A STUDY OF MOLECULAR FORMS OF THE CHOLINESTERASES WITH PARTICULAR REFERENCE TO HIRSCHSPRUNG'S DISEASE AND NEURAL TUBE DEFECTS

A thesis submitted to the University of Newcastle upon Tyne for the Degree of Doctor of Philosophy

by

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Dedicated to:

Him through whom all things were made;
without him nothing was made that has
been made. In him was life, and that
life was the light of men. Jn 1 vs 3,4

C O N T E N T S

TABLES AND FIGURES

Tables and figures are numbered on the basis of the Section to which they belong i.e. the first figure in Section 10 is Fig. 10.1, etc. In each case the corresponding legend is shown on the facing page.

PUBLICATIONS

Some of the results described in the present thesis have been published or presented in the following:-

Bonham, J.R. and Atack, J.R. [1983] A neural tube defect specific form of acetylcholinesterase in amniotic fluid. Clin Chim Acta 135, 233-237.

Bonham, J.R., Dale, G., Scott, D.J. and Wagget, J. [1985] Molecular forms of acetylcholinesterase in Hirschsprung's disease. Clin Chim Acta 145, 297-305.

Bonham, J.R., Dale, G., Scott, D.J., Scott, J.E.S. and Wagget, J. [1985] Diagnosis of Hirschsprung's disease by quantitative measurement of acetyl-cholinesterase activity in rectal mucosa. J Ped Surg [In Press].

Bonham, J.R. and Dale, G. [1985] Measurement of acetylcholinesterase molecular forms in the detection of neural tube defects. J Clin Chem and Clin Biochem 23, 579.

Dale, G., Bonham, J.R. and Wagget, J. [1983] Rectal mucosal acetylcholinesterase in Hirschsprung's disease, Poster presentation, Second International Meeting on Cholinesterases, Bled, Yugoslavia.

Bonham, J.R., Dale, G., Scott, D.J. and Wagget, J. [1985] Multiple molecular forms of acetylcholinesterase in Hirschsprung's disease. Poster presentation, National ACB Meeting, Cardiff, U.K.

ABBREVIATIONS

A_4, A_8, A_{12}	- asymmetric molecular forms of acetylcholinesterase consisting of 4, 8 and 12 catalytically active subunits respectively.
ACh	- acetylcholine
AChE	- acetylcholinesterase
ADH	- alcohol dehydrogenase
AFP	- alphafetoprotein
An	- anencephaly
AR	- analar
AThCh	- acetylthiocholine iodide
B	- the bed volume reconstitued per gram of dry gel
BChE	- butrylcholinesterase
BThCh	- butyrylthiocholine
BW284c51	- 1:5 - bis [4-allyldimethylammonium phenyl] pentan-3-one dibromide
^oC	- degree centigrade
CFA	- centrifugal fast analyser
ChE	- cholinesterase
cm	- centimetre[s]
Con A	- concanavalin A
CSF	- cerebrospinal fluid
CV	- coefficient of variation
d	- density of dry gel
DTNB	- 5,5′-dithiobis [2-nitrobenzoic acid]
EDTA	- ethylenediamine tetraacetic acid
Em	- molar extinction coefficient
Ex	- exomphalos
f	- constant eg $Re.S_{20},W = f$ [molecular mass]
g	- accelaration due to gravity
G_1, G_2,G_4	- globular molecular forms of acetylcholinesterase consisting of 1,2 and 4 catalytically active subunits respectively.
HCl	- hydrochloric acid
hr	- hour
iso-OMPA	- tetramonoisopropylpyrophosphortetramide
IUD	- intrauterine death

K	- potassium
Kd	- distribution coefficient
kDa	- kilo Dalton[s]
kg	- kilogram[s]
l	- litre[s]
LDH	- lactate dehydrogenase
M	- molar
mg	- milligram[s]
min	- minute[s]
ml	- millilitre[s]
mm	- millimetre[s]
mM	- millimolar
MOM	- multiples of the median
mU	- milli Unit[s]
μg	- microgram[s]
μl	- microlitre[s]
μm	- micro meter[s]
Na	- sodium
NaCl	- sodium chloride
NAD	- nicotinamide adenine dinucleotide
ng	- nanogram[s]
nm	- nanometre[s]
NTD	- neural tube defect
ONPG	- ortho nitrophenyl-β-D galactopyranoside
PAG	- polyacrylamide gel
PAGE	- polyacrylamide gel electrophoresis
pH	- $-\log_{10}$ [H^+ concentration]
pS	- $-\log_{10}$ [substrate concentration]
r	- mean pore size of gel
Re	- Stokes radius
RID	- radial immunodiffusion
rpm	- revolutions per minute
S	- Svedberg unit[s]
SB	- spina bifida
SD	- standard deviation
SEM	- standard error of the mean
SV	- sample volume
$S_{20,W}$	- the sedimentation coefficient for a given macromolecule measured at 20°C in distilled water
TEMED	- N,N,N′,N′-tetramethylethylenediamine

TRIS	- tris[hydroxymethyl]-aminomethane
TV	- total volume
U	- International unit[s] [micromoles per minute]
USA	- United States of America
UK	- United Kingdom
V	- volts
Ve	- elution volume
Vg	- volume not accessible to the solvent
VIP	- vasoactive intestinal peptide
Vo	- void volume
Vs	- internal volume of the stationary phase
Vt	- total volume of the gel bed
v/v	- volume/volume
w/v	- weight/volume
w/w	- weight/weight

ABSTRACT

Acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) were studied in amniotic fluid in relation to the detection of neural tube defects (NTD), and in rectal tissue in the diagnosis of Hirschsprung's disease.

An automated assay is described for measurement of AChE and BChE activity in amniotic fluid, and an increase in both is found in the presence of NTD. Analysis of AChE molecular forms by sucrose density sedimentation revealed three species with differing sedimentation coefficients and molecular masses: monomeric G_1(4.0S, 78KDa), dimeric G_2(5.5S, 126KDa) and tetrameric G_4(10.3S, 256KDa). The tetramer, G_4 is NTD specific and is largely responsible for the increase in activity seen in the quantitative assessment of total AChE and for the abnormal band identifiable by polyacrylamide gel electrophoresis in pregnancies affected by NTD. Evidence is presented which indicates that G_4 is a soluble species secreted from nerve trunks exposed as a result of the lesion.

BChE activity, the likely source of which is fetal plasma is shown to be a less specific indicator of NTD.

These results represent the first description of the structural molecular heterogeneity of AChE and BChE forms in amniotic fluid.

AChE activity was measured in rectal biopsy specimens from 213 patients in whom a diagnosis of Hirschsprung's disease was suspected. The results from this, the largest study so far reported, indicate the value of AChE measurement in the detection of the disease.

The molecular forms of AChE and BChE were investigated in resected bowel segments from patients with Hirschsprung's disease. Four species of AChE were identified: G_1(3.5S, 74KDa), G_2(5.0S, 131KDa), G_4(9.2S, 275KDa) and the asymmetric form A_{12}(16.8S, 811KDa).

In all cases there was an increase (4-14 fold) in G_4-AChE activity in the aganglionic colo-rectum. The evidence indicates that this is derived from hypertrophied nerve trunks present in the affected zone. The increase in G_4-AChE was largely responsible for the increase in total AChE activity in rectal biopsy specimens from patients with Hirschsprung's disease.

BChE molecular forms showed no consistent changes in Hirschsprung's disease.

Characterisation of the molecular forms of AChE by gel filtration and with respect to their thermal stability, sensitivity to Triton X-100 and response to substrate inhibition is also investigated.

I N T R O D U C T I O N

Section 1 : The Cholinesterases

Section 2 : Molecular Forms

Section 3 : Hirschsprung's disease

Section 4 : Neural tube defects

Section 5 : Aims of the thesis

Section 1 : The Cholinesterases

1.1 History

The existence of a cholinesterase was first proposed by Dale in 1914 who, describing the behaviour of animals previously injected with acetylcholine [ACh] wrote "In the blood at body temperature it seems not improbable that an esterase contributes to the removal of the active ester [ACh] from the circulation and the restoration of the original condition of sensitiveness". Subsequently, Loewi and Navratil [1926] were able to show that eserine delayed this recovery, they attributed this to inhibition of the enzyme normally responsible for destroying ACh. However, it was not until 1932 that Stedman et al prepared the first crude extract of cholinesterase from horse serum.

Alles and Hawes, [1940] showed that human blood contained two qualitatively different cholinesterases, one associated with erythrocytes, the other with serum. The erythrocyte associated enzyme resembled the type described later in brain by Mendel and Rudney, [1943a] and thought to act specifically on choline esters, they therefore suggested the term "specific" or "true"-cholinesterase. Later Augustinsson and Nachmansohn, [1949] introduced the term acetylcholinesterase to describe this enzyme.

In contrast, the serum enzyme was relatively non-specific with an ability to split other esters such as tributyrin and it was suggested by Mendel and Rudney, [1943b] that this be provisionally called "pseudocholinesterase".

Since this initial work, there has been a continuing widespread interest in the cholinesterases for two main reasons. Firstly their involvement in the function of nerve and muscle cells and secondly the importance of their inhibitors as insecticides and potential nerve poisons of military significance. Interest was initially focused upon the catalytic properties and histochemical localisation of these enzymes, however, in the past decade the importance of their molecular heterogeneity has prompted much interest and recent review articles reflect this trend [Massoulié and Bon, 1982 and Brimijoin, 1983].

The undoubted physiological importance of these enzymes, and the many remaining unanswered questions which surround them, guarantee continued interest in this field.

1.2 Types and nomenclature

The cholinesterases have been defined as hydrolases which, under optimal conditions, catalyse the hydrolysis of choline esters at a higher rate that that of other esters and which

are inhibited by low concentrations [10^{-5}M or less] of physostigmine [Silver, 1974].

The Enzyme Commission [1965] recommended acetylcholine hydrolase [3.1.1.7] as the systematic name for acetylcholinesterase [AChE] and acylcholine acylhydrolase [3.1.1.8] for pseudocholinesterase. While the trivial name acetylcholinesterase [AChE], has gained widespread acceptance the use of the term pseudocholinesterase to describe acylcholine acylhydrolase has been debated from its inception. The main objection has been the implication that "pseudo"- usually means false [Glick, 1945]. Several other terminologies have been suggested including non-specific, serum, Ψ , S- or butyrl-cholinesterase or simply cholinesterase. The preferred substrate for the enzyme in the majority of mammals including man is butyrylcholine and for this reason, as the present work is confined to human material, the most unambiguous name to adopt is perhaps butyrylcholinesterase [BChE]; accordingly this term is used throughout the work.

The differences between AChE and BChE are outlined in Table 1.1, some of these, particularly susceptibility to inhibition can be used to differentiate between the enzymes when measuring their respective activity in mixed suspension.

Table 1.1 The properties of AChE and BChE compared with respect to substrate specificity, inhibitor sensitivity and susceptibility to excess substrate inhibition.

Table 1.1 Properties of AChE and BChE compared

	Acetylcholinesterase [AChE] 3.1.1.7	Butyrylcholinesterase [BChE] 3.1.1.8
Preferred substrate	Acetylcholine	Butyrylcholine
Activity towards:-		
Methylcholine	+	-
Benzoylcholine	-	+
Acetyl β methylcholine	+	Slight
Inhibitors:-		
Physostigmine	Inhibited	Inhibited
Iso-OMPA	No inhibition	Inhibited
Ethopropazine	No inhibition	Inhibited
BW284c51	Inhibited	Resistant
Inhibition by excess substrate	Yes	No

Adapated, with additions from Silver, 1974

1.3 Occurrence

AChE is widely distributed in both the central and the peripheral nervous systems of mammals. In particular the observation that AChE activity in muscle appeared to be at least 3 - 6 times higher in endplate regions [Marnay and Nachmansohn, 1937] led Nachmansohn to check the enzyme activity of the electric organs of electric fish. These are related to muscle and accordingly were found to contain extra-ordinarily high concentrations of AChE [Nachmansohn and Lederer, 1939], they have proven to be an important source of the enzyme for research use.

While there are no known examples of cholinergic neurones that do not contain high levels of AChE [Lehmann and Fibiger, 1979], AChE is also present in many other structures with no known cholinergic function; examples include the dopaminergic neurones of the substantia nigra and the noradrenergic neurones of the locus coeruleus [Lehmann and Fibiger, 1979]. In addition AChE is found in cells with no clear neurological function including the endocrine cells, secreting peptide hormones [Chubb and Millar, 1984] and erythrocyte membranes.

BChE is found in many tissues including heart, liver and intestinal mucosa [Ord and Thompson, 1950]. The liver synthesises large amounts of BChE and maintains high serum activities of this enzyme, which is clinically significant more in its relation to deficiency in certain individuals [Section 1.7] than to any clear function.

Attention has been focused on AChE, which has by tradition been considered to be the functional, or true, form of the enzyme associated with nerve transmission; however, BChE also occurs at cholinergic structures such as muscle end-plates [e.g. sternomastoid, diaphragm and extraoccular muscle]. Indeed it is the predominant form of cholinesterase in the end-plates of rhesus monkeys where it accounts for 73-83% of the total activity [Barnard et al, 1971].

AChE and BChE activity have also been found in a number of biological fluids, such as the CSF of the central nervous system and, importantly from the point of this study, in the amniotic fluid surrounding the fetus [Vincent et al, 1976].

1.4 Function

An appreciation of the physiological role of AChE in synaptic transmission has developed with the recognition of the importance of acetylcholine at the neuromuscular junction [Eccles et al, 1942].

AChE can control ionic currents produced in the post synaptic membrane by hydrolysing acetyl-choline, thus destroying its potential agonist action on the receptor [Ferry et al, 1973]. The enzyme may also regulate the level of stored synaptic acetylcholine, perhaps by producing a proportion of the choline used for its synthesis [Birks and MacIntosh, 1961].

It is clear that AChE has an important function in the synaptic transmission of nerve impulses; the ratio of AChE:ACh on the receptor is carefully controlled and recurring reports suggest that the ratio of the enzymic active sites to receptor is 1:1 in electric organ and muscle [Changeux et al, 1970; Karlin et al, 1971]. Although the exact details of the role of AChE in nerve transmission is still debated, Neuman et al, [1973] have formulated an "integral model" according to which acetylcholine reacts within the membrane to amplify the small ionic currents, and produce an action potential. This proposal is in sharp contrast to the intercellular action of acetylcholine presumed by the neuro-transmitter theory [Katz, 1969]. Some evidence supports the integral model, notably that acetylcholine has never been found outside excitable cells unless eserine is added. However,

there is still insufficient evidence to discount the more widely held neurotransmitter hypothesis. This controversy merely underlines the fact that many questions surrounding the role of AChE in nerve conduction at the synapse still remain to be answered.

If the function of AChE at the synapse is not yet fully understood there is even less knowledge concerning the role of AChE outside the synapse. Several tissues not supplied with nerves, such as erythrocytes and placenta, contain large amounts of AChE, the function of which can only be speculated about. It would seem logical from this evidence alone to conclude that the physiological role of AChE is not confined to destruction of excess acetylcholine.

Recently, Chubb et al, [1980] has been able to demonstrate that AChE has peptidase activity when incubated <u>in vitro</u> with synthetic peptide substrates. These substrates include substance P, the two enkephalins and a variety of di- and tripeptides [Chubb and Millar, 1984]. It appears that AChE is able to liberate both amino- and carboxy-terminal amino-acids. This exopeptidase activity is inhibited by acetylcholine but not by puromycin [an inhibitor of other amino-peptidases]. It may well be that this ability to hydrolyse neuropeptides and their precursors is an important function of

AChE in non-cholinergic neurones and perhaps even non-neural tissue.

BChE has no clearly established physiological function. Indeed, the inhibition of this enzyme for long periods, does not appear to produce consistent pharmacological effects [Massoulié and Bon, 1982]. In addition, well known genetic variants of this enzyme, resulting in very low serum activity and subsequent problems relating to the administration of scoline, have no known effects in the absence of this drug.

Koelle, [1976, 1977a and b] has proposed a hypothesis suggesting that BChE is a precursor of AChE and is involved in the regulation of its activity. This hypothesis is based on the observation that sustained inhibition of BChE reduces the recovery of AChE after its inactivation by a phosphorylating inhibitor. On the other hand, the protection of BChE by a reversible specific inhibitor during treatment with a phosphorylating inhibitor increases the recovery of AChE in the ganglia.

There are, however, arguments against the precursor role for BChE. Firstly, continued specific inhibition of BChE in rats from six days after conception until three weeks after birth produces no observable effect on fetal development even at

muscle end-plates, and no effect on the activity of AChE [Brzin et al, 1980]. Secondly, the two enzymes are quite distinct in their thermal reactivation [Vigny et al, 1978] and in their immunoreactivity [Koelle et al, 1979]. Thirdly, at least in chickens; the mass of BChE is smaller than AChE [Allemand et al, 1981].

The observations of Koelle [1976, 1977a and b], do however demonstrate that in the cat autonomic ganglia, the regulation of the two enzymes appears to be linked.

1.5 Catalytic Properties

AChE is relatively difficult to extract from mammalian sources and most structural and kinetic studies have been performed on enzyme derived from the electric organ of the ray Torpedo marmorata and the eel Electrophorus electricus. Relative substrate specificies and other indicators suggest that AChE from a variety of species are closely related in their catalytic mechanism.

The simple model of an enzyme containing anionic and esteratic sites described by Nachmansohn and Wilson in 1951 has proven very useful. A negatively charged anionic subsite is involved in the binding of cationic substrates and inhibitors, the esteratic site includes a serine hydroxyl which is believed to be acylated during the catalytic

action [Schaffer et al, 1973]. This simple model may be extended to include one or more peripheral binding sites which when occupied can affect the stereochemistry of the active site [Fig. 1.1a].

The actual molecular architecture of the active site is believed to include a "charge relay system" [Fig.1.1b] containing an active serine. Many kinetic results are consistent with the existence of an acylated ChE intermediate complex which would be difficult to explain by alternative proposals [Krupka, 1966]. AChE is thought to react with substrates and many inhibitors according to the scheme:

$$EH + AL \rightleftharpoons EHAL \rightarrow \begin{cases} EA \xrightarrow{HOH} EH + AOH \\ HL \end{cases}$$

where EH is AChE, the H belonging to the serine hydroxyl group of the active site. This undergoes acylation to form EA. AL is a substrate/inhibitor comprising an acylating group A and leaving group L. EHAL is a reversible Michaelis-Menten affinity complex [Main, 1976].

The behaviour of AChE is consistent with Michaelis-Menten kinetics below the optimum substrate concentration but the enzyme is inhibited by higher concentrations of substrate, causing it to depart from this type of kinetics, Fig.1.2. It is unclear whether the excess substrate binds the acyl-enzyme complex, partially inhibiting deacylation, or whether deacylation

Fig. 1.1a Adapted from Rosenbery, 1975 - A diagramatic representation of AChE showing the substrate binding site containing anionic and esteratic subsites. Bis-quaternary ligands such as decamethonium will bind at anionic and peripheral sites [P_1], multiquaternary ligands such as flaxedil at other peripheral sites [P_2, P_3, P_4].

Fig. 1.1b Adapted from Main, 1976 - The "charge relay system" present in the anionic and esteratic binding subsites of the AChE molecule with acetylcholine in place.

