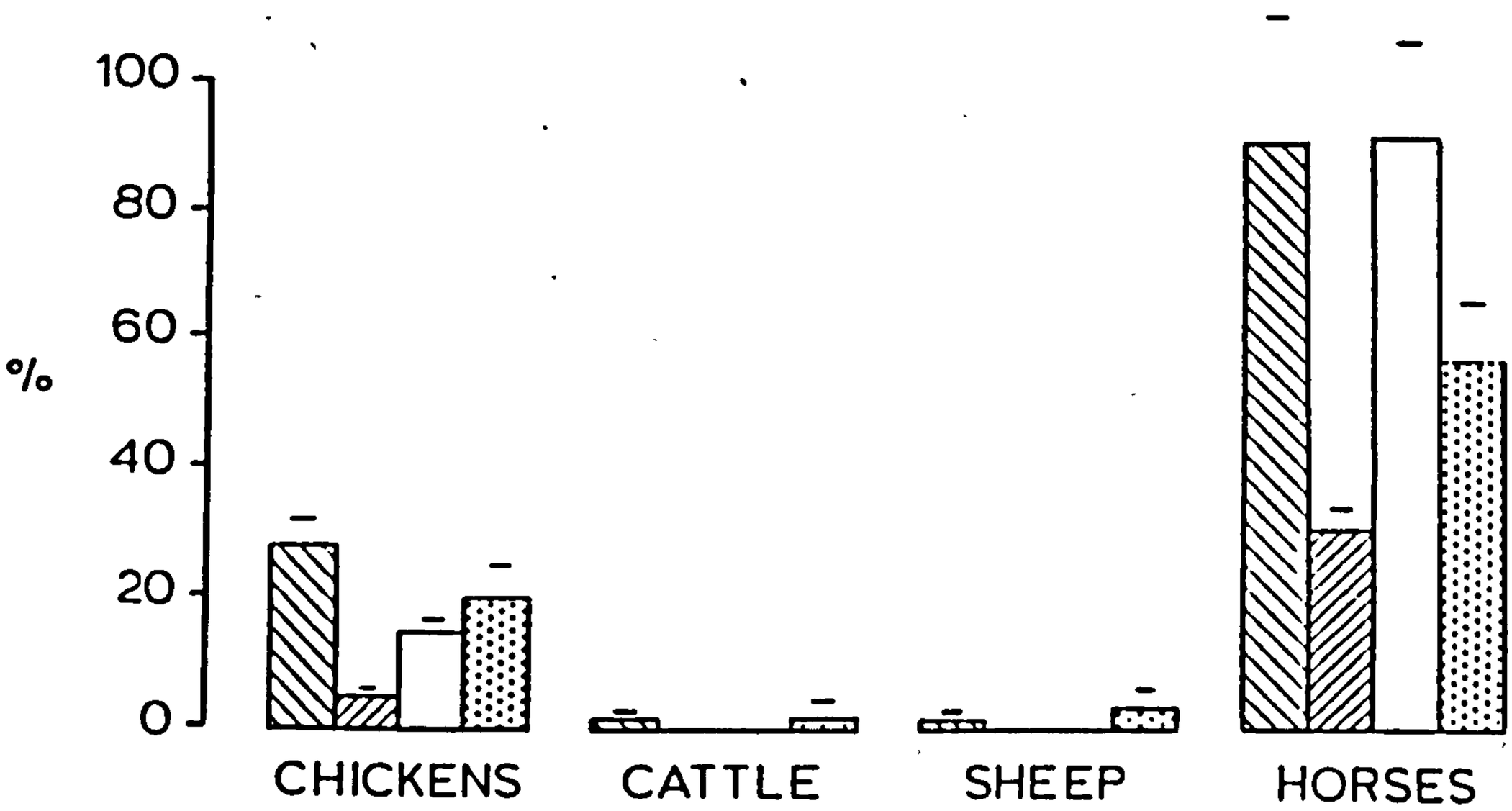

Figure 8.1a

Cholinesterase activities in domestic species expressed as % of the mean of appropriate human reference range.

= Propionythiocholine activity
= Benzoylcholine activity
= Butyrlthiocholine activity
= Succinylcholine activity

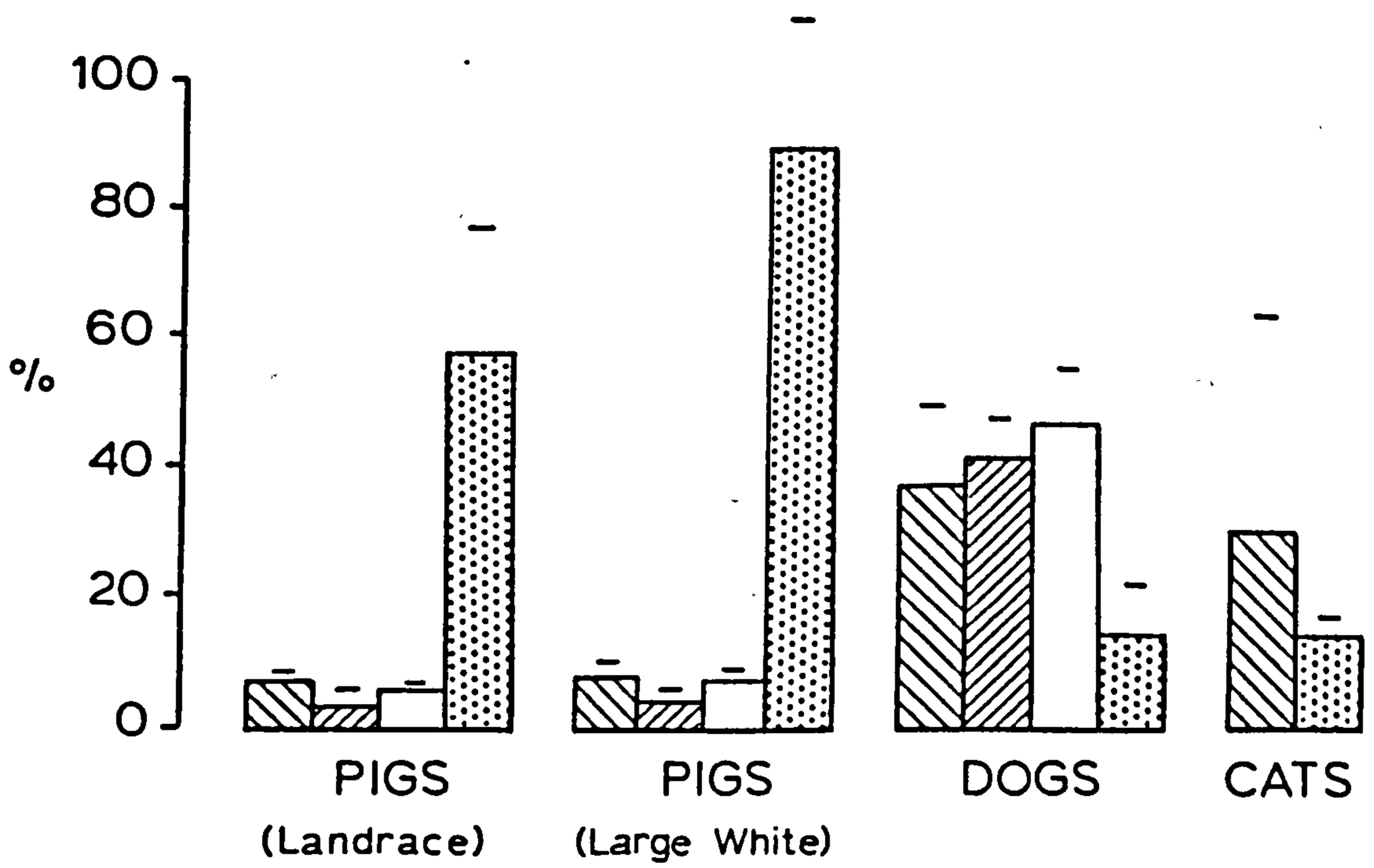

Figure 8.1b

Cholinesterase activities in domestic species expressed as % of the mean of appropriate human reference range.

= Propionythiocholine activity
= Benzoylcholine activity
= Butyrlthiocholine activity
= Succinylcholine activity

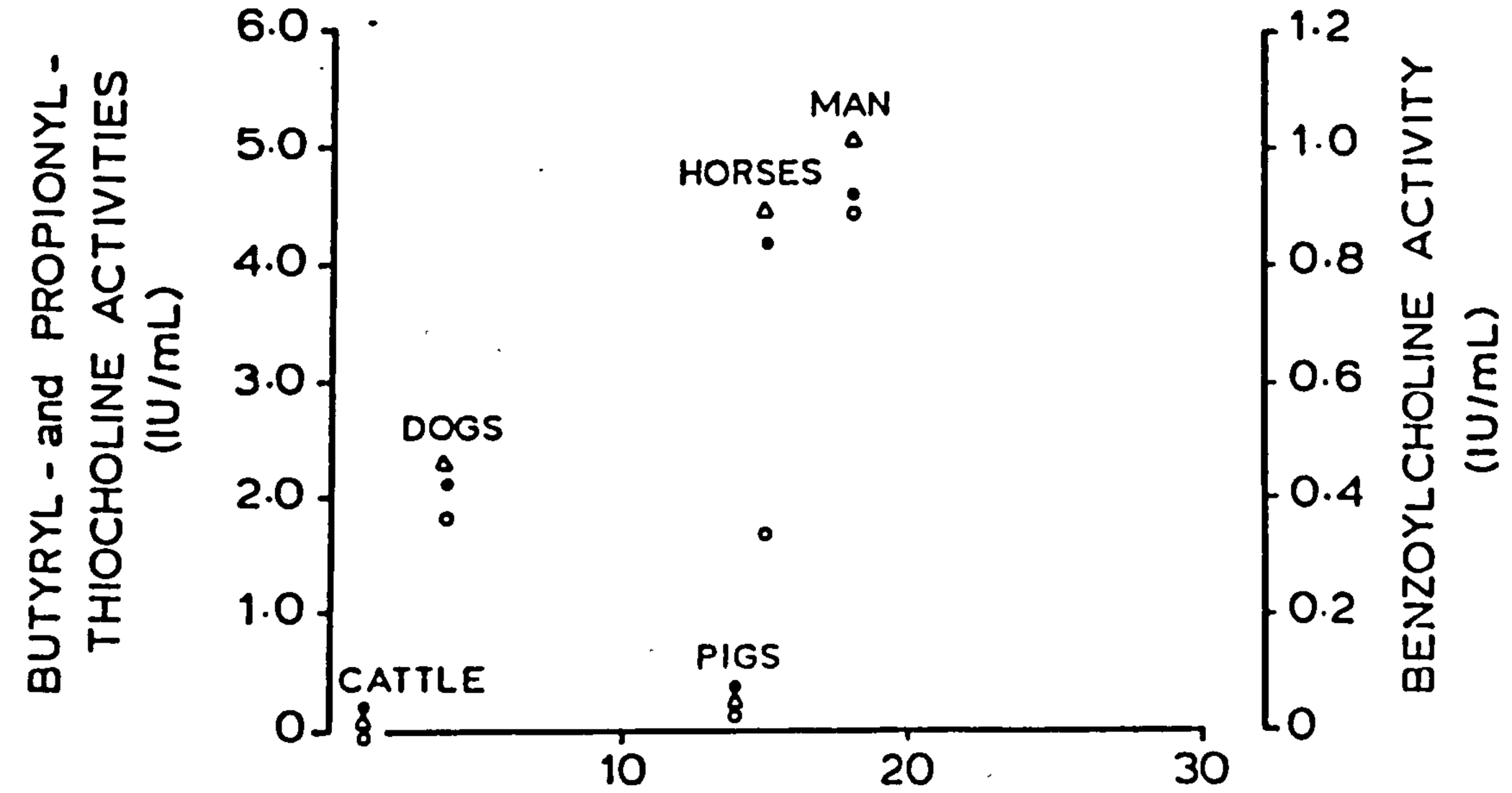

Figure 8.2

The relationship between cholinesterase activity measured using conventional substrates and tolerance to to succinylcholine in a range of species.