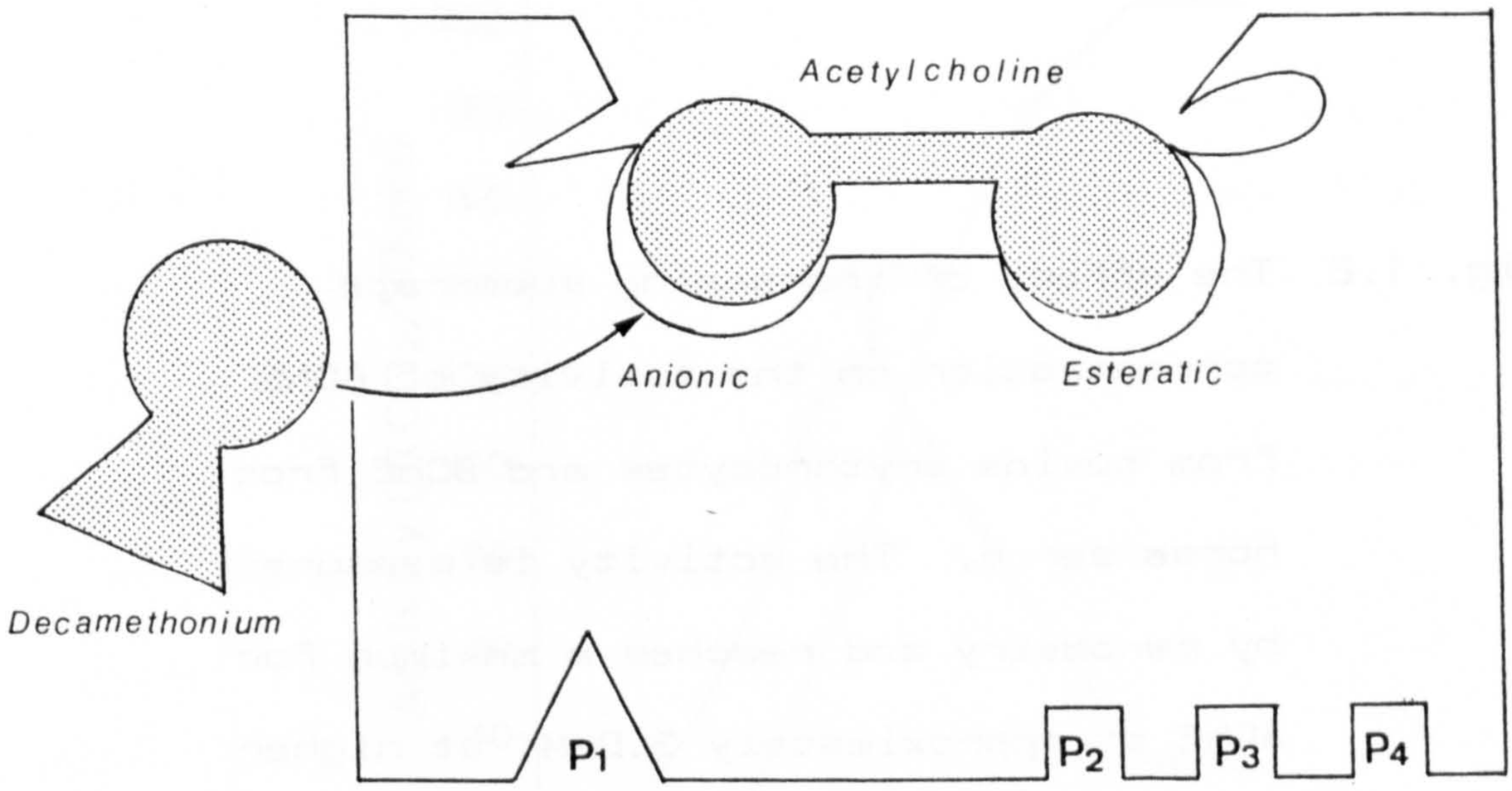


Fig. 1.1a Substrate and peripheral ligand binding in AChE

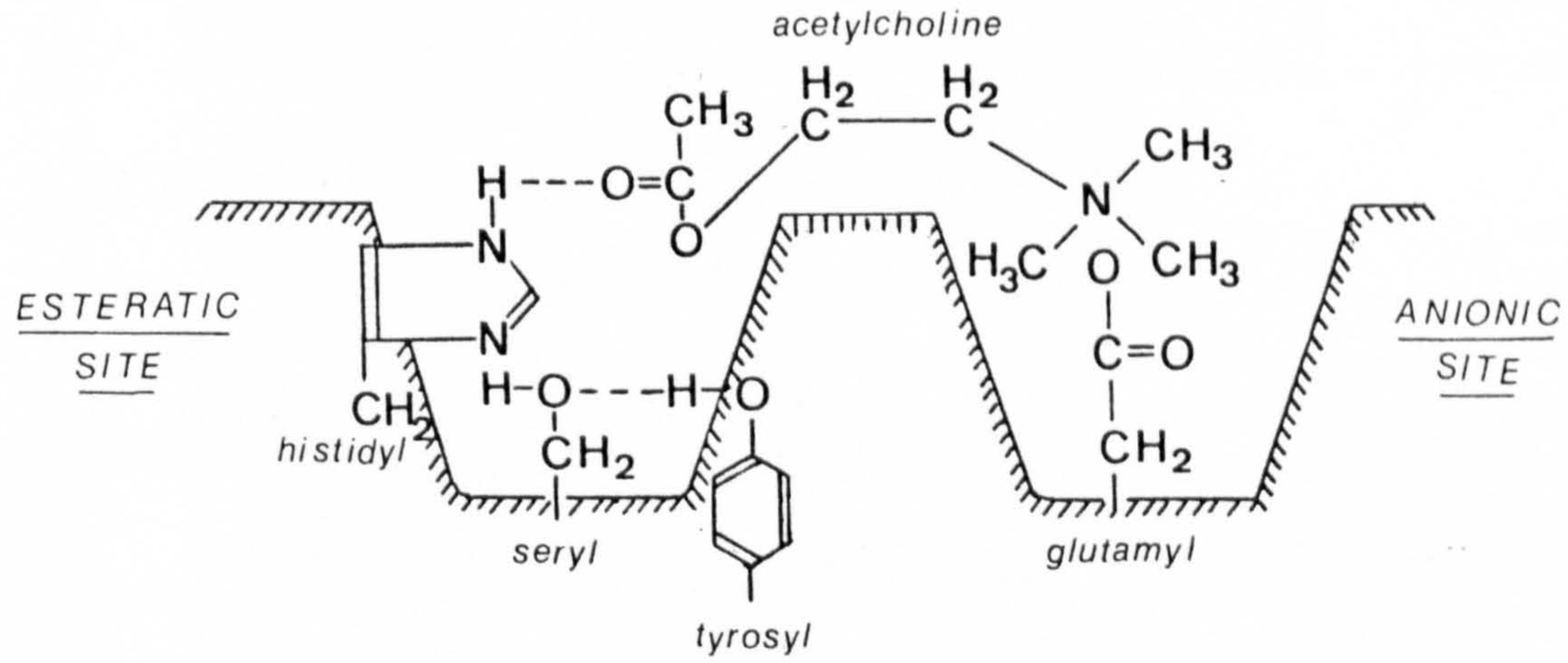


Fig. 1.1b The proposed "charge relay system" of the active site of AChE

Fig. 1.2 The effect of increasing substrate concentration on the activity of AChE from bovine erythrocytes and BChE from horse serum. The activity is measured by manometry and reaches a maximum for AChE at approximately 3.0mM, at higher concentrations the enzyme is inhibited. BChE shows no such maxima [Silver,1974].

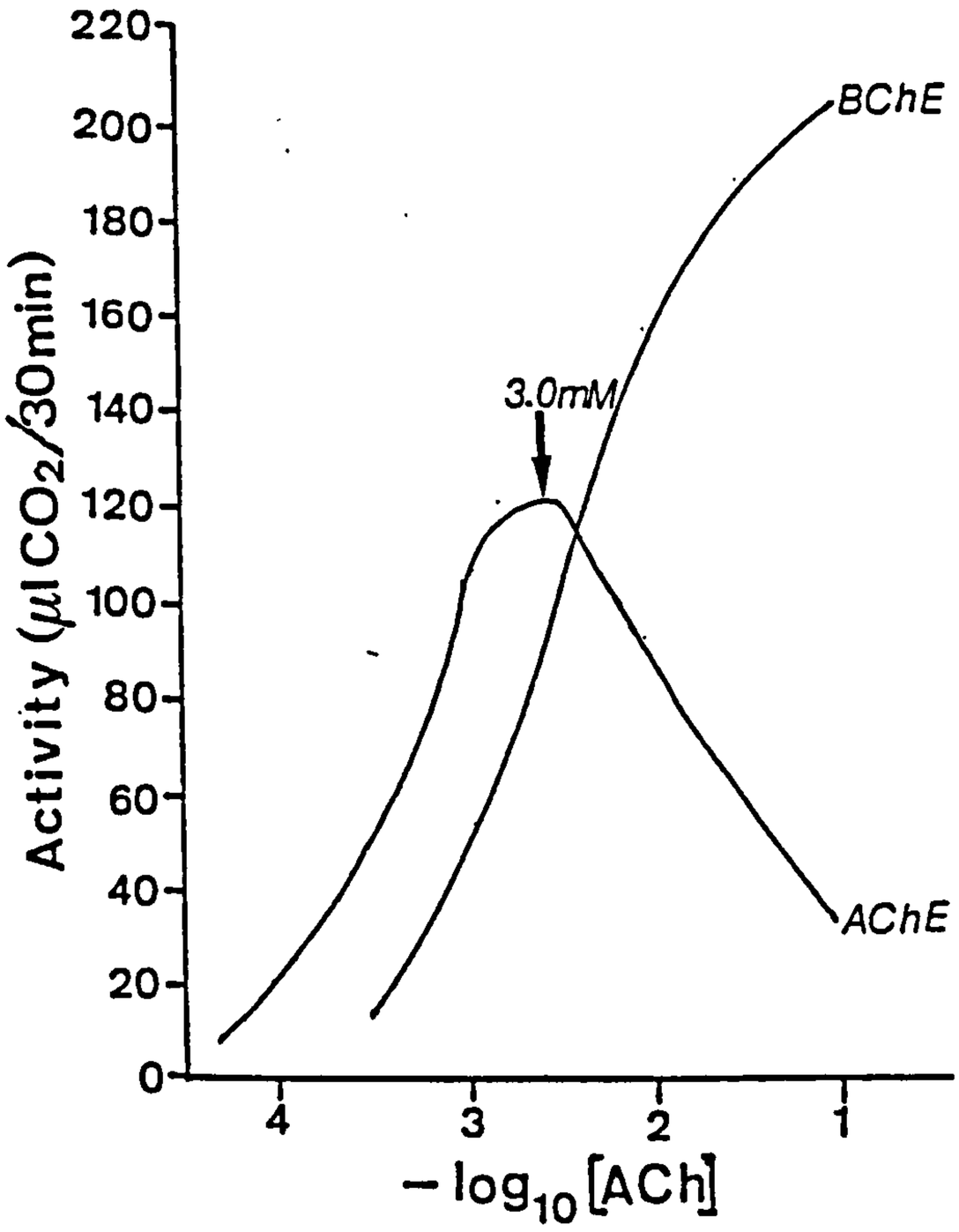


Fig. 1.2 The effect of increasing substrate concentration on AChE and BChE activity

is inhibited via allosteric effects mediated from excess substrate binding at a peripheral anionic site [Hofer and Fringeli, 1981].

Much of the foregoing discussion applies equally well to AChE and BChE, however substrate inhibition is not observed in the case of BChE, where, peripheral site binding of excess substrate appears to produce an enhanced rate of acylation and increased enzyme activity [Main, 1976].

1.6 Measurement

There have been two main approaches to assessment of cholinesterase activity: quantitative measurement of enzyme activity in solution or suspension, and histochemical localization of enzyme activity in tissue extracts.

[i] Quantitative measurement

Enzymic hydrolysis of a suitable substrate or substrate analogue can be monitored by various means:-

a) pH change [Michel, 1949] Hydrolysis of acetylcholine yields a hydrogen ion

$$CH_3COOCH_2CH_2N(CH_3)_3OH \xrightarrow[HOH]{ChE} (CH_3)_3N\ CH_2CH_2OH + CH_3COO^- + H^+$$

The pH change in the presence of excess substrate is a function of enzyme activity. This method although rarely used nowadays has the advantage of being suitable for the natural substrate, acetylcholine, or for any analogue.

b] Radiometric assay [Winteringham and Disney, 1964].

By using a ^{14}C labelled substrate such as ^{14}C-acetylcholine, the labelled acetate evolved during the reaction can be measured. The method can be adapted for use with any radiolabelled substrate provided an appropriate technique is available to separate free acid from unhydrolysed substrate.

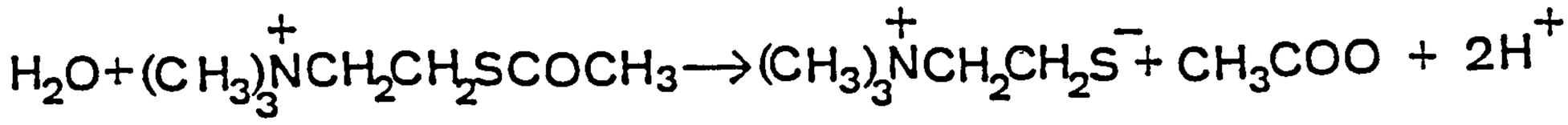
The chief advantage of the method is its sensitivity enabling detection of activities down to a level of only a few picomoles per hour. A major drawback is its relative technical difficulty and the poor precision compared with other methods.

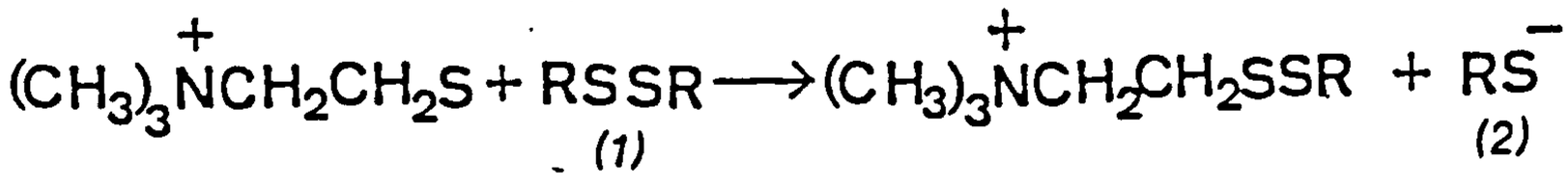
c] Spectrophotometric assays

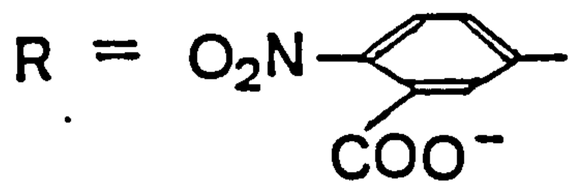
Perhaps the most widely used method of measuring cholinesterase activity is the Ellman reaction, based upon the hydrolysis of a thiol-containing substrate analogue and the detection of an evolved free thiol [Fig.1.3] by reaction with 5, 5'-dithiobis-2-nitrobenzoic acid [DTNB] to produce the yellow anion 5-thio-2-nitrobenzoate with an absorbtion maximum at 412nm [Ellman et al, 1961].

The advantages of this method are numerous; it is simple, cheap, and easily automated, with excellent precision. Provided a suitable thiol analogue can be made, most substrates can be used e.g. acetylthiocholine and butyrylthiocholine.

Fig. 1.3 The principle of the Ellman reaction which is based upon the hydrolysis of a thiol containing substrate analogue, acetylthiocholine and the detection of the evolved free thiol by reaction with 5, 5′-dithiobis-2- nitrobenzoic acid [*1*] to produce the yellow anion 5-thio-2- nitrobenzoate [2].







1 —— 5, 5′-dithiobis-2-nitrobenzoic acid

2 —— Coloured anion, 5-thio-2-nitrobenzoate ion

Fig. 1.3 The Ellman reaction

In all of these methods specific detection of either AChE or BChE in mixed suspension depends either upon the use of selective substrates or of inhibitors. Butyryl or propionylcholine and their analogues are specific for BChE; unfortunately substrates with similar specificity toward AChE are not available. Acetyl-β-methylcholine is specific for AChE and shows little cross reaction with BChE, however, its thiol analogue does not appear to have the same degree of specificity and is hydrolysed by BChE at approximately 50% of the rate shown by an equivalent concentration of acetylthiocholine [Bonham et al, 1981].

Selective inhibitors may be used to confer specificity in the assay; iso-OMPA and ethopropazine are relatively specific inhibitors of BChE, while the compound BW284c51 is a widely used selective inhibitor of AChE activity.

[ii] Histochemical measurement

Enzyme activity in tissue preparations can be demonstrated by light or electron microscopy. The means of visulaisation of the enzyme is usually dependent on the hydrolysis of the substrate to a product that reacts with another component in the medium forming an insoluble precipitate at the site of the enzyme activity. This product may have to

be converted to a coloured compound by a subsequent chemical reaction.

The most frequently used of these methods are adaptations of the initial work by Koelle and Friedenwald, [1949]. Thiocholine analogue substrates are hydrolysed and the liberated thiocholine captured by Cu^{++} ions to produce an insoluble white precipitate of copper thiocholine which can be further converted to coloured products:

$$2\ CH_3COSCH_2CH_2N[CH_3]_3OH + [NH_2CH_2COO]_2Cu + H_2O \xrightarrow{ChE}$$

acetylthiocholine — copper glycinate

$$Cu[SCH_2CH_2N[CH_3]_3OH]_2 + 2NH_2CH_2COOH + 2CH_3COOH$$

copper thiocholine — glycine — acetic acid

A commonly used modification of this original method is that of Karnovsky and Roots, [1964]. In this "direct-colouring" method, thiocholine reduces ferricyanide which is precipitated as cupric ferrocyanide - $CU_2Fe[CN]_6$, ["Hatchett's Brown"].

Appropriate substrates or selective inhibitors are used to ensure specificity.

The production of artefacts can make the interpretation of histochemical results more difficult than those of quantitative cholinesterase activity. In particular, diffusion of either enzyme or product can cause problems and in addition, the

substrate or inhibitor may not be able to penetrate certain cellular structures. Despite these shortcomings, histochemistry is an invaluable technique allowing the examination of enzyme distribution at a cellular or subcellular level in a way not otherwise possible. In addition to their application in staining tissue preparations, these histochemical methods can often be applied directly to the location of the enzyme following electrophoresis and other analytical procedures.

1.7 Cholinesterases in disease

Interest in the measurement of AChE in disease can be divided into those changes which are the result of neurological disorders and those which affect erythrocyte AChE secondary to haematological disease. Neurological disorders can also affect the level of BChE but this enzyme has more commonly been assessed in relation to liver disease or genetically related abnormality of the enzyme.

Neurological disturbance resulting in altered AChE activity

Neurological distrubance in Alzheimer's disease is accompanied by a measurable fall in CSF-AChE [Appleyard et al, 1983] conversely despite decreased cholinergic function in mania, Huntingdon's disease, depression and schizophrenia, levels of AChE in the CSF are normal [Davis et al, 1979]. Decreased activities of AChE and in particular the

absence of the end-plate form of the enzyme has been reported in a single case of myasthenic syndrome [Engel et al, 1977]. An interesting finding in dystrophic chick embryos is an increase in serum AChE, however this is not evident in the disease in man [Sketelj et al, 1983].

In Hirschsprung's disease where aganglionosis of the bowel results in nerve trunk hypertrophy there is an increase in the tissue levels of AChE [Boston et al, 1975], the basis of much of this work is discussed more fully later [Section 3.6[v]].

Similarly, in neural tube defect affected pregnancies when neural tissue is exposed to the surrounding amniotic fluid this results in an increase in the level of AChE in the amniotic fluid [Smith, A.D. et al, 1979]. Again this is discussed in greater detail in Section 4.4[iii].

<u>Altered AChE activity produced by haematological disease</u>

Haematological disorders such as paroxysmal nocturnal haemoglobinuria and ABO haemolytic disease of the newborn [Herz et al, 1972] are accompanied by a reduced erythrocyte AChE level. The activity of the red cell enzyme is also reduced in cases of organophosphorus poisoning where it is used to detect such contamination.

<u>Disorders resulting in reduced BChE activity</u>

BChE activity is most often measured in serum to detect abnormal genetic variants which occur in

0.05% of the population [Kalow and Staron, 1957]. In these cases apnoea can result following administration of certain muscle relaxants e.g. suxamethonium, it is therefore important to identify these patients to avoid future problems of this type.

BChE is produced in the liver and consequently liver disease such as viral hepatitis and alcoholic cirrhosis can reduce circulating BChE activity. This has been applied as a means of assessing liver function by several workers [Terzani et al, 1981; Sportiello et al, 1981].

In addition to liver disease certain physiological states induced by pregnancy and oral contraceptive administration can also result in reduced serum BChE levels [Areekul and Srichairat, 1981; Whittaker et al, 1971] by their effect upon hepatic synthesis.

Other disorders including leprosy [Rea and Won, 1978], tetanus [Porath et al, 1977] and carcinoma of the lung [Kaniaris et al, 1979] are also known to be accompanied by reduced BChE activity, however the factors producing this depletion are not obvious in any of these diseases. This emphasises the need to more clearly define the physiological role of BChE.

It has been suggested that BChE may be involved with lipid metabolism [Kutty et al, 1977] and one report suggests that the, BChE:High density lipoprotein cholesterol ratio, is a useful risk indicator in coronary disease [Kutty et al, 1981].

In addition to these quantitative changes in AChE and BChE activity, several reports describe qualitative changes in different disease states. These include changes in electrophoretic motility in liver cirrhosis [Matsuzaki et al, 1980] and a changes pattern of molecular forms in brain tissue in Alzheimer's disease [Atack et al, 1983].

1.8 Inhibitors

There has been great interest in understanding the mechanism of cholinesterase inhibitors and in the design of new inhibitors. Their importance is both commercial, as insecticide poisons and military as potential nerve gas substances, for these reasons they have been studied perhaps more thoroughly than any other group of enzyme inhibitors.

Cholinesterase inhibitors can be usefully classified as reversible and irreversible.

Irreversible inhibitors

There are two major classes of irreversible inhibitors - organophosphates and carbamates. These form acyl-enzyme intermediates in a manner similar to the acetyl-enzyme intermediate formed by the

natural substrate acetylcholine, but are stable to hydrolysis. Among the carbamates are the commonly used inhibitors eserine and neostigmine while the organophosphates include numerous toxic compounds such as soman, Tabun and di-isopropyl-phosphorofluoridate [DFP] which have been manufactured as chemical warfare agents.

Reversible inhibitors

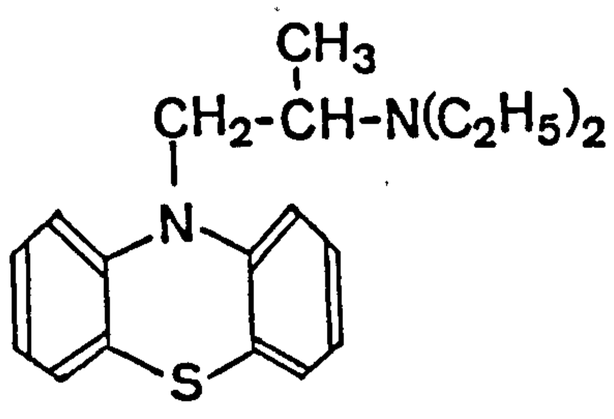
Reversible inhibitors form noncovalent bonds with active site residues; almost all contain at least one positively charged nitrogen group which binds to the anionic site. Larger substituent groups may also bind to the esteratic site or to peripheral subsites.

Some reversible inhibitors compete with the substrate and are competitive inhibitors, others bind preferentially to the enzyme acyl complex and delay deacylation, this is a non-competitive form of inhibition.

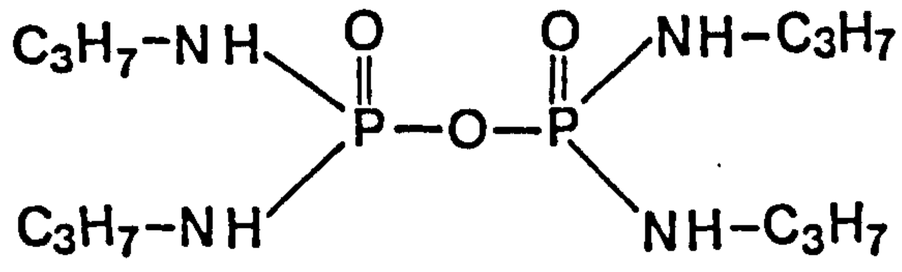
Many of the most potent inhibitors have a bis structure similar to d-tubocurarine and contain an aromatic ring. It is not clear why these structures improve the inhibitory properties of the molecule.

In addition to those reversible inhibitors that bind at the active site some compounds such as gallamine bind at peripheral anionic sites where they exert an allosteric inhibitory effect at the active site.

Of particular importance in the specific measurement of cholinesterase are those inhibitors, such as the reversible inhibitor ethopropazine and the irreversible inhibitor iso-OMPA, that preferentially inhibit BChE:

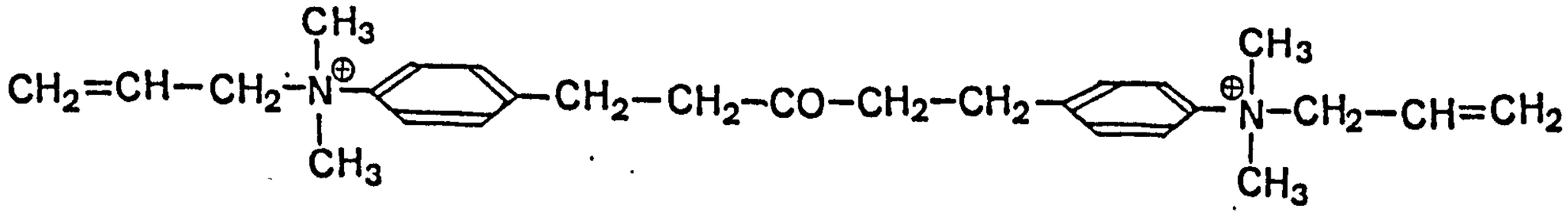


Ethopropazine



iso-OMPA

or inhibitors that selectively inhibit AChE e.g. BW284c51:



BW284c51

Section 2 : Molecular forms

2.1 General Introduction

AChE has been intensively studied over the past forty years and while much is now understood about the structure and function of this enzyme much remains to be elucidated. The study of the molecular heterogeneity of AChE has recently been the subject of renewed interest.

It has been known since the early 1960s that both AChE and BChE exist in a number of electrophoretically different forms [Bajgar and Zizkovsky, 1971]. These differences are believed to reflect changes in the carbohydrate content of the molecules rather than their amino-acid composition.