Closed circles = Propionythiocholine activity
Open circles = Benzoylcholine activity
Triangles = Butyrylthiocholine activity

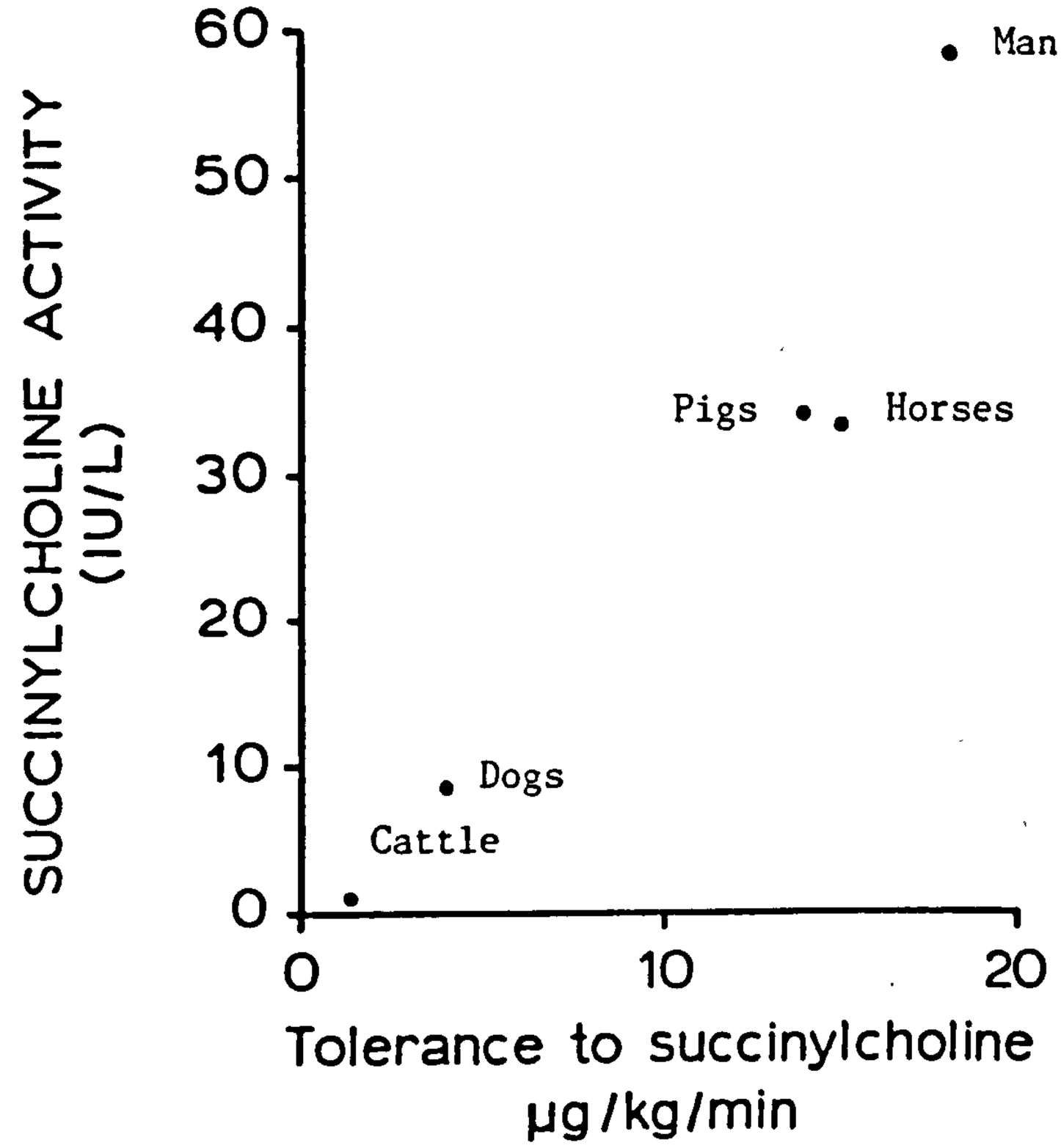

Figure 8.3

The relationship between cholinesterase activity measured with succinylcholine as the substrate and tolerance to succinylcholine in a range of species.

CHAPTER 9

CONCLUSIONS.

1. A simple, precise "reaction rate" assay for plasma cholinesterase based on a succinylcholine substrate has been developed. It employs only readily available reagents and equipment and therefore is suitable for use in any routine clinical chemistry laboratory.

2. It's ability to define individuals at risk of succinylcholine sensitivity and identify those who had experienced apnoea was assessed and it performed better than a propionylthiocholine-based assay, previously shown to be the best available for this purpose [Evans and Wroe 1978].

3. It was applied to genotypically normal patients with apparently abnormal reactions to succinylcholine in an attempt to identify previously unrecognised, abnormal forms of cholinesterase which could hydrolyse conventional assay substrates but not succinylcholine. No such variants of cholinesterase were identified and it was concluded that if these forms exist their numbers are small.

3. The kinetics of the succinylcholine-cholinesterase interaction were studied and results showed that the hydrolysis of succinylcholine by cholinesterase does not obey Michaelis-Menten kinetics. The observed kinetics were consistant with previous observations, using other

substrates, that cholinesterase has two active sites one with high the other with low Km and Vmax values. The average value for the Km1 site (the site responsible for the hydrolysis of succinylcholine at pharmacological concentrations) for usual cholinesterase was 92.4 umol/L and for the atypical enzyme was 6.922 mmol/L which are consistant with clinical observations of reactions to succinylcholine.

4. Serum cholinesterase was measured in 54 subjects with clinically graded liver disease. The succinylcholine-based assay was compared with three conventional assays using propionylthiocholine, benzoylcholine and butyrylthiocholine. All assays identified patients with severe liver disease but the succinylcholine-based assay identified more with moderate/mild disease and therefore is the most clinically useful.

5. The succinylcholine-based assay was applied to plasma from neonates and infants in an attempt to clarify reasons for their relative resistance to succinylcholine. Premature neonates were found to have lower cholinesterase activities than adults. This is in agreement with other workers reporting a transient cholinesterase deficiency in this group. The infants were found to have higher mean cholinesterase activities than adults when measured using succinylcholine as the substrate. This may contribute to their relative resistance to the drug but the range of

values observed make it unlikely that this is the only factor.

6. Cholinesterase measurements made in a variety of animal species using a range of substrates including succinylcholine indicate that there is a diverse pattern of substrate specificity. However only when succinylcholine is used as the substrate for assay of cholinesterase does enzyme activity correlate with tolerance to it´s muscle relaxant properties.

REFERENCES

Abernathy,MH,George,PM,Melton,VE (1984)
A new succinylcholine-based assay of plasma cholinesterase
Clin Chem 30:192

Agarwal,DP,Goedde,HW (1976)
Thin layer chromatograpic separation of ^{14}C labelled succinyldicholine, succinylmonocholine and choline
J Chromatog 121 :170

Agarwal,DP,Schwenkenbecher,S,Srivistava,LM,Goedde,HW (1975)
Spectrophotometrische bestimmungsmethode fur seruncholinesterase (EC 3.1.1.8). Varianten mit succinylbischolin als substrat
Z Klin Chem Klin Biochem 13:133

Agarwal,DP,Srivistava,LM,Goedde,HW (1976)
A note on suxamethonium sensitivity and serum cholinesterase variants
Hum Genet 32:85

Alles,GA,Hawes,RC (1940)
Cholinesterase in the blood of man
J Biol Chem 133:375

Anon. (1973)
Suxamethonium apnoea
Lancet 1:246

Anon. (1984)
Succinyldicholine and game culling
J South African Vet Ass 55:156

Augustinsson,KB (1948)
Cholinesterase. A study in comparative enzymology
Acta Physiol Scand 15 Suppl 52:1

Augustinsson,KB (1955)
A titrimetric method for the determination of plasma and red blood cell cholinesterase activity using thiocholine esters as substrates.
Scand J Clin Lab Invest 7:284

Augustinsson,KB (1959a)
Electrophoresis studies on blood plasma esterases. I. Mammalian plasmata
Acta Chem Scand 13:571

Augustinsson,KB (1959b)
Electrophoresis studies on blood plasma esterases. II. Avian, reptilian and piscine plasmata
Acta Chem Scand 13:1081

Augustinsson,KB (1959c)
Electrophoresis studies on blood plasma esterases. III. Conclusions
Acta Chem Scand 13: 1097

Augustinsonn,KB (1973)
Molecular properties of serum cholinesterase
Hoppe-Seylers Z. Physiol Chem 354:1

Barenghi,L,Ceriotti,F,Luzzana,M,Ripamonti,M,Mosca, A,Bonini,PA (1986)
Measurement of erythrocyte acetylcholinesterase and plasma cholinesterase activity by a differential pH technique
Ann Clin Biochem 23:538

Bauld,HW,Gibson,PF,Jebson,PJ,Brown,SS (1974)
Aetiology of prolonged apnoea after suxamethonium
Br J Anaesth 46:273

Berger,J,Kock,M,Cunningham,C,Dodson,N (19830
Chemical restraint of wild horses: Effects on reproduction and social structure
J Wildlife Dis 19(3):265

Bergmann,F,Segal,R,Shimoni,A,Wurzel,M (1956)
The pH-dependence of enzyme ester hydrolysis.
Biochem J 63:684

Berry,WK (1960)
Cholinesterases in human serum
Biochim Biophys Acta 39:346

Bessey,OA,Lowry,OH,Brock,MJ (1946)
A method for the rapid determination of alkaline phosphatase with five cubic millimetres of serum
J Biol Chem 164:321

Beuchner,HK,Harthoorn,AM,Lock,JA (1960)
Control of african wild animals
Nature 185:47

Biggs,HG,Carey,S,Morrison,DB (1958)
Simple colorimetric method for measuring activities of cellular and plasma cholinesterase.
Am J Clin Path 30:181

Billings,BH,Haslam,R,Wald,N (1971)
Bilirubin standards and the determination ofbilirubin by manual and Technicon auto analyser methods
Ann Clin Biochem 8:21

Blow,DM,Birktoft,JJ,Hartley,BS (1969)
Role of a buried acid group in the mechanism of action of chymotrypsin
Nature 221:337

Bourne ,JG (1953)
Long action of suxamethonium (succinylcholine) chloride
Br J Anaesthesia 25:116

Bourne,JG,Collier,HOJ,Somers,GF (1952)
Succinylcholine (sucinoylcholine); muscle relaxant of short action
Lancet i:1225

Boutin,D,Brodeur,J (1971)
Human serum pseudocholinesterases. Molecular weight estimation of a sub-unit structure
Can J Physiol Pharmacol 49:777

Bowers,GN,McComb,RB (1975)
Measurement of total alkaline phosphatase activity in human serum
Clin Chem 21:1988

Brown ,SS,Kalow,W,Pilz,W,Whittaker,M,Waronick,LL (1981)
The plasma cholinesterases: a new perspective.
Adv Clin Chem 22:1

Buechner,HK,Harthoorn,AM,Lock,JA (1960a)
Control of African wild animals
Nature 185:47

Buechner,HK,Harthoorn,AM,Lock,JA (1960b)
The immobilisation of African animals in the field, with special reference to their transfer to other areas (U.S. Translocation)
Proc Zool Soc Lond 135:261

Chawhan,RN,Runwal,KP,Zarvar,PB,Jadhav,AB (1982)
Pseudocholinesterase in acute MI.
Ind Heart J 34:21

Child,CG (1964)
In: " The liver and portal hypertension". Publ; Saunders Philadelphia. pp50

Clitherow,JW,Mitchard,M,Harper,NJ (1963)
The possible biological function of pseudocholinesterase.
Nature 199:1000

Cook,DR (1981)
Muscle relaxants in infants and choldren
Anesth Analg 60:335

Crookshank,HR,Palmer,JS (1978)
Comparison of two procedures for determination of cholinesterase in livestock
Clin Toxicol 13:557

Cucuianu,M,Popescu,TA,Haragus,S (1968)
Pseudocholinesterase in obese and hyperlipemic subjects
Clin Chim Acta 22:151

Cucuianu,M,Popescu,TA,Opincoru,A,Haragus,S, (1975)
Serum pseudocholinesterase and ceruloplasmin in various types of hyperlipoproteinaemia
Clin Chim Acta 59:19

Dale,HH (1914)
The action of certain esters and ethers of choline and their relation to muscarine
J Pharmacol Exp Therap 6:147

Das,PK,Liddell,J (1970a)
Purification and properties of human serum cholinesterase.
Biochem J 116:875

Das,PK,Liddell,J (1970b)
Value of butyrlthiocholine assay for the identification of cholinesterase variants
J Med Genet 7:351

Davies,RO,Marton,AV,Kalow,W (1960)
The action of normal and atypical cholinesterase of human serum upon a series of esters of choline.
Can J Biochem Physiol 38:545

De La Huerga,J,Yesinik,C,Popper,H (1952)
Colorimetric method for the determination of serum cholinesterase
Am J Clin Pathol 22:1126

Delbruck,A,Henkel,E (1969)
A rare genetically determined variant of pseudocholinesterase in two German families with high plasma cholinesterase activity
Eur J Biochem 99:65

Dietz,AA,Rubinstein,HM,Lubrano,T (1973)
Colorimetric determination of serum cholinesterase and its genetic variants by the propionylthiocholine dithiobis (nitrobenzoic acid) procedure
Clin Chem 19;1309

Dittmer,JC,Wells,MA (1969)
Quantitative and qualitative analysis of lipids and lipid components
Methods Enzymol 14:482

Drew,AH,Rundle,AT (1977)
Serum cholinesterase (pseudo cholinesterase) in Downs syndrome I. Phenotype frequencies at the E1 and E2 loci.
J Mental Deficiency Res 21:181

Dubbs ,CA (1966)
Ultrasonic effects on isoenzymes
Clin Chem 12:181

Earl,CJ,Thompson,RHS (1952)
The inhibitory action of tri-ortho-cresyl phosphate on cholinesterase
Br J Pharmacol 7:685