Polymorphism based upon molecular size and distinguishable by sedimentation coefficient was first described in electric fish by Massoulié and Rieger, [1969] and later in mammals by Hall, [1973]. The study of these "molecular forms" of AChE has dominated research in this area over the last ten years. Unlike electrophoretically defined species, distinct quaternary structures have been proposed for each of these differently sedimenting forms and this has allowed a reasonably rational and physiologically significant classification [Massoulié and Bon, 1982]. In addition there appears to be a considerable degree of homology

between the heterogeneity found in different species [Brzin et al, 1983].

Much effort has been directed to assigning particular cellular and sub-cellular locations to individual forms of the enzyme. However, while some species do predominate at particular sites [Brimijoin, 1983], they cannot be exclusively identified with these locations.

AChE species also differ in their relative hydrophobicity suggesting the presence of a distinct hydrophobic sequence in some molecules which is probably responsible for attachment of the enzyme to the cell membrane [Massoulié and Bon, 1982].

All the possible combinations of size, charge and hydrophobic diversity clearly create a very complex analytical picture, however, it may well be that the physiological flexibility conferred by such a system is indispensible to the function of the enzyme "in-vivo".

BChE, displays an entirely parallel set of size isomers closely related to the AChE forms [Brimijoin, 1983]. The significance of this heterogeneity is even less well understood than that of AChE.

2.2 Structural characteristics

The structural characteristics of the molecular forms of AChE have been most studied in the electric organs of Electrophorus and Torpedo marmorata. It appears that the basic building block of the enzyme is a globular monomer with a molecular mass of

approximately 80 kilo-Daltons [kDa]. This monomer can form a dimer via a single interchain disulphide bond; the two dimers are associated to form tetramers under the influence of Van der Waals forces [Brimijoin, 1983].

Tetramers may have three stranded "tails" attached. These can be visualised by electron microscopy and resemble collagen both immunologically and chemically as well as being hydrolysed by collagenase. The tails are connected directly to one of the dimers in the tetrameric head by means of a disulphide bond. Each tail can make three such junctions [Brimijoin, 1983].

Six major forms of AChE are therefore possible [Fig.2.1]. These include a globular series of monomer, dimer and tetramer [G_1, G_2 and G_4] and a collagen tail-containing asymmetric series designated A_4, A_8 and A_{12} - in each case the subscript denotes the number of catalytic subunits present in the molecular structure [Bon and Massoulié, 1976]. Although these molecular inter-relationships were worked out for the AChE of Electrophorus the scheme appears to be generally applicable to all tissues and species so far studied including mammalian enzyme.

While the molecular masses of the forms range from 80 kDa [G_1] to 1,100 kDa [A_{12}] the asymmetry of the collagen tail retards the sedimentation rate on ultracentrifugation and S-values

Fig. 2.1 A diagramatic representation of the six major forms of AChE in Electrophorus electricus [Brimijoin, 1983]. Globular and asymmetric forms are shown as G_1, G_2, G_4, A_4, A_8 and A_{12} repsectively. In each case the subscript denotes the number of catalytically active subunits present in the molecule.

Fig. 2.1 The quaternary structure of the six major forms of AChE in Electrophorus electricus

show a more limited range from 3.5 to 20S. Sedimentation coefficients of AChE forms show remarkably little variation among different tissue types and animal species [Table 2.1].

The larger asymmetric forms can be converted to monomers, dimers, tetramers and tail fragments revealing information about their quaternary structure, [Fig. 2.2]. In A_{12} from Electrophorus each of the three tetramers are attached to one strand of a collagen triple helix; half of the 80 kDa subunits are linked to each other via disulphide bridges, but not covalently linked to the tail [Fig.2.2]. They are detached by Sodium dodecyl sulphate [SDS]. Trypsin cleaves the tail close to the point of attachment, releasing intact tetramers which sediment at 11S and appear to be identical to the G_4 globular form. Pepsin totally digests the catalytic subunits leaving behind a fragment corresponding to the collagen tail. Collagenase treatment at 20°C results in the hydrolysis of the distal portion of the tail, removing about half the collagen sequence. The collagenous tail has a molecular mass of approximately 135 kDa,each strand of this triple helix comprising approximately 45 kDa [Silman and Anglister, 1980]. Studies of A_{12} species in mammalian motor nerve and skeletal muscle suggest similar structural properties and collagenase sensitivity.

Table 2.1 The sedimentation coefficients of AChE and BChE molecular forms from different species.

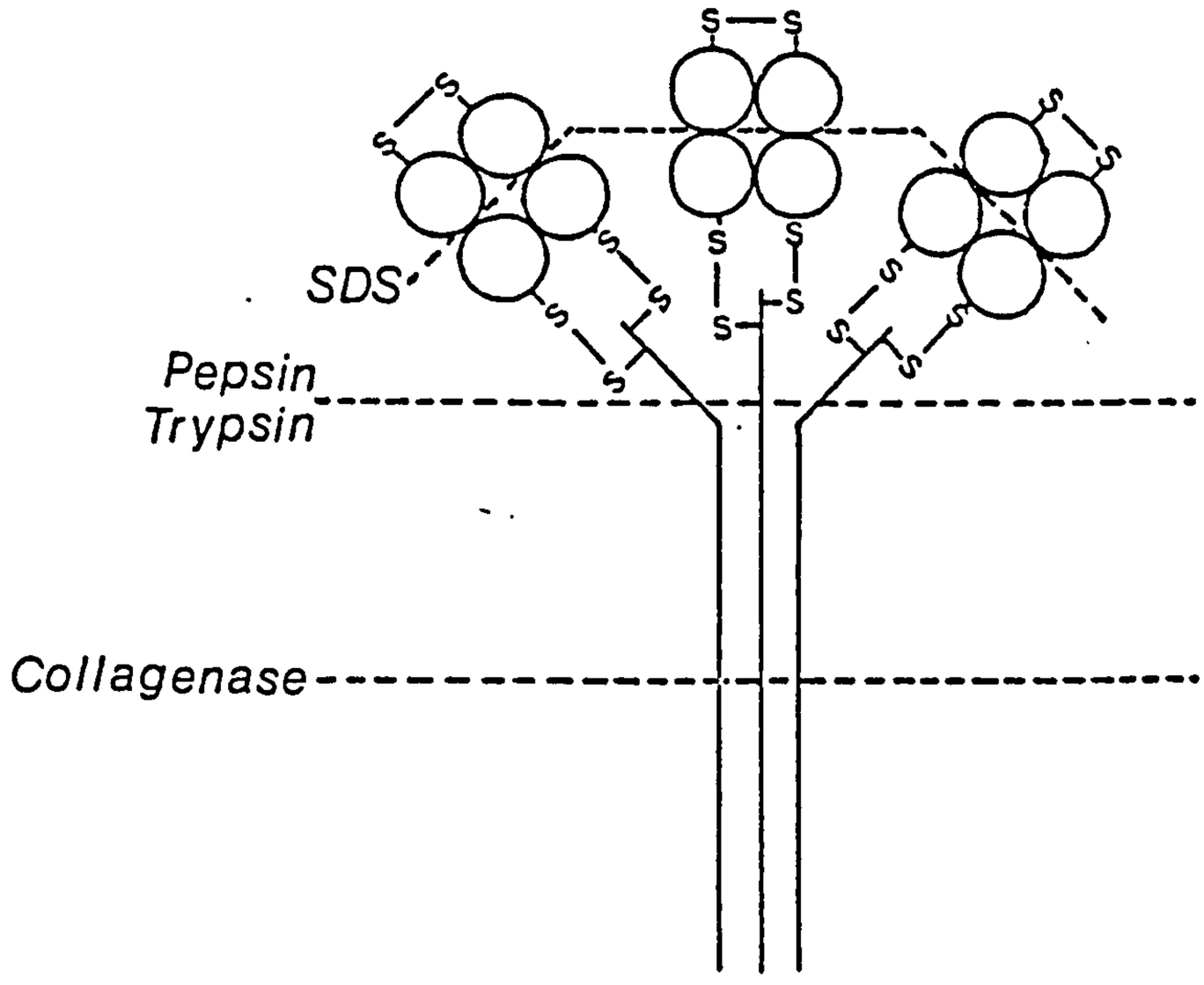
Table 2.1 Sedimentation coefficients of AChE and BChE molecular forms in different species.

	G_1	G_2	G_4	A_4	A_8	A_{12}
AChE	Sedimentation coefficient:					
Fish						
Torpedo	3.6	5.6	11.0	9.4	13.8	17.5
Electrophorus	5.3	7.7	11.8	9.1	14.2	18.4
Amphibia						
Frog	5.4	-	11.2	-	13.6	17.3
Bird						
Chick	5.4	7.9	11.8	8.5	13.3	20.0
Pigeon	4.3	6.3	11.8	-	15.1	19.3
Mammals						
Cat	4.3*	5.8	10.8*	8.8	13.0	16.2
Cow	3.9*	6.7*	10.7*	8.7	13.0	17.1
Man	3.5	6.0	11.0	9.5	13.3	16.7
BChE						
Bird						
Chick nerve	4.2	6.4	11.3	-	-	19.0
Chick muscle	4.9	7.1	11.1	-	-	18.6
Mammal						
Mouse serum	5.0	-	11.0	-	-	-

*Values denoted thus are mean values

Table adapted from Brimijoin, [1983] with additional information from Chatonnet and Bacou, [1983]; Lee et al, [1982]; Edwards and Brimijoin, [1983]; Allemand et al, [1981]; Couraud et al, [1982].

Fig. 2.2 The manner in which: collagenase, pepsin trypsin and SDS cleave A_{12} AChE from Electrophorus electricus. Adapted from Silman and Anglister, 1980.



A_{12}

Fig. 2.2 A diagramatic representation of the effect of treatment with various agents on the quaternary structur of A_{12}-AChE

In some species (Torpedo) a non-collagenous, structural subunit of approximately 100 kDa has also been identified. The function of this subunit is unclear but it may well be involved in locating the enzyme onto the surface of the basal lamina and may even by a component of it (Lee and Palmer, 1982).

Some of the globular molecular forms G_1, G_2 and G_4 appear to contain a hydrophobic amino-acid sequence, susceptible to pronase digestion; it has been suggested that its removal alters the ability of these forms to associate with lipid membranes and that this sequence must be removed before the globular forms can have a "tail-unit" added (Massoulié and Bon, 1982).

There is a strong indirect evidence, including their kinetic and immunological properties, that G_1, G_2, G_4, A_4, A_8 and A_{12} are products of the same gene and vary in post-transcriptional assembly alone.

In addition to heterogeneity due to these molecular associations, AChE also contains a large number of sialic acid containing carbohydrate residues. Similarly, BChE has a substantial proportion of carbohydrate,, each subunit being calculated to contain 103 sugar residues and 508 amino acids. Assuming that each oligosaccharide chain contains two sialic acid terminii, one can estimate that a minimum of nine oligosaccharide chains are attached to each subunit (Lockridge et al, 1979).

BChE molecular forms exist in a series homologous with those of AChE and including G_1, G_2 and G_4 globular and A_4, A_8, A_{12} asymmetric forms [Vigny et al, 1978]. In human tissue monomeric G_1 BChE has a slightly larger molecular mass of 90 kDa compared with AChE. G_2 is 180 kDa and G_4 340 kDa. The S values of these forms are correspondingly marginally higher than their AChE counterparts [Lockridge et al, 1979].

2.3 Distribution

As described in Section 1.3 cholinesterases are widespread in both nervous and non-nervous tissues. It is true that while AChE can be demonstrated in non-cholinergic neurones the highest levels are found in association with cholinergic structures [Brzin, 1983].

While, for a given tissue, the distribution of molecular forms are somewhat species dependent, certain generalisations can be applied to their distribution which are independent of this species variation and these are outlined below:

Nervous tissue

The overwhelming bulk of the AChE of nervous tissue is of the globular type. In mammalian brain

80-90% of the activity is typically of the G_4 form with most of the remainder occurring as G_1. Tailed asymmetric forms do exist in the brain but comprise only about 0.02% of the total activity [Brimijoin, 1983]. Peripheral nerve is more variable although again G_4 typically predominates with asymmetric forms rarely comprising more than 10% of the activity. Nerve axons, both pre- and post-synaptic, are particularly rich in the G_4 species, whereas cell bodies, in the case of cilliary ganglia, contain a greater proportion of G_2 [Couraud et al, 1980].

In order to investigate the subcellular distribution of the enzyme, combined inhibitor studies have been employed. BW 284c51, a reversible AChE inhibitor, will not enter cells and therefore will protect only externally located enzyme from further irreversible inhibition with DFP [Rotundo and Famborough, 1982]. Results from studies such as this suggest that G_1 and G_2 forms are almost exclusively intracellular whereas G_4 and A_{12} appear to be predominantly externally located [Brimijoin, 1983].

Secretion of AChE by nerves was suggested by Skangiel-Kramska and Niemerko, [1975]. It was later demonstrated in rats that the major proportion of the secreted form released from phrenic-nerve was G_4, and in every known case, even when cells are

richer in G_1 and G_2, G_4 still accounts for the majority of secreted enzyme [Brimijoin, 1983].

The parallelism of AChE and BChE forms can be seen in nervous tissue. Even neurones, which classically were considered to contain only AChE, have been shown to have BChE activity. This suggests some neural function for this enzyme [Brimijoin, 1983].

Muscle tissue

AChE activity from innervated skeletal muscle is distinctive in containing a large proportion of the asymmetric forms of the enzyme, especially A_{12}. This A_{12} species predominates at the muscle end-plate, and is a particularly efficient structure for the hydrolysis of acetylcholine [Fig.2.3]. Indeed Hall, in 1973 originally suggested that A_{12} AChE could be used as a marker for the muscle end-plate in rat. Since then, however, A_{12} enzyme has been demonstrated at other sites, and other forms including globular species have been identified at the end-plate region.

There is evidence that the AChE molecular form profile varies with the muscle type. There are large differences between fast and slow twitch muscle, the latter containing less G_4 and more A_8 - this may reflect differences in the ACh hydrolysis rates necessary in muscle types with different depolarization periods [Groswald and Dettbarn, 1983].

Fig. 2.3 Adapted from Thompson, 1980 - A diagramatic representation of the neuromuscular junction showing the distribution of AChE molecular forms.

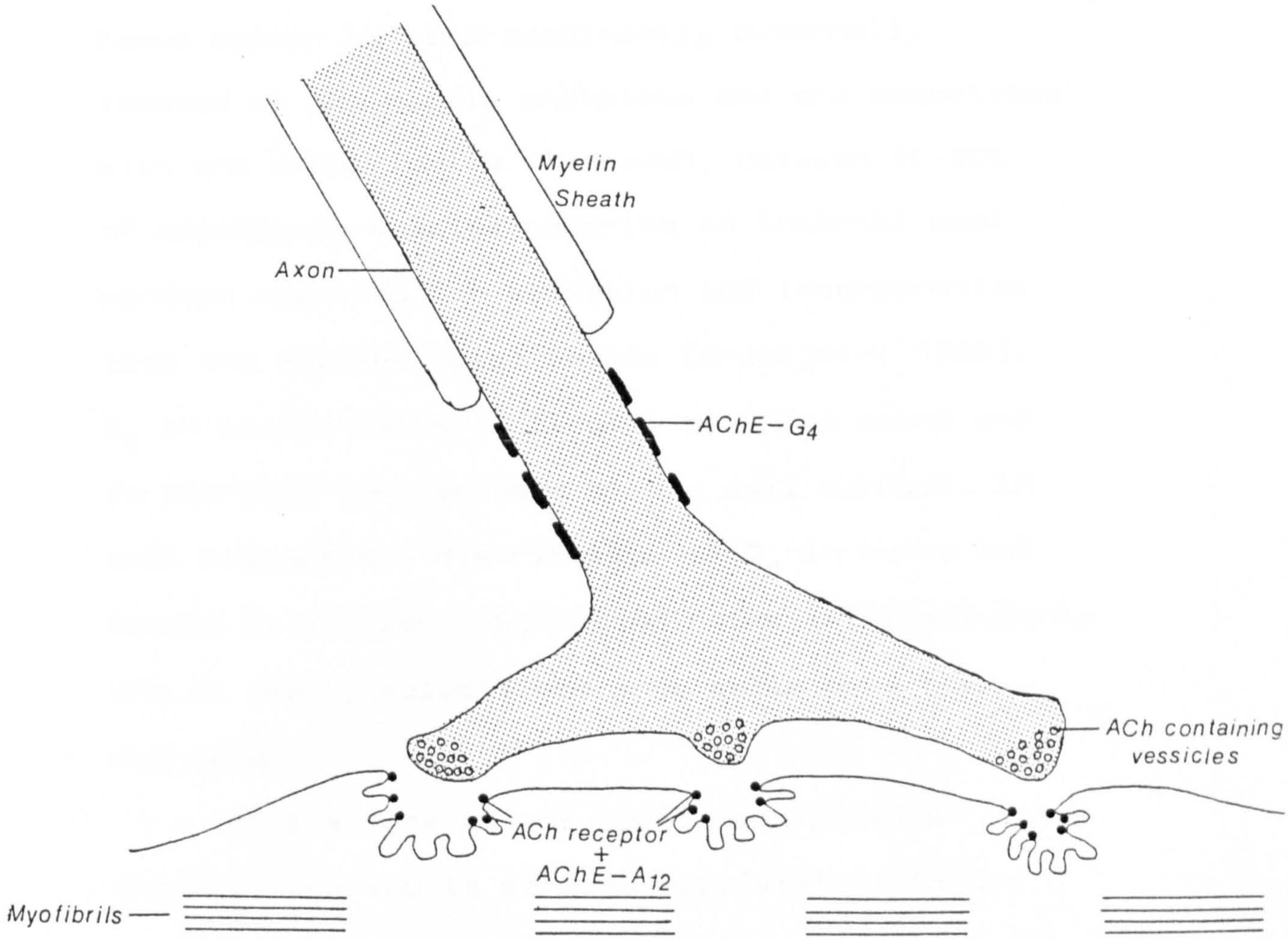


Fig. 2.3 Molecular forms of AChE at the neuromuscular junction

Asymmetric, collagenase sensitive, AChE forms appear to be predominantly externally located at the muscle end-plate and are associated with the basal lamina. However, between 10-20% of asymmetric species comprise an internal pool perhaps destined for secretion and incorporation into the extracellular matrix [Brimijoin, 1983]. G_4 in muscle cells is largely membrane bound and is the main form exposed on the cell surface, in this respect the distribution of G_4 in nerve and muscle is similar. G_1 predominates intracellularly and is easily solubilised once cells have been disrupted.

BChE exists at the muscle end-plate as the A_{12} form and is structurally similar to the corresponding A_{12} AChE species [Jedrzejczyk et al, 1981]. In newborn rats BChE is predominant in the end-plate region; as the animal matures however, AChE activity begins to increase and BChE decline. At least some of this BChE activity is found in the numerous Schwann cells which overlie the nerve endings of motor end-plates. The decrease in BChE activity postnatally coincides with a reduction in the number of nerve endings which takes place between 10 and 15 days post partum [Brzin et al, 1981].

<u>Blood, cerebrospinal fluid and amniotic fluid</u>

Human erythrocyte membrane is rich in AChE

which in the presence of non-denaturing detergents exists solely in the G_2 form with a molecular mass of 151 kDa [Ott et al, 1982]. The enzyme is firmly attached to the erythrocyte membrane but can be removed by non-ionic detergents such as Triton X-100.

Cerebrospinal fluid [CSF] contains soluble AChE which corresponds mainly to the G_4 form in man [Massoulié and Bon, 1982]. It is probable that this derives by direct secretion from nerve tissue into the surrounding CSF [Scarsella et al 1979].

Serum also contains soluble AChE in some species. In rat this is again predominantly G_4 [Massoulié and Bon, 1982]. The preponderant BChE species in human serum is the tetrameric G_4 BChE [Lockridge et al, 1979].

Amniotic fluid contains both soluble AChE and BChE activity in low levels [Smith, A.D. et al, 1979, Milunsky et al, 1979]. The activity of both are increased in pregnancies affected by neural tube defect [NTD] where the exposed neural tissue augments the activity of these enzymes in the amniotic fluid. The molecular forms present in amniotic fluid in normal and NTD affected pregnancies have not been previously described and their description and application in diagnosis of fetal abnormality are included within this thesis.

2.4 Dynamics

AChE and BChE are not static within the cell but rather are synthesised, assembled, transported and released in a way tailored to the cell and the function they are to perform. Each molecular form is distinct in the way in which it is handled and processed by the cell. Interconversion between these forms being an integral part of this overall fate of the enzyme in the cell. These are outlined below:

Transport

AChE is transported bi-directionally in nerve. The velocity can be calculated by nerve trunk transection and monitoring the rates of accumulation. Anterograde and reterograde rates differ, having been found to be about 400 and 260 mm/day respectively [Lubinska and Niemerko, 1971].

Interruption of the distal flow in nerve axons either by local cooling, ligature or crush injury has allowed the accumulation rates for different molecular forms to be determined. A_{12} although not the major intracellular form, accumulates most rapidly. In contrast G_4 which represents 90% of the activity in sciatic nerve and comprises over 80% of the distally transported enzyme, accumulates less rapidly than A_{12}. G_1 and G_2 forms are the most slowly accumulating species [Brimijoin, 1979] and

it seems probable that they are conveyed by a different mechanism from the larger species.

The proportion of A_{12} returned to the cell body by retrograde transport is only 24% of that distally conveyed [Brimijoin, 1979]. This suggests that A_{12} is primarily destined for incorporation into synaptic structures at the nerve ending. A large proportion of G_4 is stationary in the nerve axon and this supports the hypothesis that its major fate is insertion into the axolemma [Brimijoin et al, 1978].

BChE molecular forms like those of AChE appear to be subject to transport within the nerves. In chick sciatic nerve [Couraud et al, 1982] all of the A_{12} and some of the G_4 move with the slow phase of axonal transport while G_1 and G_2 remain stationary. This would be consistant with G_1, G_2 and a major proportion of G_4 BChE being associated with the Schwann cells.

<u>Interconversion and release of AChE</u>

It is tempting to suggest that larger AChE molecular forms are assembled from G_1, the initial translation product. Indeed evidence gained from observing embryonic development of molecular forms lends some support to this, since G_1 appears before larger forms in rat fetal brain [Reiger and Vigny, 1976].

The most convincing studies in this area involve irreversible inhibition of existing forms and observation of the order of their reappearance in the cell. A rapid increase of G_1 with delayed appearance of other forms is strong confirmatory evidence that G_1 is the initial building block [Reiger et al, 1976]. Gisiger and Vigny, [1977] demonstrated a sequential reappearance of molecular forms in the order G_1, G_4, A_{12}. The species A_4 and A_8 are probable intermediates but were not clearly demonstrated - perhaps due to their low concentration.

Not all G_4 ends up as A_{12} and it may be that branches in this pathway further modify these molecules for insertion into the cell membrane or secretion. Based upon inhibitor studies and the subsequent reemergence of AChE in particular cell fractions, Rotundo and Famborough, [1982] have postulated an outline model for AChE secretion from muscle cells [Fig.2.4]. The details may differ for each molecular form, depending upon its ultimate fate, but without pulse labelling studies or some alternative it is difficult to elucidate this for each species.