Eastham,RD (1978)
In:" Biochemical values in clinical medicine"
Ed. R.D. Eastham. Wright, Bristol. 6th Edition. pp180

Ecobichon,DJ,Kalow,W (1963)
The effect of sialidase on pseudocholinesterase types
Can J Biochem 41 :969

Ehrich,JHH,Rothganger,S (1987)
Cholinesteraseaktivitat und proteinkonzentration im serum von fruh- und neugeborenen
Klin Padiat 199:98

Ellis,FR,Cain,PA,Harriman,DGF,Toothill,C (1978)
Plasma cholinesterase and malignant hyperpyrexia
Br J Anaesth 50:86

Ellman ,GL,Courtney,KD,Andres,V,Featherstone,RM (1961)
A new and rapid colorimetric determination of acetyl cholinesterase.
Biochem Pharmacol 7:88

Erdos,EG,Foldes,FF,Baart,N,Shanor,SP (1957)
Activating effect of tryptamine, benzylamine and histamine on plasma cholinesterase
Fed Proc Am Soc Exp Biol 16:294

Erdos,EG,Foldes,FF,Baart,N,Zsigmond,EK,Zwartz,JA, (1959)
The accelerating effects of narcotic analgesics on the hydrolysis of aromatic substances by human plasma cholinesterase
Biochem Pharmacol 2:97

Erdos,EG,Foldes,FF,Zsigmond,EK,Baart,N,Zwartz,JA (19580
Acceleration of plasma cholinesterase activity by quaternary ammonium salts
Science 128:92

Evans,DB,Lehmann,H (1971)
Pseudocholinesterase activity in liver transplantation
Lancet 1:1040

Evans,FT,Gray,PWS,Lehmann,H,Silk,E (1952)
Sensitivity to succinylcholine in relation to serum cholinesterase
Lancet 1:1229

Evans,RT,Wroe,JM (1978)
Is serum cholinesterase activity a predictor of succinylcholine sensitivity? An assessment of four methods
Clin Chem 24:1762

Evans,RT,Macdonald,R,Robinson,A (1980)
Suxamethonium asssociated apnoea associated with plasma pheresis
Anaesthesia 35 :198

Evans,RT,Wroe,JM (1980)
Plasma cholinesterase activity in early pregnancy. Their interpretation in a cause of suxamethonium induced apnoea
Anaesthesia 35:651

Evans,RT,Iqbal,J,Ellis,FR,Halsall,PJ,Dietz,AA,Lubrano,T, Rubinstein,HM, (1981)
Collaberative study of the frequency of the fluoride resistant variant in patients with malignant hyperpyrexia.
Br J Anaesth 53:245

Evans,RT (1986)
Cholinesterase phenotyping: clinical aspects and laboratory applications.
CRC; Critical Rev in Clin Lab Science 23:1

Fishtal,A,Evans,RT,Chapman,CN (1972)
An original approach to the diagnosis of scoline induced apnoea
J Clin Path 25:222

Forbat,A,Lehmann,H,Silk,E (1953)
Prolonged apnoea following injection of succinylcholine
Lancet 2 :1067

Funnell,HS,Oliver,WT (1965)
Proposed physiological function for plasma cholinesterase
Nature 208:689

Gaffney,PJ Jr (1970)
Human serum cholinesterase I Partial purification and nature of the heterogeneity of this system
Biochim Biophys Acta 207; 465

Gall,EM,Roth,E (1957)
Spectrophotometric methods for the determination of cholinesterase activity
Clin Chem Acta 2:316

Ganga,CC,Heyduk,JV,Marx,GF,Sklar,GS (1982)
A comparison of the response to suxamethonium in the post partum gynaecological patient
Anaesthesia 37:903

Garry,PJ,Dietz,AA,Lubrano,T,Ford,PC,James,K,Rubinstein,HM (1976)
New allele at cholinesterase locus I
J Med Genetics 13:38

Garry,PJ,Routh,JI (1965)
A micro method for serum cholinesterase
Clin Chem 11;91

Ghooi,AM,Malaviya,GN,Kashyap,A (1980)
A comparative study of LDH and pseudocholinesterase in sera of cancer patients; a preliminary report.
Ind J Cancer 17:31

Glick,D (1948)
Controversy on cholinesterase
Science 102:100

Goedde,HW,Agarwal,DP (1978)
Pseudocholinesterase variation
Hum Genet Suppl 1;44

Goedde,HW,Held,KR,Atland (1968)
Hydrolysis of sucinyldicholione and succinylmonocholine in human serum.
Mol Pharmacol 4:274

Gross,O,Audetat,V,Bircher,J (1978)
Serumcholinesterase und leberfunction. Ein vergleich mit BSP-elimination, Galactose-eliminationskapazitat und mit serumalbuminkozentration
Schweiz Med Wschr 108;1389

Hall,LW,Clarke,KW (1983)
In: " Veterinerary Anaesthesia". Eds. L.W. Hall and K.W. Clarke. 8th Edition. Balliere Tindall, London.

Hall,LW,Lehmann,H,Silk,E (1953)
Response in dogs to relaxants derived from succinic acid and choline
Br Med J 1:134

Hansson,CH (1957)
Blood cholinesterase activity in relation to tolerance for succinylcholine
Acta Pharm Tox 14:6

Harris,H,Whittaker,M (1961)
Differential inhibition of human serum cholinesterase with fluoride: Recognition of two new phenotypes
Nature 191;496

Harris,H,Hopkinson,DA,Robson,EB (1962)
Two dimensional electrophoresis of pseudocholinesterase components in normal human serum.
Nature 196:1296

Harris,H,Robson,EB (1963)
Fractionation of human serum cholinesterase components by gel filtration
Biochim Biophys Acta 73:649

Harris ,H,Whittaker,M (1963)
Differential inhibition of "usual" and "atypical" serum cholinesterase by NaCl and NaF.
Ann of Hum Genet 27:53

Hastings,FL (1966)
The study of horse and human serum cholinesterase substrate reactions.
Ph.D. Thesis. North Carolina State University, Rayleigh.
Quoted by Brown et al. 1981.

Haupt,H,Heide,K,Zwisler,O,Schwick,HG (1966)
Isolierung und physikalischchemische charakterisierung der cholinesterase aus humanserum
Blut 14 :65

Heilbron,E (1958)
The pS-activity curve of serum fraction IV-6-3 studied with butyrylcholine at pS above 3.
Acta Chem Scand 12;1879

Heilbron,E (1965)
Action of fluoride on cholinesterase. I. On the mechanism of inhibition
Acta Chem Scand 19:1333

Hobbiger,F,Peck,AW (1969)
Hydrolysis of suxamethonium by different types of plasma
Br J Pharmacol 37:258

Hobbiger,F,Peck,AW (1971)
Relationship of enzymic hydrolysis of suxamethonium in plasma and tissues: Studies in cats
Br J Pharmacol 43:341

Hodges,RJH,Harkness,J (1954)
Suxamethonium sensitivity in health and disease
Br Med J 2:18

Hoftsee,BHJ (1960)
Mechanism of action of bivalent metal ions and of phenothiazine derivatives on serum cholinesterase
J Pharmacol Exper Therap 128:299

Hoover,DB,Kosa,J,Colasanti,BK,Craig,CR (1976)
A modified assay for cholinesterase
Microchemistry J 21:267

Hunt,AH,Lehmann,H (1960)
Serum albumin, pseudocholinesterase and transaminase in the assessment of liver function before and after venous shunt operations.
Gut 1:303

Hutchinson,AO,Widdowson,EM (1952)
Cholinesterase activities in the serum of healthy British children
Nature 169:284

Jansz,HS,Brons,D,Warringa,MCPJ (1954)
Chemical nature of the DFP binding site of pseudocholinesterase
Biochim Biophys Acta 34:563

Jendrassik,L,Grof,P (1938)
Vereinfachte photometrisch methoden zur bestimmungs des blutbilirubins
Biochem Z 297;81

Johnson,JK,Whitehead,TP (1965)
Rapid estimation of serum cholinesterase using the Astrup microequipment
J Clin Path 18:435

Juhl,P (1968)
Human plasma cholinesterase isoenzymes
Clin Chem Acta 19:205

Kalow,W,Lindsay,HA (1955)
A comparison of optical and manometric methods for the assay of human serum cholinesterase
Can J Biochem 33:568

Kalow,W,Genest,K (1957)
A method for the detection of atypical forms of human serum cholinesterase. Determination of dibucaine numbers
Can J Biochem 35:338

Kalow,W,Gunn,DR (1957)
The relationship between dose of succinylcholine and duration of apnoea in man
J Pharmacol Exp Therap 120:203

Kalow,W,Staron,N (1957)
On distribution and inheritance of atypical forms of human serum cholinesterase as indicated by dibucaine numbers
Can J Biochem 33;568

Kalow,W,Gunn,DR (1958)
Some statistical data on atypical cholinesterase of human serum.
Ann Hum Genet 23:239

Kalow,W (1959)
Cholinesterase types. In " Biochemistry of Human Genetics". Eds. Wolstenholme GEW and O´Connor CN. Churchill London pp39.

Kalow,W (1962)
Hereditable facors recognised in man by the use of drugs.
In "Pharmacokinetics; heredidity and the response to drugs." Saunders, Philadelphia. pp69

Kalow,W (1965)
Contribution of hereditary factors to response to drugs
Fed Proc24:1259

Keeser,E (1938)
Uber die medikamentose beeinflussing der cholinesterase
Klin Wschr 17:1811

Kessler,G,Rush,R,Leon,L,Delea,A,Cupiola,R (1971)
Automated 340nm measurement of sGOT, sGPT and LDH
In: Advances in automated analysis. Technicon International Congress 1 :67

Kanaris,P,Fassoulaki,A,Liarmakopolou,K,Dermitzakis,E (1979)
Serum cholinesterase levels in patients with cancer
Anesth Analg (Cleveland) 58:82

King,J,Griffin,D (1973)
Differentiation of serum cholinesterase variants by succinylcholine inhibition
Br J Anaesth 45:450

Koelle,WA,Koelle,GB,Smyrl,EG (1973)
Effects of persistent selective supression of ganglionic butyrylcholinesterase on steady state and regenerating levels of acetylcholinesterase; implications regarding function of butyrylcholinesterase and regulation of protein synthesis.
Proc Natl Acad Sci. USA 73,:2936

Kutty,KM,Jacobs,JC,Hutton,CJ,Davis,PJ,Peterson,SC (1975)
Serum beta-lipoprotein: Studies in a patient and in guinea pigs after the ingestion of organophosphorus compounds
Clin Biochem 8:379

Kutty,KM,Reheendran,R,Murphy,D (1977)
Serum cholinesterase, function in lipoprotein metabolism
Experientia 33:420

Kutty,KM,Rowden,G,Cox,AR (1973)
Interrelationship between serum beta-lipoprotein and cholinesterase.
Can J Biochem 51:883

La Du,BN,Snady,H (1971)
Esterases of human tissues.
Handbook of Pharmacology No28. Publ; Springer-Verlag,New York ppl.

La Motta,RV,Williams,HM,Wetstone,HJ (1957)
Studies of cholinestease activity. II. Serum cholinesterase in hepatitis and cirrhosis
Gastroenterology 33:50

La Motta,RV,McComb,RB,Wetstone,HJ (1965)
Isozymes of serum cholinesterase; a new polymerisation sequence.
Can J Physiol Pharmacol 43:313

La Motta,RV,Woronick,CL,Reinfrank,RF (1970)
Multiple forms of serum cholinesterase: Molecular weights of the isoenzymes
Archiv Biochem Biophys 136:448

La Motta,RV,Woronick,CL (1971)
Molecular heterogeneity of human serum cholinesterase
Clin Chem 17:135

Lam,VMS (1977)
Pseudocholinesterase of snake sera
Comparative Biochem Physiol 58c:129

Lawrence,SH,Melnick,PJ (1961)
Enzymatic activity related to human serum beta lipoproteins: Histochemical, immuno-electrophoretic and quantitative studies.
Proc Soc Exp Biol Med 107:998

Laxenaine,ML,Sigiel,M (1976)
Le suxamethonium
Anaesthetics Anal Rean 33 :11

Lee,C (1975)
Dose relationship of phase II tachyphylaxis and train of four fade in suxamethonium induced dual neuromuscular blockade in man
Br J Anaesth 47:841

Lehmann,H,Silk,E (1953)
Succinylmonocholine
Br Med J 1:767

Lehmann,H,Liddell,J (1969)
Human cholinesterase (pseudocholinesterase): genetic variants and their recognition
Br J Anaesth 41:235

Liddell,J,Lehmann,H,Silk,E (1962)
A "silent" pseudocholinesterase gene
Nature 193:561

Liddell,J,Newman,GE,Brown,DF (1963)
A pseudocholinesterase variant in human tissues
Nature 198:1090

Lockridge,O,Bartels,CF,Vaughan,TA,Wong,CK,Norton,SE,Johnson,LL (1987)
Complete amino acid sequence of human serum cholinesterase.
J Biol Chem 262:549