AChE is actively secreted [Section 2.3] from nerve tissue. G_4 in particular is secreted by rat superior cervical ganglia [Gisiger and Vigny, 1977]. It has been postulated that neuronal

Fig. 2.4 Adapted from Rotundo and Fambrough, 1982 -
A hypothetical model of AChE and acetylcholine-receptor metabolism in cultured muscle cells.

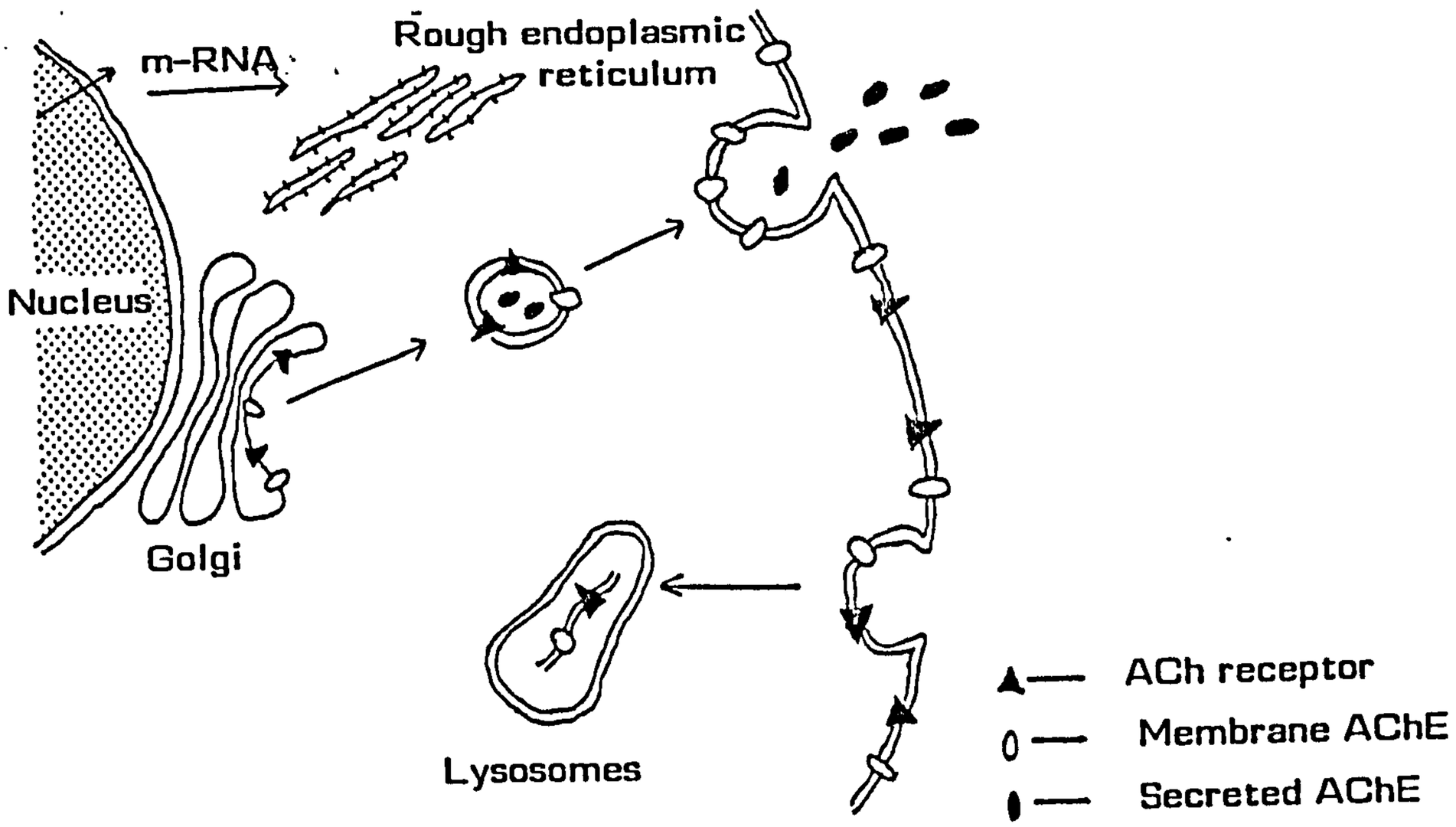


Fig. 2.4 AChE and ACh-receptor metabolism in muscle

release is the probable source of the soluble G_4 AChE found in the CSF; its concentration is increased by stimulation of the sciatic nerve [Chubb et al, 1976]. This neuronal origin may also account for the AChE found in amniotic fluid in pregnancies affected by neural tube defect and its presence in the extracellular space of mammalian central and peripheral nervous tissue.

2.5 Extraction

AChE molecular species may be either soluble or membrane bound and have different cellular locations so that extraction conditions vary depending on the location and nature of the species under study.

Asymmetric forms attached by a collagen tail to the basal lamina or synaptic cleft material, can be removed by buffer systems containing high salt concentrations e.g. 1 M NaCl. Addition of EDTA has also been shown to aid their removal [Interossa et al, 1981]. They can also be removed by cleavage of the "tail" with collagenase and this has been utilised as a method of extraction in low salt conditions [Silman and Anglister, 1980].

The means necessary for extraction of globular AChE forms depends upon the degree of membrane binding. Soluble species present either in extracellular matrix

or intracellularly can be released in low salt buffers e.g. 100 mM NaCl simply by adequate cell disruption. Membrane bound species such as the G_2 from erythocytes and the G_4 in the axolemma require a detergent-containing medium, such as Triton X-100 for their removal. In rabbit sciatic nerve, for instance, 25-32% of AChE activity is solubilized by homogenisation in TRIS buffer without Triton, whereas the remainder requires 0.1% v/v Triton [Brimijoin, 1979].

If it is necessary to solubilise all AChE forms, an extraction buffer containing a high salt concentration together with Triton and EDTA is capable of extracting both soluble and asymmetric forms, provided that cells and subcellular particles have been adequately disrupted for the buffer to gain access to all regions where AChE is located.

When Triton is present in the extraction medium in excess of the critical micellar concentration [CMC], $\sim$ 0.015%, it is possible that the enzyme may exist in protein - detergent complexes whose configuration reflects the characteristics of the detergent rather than the form in which the protein exists "in-vivo" [Rueb and Lieflander, 1981]. While not discounting this possibility Massoulié and Bon, [1982] reviewing several inhibitor studies,

conclude that "the composition of molecular forms in solution reflected the state of the enzyme in-situ".

2.6 Methods of separation

The molecular forms of AChE and BChE in the series G_1, G_2, G_4 and A_4, A_8, A_{12} vary both in molecular mass and in molecular size [as reflected in the Stokes radius of each species]. In addition to these size differences there is electrophoretic heterogeneity within distinct quaternary structures [Lazar and Vigny, 1980]. In order to classify forms on the basis of quaternary structure alone, methods which allow separation on the basis of molecular size or mass, and largely ignore charge differences, avoid a bewildering heterogeneity.

Characteristics such as thermal stability, immunogenicity and selective inhibitor sensitivity peculiar to a particular molecular form, could theoretically also be used. In practice, the most useful techniques are those that depend upon the hydrodynamic properties of the different forms, such as density gradient sedimentation, gradient polyacrylamide gel electrophoresis and gel filtration. These are discussed in turn below:

Density gradient sedimentation

Density gradient sedimentation, first applied to the separation of proteins by Martin and Ames, [1961]

was suggested by Brimijoin, [1983] to be, "by far the most convenient method", for analysis of cholinesterase molecular forms in tissue extracts. Certainly over the past ten years it has gained widespread acceptance as the method of choice in most situations.

In this technique a sample is layered on the top of a medium of progressively increasing density most commonly a sucrose gradient. This stabilizes the bands against convection and provides a gradient of increasing viscosity. The sedimentation rate [V] is dependent upon the size and shape of the molecule and the centrifugal field [$\omega^2 r$], where ω is the angular velocity in radians per second and r is the radius in cm. The sedimentation coefficient S, is given by the relationship:

$$S = \frac{V}{\omega^2 r}$$

For macromolecules, S is of the order of 10^{-13} seconds and the Svedberg unit [S] is defined as being equal to 10^{-13} seconds. In order to permit standardisation, Svedberg and Pederson, [1940] defined a standard sedimentation coefficient which would obtain for a given macromolecule in water at $20^{o}C$ [$S_{20,W}$]. These values can be determined either by comparison with the migration of known markers or by

calculation from pre-computed tables [McEwen, 1967]. More recently Young, [1978] has adapted the equation of Fundig and Steensgaard, [1973] for use in a computer program which gives a greater degree of flexibility to the parameters which can be defined than tables would allow.

The S values of the AChE molecular forms are, with the exception of G_4 and A_4 [A_4 is usually a very minor component], conveniently evenly spaced between 3 and 20S [Table 2.1].

Gel filtration

Macromolecules can be separated on the basis of their molecular size by the degree to which they partition within or are excluded from the stationary phase contained in a suitable gel matrix. The distribution coefficient [Kd] between the stationary and mobile phases can be calculated as:

$$Kd = \frac{Ve - Vo}{Vs}$$

where, Ve is the elution volume of the solute, Vo is the void volume and Vs is the volume of the stationary phase.

When the molecules under study are globular, elution will be inversely proportional to their molecular mass. However when they are asymmetric this relationship may be disturbed and the apparent molecular mass will appear larger than the true molecular mass.

In order to calculate the true molecular mass, the Stokes radius is calculated by the method of Ackers, [1964] or Siegel and Monty, [1966] and S_{20},W data obtained by sucrose density sedimentation.

Molecular mass can be calculated according to:

$$Re.S_{20},W = f[\text{Molecular mass}]$$

where f is a constant. Hence not only is gel filtration a useful tool for separation and identification of cholinesterase molecular forms but it also allows the indirect calculation of the true molecular mass of these forms when the data is used in conjunction with that gained from sucrose density sedimentation.

Polyacrylamide gradient gel electrophoresis

Separation of proteins is achieved on the basis of molecular size. In addition their apparent molecular mass can be determined [Slater, 1969].

Under the influence of a voltage gradient proteins migrate into regions of progressively higher gel concentration and thus smaller, average pore size. The decreasing pore size increasingly restricts the migration of the protein. If electrophoresis is continued until the protein migration has virtually ceased proteins of low charge density will catch up to those of similar size but higher charge density.

Thus, the final migration position is a function of size and shape. Migration position is usually

referred to in terms of Rf, distance migrated by the protein/length of the gel slab. A plot of Rf Vs log [molecular mass] is approximately linear. Apparent molecular mass can therefore be determined by comparing migration distances with those of standard proteins. There are several reports in the literature where this technique has been used to identify AChE and BChE molecular forms [McIntosh and Plummer, 1973; Wright and Plummer, 1973; Vidal et al, 1978; McIntosh and Plummer, 1976; Rakonczay et al, 1981b; Ortonne et al, 1979]. Just as in gel filtration, if the molecules under study are asymmetric their molecular mass will be overestimated.

Cholinesterase bands can be visualised in the gel slabs and adaptation of one of the reactions employed for histochemical localisation of the enzyme, for instance the Karnovsky and Roots, [1964] reaction - Section 1.6.

Polyacrylamide gel electrophoresis [PAGE and Iso-electric focusing

PAGE using continuous buffer systems [Clarke, 1964] in rods or gel slabs has been extensively used to separate AChE and BChE forms for more than twenty years [Bajgar and Zizovsky, 1971]. While this technique does not separate on the basis of quaternary structure alone but also reflects charge

diversity, it is still a powerful technique. Charge heterogeneity can occur within a single molecular form identified by, for instance, gel filtration [Wright and Plummer, 1973] nevertheless, in particular locations individual electrophoretic forms may have important physiological significance. For instance, the AChE released from brain into the CSF is of primarily one electrophoretic form [Chubb et al, 1974]. It may therefore sometimes be helpful to adopt electrophoretic techniques in addition to other methods.

In particular PAGE has been widely used diagnostically in the detection of neural tube defect affected pregnancies by identification of an abnormally migrating band in the amniotic fluid [Smith, A.D. et al, 1979].

Isoelectric focusing first described in polyacrylamide gels by Dale and Latner, [1968] will separate molecules on the basis of their isoelectric point alone, this therefore reflects charge diversity. Molecules of differing charge are very efficiently distinguished and consequently in human erythrocyte AChE for example thirteen distinct bands can be identified [Biagioni et al, 1982].

Other methods of separation

Any property characteristic of a given molecular

form may be used to identify that form in mixed solution.

There is evidence from thermal stability studies [Edwards and Brimijoin, 1983] that the G_1 form of the enzyme is the most thermally labile species and it has been suggested that this might form the basis of a method that could replace density gradients as a means of identifying and quantitating this particular molecular form [Edwards and Brimijoin, 1983].

While to a first approximation, the molecular forms of AChE are enzymologically equivalent, one study indicates that G_1 from rat tissue may have a different Km [Bon and Massoulié, 1976] from other forms. Similarly it has been suggested that "isoenzymes" of BChE may vary in their response to organophosphorus inhibition, however, in that particular study it was unclear whether these BChE species denoted ChEI and ChEII, corresponded in any way to other classification systems [Chemnitius et al, 1982].

It may be that during assembly of higher molecular forms from the basic monomer certain parts of the molecule are exposed or obscured. Hence, theoretically it may be possible to raise a range of monoclonal antibodies which would allow each form to be specifically identified. There is as yet little

suggestion that this is imminent, however one report demonstrates that membrane bound and soluble forms of AChE differ immunologically [Zanetta et al, 1981]. This kind of approach may in the long term prove the most rewarding as it would permit immunohistochemical study of enzyme location as well as quantitative molecular form analysis.

2.7 Stability

It is acknowledged that under certain conditions molecular forms of AChE may inter-convert by either association or dissociation. However, under neutral aqueous conditions this process is slow enough to allow analysis to reflect accurately the distinct cellular components present at the time of extraction. It has been reported that G_4 remains unchanged during storage at $4^{o}C$ for up to ten days [Massoulié and Bon, 1982]. In the same series of experiments G_1 was shown to associate to produce a 7.5S form, this could be prevented by the addition of Triton X-100. It is possible to isolate and purify individual molecular forms and consequently these can be thought of as stable molecular entities [Massoulié and Bon, 1982].

Endogenous proteases may act upon the collagen containing molecular species such as A_{12} to produce

an increase in the globular fraction. This is less of a problem in nervous tissue [Couraud, 1980] than in muscle extracts. Addition of a mixture of protease inhibitors such as that suggested by Lai et al, [1983] can prevent this autolysis in either situation.

2.8 Properties

Each molecular form has intrinsic properties related to its molecular architecture. In addition the presence of particular sequences or carbohydrate substituent groups confer hydrophobic or solubility differences which give rise to diversity within a particular molecular form.

The secreted soluble forms, predominantly G_1 and G_4 do not interact with detergents, however, a fraction of cellular G_1 and G_4 can also be solubilised without detergent, these do interact with Triton and their S value in the presence of this detergent is altered. G_4 from a T_{28} neuroblastoma x sympathetic neuron hybrid has a sedimentation coefficient of 10.2S in the absence of detergent but 9.7S if detergent is present; G_1 is similarly affected from 4.5S to 3.2S [Lazar and Vigny, 1980]. This adds to the diversity of the simple G_1, G_2, G_4 nomenclature [Fig. 2.5].

Molecular forms also vary in their thermolability. G_1 forms of AChE and BChE are markedly more sensitive

Fig. 2.5 A diagramatic representation of the diversity attributable to hydrophobic interactions present within globular AChE species.

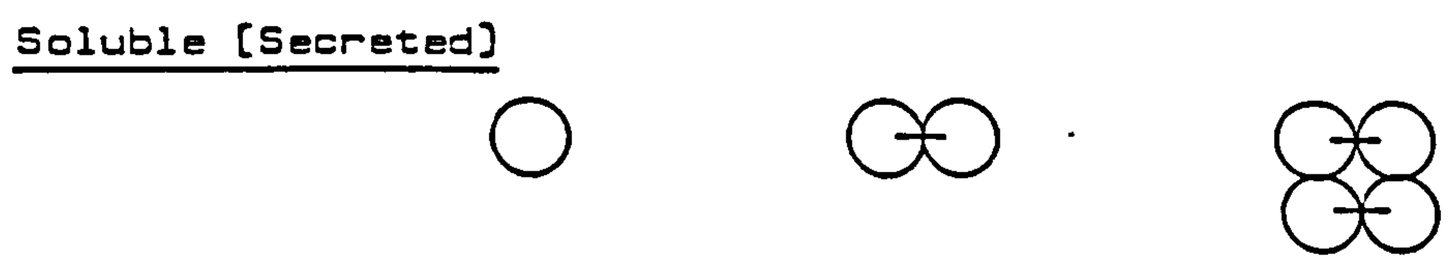


Fig. 2.5 Hydrophobic diversity within globular AChE species

to heat than the larger forms. Ten to fifteen minutes at 51°C will ensure 95% inactivation of G_1 without effect on other forms [Edwards and Brimijoin, 1983].

Immunologically it is possible to raise monoclonal antibodies with much greater affinity for dimeric 5.6S AChE from Torpedo than the catalytic subunits of the collagen tail containing forms from the same source [Doctor et al, 1983].

As referred to in Section 2.6 there is considerable electrophoretic heterogeneity within distinct quaternary structures.

This diversity of behaviour may simply reflect obligatory structural change dictated by the quaternary structure. However, it seems likely that it also reflects necessary adaptation for biological function "in vivo".

2.9 Function

It is difficult at present to assign a distinct function to individual molecular forms. They are catalytically equivalent and therefore probably do not directly affect the regulation of enzyme activity but may provide a means of adaptation to different subcellular and extracellular locations. In particular the condensation of multiple catalytic units in the A_{12} is reasonably viewed as a modification

to increase packing density and promote the efficient termination of acetylcholine mediated signals [Brimijoin, 1983]. The function of the globular species, predominantly G_4, located in the presynaptic nerve axon remains obscure. Smaller forms such as G_1 may simply be the biosynthetic precursors of larger molecules, however, it is unlikely that this is their sole "raison d'etre" and there is evidence that G_1 is actively secreted [Massoulié et al, 1984].

It is probable that a fuller understanding of the physiological significance of this heterogeneity will have to await improved analytical techniques such as immunohistochemistry employing specific monoclonal antibodies directed against individual molecular forms.

Section 3: Hirschsprung's Disease

3.1 History

Hirschsprung's disease is a congenital disorder of the bowel caused by the absence of ganglion cells in both Auerbach's intramyenteric and Meissner's submucosal plexuses. The disease is named after Harald Hirschsprung who in 1886 described a massive dilatation and hypertrophy of the colon in two infants at autopsy [Weinberg, 1975].

The remaining history of the disease is summarised by Weinberg, [1975] he describes how Tittel in 1901, first reported an abnormality of the myenteric plexus; this was considered only part of the abnormality which he believed also included proximal dilatation as a causal feature. In 1920 Dala Valle published the first thorough histological study of the colon in the disease documenting an abnormal distal plexus in a ten month old affected infant. Despite this, reports continued to view the proximal dilated bowel as the basic abnormality and treat accordingly.

It was not until 1948 that Whitehouse and Kernohan firmly established the cause of the disease as due to aganglionosis of the distal bowel and in the same year Swenson and Bill reported the first successful operative correction of

Hirschsprung's disease by removal of the aganglionic segment and preservation of the sphincter mechanism.

3.2 Clinical presentation

A prominent feature of Hirschsprung's disease is failure to pass meconium within the first twenty four hours of life. This is followed by the gradual development of abdominal distention and subsequently, vomiting due to intestinal obstruction. It has been described as "the commonest single cause of admission to hospital for obstruction in the newborn period" [Jones, 1970].

Diagnosis on clinical grounds alone in the immediate neonatal period is often confusing with a bizarre combination of obstruction or constipation followed by explosive diarrhoea-like stools. There are periods of abdominal distention and vomiting although at times the baby may appear perfectly normal [Schnaufer, 1976]. If the condition remains untreated during childhood, there is a gradual increase in the degree of constipation and progressive distention of abdomen. The distended colon may be palpated and often visualized by its impression on the abdominal wall, occasionally active peristalsis may also be seen. Anorexia, lassitude, poor nutrition and retardation of growth are associated features. The child gives the appearance of being undernourished with a gross

potbelly and wasted extremities [Singleton et al, 1977].

The differential diagnosis in the older child is often between Hirschsprung's disease and chronic or psychogenic causes of constipation. Clinical history and physical examination may help differentiate these alternatives. In Hirschsprung's disease digital examination of the rectum usually reveals increased tone in an empty rectum, into which faeces has not been able to enter. Typically there will never have been any encoporesis, neither will the child have ever passed a normally formed stool. There is frequently a history of chronic ill health secondary to malnourishment [Schnaufer, 1976].

3.3 Occurrence and genetics

The largest study to date, of 1628 cases of Hirschsprung's disease [Ikeda and Goto, 1984] over the five year period 1978-1982 in Japan, shows an incidence of the disease of 1 in 4697 live births. The length of the aganglionic segment varied from very short [confined to the rectum distal to peritoneal reflex] to total intestinal aganglionosis affecting small bowel and colon. The proportion of patients affected by aganglionosis of varying lengths is shown [Fig. 3.1]. In approximately 80% of cases the aganglionosis region is confined to

Fig. 3.1 Adapted from Ikedo and Goto, 1984.

The number of patients from a total of 1562 affected by aganglionosis of varying lengths. The proportion of the total is expressed as a percentage.