Lockridge,O,Eckerson,HW,La Du,BN (1979)
Interchain disulfide bonds and subunit organisation in human serum cholinesterase
J Biol Chem 254 :8324

Lockridge,O,La Du,BN (1978)
Comparison of atypical and usual serum cholinesterase. Purification, number of active sites, substrate affinity and turnover number.
J Biol Chem 253:361

Lockridge,O,La Du ,BN (1986)
Amino acid sequence of the active site of human serum cholinesterase from usual, atypical and silent genotypes
Biochemical Genetics 24:485

Loewi,O,Navratil,E (1926)
Uber humorale ubertragbarkeit der Herznervenwirkung. XI. Uber den mechanismus der vaguswirkung von physostigmin und ergotamin.
Pflugers Archives 214:689

Lui,LMP,De Cook,TH,Goudsouzian,NG,Ryan,JF,Lui,PL (1981)
Dose response to intramuscular succinylcholine in children
Anesthesiology 55:599

Mackey,CL (1982)
Anticholinesterase insecticide poisoning
Heart Lung 11:479

Main,AR (1983)
Kinetic and structural relationship of transition monomeric and oligomeric carboxyl and choline esters.
J Envirom Sci and Health B18:29

Main,HR (1976)
Structure and Inhibitors of Cholinesterase. In "Biology of cholinergic function". Eds. AM Golding & I Hanin. Raven Press. New York. pp 69

McArdle,B (1940)
The serum cholinesterase in jaundice and diseases of the liver
Q J Med 9:107

McComb,RB,La Motta,RV,Wetstone,HJ (1964)
Studies of cholinesterase activity. VII. Kinetic constants of serum cholinesterase in normal populations and those with neoplasms
J Lab Clin Med 63:827

McComb,RB,La Motta,RV,Wetstone,HJ (1965)
Procedure for detecting atypical cholinesterase using o-nitrophenylbutyrate as substrate.
Clin Chem 11:645

McOsker,DE,Daniel,LJ (1959)
A colorimetric micromethod for the determination of cholinesterase
Arch Biochem Biophys 79:1

Meade,BW,Rosalki,SB (1963)
Serum activity in normal pregnancy and the newborn
J Obst Gynae British Commonwealth 70:693

Mendel,B,Mundell,DB (1943)
Studies of cholinesterase II. A method for the purification of pseudocholinesterase from dog pancreas.
Biochem J 37:64

Mendel,B,Rudney,H (1943)
Studies of cholinesterse I. Cholinesterase and pseudo-cholinesterase.
Biochem J 37:59

Mendel,B,Mundell,DB,Rudney,H (1943)
Studies of cholinesterase III. Specifications for true cholinesterase and pseudocholinesterase.
Biochem J 37:473

Merin,RG (1986)
Succinylcholine is different in humans than in dogs
Anesthesiology 65:452

Michel,HO (1949)
Electrometric method for determination of red blood cell and plasma cholinesterase activity
J Lab Clin Med 34:1564

Mirakhur,RK,Elliot,P,Lavery,TD (1984)
Plasma cholinesterase activity and the duration of suxamethonium apnoea in children
Ann Roy Coll Surg Engl 66:43

Motulsky,AG (1964)
Pharmacogenetics
Prog Med Genet 3:49

Muensch,H,Goedde,HW,Yoshida,A (1976)
Human serum cholinesterase subunits and number of active sites of the major component
Eur J Biochem 70:217

Myers,DK (1953)
Studies on cholinesterase. IX. Species variation in the specificity patterns of the pseudocholinesterases
Biochem J 55: 77

Navarette,JI,Lisker,R,Perez-Briceno,R (1979)
Serum atypical pseudocholinesterase and leprosy
Int J Dermatol 18:822

Neitlich,HW (1966)
Increased plasma cholinesterase activity and succinylcholine resistance: a genetic variant
J Clin Invest 45:380

Nugent,SR,Laravuso,R,Rogers,MC (1979)
Pharmacology and use of muscle relaxants in infants and children
J Pediat 94:481

Ord,MG,Thompson,RHS (1952)
Pseudo-cholinesterase activity in the central nervous system
Biochem J 51:245

Pavlic,M (1967)
The inhibitory effect of tris on the activity of cholinesterases
Biochim Biophys Acta 139:133

Pickard ,J (1976)
Purification de la cholinesterase du serum human par affinite.
Crit Rev Academ Sci (Paris) 282:235

Prellwitz,VW,Knapp,S,Muller,D (1976)
Vergleich von methoden zur aktivitatsbestimmung der serum cholinesterasen (acylcholin-acylhydrolase EC 3.1.1.8) und deren diagnostische wertigkeit
J Clin Chem Clin Biochem 14 :93

Propert,DN,Brackenbridge,CJ (1976)
The relationship of sex, age, smoking status, birth rank and parental ages to pseudocholinesterase activity and phenotypes in a sample of Australian caucasian adults.
Human Genetics 32:181

Pugh,RNH,Murray-Lion,IM,Dawson,JL,Pietroni,MC, Williams,R (1973)
Transection of the oesophagus for bleeding oesophageal varices
Brit J Surg 60:646

Rea,TH,Ng,WG (1978)
Serum pseudocholinesterase variants in mexican burn patients with lepromatous leprosy
Int J Leprosy 46:333

Reinhold,JG,Tourigny,LG,Yonan,VL (1953)
Measurement of serum cholinesterase activity by a photometric indicator method
Am J Clin Path 23:645

Rimbach,E,Dacic,Z (1963)
Serum cholinesterase in normal pregnancy, postpartum period and during puerperium
Zbl Gynak 85:811

Robertson,GS (1966)
Serum cholinesterase deficiency II; pregnancy
Br J Anaesth 38:361

Rodkey,FL (1965)
Measurement of serum albumin using bromocresol green.
Clin Chem 11:478

Rubinstein,HM,Dietz,AA,Lubrano,T (1978)
$E1^K$ another quantitative variant at cholinesterase locus I.
J Med Genet 15:27

Sastry,BVR,White,EC (1968)
Cholinesterase hydrolysis and substrate inhibition of lactoylcholines
J Med Chem 11:528

Sastry,BVR,Chiou,CY (1968)
Molecular aspects of the interactions of halogenoacetyl cholines with cholinesterase.
Biochim Biophys Acta 167:339

Sategna-Guidetti,C,Bianco,L,Durazzo,M (1980)
Pseudocholinesterase activity in Crohn´s disease.
Lancet 2:534

Scmidinger,ST,Held,KR,Goedde,HW (1966)
Hydrolysis of succinyldicholine by pseudocholinesterase at low concentrations
Human Genetik 2:221