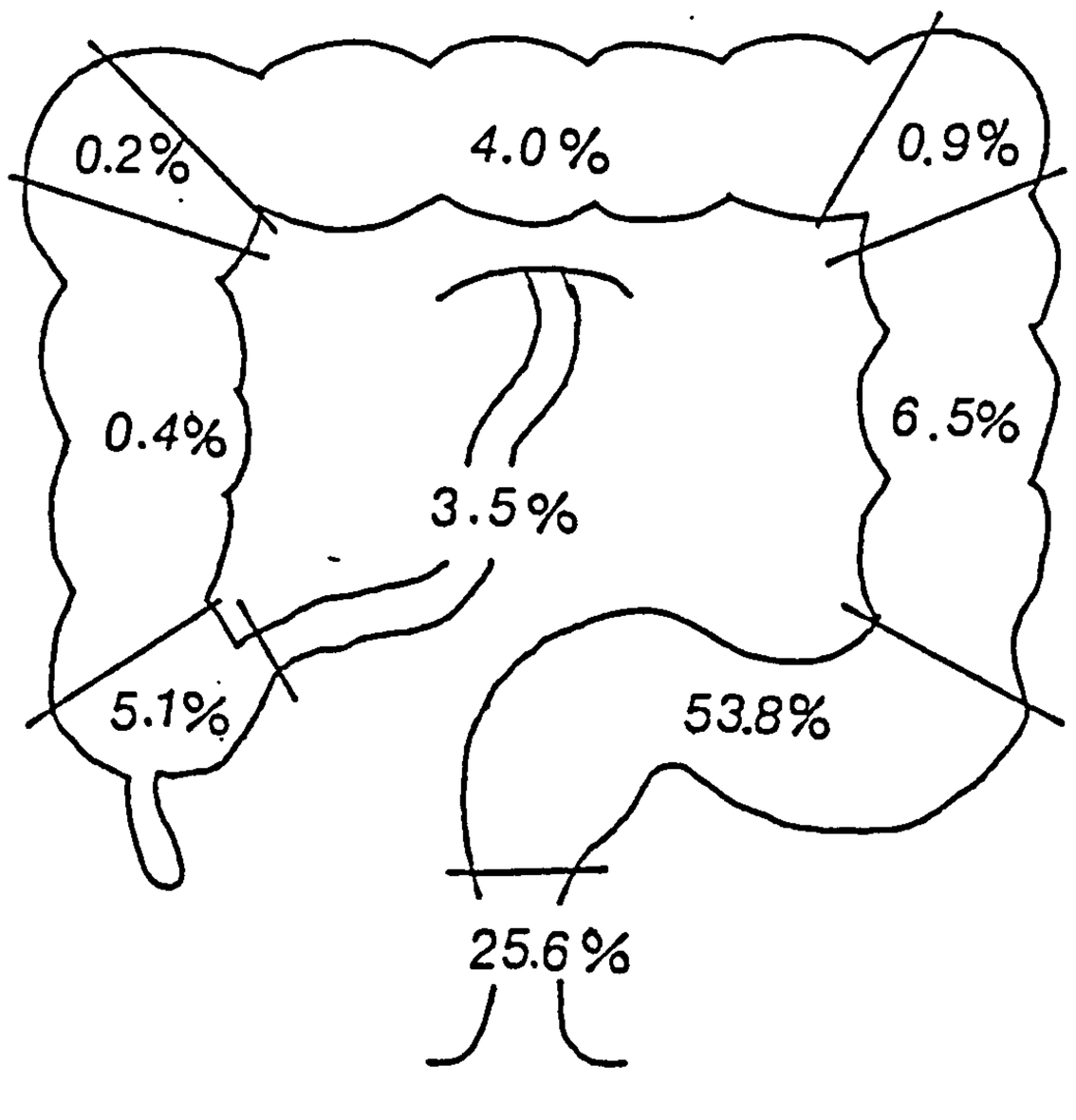


Fig. 3.1 The proportion of patients affected by aganglionosis of varying lengths

rectosigmoid. The length of the affected segment appeared to alter the male:femal ratio; 3.5:1 in lesions confined to the lower rectum falling to 0.8:1 in those cases which include small bowel and colon.

Familial involvement in this study was 3%, although this increased to 11.3% in cases of total colonic aganglionosis. Passarge, [1967] showed an overall risk for siblings of a male patients to be 2.6% but for siblings of a female patient this rose to 7.6%. There is no evidence of a simple Mendelian genetic inheritance [Passarge, 1967].

Ikeda and Goto, [1984] report that 14% of patients have some associated abnormality, the most common of these being Down's syndrome, occurring in 2.9% of patients, others include cardiac anomolies at 2.1% and inguinal hernia.

A previous large study [1196 patients] [Kleinhaus et al, 1979] performed in the USA showed results similar to the Japanese report. The only point of variance was the increased overall familial involvement, assessed at 7.0%. In long segment disease, where aganglionosis extended into the caecum, this increased to 21%.

A recent UK study of 103 documented cases gathered over a 15 year period in South West

Scotland, [Orr and Scobie, 1983] showed similar results to both of these larger studies. The overall incidence quoted was 1:4,500 live births with a male preponderance of 4:1. The overall familial involvement was 6%. The distribution of the extent of aganglionosis was also similar, 15% with ultrashort segment disease, 67% with short segment involvement and 18% with a long affected segment.

3.4 Aetiology

The cause of the aganglionosis in Hirschsprung's disease has not been unequivocally defined. The most widely accepted theory remains that of Okamoto and Ueda, [1967] who postulate a failure of the normal craniocaudal migration of ganglion cells during embrylogical development.

Okamoto demonstrated in normal human embryos that by five weeks vagal fibres have already reached the upper oesophagus, at six weeks neuroblasts can also be seen, by eight weeks all but half of the colon has neuroblasts present and by twelve weeks the entire myenteric plexus with intrinsic ganglia is present along the whole gut. The submucosal [Meissner's plexus] [Fig. 3.2] is formed by migration of neuroblasts across the circular muscle layer, again proceeding in a

Fig. 3.2 Adapted from Ham, 1974 - A transverse section of the normal gut wall. The major anatomical features are shown including the position of the submucosal [Meissner's and Henle's] and the intra-myenteric [Auerbach's] nerve plexuses.

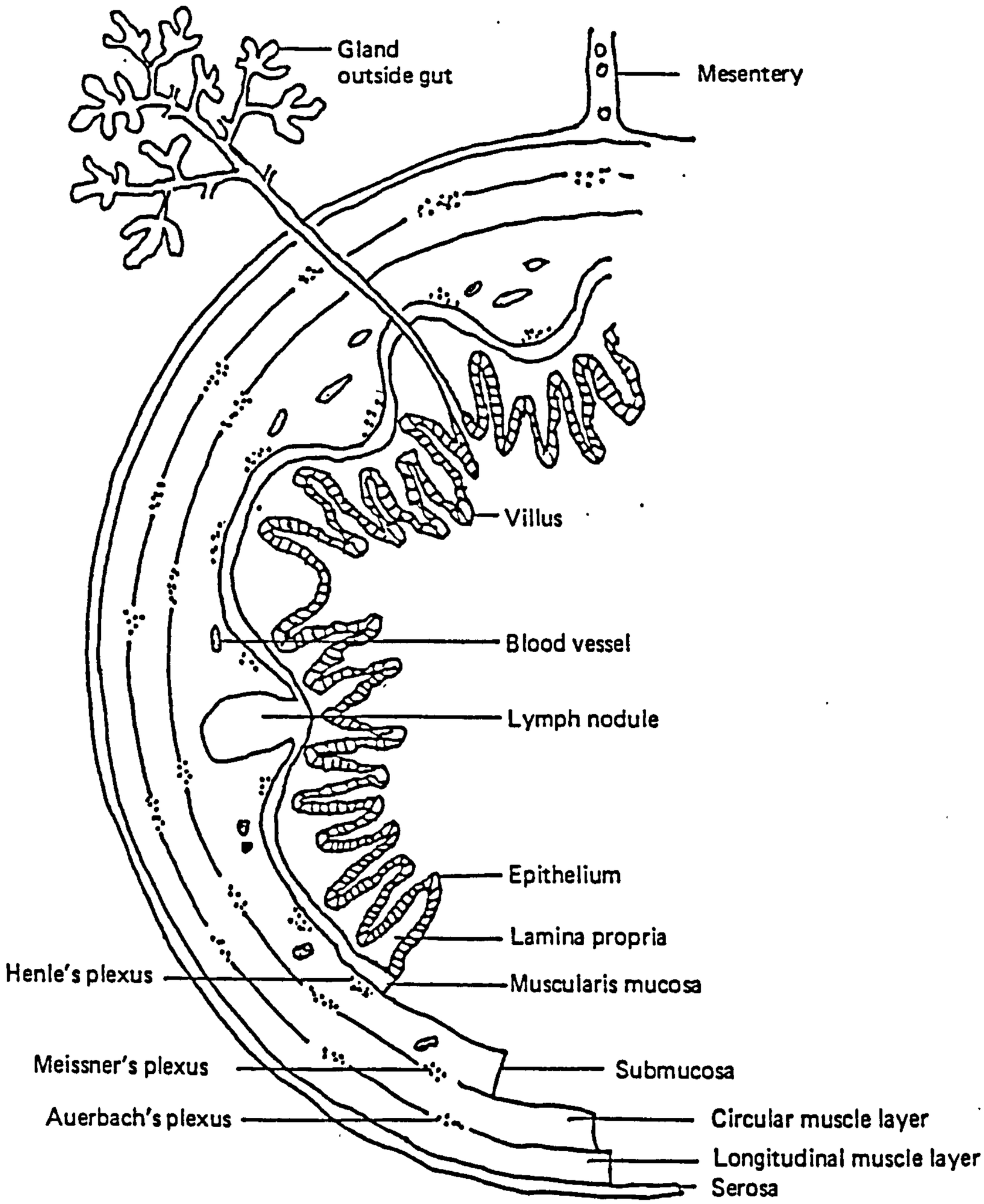


Fig. 3.2 A transverse section through normal gut wall showing nerve plexuses

craniocaudal progression. In Hirschsprung's disease, the earlier the termination of these migratory processes the longer the segment of gut which will finally remain aganglionic.

In addition to this single vagal hypothesis other theories include a dual vagal and pelvic origin for ganglia of the myenteric plexus. Certainly extrinsic cholinergic nerve fibres of sacral origin are demonstrable in the plexus, but it seems likely that the dual origin of ganglia may only apply in chickens, where the anatomy of the terminal digestive tract is significantly different [Okamoto et al, 1982].

Failure of craniocaudal migration of ganglia would exclude acquired aganglionosis as a possible cause of Hirschsprung's disease nor would it permit segmental aganglionosis [skip lesions]. While reliable reports of these phenomena are rare they do exist. Acquired aganglionosis, a condition where, ganglia are clearly visible in specimens taken at birth or shortly afterwards and yet cannot be identified in biopsies made later in life, has been described [Touloukian and Duncan, 1975; Towne et al, 1979; Dimler, 1981]. Similarly, reports of segmental aganglionosis keep occurring

in the literature; perhaps one of the most convincing was a single case where terminal ileum and ascending colon were aganglionic, transverse colon contained normal ganglia and descending colon and rectosigmoid was totally aganglionic [Martin et al, 1979].

In addition to a failure of craniocaudal migration other mechanisms of pathogenesis have been suggested for Hirschsprung's disease including ischaemia [Earlam, 1972]. This pathogenesis may underly the atypical pathologies such as acquired aganglionosis and skip lesions.

3.5 Pathophysiology

In the normal colon motility is regulated by the intrinsic automonic nervous system. This consists of submucosal [Meisner's and Henle's] and the myenteric [Auerbach's plexuses],[Fig. 3.2]. These have the appearance of a broad stranded network in polygonal array with ganglion cells at the junction of neural strands. The meshwork consists of Schwann cells supporting various nerve cell processes [Weinberg, 1975]. The neurones of the enteric plexuses are largely cholinergic, however sympathetic fibres ending in a basket work about the ganglia are also

present. In addition a third component has been described, these neurones which depend upon purines as their transmitter molecules are postulated to act directly upon bowel muscle in an inhibitory fashion [Burnstock, 1972].

Cholinergic nerve fibres in the normal plexus derive largely from vagal origin, however, extrinsic nerve bundles also enter through the longitudinal muscle coat. The extrinsic nerve trunks are surrounded by a perineurium and have a more compact appearance than the plexus itself; they immediately ramify and blend with the intrinsic plexus structure.

The way in which differing nerve types inter-relate and control muscle contraction is poorly understood. In general, cholinergic nerves are excitatory whereas adrenergic nerves are believed to have both excitatory and inhibitory characteristics. Purinergic nerves, as defined by Burnstock, [1972] are suggested to have a direct inhibitory effect [Fig. 3.3].

In Hirschsprung's disease ganglion cells are absent from all layers of bowel. The delicate neural matrix is replaced by hypertrophic nerve trunks which stain densely for cholinesterase

Fig. 3.3 Adapted from Weinberg, 1975 - A diagramatic representation of the innervation of normal and aganglionic bowel. Cholinergic, adrenergic and purinergic [as defined by Burnstock, 1972] nerves are illustrated as they are believed to act upon the smooth muscle cells of the gut wall.

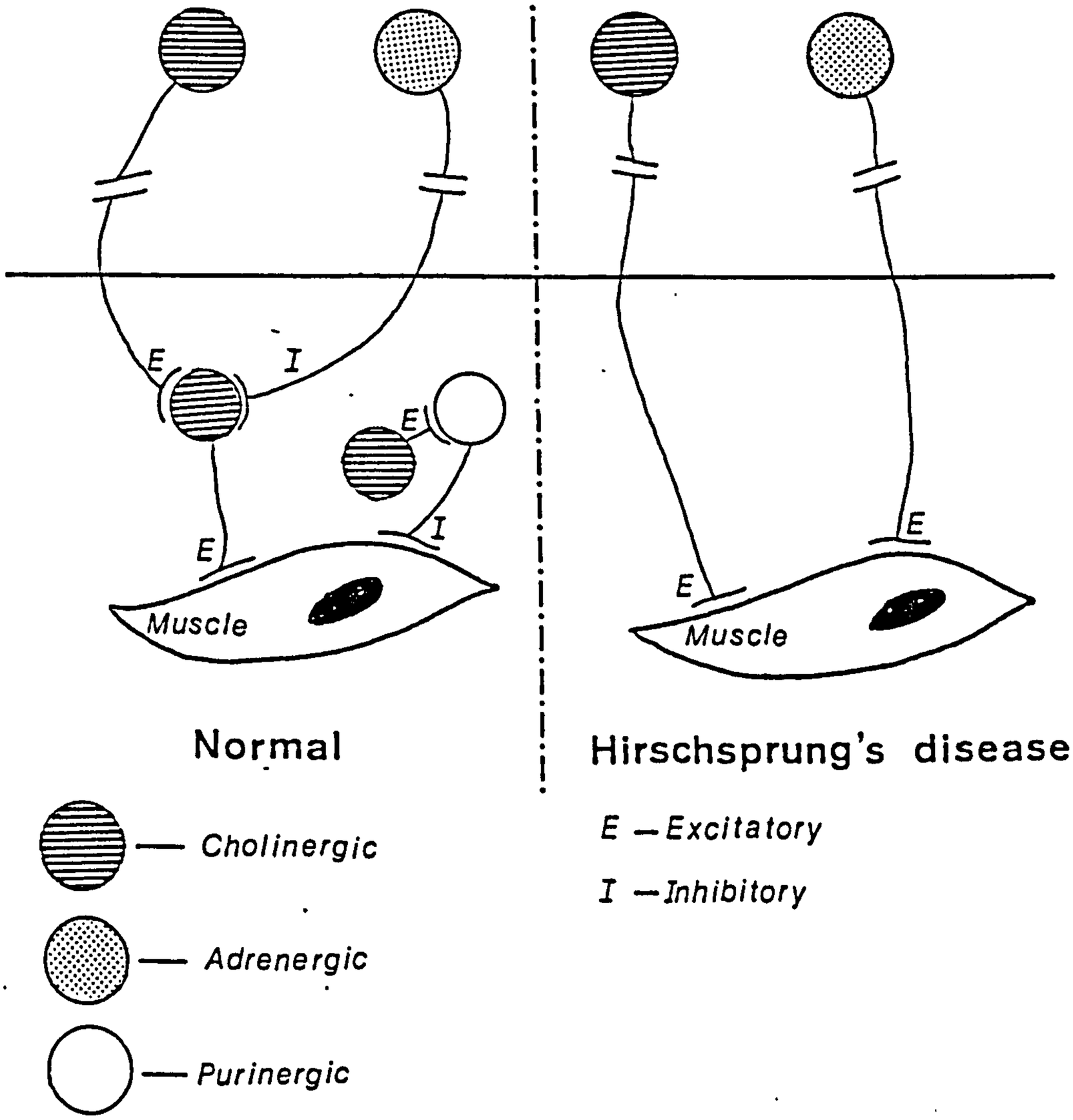


Fig. 3.3 A diagramatic representation of the innervation in normal and aganglionic bowel

activity and ramify throughout muscle and mucosal layers. These are of extrinsic origin [Okamoto et al, 1982] perhaps deriving from the sacral region of the spinal chord. There is also an increase in adrenergic innervation [Touloukian et al, 1973].

The aganglionic region in Hirschsprung's disease is in a constant state of contraction and several hypotheses have been devised to account for this. The most simple suggests that in accord with Cannons Law,"any denervated smooth muscle will remain in a constant state of contraction", certainly ACh-receptor remains intact in Hirschsprung's disease and increased sensitivity to acetylcholine may be partly responsible for the observed spasm [Ikawa et al, 1980]. Other workers have suggested that the lack of a non-adrenergic, possibly purinergic, inhibitory system is the major cause of the contraction [Richardson, 1975].

An additional factor relating to the altered innervation pattern in Hirschsprung's disease and contributing to the obstruction is the paradoxical distal contraction of the internal anal sphincter in response to luminal distension [Lawson and Nixon, 1967].

In the region adjacent to the anal sphincter in normal gut there is an absence of ganglia and some degree of nerve trunk hypertrophy. The resultant muscle response is believed to be partially responsible for the normal maintenance of sphincteric tone. Consequently, in Hirschsprung's disease we may be observing a normal physiological response in an abnormal situation.

In addition to the increased cholinesterase activity present in the aganglionic region secondary to the nerve trunk hypertrophy there are other disturbances of neural activity. Acetylcholine is increased almost three fold in the aganglionic region [Ikawa et al, 1980]. Similarly there is a two to three-fold increase in catecholamine concentration in the affected segment [Ikawa et al, 1980]. Conversely levels of vasoactive intestinal peptide [VIP] are reduced, VIP and substance P sensitive nerves are similarly depleted [Bishop et al, 1981].

The derangement includes not only abnormality of the normal direct smooth muscle innervation but also disturbances of normal control mechanisms and the overall pathophysiology of the disease is clearly complex.

3.6 Diagnosis of Hirschsprung's disease

The early diagnosis of Hirschsprung's disease is important both to improve the long term outlook for the affected child, by avoiding the malnourishment and chronic ill health with which the disease is associated [Section 3.2], and more acutely to avoid the life threatening development of enterocolitis. Delay in diagnosis can result in increased incidence of enterocolitis [Ikeda and Goto, 1984], this condition occurs preoperatively in 15-30% of children with Hirschsprung's disease [Ikeda and Goto, 1984; Kleinhaus et al, 1979] and carries a mortality rate of 30% [Kleinhaus et al, 1979]. While 83% of patients are diagnosed within the first year of life [Ikeda and Goto, 1984] a much smaller proportion are diagnosed within the first month - only 15% in the American Association of Pediatrics study [Kleinhaus et al, 1979]. Several approaches have been used to establish a diagnosis. When considering these it is important to remember the need for diagnostic accuracy early in life. Techniques used include: X-ray, barium enema, rectal manometry, histological demonstration of ganglia, histochemical demonstration of hypertrophic nerve trunks, measurement of AChE activity in rectal

tissue and measurement of AChE activity in both erythrocytes and serum. These methods are discussed in Sections 3.6[i]-[vi].

Several other disorders resemble Hirschsprung's disease and may prove misleading, these include neuronal colonic dysplasia [Puri et al, 1977] a specific deficiency of argyrophyll neurones [Tanner et al, 1976], chronic adynamic bowel [Kapila et al, 1975], hypoganglionosis [Weinberg, 1975] and Chagas disease [Singleton et al, 1977].

In addition Hirschsprung's disease may have atypical presentations; the long segment form is frequently missed [Careskey et al, 1982] and very short segment disease is sometimes mistakenly labelled as psychogenic constipation [Orr and Scobie, 1983].

Ideally, any satisfactory diagnostic technique should be able to differentiate similar conditions and identify unusual presentations of the disease.

3.6[i] Radiology

Radiology, including both plain abdominal X-ray and radiopaque enema, is probably the most commonly used investigative technique in Hirschsprung's disease, but alone it is rarely diagnostic [Lavery, 1983]. The aim is to demonstrate a clear funnel-shaped transitional zone between a dilated ganglionic and narrowed aganglionic bowel.

False positive [Lake et al, 1978; Kekomaki et al, 1979] and false negative [Lavery, 1983] results have both been described. In particular it is often difficult to differentiate low rectal aganglionosis from functional constipation; consequently very short segment disease may be missed [Weinberg, 1975]. A further limitation to the technique is the difficulty of interpretation early in life, particularly within the first month [Lavery, 1983]. Following colostomy the investigation is of little value as the typical distention of the ganglionic bowel is no longer present.

An advantage of the technique is the possible assessment of the length of the aganglionic segment.

3.6[ii] Rectal Manometry

Schuster first described an abnormal sphinteric response to rectal stimulation in Hirschsprung's disease in 1965 [Schuster et al, 1965]. In normal subjects, distension of the rectal ampulla by an inflated balloon produces reflex relaxation of the internal anal sphincter and contraction of the external sphincter. Similar rectal distension in patients with Hirschsprung's disease produces a reflex contraction of the internal sphincter with a normal response of the external sphincter.

This phenomenon has been applied diagnostically by inflating with air a series of balloons inserted into the anal canal [Fig.3.4] and monitoring the pressure response of internal and external sphincters. The false positive rate has been reported at 5-10% [Kekomaki et al, 1979; Schnaufer, 1976; Meunier et al, 1978] and the false negative rate at 1-2% in children over one month [Meunier et al, 1978; Schnaufer, 1976; Kekomaki et al, 1979]. Equivocal results are also common [Schnaufer, 1976].

Despite the appreciable error rate this technique has gained an undeserved widespread acceptance, perhaps because the test is usually performed by the clinician and results obtained immediately.

Major problems occur in the neonatal period, during which, in one study 26% were incorrect [Meunier et al, 1978]. In particular, false negative rates of up to 33% have been reported [Davies et al, 1981]. This may be due in part to the low sensitivity of the measuring devices and the relative shortness of the functional canals [Tamate et al, 1984]. Immaturity of the reflex has also been suggested to explain these discordant results in neonates [Meunier et al, 1978].

Total colonic aganglionosis also frequently

Fig. 3.4 Adapted from Weinberg, 1975 - A diagramatic representation of the rectal manometry procedure. Inflation of the rectal balloon induces reflex relaxation of the internal sphincter in the normal and reflex contraction of the sphincter in Hirschsprung's disease.

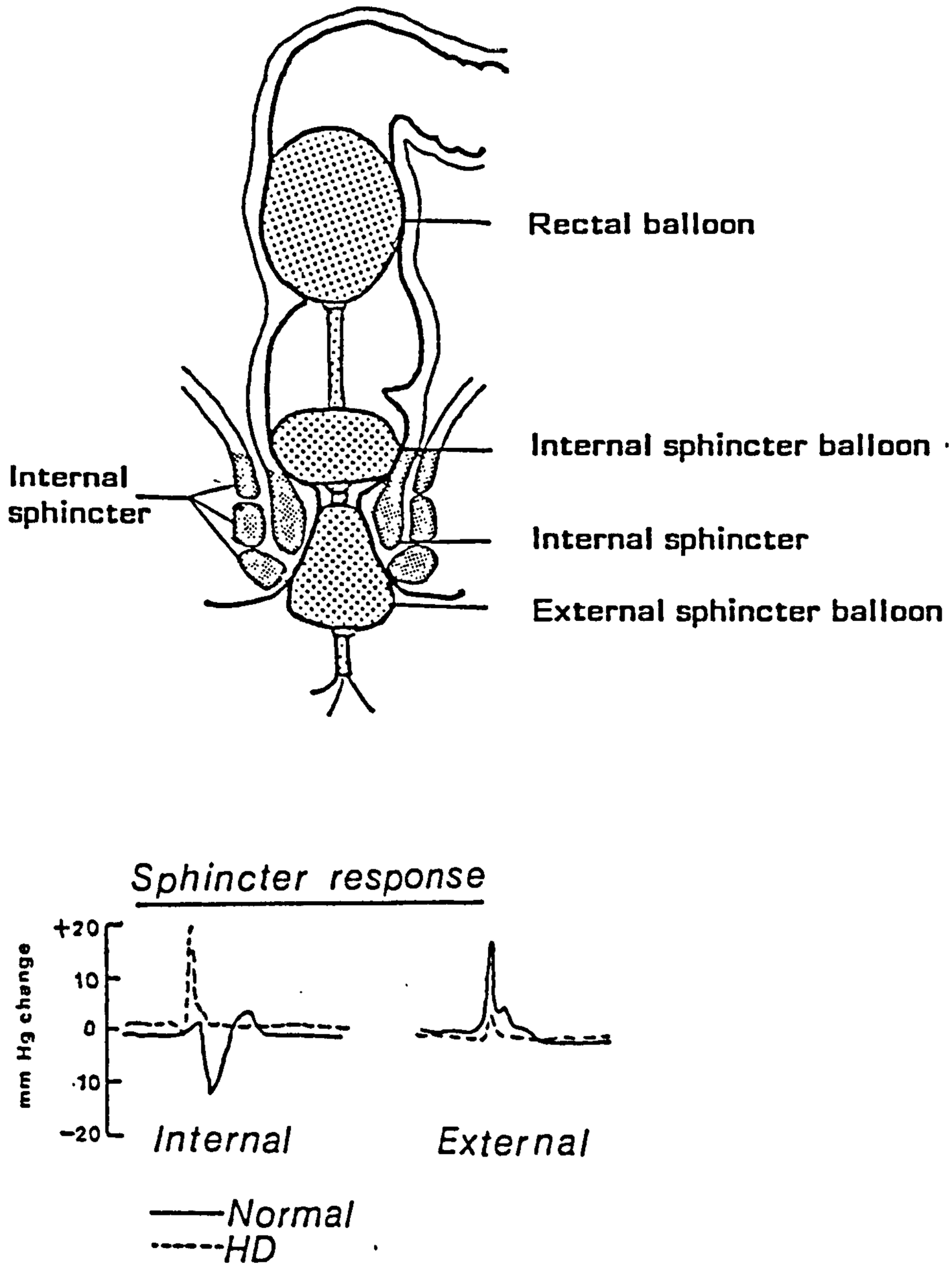


Fig. 3.4 A diagramatic representation of rectal manometry

produces false negative results, in one report this amounted to five of the six cases studied [Davies et al, 1981]. Rectal manometry is therefore far from reliable, particularly in the very important neonatal period, when ideally diagnosis should be established.

3.6[iii] Histology :

The histological demonstration of the absence of ganglion cells in a full thickness biopsy taken 2-3cm above the pectinate line [Fig.3.5] remains the definitive diagnostic procedure for Hirschsprung's disease.

Unfortunately there are several practical difficulties with this technique. Firstly, it is markedly invasive requiring a general anaesthetic. In addition, the development of scar tissue may prejudice subsequent surgery. It is therefore an unsuitable procedure for excluding Hirschsprung's disease in the otherwise normal, chronically constipated child. Secondly, the pathologist may have to examine up to 100 sections before there is a reasonable certainty that they are entirely absent.

Provided ganglia are not misidentified, false negatives are by definition impossible. Given adequate tissue false positive results are unlikely, however, equivocal results from poor specimens may sometimes be reported.

Fig. 3.5 Adapted from Weinberg, 1975 - A diagramatic representation of the rectal wall. The usual site of biopsy employed in the diagnosis of Hirschsprung's disease is shown in relation to the pectinate line.

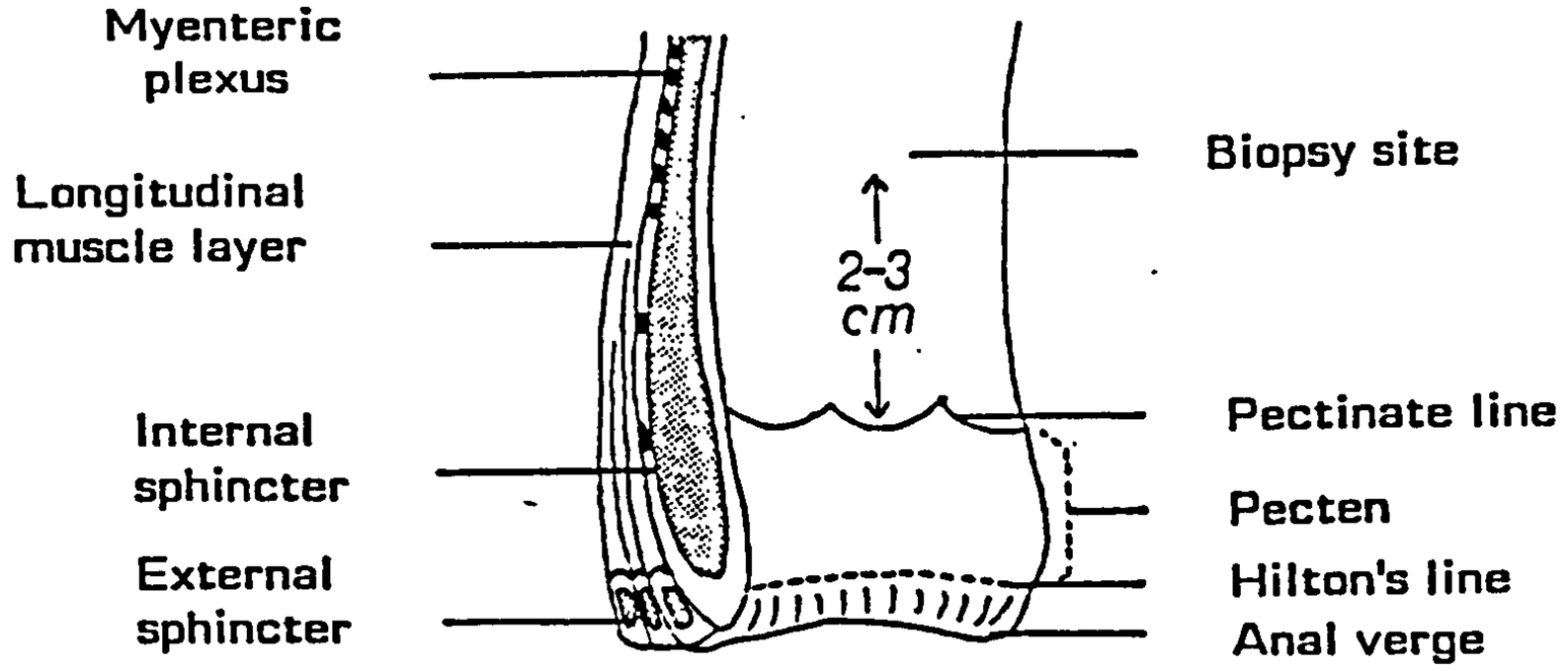


Fig. 3.5 A diagramatic representation of the rectal wall showing the usual site of biopsy

In order to make the investigation somewhat less invasive Bodian, [1960] advocated the use of mucosal biopsy for the diagnosis of Hirschsprung's disease. Dobbins and Bill, [1965] further reduced the amount of tissue required by using serial sections from suction biopsy of rectal mucosa. Provided submucosa was present it was usually possible to establish a diagnosis, but potential problems could arise in regions of hypoganglionosis.

In addition to the usual paraffin-embedded specimens, frozen sections have also been employed. These are not adequate for initial diagnosis but useful for defining the limit of aganglionosis once a diagnosis has been established [Ardrassy et al, 1981] especially peroperatively in order to determine the position of colostomy.

3.6[iv] Histochemistry

While the introduction of suction biopsy techniques made the procedure safer for the patient and simpler for the surgeon, the reduced size of the specimen presented the pathologist with considerable difficulties. In an attempt to overcome these, histochemical techniques depending upon the demonstration of hypertrophied, cholinesterase staining, nerve trunks were introduced [Meier-Ruge et al, 1972]. In addition to the use of this positive criterion of nerve trunk hypertrophy, ganglia if

present, are also easily recognizable in cholinesterase preperations [Lake et al, 1978]. The staining procedure normally used is that of Karnovsky and Roots, [1964] [Section 1.6[ii]]. The method is simply, reliable and takes under two hours to perform. It has been suggested that the biopsy specimen should contain at least some muscularis mucosae.

False negative and false positive results do occur and the combined mean error rate is 8.4% based on those studies shown in Table 3.1. The false negative rate is about five times the false positive rate and several explanations have been suggested to account for the former. These include, the biopsy being made proximal to very short segment disease and the biopsy being too superficial i.e. mucosae and lamina propria only. In some cases of Hirschsprung's disease nerve trunk hypertrophy is confined to the muscularis mucosae and immediately adjacent lamina propria [Chow et al, 1977]; in these cases superficial biopsy would give a false negative result. However, the more exacting the requirement for biopsy sampling, the greater the number of samples reported as equivocal due to poor biopsy. While many authors do not record the number of equivocal results it is appreciable, Table 3.1.

False positives with abnormal nerve trunks in the presence of ganglia, probably reflect some

Table 3.1 The summarised results of a number of surveys from 1972-1984 reporting the use of histochemistry in the diagnosis of Hirschsprung's disease. The false positive, false negative and equivocal result rates are shown, where reported, in each case.

Table 3.1 The performance of histochemically determined AChE in the detection of Hirschsprung's disease

Report	Number of patients	False positive rate [%]	False negative rate [%]	Equivocal sample rate [%]
Meier-Ruge et al, 1972	174	0	-	3.4
Elema et al, 1973	46	0	-	-
Chow et al, 1977	81	28	-	-
Almoyna et al, 1978	60	0	8.3	-
Lake et al, 1978	168	0	0	-
Morikawa et al, 1979	25	0	0	0
Kekomaki et al, 1979	60	2.0	0	-
Nezlof et al, 1981	123	2.1	7.6	11.0
Coker-Huntley et al, 1982	58	6.1	0	5.2
Hamoudi et al, 1982	[131]*	0	29.0	21.0
Plenat et al, 1982	115	8.2	17.4	6.0
Lake and Claireaux, 1983	800	-	0.4	-
Ziegler et al, 1984	524	-	0.5	1.5
	Mean	4.2	6.3	6.9
	Range	0-8.2	0-29.0	0-21.0

Combined average mean error rate = 8.6%

*Refers to number of specimens rather than number of patients

Based upon Coker-Huntley, [1982] with additions

alternative pathology, such as the neuronal colonic dysplasia described by Meier-Ruge, 1974. In this condition there are structural changes in the myenteric plexus with some increase in the number of AChE positive nerves.

Histochemistry then, represents a safe, relatively rapid and useful test for the diagnosis of Hirschsprung's disease. It can be applied more widely than the combination of full thickness biopsy and conventional histology but problems do arise due largely to sampling difficulties and consequently a proportion of Hirschsprung's disease cases are missed.

3.6[v]Quantitative measurement of tissue acetylcholinesterase

While histochemical assessment of nerve trunk hypertrophy is a useful aid in the diagnosis of Hirschsprung's disease, it is somewhat subjective requiring a high degree of interpretative skill.

In 1975, Boston et al reported quantitative measurement of AChE in homogenised suction biopsy specimens as a diagnostic test. This was later modified and improved by Dale et al, [1977] and gives a reliable, highly significant [$p<0.001$] separation between Hirschsprung's disease and other causes of chronic constipation. The method described by ourselves [Dale et al, 1977] has

gained widespread acceptance; the results obtained by various authors are shown in Table 3.2.

The measurement of percentage AChE [% AChE], that is the AChE activity expressed as a percentage of the total cholinesterase activity, is a useful additional parameter avoiding sources of error, such as measurement of the weight of small [<2.0mg] biopsy specimens and the effect of dehydration. Percentage AChE shows a surprising consistency between different reports - Table 3.2.

The method is safe, using a non-toxic inhibitor of BChE; reliable and rapid, taking approximately 30 minutes to perform. Table 3.2 shows that while results are encouraging the studies so far have been small, the largest only including 13 cases of Hirschsprung's disease [Yangihara et al, 1983]. Therefore, part of this thesis, before going on to explore the molecular forms of AChE involved in this increase, is devoted to a larger study comprising 50 cases of Hirschsprung's disease and a larger number of control samples.

It was reported by Bajger and Hak, [1979] that not only were tissue levels of AChE elevated in the disease but that also an electrophoretically distinct band of activity could be observed. When the research leading to this thesis was commenced the molecular

Table 3.2 Quantitative acetylcholinesterase measurement in rectal tissue biopsy specimens in the diagnosis of Hirschsprung's disease. The results obtained by several authors from 1975 to 1984 are shown.

Table 3.2 Quantitative assessment of AChE in the diacnosis of Hirschsprung's disease

Study	No. of patients	AChE $\mu U \times 10^{-1}/g$	p-value	Percentage AChE*	p-value
Boston et al, 1975[1]	12 [HD]	10.5 ± 6.9	<0.001	-	-
	7 [Cont]	34.3 ± 18.1			
Dale et al, 1977[2]	12 [HD]	6.3 ± 3.5	<0.001	50.8 ± 7.7	<0.001
	6 [Cont]	23.9 ± 5.3		78.5 ± 6.9	
Dale et al, 1979a[2]	56 [Cont]	5.0 ± 2.2	<0.001	54.0 ± 11.0	<0.001
	12 [HD]	30.5 ± 15.1		81.0 ± 7.0	
Bajgar & Hak, 1979[4]	6 [Cont]	10.96 -	-	-	-
	12 [HD]	25.8 -			
Patrick et al, 1980[2]	101 [Cont]	2.7 ± 1.4	-	54.6 ± 10.5	-
	9 [HD]	15.2 ± 9.0		78.9 ± 6.8	
Yangihara et al, 1983[2]	7 [Cont]	3.0 ± 1.7	<0.001	40.7 ± 13.2	<0.001
	13 [HD]	7.1 ± 3.9		65.9 ± 15.8	
Rakonczay & Németh, 1984[3]	7 [Cont]	3.9 ± 0.5	<0.001	45.1	-
	4 [HD]	25.3 ± 5.7		87.8	
Goto et al, 1980[2]	50 Anterior wall [aganglionic]	92.4 ± 54.6	<0.001	64.7 ± 12.8	<0.001
	50 Posterior wall [ganglionic]	28.3 ± 14.0		28.5 ± 10.2	

1 - Assayed without a specific BChE inhibitor

2 - The method of Dale et al, 1977

3 - As the method of Dale et al, 1977 using iso-CMPA in place of lysivane and measuring at 37°C

4 - The method of Ellman et al, 1961

[Cont] - Control sample, no evidence of Hirschsprung's disease

[HD] - Confirmed Hirschsprung's disease

- not stated

* - percentage AChE = [AChE/AChE + BChE] x 100

forms of AChE present in rectal tissue had not yet been identified, but during the course of the study a report was published indicating that the major species in aganglionic biopsy tissue was the 10S, tetrameric form of the enzyme [Rackonczay and Németh, 1984]. This substantiated the findings of the present study. The work described in this thesis characterises more fully the 10S form and how its levels together with those of other AChE molecular forms, change in the transition from ganglionic to aganglionic bowel.

Quantitative measurement of AChE is liable to the same sampling errors as those described for the histochemical technique. It has been suggested [Lake et al, 1979] that problems with the quantitative assay may be encountered because the material in which the enzyme is being measured is not microscopically assessed and that certain specimens may contain lymphoid follicles, etc. The most powerful counter argument is that in over 250 samples so far reported [Table 3.2],this criticism has not been found to be a practical difficulty.

3.6[vi] Erythrocyte and serum acetylcholinesterase

The increase in rectal tissue AChE in Hirschsprung's disease is well attested [Sections 3.6[iv], 3.6[v]]. In order to determine whether or not this is reflected in an increased serum level of AChE,

perhaps due to tissue release and whether the disease has any effect upon erythrocyte AChE these activities were measured by Boston et al, 1978.

The methods used were adequate for erythrocyte AChE, but entirely inappropriate for measurement of serum AChE, in fact in the title to the paper the authors refer to serum AChE, whereas in the results they refer to serum pseudocholinesterase, apparently without differentiation. The assay method includes no selective inhibitor of serum BChE and therefore more properly reflects BChE than AChE. They report a mean increase in erythrocyte AChE and serum BChE, Table 3.3, with p-values of $p<0.02$ and $p<0.05$ respectively. There was considerable overlap between groups and the method therefore has little practical diagnostic value.

This work remained unsubstantiated until 1983, when conflicting reports began to emerge. Okasara et al, [1983], using the original assays of Boston et al, [1978] demonstrated a significant increase in serum AChE, $p<0.05$ in Hirschsprung's disease but failed to find any increase in erythrocyte AChE. Ya-xiong et al, [1984] on the other hand, found an increase in erythrocyte AChE, $p<0.001$, but no difference in serum AChE.

Yanagihara et al, [1983] again using the original assay methods of Boston et al, [1978] found a

Table 3.3 Erythrocyte AChE and serum cholinesterase measured in an attempt to investigate their application in diagnosis of Hirschsprung's disease. Significant differences between groups, where they occur are reported by their p-value.

Table 3.3 Erythrocyte AChE and serum cholinesterase in patients with Hirschsprung's disease

Study	Group	Number in group	Erythrocyte AChE	p-value	Serum Cholinesterase	p value
Boston et al, 1978	Control	10	0.73 U^1_1 -	<0.02	5.0 U^1_1 -	<0.05
	HD	12	1.39 U^1_1 -		11.7 U^1_1 -	
Okasora et al, 1983	Control	16	2.79 ± 0.34 U^1_1	n.s.	17.25 ± 0.61 U^1_1	<0.01
	HD	9	3.09 ± 0.12 U^1_1		22.6 ± 0.96 U^1_1	
Yanagihara et al, 1983	Normal	30	1.12 ± 0.54 U^1_1		8.6 +± 2.2 U^1_1	
	IC	7	1.19 ± 0.59 U^1_1		12.2 ± 3.7	
	HD	13	1.86 ± 0.39 U^1_1	n.s.	14.8 ± 5.8	n.s.
Ya-xiong et al, 1984	Control	127	73.51 ± 9.36 U^2_2	<0.001	16.89 ± 8.86 U^2_2	n.s.
	HD	31	91.24 ± 10.24 U^2_2		14.49 ± 4.04 U^2_2	
Bamforth et al, 1985	Normal	53	7,150 ± 4,400 U^3_3	n.s.	- -	-
	HD	8	9,850 ± 3,310 U^3_3			

HD - Hirschsprung's Disease

IC - Idiopathic constipation

n.s. - no significant difference

[1] Units as defined by Boston et al, 1978

[2] Units as defined by Ya-xiong et al, 1984

[3] Units as defined by Bamforth et al, 1985

significant increase in both erythrocyte AChE p<0.01 and serum cholinesterase p<0.01, when Hirschsprung's disease patients were compared with normal patients but no increase in either when they were compared with a control group of patients with idiopathic constipation. The most recent report, Bamforth et al, [1985] in seven children with, and 53 without the disease failed to find any significant increase in erythrocyte AChE.

The increases in erythrocyte AChE if valid are difficult to rationalise while measurement of serum AChE requires better methodology than has so far been applied in order to confirm or deny any increase.

In all of these studies there was considerable overlap between groups and therefore it is doubtful whether the assays in their present form have any diagnostic usefulness in Hirschsprung's disease.

3.7 Treatment

In many cases Hirschsprung's disease is diagnosed within the first few months of life. The aim of treatment is to relieve the obstruction and in particular to avoid the risk of enterocolitis.
A defunctioning temporary colostomy is usually performed proximal to the aganglionic segment. The child is then usually allowed to grow unitl a weight of 10-15 kg is achieved and normal health is restored.

In older children a colostomy is not always necessary prior to definitive treatment but is believed by some to reduce the subsequent risk of infection at operation.

The treatment of choice is almost invariably surgical and three basic operative procedures or variations of these predominate [Fig.3.6]:-

[i] Rectosigmoidectomy with end to end anastomosis, Swenson and Bill, [1948]

[ii] Reterorectal transanal pull through with side to side anastomosis, Duhamel, [1956]

[iii] Endorectal pull through, Soave, [1964]

Individual procedures are favoured by different surgeons for their avoidance of post operative complications and the maintenance of adequate anal sphincter function.

In some cases of very short segment disease sphincterotomy may be corrective, without the need for a pull through procedure [Lavery, 1983].

Total intestinal aganglionosis from duodenum to rectum, presents a particularly difficult problem. When sufficient length of gut is resected to ensure motility, adequate absorption may be impossible. In a recent paper [Potts et al, 1983] referring to eleven previously reported cases, all of whom subsequently died, a medical approach is suggested involving the use of caerulein. This drug is known to produce

Fig. 3.6 Adapted from Schnaufer, 1976 - A diagramatic representation of the major operative procedures currently used in the correction of Hirschsprung's disease.

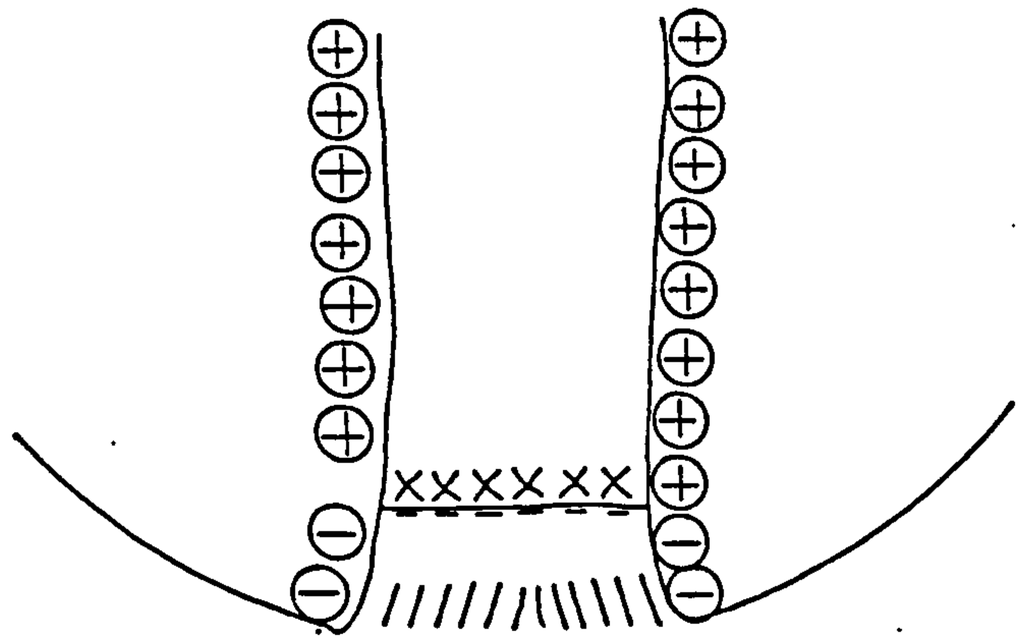
Swenson - rectosigmoidectomy with end to end anastomosis [Swenson and Bill, 1948].