Sekul,AA,Holland,WCE,Breland,AE (1962)
Enzymic hydrolysis of saturated and unsaturated esters of choline
Biochem Pharmacol 11:487

Sidell,FR,Kaminskis,A (1975)
Influence of age,sex and oral contraceptives on blood cholinesterase activity
Clin Chem 21:1393

Silk,E,King,J,Whittaker,M (1979)
Assay of cholinesterase in clinical chemistry
Ann Clin Biochem 16:57

Silver,A (1974)
In "The biology of cholinesterase". North Holland Publishing Company, Amsterdam. pp443

Silvestri, G (1977)
New techniques to measure blood cholinesterase activity in domesticated animals
Am J Vet Res 38:659

Simon,NM,del Greco,F,Dietz,AA,Rubinstein,HM (1969)
Serum cholinesterase deficiency in renal failure.
Trans Am Soc Artificial Internal Organs 15:328

Singh,DS,Shulka,PK,Gupta,JP,Dube,B (1976)
Serum cholinesterase in hepatic disoeders
J Ind Med Assoc 66:49

Somorin,O (1978)
Cholinesterase assay by gas-solid chromatography
Annals Biochem 88:442

Srinkantia,SG,Jacob,CM,Reddy,V (1964)
Serum enzyme levels in protein-calorie malnutrition. Studies in children with kwashiokor and marasmus.
Am J Dis Childhood 1:236

Stedman,E,Stedman,E,Easson,LH (1932)
Choline-esterase. An enzyme present in the blood serum of the horse.
Biochem J 26:2056

Strauss,AA,Modanlov,HD (1986)
Transient plasma cholinesterase deficiency in preterm infants
Dev Pharmacol Therap 9:82

Surgenor,BM,Strong,LE,Taylor,HL,Gordon,RSJR,Gibson,DM (1949)
The separation of cholinesterase, mucoprotein and metal combining protein into subfractions of human plasma.
J Am Chem Soc 71:1223

Svensmark,O (1961)
Human serum cholinesterase as a sialo protein
Acta Physiol Scand 52:267

Svensmark,O,Kristensen,P (1963)
Isoelectric point of native sialidase treated human serum cholinesterase
Biochim Biophys Acta 67:441

Takayama,M,Itoh,S,Nagasaki,T,Tanimizu,I (1977)
A new enzymatic method for determination of serum choline containing phospholipids
Clin Chim Acta 79:93

Thompson,JC,Whittaker,RM (1965)
Pseudocholinesterase activity in thyroid disease
J Clin Path 18:811

Tourtellotte,WW,Odell,LD (1950)
Plasma acetylcholinesterase activity
Am J Obstet Gynecol 601343

Turner,JM,Hall,RA,Whittaker,M,Kricka,LJ (1984)
Effects of storage and repeated freezing and thawing on plasma cholinesterase activity
Ann Clin Biochem 21:363

Uete,T,Miyamoto,V,Ohinshi,M,Shimano,N (1972)
Spectrophotometric micromethod for measuring cholinesterase in serum and plasma
Clin Chem 18;454

Vaccarezza,JR,Wilson,JA,Bochi,AA (1966)
A new test for the diagnosis of neoplastic disease. Further investigation of cholinesterase in plasma, whole blood and blood cells in cancer of the lung, extrapulmonary tumours and tuberculosis.
Diseases of the Chest 49:444

Viby-Mogensen,J,Hanel,HK,Hansen,E,Sorensen,B,Graae,J (1975)
Serum cholinesterase activity in burned patients I. Biochemical findings.
Acta Anaesthes Scand 19:159

Viby-Mogensen,J,Hanel,HK (1978)
Prolonged apnoea after suxamethonium. An analysis of the first 225 cases reported to the Danish cholinesterase research unit.
Acta Anaesth Scand 22; 371

Viby-Mogensen,J (1980)
Correlation of succinylcholine duration of action with plasma cholinesterase activity in subjects with the genotypically normal enzyme
Anesthesiology 53:517

Viby-Mogensen,J (1983)
Cholinesterase and succinylcholine
Danish Med Bull 30:129

Vorhaus,LJ,Kark,RM (1953)
Serum cholinesterase in health and disease
Am J Med 14:707

Wakid,NW,Tubbeh,R,Baraka,A (1985)
Assay of serum cholinesterase with succinylcholine and propionylthiocholine as substrates
Anesthesiology 62:504

Walts,LF,Dillon,JB (1980)
The response of newborns to succinylcholine and d-tubocurarine
Anesthesiology 31:35

Way,RC,Hutton,CJ,Kutty,KM (1975)
Relationship between serum cholinesterase and low density lipoprotein in children with nephrotic syndrome
Clin Biochem 8:103

Wetherall,JR,French,MC (1986)
The hydrolysis of succinyldithiocholine and the related thiocholine esters by human plasma and purified cholinesterase.
Biochem Pharmacol 35:939

Whittaker,M, (1968a)
The pseudocholinesterase variants. Differentiation by means of alkyl alcohols
Acta genet (Basel), 18:325

Whittaker,M (1968b)
Differential inhibition of human serum cholinesterase with n-butyl alcohol: Recognition of new phenotypes
Acta genet (Basel) 18:335

Whittaker,M (1968c)
Differential inhibition of human serum cholinesterase with n-butylalcohol; recognition of a new phenotype
Acta Genetica et Stat med,18,235,340,

Whittaker,M,Vickers,MD (1970)
Initial experiences with the cholinesterase research unit
Br J Anaesth 42:1016

Whittaker,M,Spencer,R (1977)
Plasma cholinesterase variants in patients taking lithium therapy
Clin Chim Acta 75:421

Whittaker,M,Berry,M (1977)
The plasma cholinesterase variants in mentally ill patients
Br J Psych 130,:397

Whittaker ,M,Spencer R Searle,J (1977)
Plasma cholinesterase and malignant hyperthermia.
Br J Anaesth 49:393

Whittaker,M,Britten,JJ (1987)
E_1^h, a new allele at cholinesterase Locus 1.
Hum Hered 37:54

Wright,PG,De Vos,V,Marcus,E,Gunhao,M,Hattingh,J (1981)
Species variation in plasma cholinesterase activity
Comp Biochem Physiol 70(2):289

Yoshida,A,Motulsky,AG (1969)
A pseudocholinesterase variant (E. Cynthiana) associated with elevated plasma enzyne activity.
Am J Hum Genetics 21:486

Zsigmond,EK,Davis,JR (1971)
Plasma cholinesterase activity in newborns and infants
Can Anaesth Soc J 18:278