Duhamel - reterorectal transanal pull through with side to side anastomosis [Duhamel, 1956].

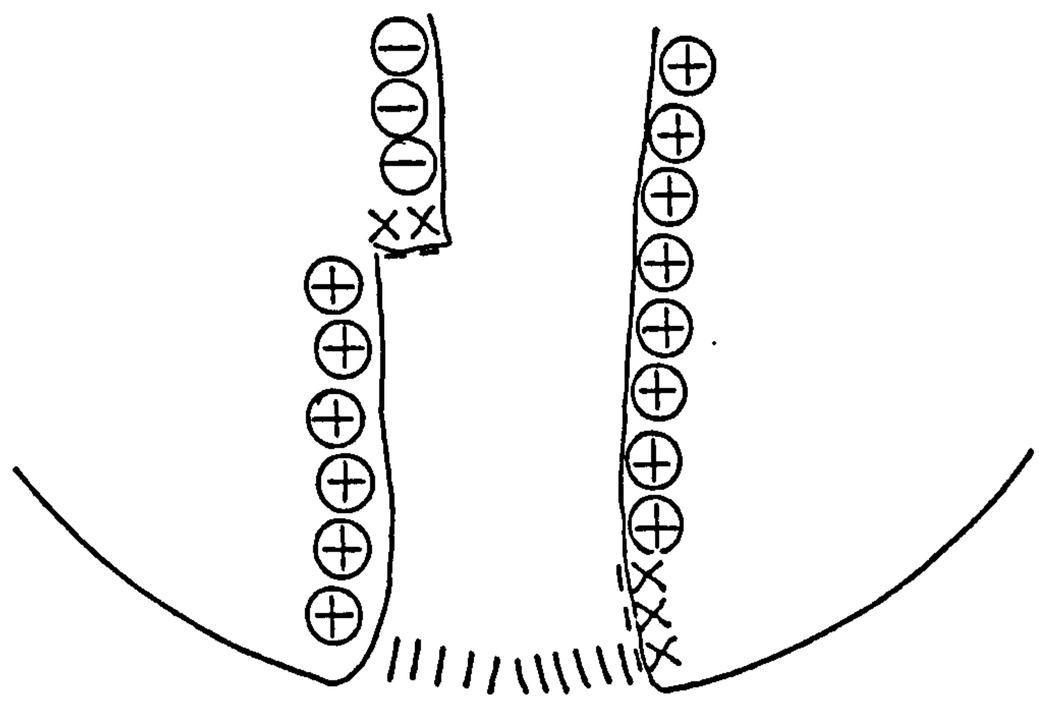
Soave - Endorectal pull through [Soave, 1963].

Regions of aganglionosis are shown as ⊖ and regions where ganglia are present as ⊕ .

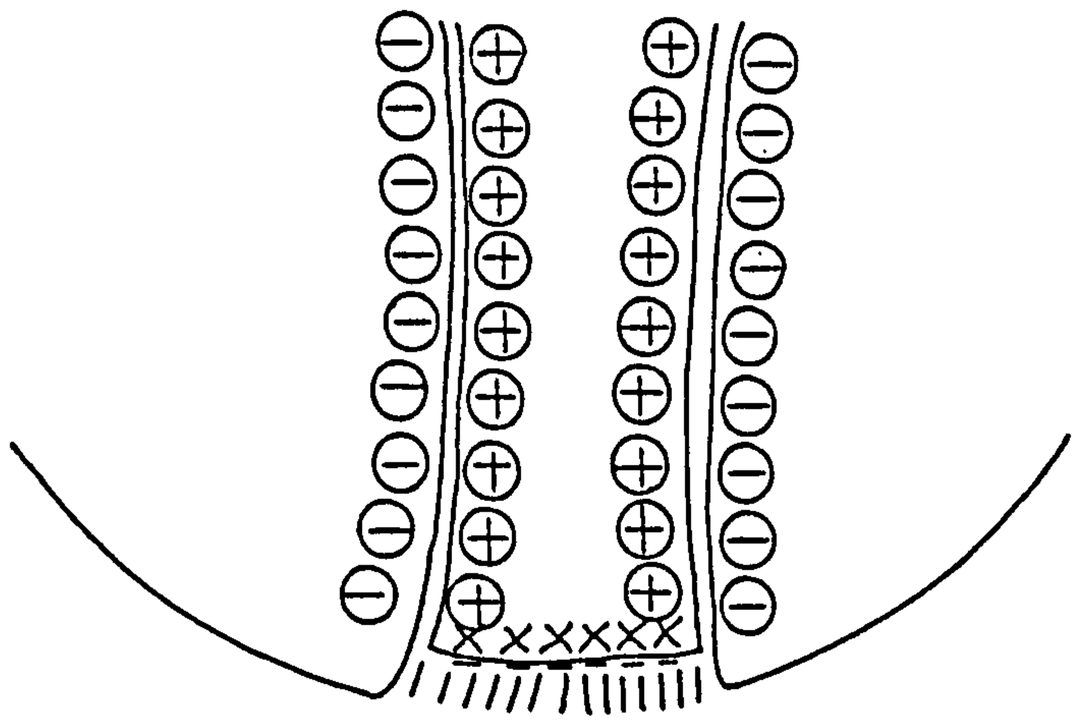
The position of suture lines in each case are represented by xxxx.



Swenson



Duhamel



Soave

⊖ — Aganglionic

⊕ — Ganglia present

XXXXX — Suture line

||||||||||| — Anal verge

Fig. 3.6 Operative procedures used in the correction of Hirschsprung's disease.

smooth muscle contraction in denervated muscle. In a single case where this type of treatment has been attempted the child succumbed due to respiratory aspiration caused by vomiting, the authors suggest however, that this may represent an alternative in an otherwise fatal condition.

In general, the surgical treatment of Hirschsprung's disease is effective and provides a relatively trouble free solution to a serious and often fatal condition. Early unequivocal diagnosis is therefore of the utmost importance.

Section 4 : Neural Tube Defects

4.1 History and anatomy

Neural tube defects [NTD] have long been known to mankind and the pre-historic art of many cultures depict forms suggestive of these disorders. Certainly, they were recognized by Aristotle and Plato [Shurtleff and Lamers, 1978] who recommended infanticide in such cases. The first recorded attempt at corrective surgery is attributed to Forestus, who in 1610, operated by ligating the cystic sac present in untreated spina bifida. Thus began the long and difficult period of medical intervention in the management of these harrowing and often controversial developmental abnormalities.

The diversity of disorders which can be grouped under the general heading of NTD range from simple meningocele without neurological diorder [10% of spina bifida cases [Nixon, 1978] to anencephaly with absence of much of the forebrain and calvarium.

Recognizable differentiation of the nervous system begins in utero prior to the thirtieth day of gestation. The primordial neural plate forms a mature brain and spinal chord. It is currently believed that incomplete tubular formation in the developing head results in anencephaly, whereas

a similar anomaly occuring in the spinal chord produces spina bifida [Macri et al, 1981].

At birth the plaque which covers the region of exposed spinal chord in spina bifida is translucent and lies flat with the surrounding skin. However, without closure this plaque becomes keratinised and swells becoming filled with CSF, this gives rise to the descriptive classification, "spina bifida cystica".

4.2 Aetiology

The exact underlying cause of NTD remains unclear [Macri et al, 1981]. Its occurrence appears to contain both genetic and environmental elements. Mendelian genetics cannot be demonstrated, however, there is a strong familial tendency and the risk of an individual with an NTD having offspring affected by the disease is around 5% [Siggers, 1978]

An environmental element, perhaps related to dietary intake can also be demonstrated, the recurrence rate of NTD in women who have already conceived an affected child, approximately 5% in the British population [Siggers, 1978], can be reduced to less than 1% when a multivitamin supplement containing folate, ascorbic acid and riboflavin is administered immediately prior to conception and continued throughout pregnancy [Smithells, 1982].

It has been suggested by Seller, [1983] that the disorder may be attributable to a deficiency in DNA synthesis resulting in an overall decreased production of nerve cells in the developing embryo. The author further suggests that this deficient DNA synthesis is caused by an inadequate supply of co-factors such as folate, zinc, and vitamin B_{12}. Much of the evidence for this hypothesis is based upon the observation that inhibitors of DNA synthesis given to a strain of NTD producing mice at the correct gestational age alter development in such a way that the lesion no longer occurs. The argument follows that DNA synthesis being halted in the developing fetus allows the build up of deficient co-factors so that when synthesis recommences fetal growth is synchronised and the specific neural lesion does not occur. Clearly, the experiments are far from conclusive proof of such a theory but nevertheless they do point toward some metabolically based abnormality which it may be possible to correct.

The adequate development of a neural tube requires more than the growth of neural tissue and it has been suggested that the primary failure may be mesodermal rather than neurological [Nixon, 1978].

The exciting possibility remains that if some co-factor or other metabolic deficiency is the primary causative factor, then this disorder may be avoidable by suitable replacement therapy.

4.3 Occurrence and genetics

Neural tube defects appear to be inherited in a non-Mendelian multifactorial way. Consequently the recurrence risk is linked to the incidence in the population. In the USA, where the disease is seen in 2 per 1000 live births, the recurrence risk is 2%, whereas in Glasgow, where the incidence in the whole population is 5 per 1000 the frequency with which it recurs is 5% [Haddow and Miller, 1982].

The overall frequency is known to vary widely between countries, from 9.7 per 1000 in Dublin [Coffey and Jessop, 1957] to 0.9 per 1000 in Japan [Neel, 1958]. The occurrence also varies between ethnic groups within the same country. It is substantially less in the negro [Windham and Edmonds, 1982] population in the USA than in the while population. In England and Wales there is a distinct regional variation in frequency, Fig. 4.1, with the highest rates found in Wales.

Fig. 4.1 Adapted from Alberman, 1978. The geographical variation in England and Wales of the rates of occurrence of NTD. These are expressed per thousand live births.

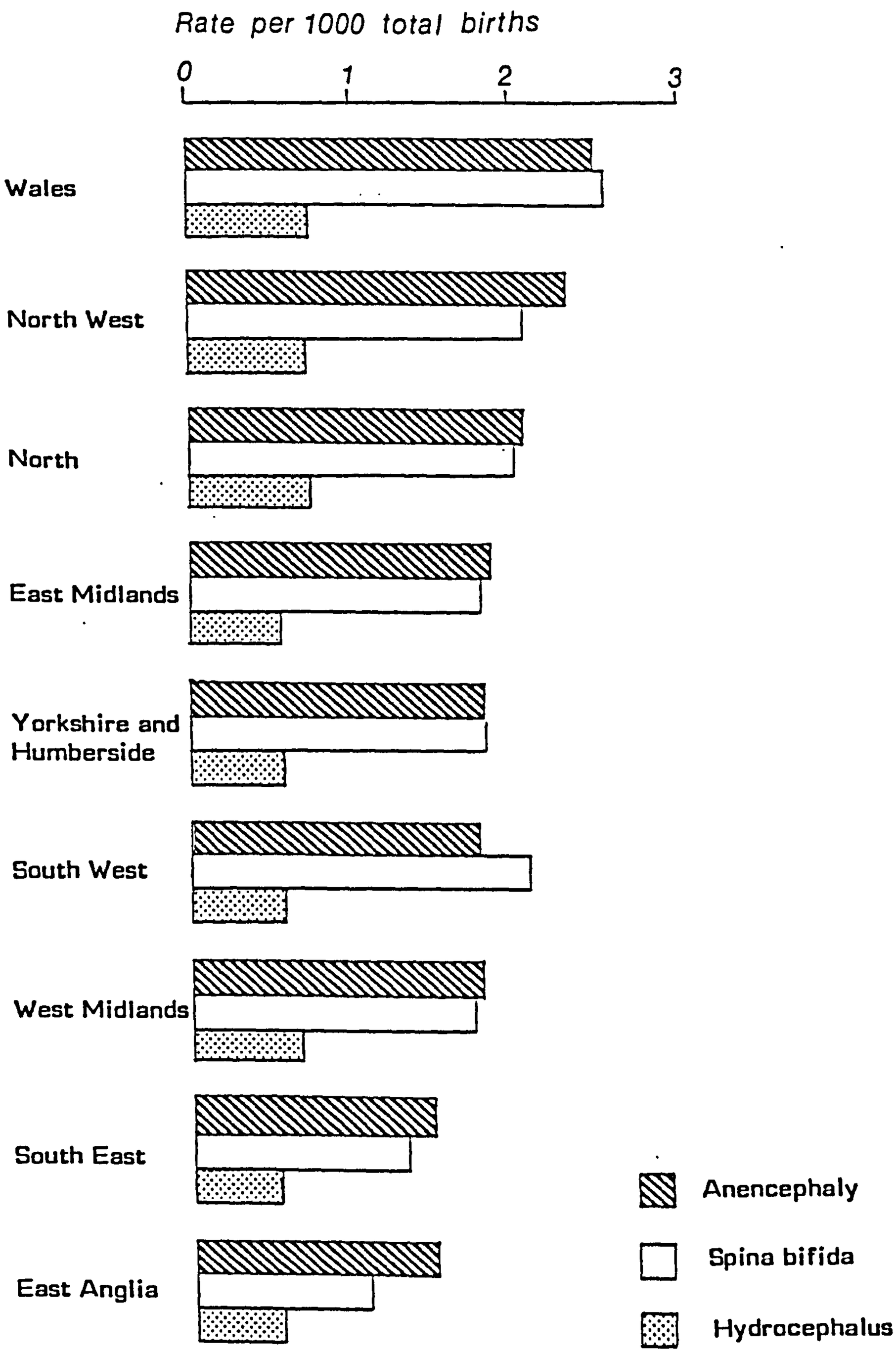


Fig. 4.1 The rate of occurrence of NTD.

It has been suggested that environmental factors may play a part in this distribution and in the higher incidence observed in social groups III, IV and V [Smithells, 1982].

The rate of anencephaly to spina bifida varies but in most studies is approximately 1:1. Twenty per cent of cases of spina bifida have a closed lesion and are undetectable by most antenatal screening programmes [Smith, 1982]. Other fetal abnormalities are frequently mistaken for NTD in detection programmes and chief among these are the abdominal wall defects such as exompahlos. The frequency of this disorder has been reported as 0.3-0.4:1000 [Wald et al, 1980], but individual studies have claimed a higher incidence [Read et al, 1982].

In the UK the recurrence rate for a woman with one previous NTD child is approximately 5%; if there have been two affected children this is increased to 10%. If the parent themselves have NTD the risk is 5% or 1% if the familial involvement is limited to a second degree relative [Siggers, 1978].

Several recent reports have shown an overall decline in the incidence of NTD in the UK and USA [Windham and Edmonds, 1982; Ferguson-Smith,

1983]. In the UK this might be partially accounted for by the antenatal detection and termination of affected pregnancies. However, swings in the past have been noted; in Dublin in 1900 the rate was 1:1000 and this increased to 9:1000 by 1960 [Windham and Edmonds, 1982]. Prenatal diagnosis is doubtless a partial explanation for the recent decline in incidence in the UK, but in the period 1975 to 1980 there has been a 59% drop and antenatal diagnosis is not sufficient to account for all of this [Ferguson-Smith, 1983].

4.4 Prenatal detection of Neural Tube Defects

Detection of NTD in utero has largely depended upon the identification of substances in the amniotic fluid whose concentration is increased in the presence of the lesion. The difficulties and risks associated with amniocentesis restrict its use to selected populations at increased risk.

The actual danger from amniocentesis is hard to define because of the problem in obtaining a suitably matched reference group. However, the best studies available suggest a 1 to 1.5% risk of fetal mortality and some increased risk of fetal morbidity including skeletal deformity

[Turnbull, 1978]. The risk to the mother is slight although maternal death attributable to amniocentesis has been described [Turnbull and MacKenzie, 1983].

Identification of groups with increased risk of NTD allows amniocentesis to be more effectively applied. Two such groups commonly offered prenatal diagnosis in the U K are:

a] Women with a previous NTD affected child.

b] Women with a raised serum alphafetoprotein [MSAFP] level.

Once these groups have been successfully identified the most appropriate amniotic fluid marker for NTD must be decided upon. Several problems must be born in mind when choosing such a suitable marker:

a] The level of many components of amniotic fluid change throughout pregnancy, marked change with gestational age can make interpretation difficult where dates are not accurately established. Ideally the marker concentration would be idependent of gestational age.

b] Blood staining of amniotic fluid is common. 10% of all amniotic fluids [Smith, 1982] and, most importantly, 40% of those associated with anencephaly, and 20% of those with open spina bifida are blood stained [Haddow and

Miller, 1982]. The NTD marker used should be insensitive to moderate amounts of blood from either fetal or maternal origin.

c] Other fetal malformations are sometimes encountered and some of these, such as exomphalos, are surgically correctable. Ideally, the concentration of the substance measured should not be affected by the presence of non-NTD lesions.

d] Practical considerations dictate that adequate methods be available for the substance to be measured. Frequently large numbers of samples will need to be processed particularly if assessment is linked to a whole population screening programme, such as maternal serum AFP. The method must ideally be cheap, reproducible, rapid, suitable for automation, use a small amount of amniotic fluid and the substance must be stable for a reasonable time span to allow measurement and repeat as necessary.

e] In an ideal situation all false positive results are unacceptable, in practise the false positive rate must be very low compared with the true positive rate in the population under study. Where amniotic fluids taken for

chromosomal studies are assessed the false positive rate must be very much less than 0.5%, the incidence of NTD in this population.

f] False negative results are arguably less of a problem than false positives, however, if the original requirement to detect NTD is accepted then a significant false negative rate would severely handicap the usefulness of the test.

It is unlikely that any single marker would perfectly fulfil all the above requirements. It may be necessary to use two assays in conjunction. For instance, a test with a low false negative rate followed by one with a low false positive rate to be performed on all initially positive results.

The increasing resolution available with ultrasonography may make it possible in the future to depart from indirect methods of measuring a substance in the amniotic fluid. Recent results assessing the usefulness of ultrasound in diagnosis of anencephaly and spina bifida without the use of amniotic fluid measurement are encouraging [Campbell and Pearce, 1983]. At present, however, the technique is more useful as an adjunct than in the definitive detection of NTD.

In the following pages individual tests used in the detection of NTD are discussed in greater

detail and a suggested scheme of how they could be successfully used in conjunction is outlined.

4.4[i] Ultrasonography

In the 1970's static scanning with its greater resolving power was exclusively used for detection of fetal abnormalities. However, more recently the resolution of real time scanning has improved to match static scanning and has several advantages [Campbell and Pearce, 1983]:-

a] Avoidance of movement artefacts.

b] The study of organs and limbs during movement.

c] Three-dimensional conceptual images can be built up.

At present ultrasound has two major applications in detection of NTD. Firstly, measurement of bi-parietal diameter in accurately defining gestational age. This can be performed in order to help the interpretation of maternal serum AFP [MSAFP] data, for which gestational age makes a significant difference. Secondly, subsequent to a high MSAFP or in cases where there has been a previous NTD high resolution ultrasound can be used to detect any fetal abnormality and to help in performance of amniocentesis.

The evidence is convincing that ultrasound localisation of fetus and placenta is necessary

for safe amniocentesis and reduces the number of blood stained samples obtained [Kerenyi and Walker, 1977].

The direct use of ultrasound in detecting fetal abnormality is valuable; in a recent study [Campbell and Pearce, 1983] 41 cases of anencephaly were detected without any error and 90 cases of spina bifida, of which five were closed lesions, produced only six false negative and one false positive result.

The future of ultrasound looks promising, at present its use in differentiating NTD lesions from other fetal malformations, in accurate dating of pregnancy and in guiding amniocentesis makes carefully performed ultrasonography a valuable contributor to any pre-natal detection programme for NTD.

4.4[ii] Alphafetoprotein measurement

Alphafetoprotein [AFP], an oncofetal protein with a molecular mass of approximately 70 kDa was first described in the serum of human fetuses in 1956 [Bergstrand and Czar, 1956]. In the first trimester it is largely produced by the yolk sac and after this by the fetal liver. Concentration in the fetal serum peaks at the end of the first trimester, at 3000 mg/l and declines thereafter

until birth when levels are in the range 10-50 mg/l [Brock, 1983].

In the normal fetus, AFP enters amniotic fluid by fetal micturation [Weiss et al, 1976], however, in the NTD-affected fetus a more direct communication may be available and consequently amniotic fluid AFP levels are elevated. The use of amniotic fluid AFP in prenatal detection of NTD was first reported by Brock and Sutcliffe in 1972. It soon became apparent that in the normal fetus change in the concentration of AFP in amniotic fluid reflected that of the fetal serum concentration, albeit at a much reduced level [Brock, 1974].

Later it was recognised that transfer across amniotic membranes occurred and this allowed the measurement of AFP in maternal serum [MSAFP]. Paradoxically levels of MSAFP rose during the second trimester while those in amniotic fluid fell [Fig.4.2]. It has been suggested that this is due to the rapidly expanding surface area of the amniotic membranes permitting more AFP to diffuse across [Haddow and Miller, 1982].

Maternal serum AFP is most commonly measured by radioimmunoassay and can be used in whole population screening to identify patients with a

Fig. 4.2 The variation in AFP levels in amniotic fluid and maternal serum with gestational age is shown based on data from Laing, 1985 and Timothy, 1985 (Personal communications).

Overlap is shown between unaffected, open spina bifida and anencephalic pregnancies both for maternal serum AFP and amniotic fluid AFP results. The choice of intervention point between two and three multiples of the median [MoMs] determines the proportion of false-positive and false-negative results. Adapted from Ferguson-Smith, 1983.

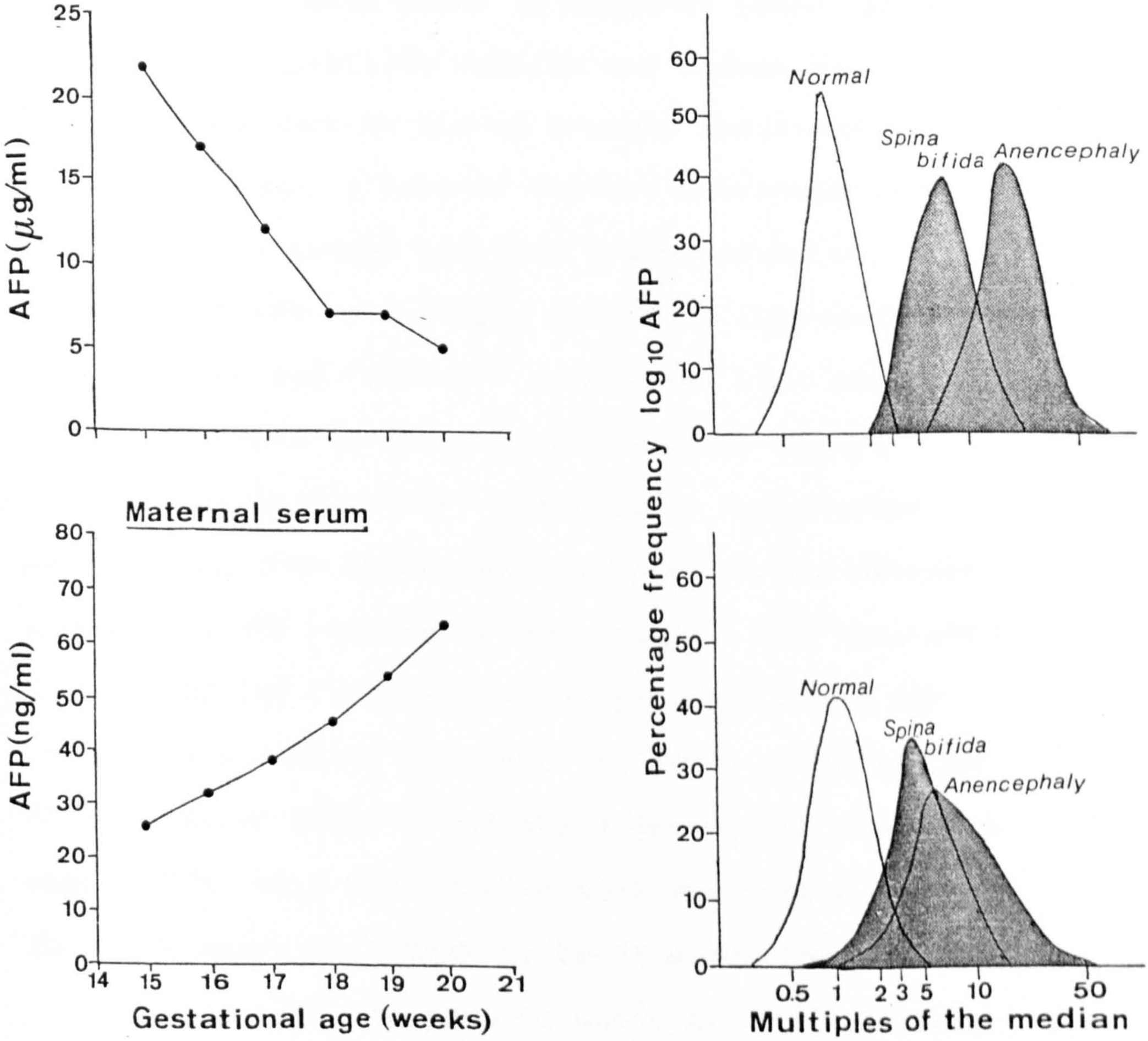


Fig. 4.2 Variation in the level AFP in amniotic fluid and maternal serum.

sufficiently high risk NTD to justify subsequent amniocentesis. This is usually in the order of 5% of patients in whom MSAFP is measured [Macri et al, 1981]. False positive results are common and in 20% of cases this is due to wrongly estimated gestational age, a further 25% are attributable to threatened or missed abortion [Ferguson-Smith, 1983] and 5-15% to multiple pregnancy [Leonard, 1981]. Depending on the "cut-off" used MSAFP will detect 70-80% of NTD's in the population under study.

Amniotic fluid AFP measurement has greater specificity than MSAFP despite some overlap between normal and NTD results [Brock, 1983]. The "cut-off" limits applied in interpreting amniotic fluid AFP results are usually described in terms of multiples of the median [MOM] or standard deviations above the mean. They vary with gestational age from 2.5MOM at 13-15 weeks to 4.0MOM at 22-24 weeks [Brock, 1983].

False positive results occur at approximately 0.5% and false negatives at 2% [Collaborative AFP Study, 1979]. If these false positive and negative rates are considered to be acceptable three major problems remain in using amniotic fluid AFP in diagnoses of NTD:-

a] Uncertin gestational age creates a fundamental difficulty for interpretation.

b] Fetal blood contamination invalidates results.

c] Other fetal malformations are found to be associated with elevated AFP levels [Table 4.1].

To lessen the effects of these interfering factors, ultrasound dating of pregnancy has been used to improve the precision of the estimation of gestational age. The Kleihauer test to identify fetal blood is performed on blood stained samples and ultrasound may be used to identify other fetal abnormalities.

With all these precautions the resultant false positive rate of 0.5% is still high, particularly if AFP is measured in amniotic fluids from pregnancies screened for chromosomal abnormalities arising due to advanced maternal age. In this population the risk of NTD is approximately 0.5%, equivalent to the false positive rate of the test and therefore the number of normal fetuses terminated is the same as those with the defect.

AFP estimation has made a very significant contribution to the identification of fetal abnormality in utero. Its measurement in maternal serum makes voluntary whole population screening feasible, indeed it is practised in many regions throughout the UK.

The major problems which remain are the high false positive rate, the difficulty in interpretation of blood contaminated samples and the uncertainty

Table 4.1 Fetal conditions which may give rise to an increased amniotic fluid AFP level. Exomphalos and congenital nephrosis in particular can be confused with NTD in antenatal detection. Adapted from Brock, 1978.

Table 4.1 Fetal conditions in which elevations of amniotic fluid AFP may be seen early in pregnancy

Probable
Anencephaly
Spina bifida (open)
Intrauterine Death
Congenital Nephrosis
Exompahlos
Fetal Teratoma

Possible
Turner's Syndrome
Duodenal Atresia
Oesophageal Atresia
Polycystic Kidneys
Fallot's Tetralogy
Annular Pancreas
Congenital skin defects
Epidermolysis Bullosa
Hydrocephalus

of determining gestational age - all of which can lead to the abortion of normal fetuses. Recent developments have centred upon the analysis of other markers in amniotic fluid either as an adjunct to AFP measurement or as a more reliable alternative.

4.4[iii] Cholinesterase measurement

AChE is secreted by nerve trunks and found in soluble forms in the CSF [Section 2.3] and therefore it might be expected that in NTD, where the lesion exposes nervous tissue and CSF to direct contact with amniotic fluid, AChE levels would be significantly increased in the presence of the abnormality.

Shortly before our initial report of the preliminary work [Dale et al, 1979b], other workers [Smith A.D. et al, 1979; Chubb et al, 1979] independently reported similar findings. These three reports showed that both AChE and BChE were increased in amniotic fluid of NTD affected pregnancies; in our own study [Dale et al, 1979b] this was without overlap in non-bloodstained fluids.

In addition to this quantitative increase, gel electrophoresis revealed a distinct band of enzyme activity in the amniotic fluid of NTD affected pregnancies [Smith, 1982]. This had similar characteristics of mobility and inhibitor sensitivity

to AChE present in CSF and released by neurones in culture.

Further work with chick embryos with NTD suggested that AChE might enter the amniotic fluid by direct secretion from nerve axons or by CSF leakage [Pilowsky et al, 1982]. The source of AChE activity in normal amniotic fluid is unclear but a placental origin has been suggested.

The application of cholinesterase measurement in amniotic fluid has followed two distinct routes:

a] Quantitative measurement either of total cholinesterase activity [AChE + BChE] or of AChE alone.

b] Qualitative identification of an abnormal band of AChE activity on gel electrophoresis.

Both approaches offer advantages and suffer from drawbacks. The application of quantitative and qualitative techniques may ultimately prove distinct and complimentary. The methods are discussed below:-

a] <u>The quantitative approach</u>

The measurement of total cholinesterase activity can be dismissed early in the discussion, its main proponent has been Milunsky [Milunsky et al, 1979; Milunsky, 1980], although one other group has subsequently reported its use [Simpson, 1983]. The inherent simplicity and sensitivity of this method are greatly outweighed by an inability to distinguish

non-NTD lesions and by inordinate sensitivity to blood staining of either fetal or maternal origin.

Quantitative measurement of AChE directly either by selective inhibition of BChE, or indirectly by inhibition of AChE has been reported by many groups, with similar results [Table 4.2]. When compared with AFP the advantages of the method are numerous. It is very reproducible, simple, rapid [taking less than 30 minutes], easily automated [Moreau et al, 1982] and cheap [less than 1p per test]. It is relatively unaffected by gestational age and consequently one reference range can be used [Hodgson et al, 1981]. There are conflicting reports of false positive and false negative rates - ranging from zero for both [Dale et al, 1981] to 8% and 32% respectively [Voigtlander et al, 1981]. Similar controversy exists over the affect of blood staining [Chubb and Pilowsky, 1979; Voigtlander, 1981] although there is general agreement that it is superior to AFP in this respect.

Several reports suggest that some AFP positive non-NTD lesions, such as exomphalos, can be identified by high BChE/AChE ratios [Dale et al, 1981; Bonham et al, 1981], with AChE levels either marginally increased or showing no increase at all [Guibaud et al, 1982].

Table 4.2 Quantitative measurement of AChE activity in amniotic fluid in the detection of neural tube defects. The mean activity standard deviation and range is shown when reported.

Table 4.2 Quantitative AChE messurement in amniotic fluid

Reference	n	Mean ±SD	Range	Gest. Age	Outcome	Method
		U/l	U/l	Weeks		
Chubb et al, 1979a	77	2.8 -	0.2 - 8.9	14-23	Normal	Smith A.D., et al, 1979
	1	4.5 -	-	21	SB	
	1	23.2 -	-	20	An	
Dale et al, 1979 b	150	3.7 ± 1.3	1.4 - 7.8	12-25	Normal	Dale et al, 1979b
	7	- -	8.9 - 15.2	-	NTD	
Smith A.D., et al, 1979	56	2.6 ± 1.3	0.9 - 8.6	14-23	Normal	Smith A.D., et al, 1979
	8	9.9 -	6.2 - 17.0	-	An	
	8	7.2 -	4.8 - 11.7	-	SB	
Seller et al, 1980	71	2.1 ± 1.1	0.5 - 4.8	15-20	Normal	Smith A.D., et al, 1979
	16	14.9 ±10.0	5.9 - 41.3	-	An	
	9	8.0 ± 4.6	4.0 - 15.8	16-19	SB	
Dale et al, 1981	101	2.6 ± 1.1	0.4 - 5.8	14-24	Normal	Dale et al, 1979b
	10	15.0 ± 3.3	10.2 - 19.5	-	An	
	26	10.3 ± 3.5	5.5 - 20.4	-	SB	
	5	9.4 ± 4.8	2.7 - 15.6	-	Ex	
Bonham et al, 1981	50	2.0 ± 0.9	0.6 - 4.2	15-25	Normal	Modified Dale et al, 1979b
	5	8.0 -	5.1 - 8.6	"	An	
	9	6.3 -	4.8 - 9.7	"	SB	
	2	3.9 -	2.6 - 5.1	"	Ex	
Webb et al, 1981	68	3.4 ± 1.6	0 - 8.8	14-27	Normal	Dale et al, 1979b but lower centrifugation speed
	4	13.2 -	3.3 - 21.0	"	An	
	4	7.7 -	6.2 - 9.7	"	SB	
	1	3.6 -	-	"	Ex	
Hodgson et al, 1981	204	3.0 ± 1.6	-	14-27	Normal	Smith A.D., et al, 1979
	7	16.9 -	8.4 - 30.0	-	An	
	3	38.2 -	4.6 -102.0	-	SB	
Voigtlander et al, 1981	70	2.9 -	0.9 - 9.0	14-27	Normal	Smith A.D., et al, 1979
	10	8.1 -	8.2 - 19.3	16-24	An	
	3	4.9 -	2.8 - 7.1	18-20	SB	
	1	6.1 -	-	17	Ex	
Coombes et al, 1982	49	3.9 ± 1.2	-	16-23	Normal	Dale et al, 1979b
	46	15.9 -	6.6 -109.0	16-22	NTD	
	6	15.3 -	2.9 - 48.3	18-22	Ex	
Rorive et al, 1982	14	1.4 ±	0.5 - 3.2	14-24	Normal	Smith A.D., et al, 1979
	6	6.0 -	3.2 - 8.7	-	An	
	4	3.9 -	2.7 - 6.2	-	SB	
Guibaud et al, 1982	127	2.7 ± 1.5	0.0 - 7.1	16-24	Normal	Dale et al, 1979b
	17	8.0 ± 6.7	1.0 - 28.7	-	NTD	
Legge & Potter, 1983	146	1.5 ± 0.7	-	13-21	Normal	Dale et al, 1979b
	8	9.3 ± 3.6	-	-	An	
		5.1 ± 0.4	-	-	SB	
Lamedica et al, 1983	412	4.1 ± 1.3	-	15-28	Normal	Dale et al on CFA 1979b
	4	11.6 -	10.2 - 13.2	-	An	
Moreau et al, 1983	92	3.4 ± 1.3	1.4 - 7.5	17-20	Normal	Dale et al on CFA 1979b
Aitken et al, 1984	210	2.06± 1.06	0.1 - 7.3	14-26	Normal	Smith A.D. et al, 1979
	159	7.68± 5.24	0.5 - 31.7	-	An	
	96	5.60± 2.73	0.14- 14.7	-	SB	
	14	1.74± 2.6	0 - 10.7	-	Ex	
Wyvill et al, 1984	1410	5.6 -	-	14-22	Normal	Hullin et al, 1981
	32	18.2 -	-		An	
	28	11.9 -	-		SB	

An - Anencephaly SB - Spina Bifida Ex - Exomphalos CFA - Centrifugal fast analyser

NTD - Neural tube defect

The chief attraction of the quantitative method include its objectivity and the speed and simplicity of the assay.

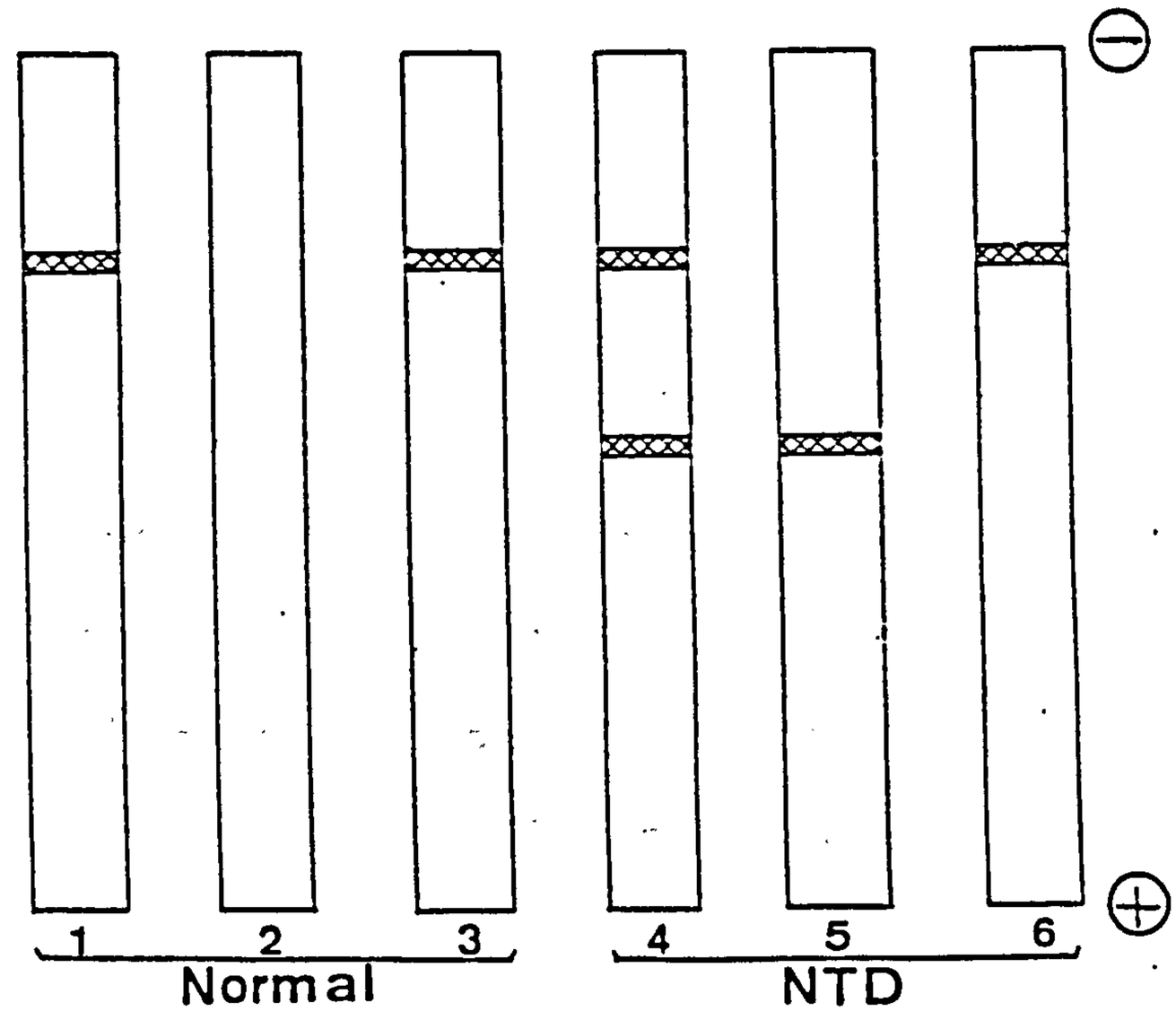
b) Qualitative measurement of AChE

The original paper reporting a quantitative change in AChE activity in amniotic fluid [Smith,A.D. et al, 1979] also reported an abnormal band in gel electrophoresis when the amniotic fluid of NTD pregnancies was examined. This faster migrating form, [Fig.4.3] was sensitive to AChE inhibitors such as BW 284c51 [Section 1.8].

This technique has been thoroughly explored in detection of NTD and is specific in its prediction of the disease with low false positive and negative rates even in selected cases with anomalous AFP results [Table4.3]. It is relatively insensitive to blood staining with some reports suggesting no effect [Buamah et al, 1980], later studies however, confirm that heavy blood staining does produce false positive results and that fetal plasma was responsible for this interference [Barlow et al, 1982b].

The technique has disadvantages when compared with the quantitative method. It is time consuming and not amenable to automation. The identification of a fast-moving band is somewhat subjective. Cross contamination between gels is possible [Smith, 1982].

Fig. 4.3 Adapted from Smith, 1982 - Polyacrylamide gel electrophoresis of amniotic fluid from normal and NTD affected pregnancies - stained for cholinesterase activity. The effects of selective inhibition of AChE [due to BW284c51] and BChE [due to ethopropazine] are also illustrated.



Normal
- 1 — Without inhibitors
- 2 — With ethopropazine
- 3 — With BW284c51

NTD
- 4 — Without inhibitors
- 5 — With ethopropazine
- 6 — With BW284c51

Fig. 4.3 Polyacrylamide gel electrophoresis of amniotic fluid from normal and NTD affected pregnancy

Table 4.3 A summarised assessment of the results of the AChE gel test (polyacrylamide gel electrophoresis of amniotic fluid) in the detection of NTD from 1979 to 1984. Total numbers of false positive and false negative results are shown.

Table 4.3 False positive and negative results in the AChE gel test used in detection of NTD

Study	True Negative	False Positive	True Positive	False Negative	Comments
Smith A.D., et al, 1979	17	0	20	0	
Brock & Hayward, 1980	169	0	30	0	
Buamah et al, 1980	140	0	61	0	
Seller & Cole, 1980	130	7	35	0	*Some blood-stained samples
Collaborative AChE Study, 1981	125	8	813	4	*7 blood-stained samples
Haddow et al, 1981	153	1	51	1	*1 blood-stained sample
Macri et al, 1981	115	0	39	0	
Seller & Barry, 1981	1000	1	-	-	
Voigtlander et al, 1981	71	0	19	0	
Webb et al, 1981	68	0	7	0	
Barlow et al, 1982a	60	0	17	0	
Coombes et al, 1982	49	0	46	0	
Davidson et al, 1982	40	0	18	2	Both false neg. results third trimester
Guibaud et al, 1982	191	0	20	0	
Haddow & Miller, 1982	153	1	50	1	*Fetal blood-staining in 1 FP
Milunsky & Sapirstein, 1982	106	9	66	0	*6 blood-stained samples
Norgaard-Pederson et al, 1982	170	0	24	0	
Read et al, 1982	853	2	47	0	
Goldfine et al, 1983b	128	0	-	-	
Crandall et al, 1983	333	3	47	0	*2 blood-stained samples
Simpson, 1983	-	2	37	0	
Van Regemorter et al, 1983	33	0	10	0	
Aitken et al, 1984	3054	33	170	0	
Wyvill et al, 1984	1408	8	60	0	
	8566	75	1687	8	

The test gives false positive results with non-NTD lesions [Table 4.4; in particular, in 75% of cases of the surgically correctable condition exomphalos an AChE band indicative of NTD is visible. This is less of a problem in the quantitative assay. Attempts have been made to improve the technique by densitometric scanning of the gels. This improves the objectivity of the test and preliminary results are encouraging [Goldfine et al, 1983a; Goldfine et al, 1983b]. The interpretation of other "extra bands", so far ignored, may be of value in identifying non-NTD lesions such as exomphalos [Jones and Evans, 1983].

The major contribution of the AChE gel test lies in reducing the false positives generated by AFP measurement [Haddow et al, 1981; Milunsky and Sapirstein, 1982; Collaborative AChE Study, 1981]. It is estimated that these may be reduced by 90% without causing a significant number of true positives to be missed [Collaborative AChE Study, 1981].

It is not yet clear whether AChE might eventually replace AFP as the primary diagnostic test [Brock, 1983]. If this were to be the case, then the quantitative method would be the method of choice [perhaps used in combination with a more specific test for secreted AChE activity such as the gel test to check positive results].

Table 4.4 Fetal abnormalities which give rise to false positive AChE gel test results.

Table 4.4 False positive cel test results arisinc due to non-NTD malfcrmations .

Study	Abdominal Wall Defect	Intrauterine Death	Turner's Syndrome	Macerated Fetus	Congenital Nephrosis	Fetus papyraceus	Exstrophy of Cloaca	Teratoma	Trisomy E,18,21	Potter's Syndrome	Congenital Heart Defect	Prune Belly Syndrome	Cystic Hygrome	Meckel's Syndrome	Upper Gastro-intestinal atres
Brock & Hayward, 1980	4/4	4/4	1/1	-	0/6	1/1	2/2	-	0/3	-	-	-	-	-	-
Bummah et al, 1980	4/4	3/3	-	-	-	-	-	-	-	-	-	-	-	-	-
Seller & Cole, 1980	-	4/4	-	-	-	-	-	-	-	-	-	-	-	-	-
Collaborative AChE Study, 1981	47/63	-	4/4	-	0/11	-	-	1/3	1/3	0/1	1/1	-	-	-	-
Haddow et al, 1981	5/5	-	1/1	1/1	-	-	-	-	-	-	-	-	-	-	-
Macri et al, 1981	3/4	-	-	-	0/2	-	-	-	-	-	-	-	-	-	-
Voigtlander et al, 1981	2/2	2/2	-	-	0/1	-	-	-	-	0/1	-	-	-	-	-
Barlow et al, 1982a	4/6	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Coombes et al, 1982	6/6	-	-	-	0/2	-	-	-	0/1	-	-	-	-	-	-
Crandall et al, 1982	5/11	5/10	-	-	0/1	-	-	-	-	-	-	-	3/8	-	-
Davidson et al, 1982	-	-	-	1/1	-	-	-	-	-	-	-	-	-	-	-
Haddow & Miller, 1982	5/5	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Milunsky & Sapirstein, 1982	-	-	-	-	0/1	-	-	-	0/2	-	-	-	-	1/2	-
Norgaard Pederson et al, 1982	4/6	2/2	-	-	-	-	-	-	-	-	-	-	-	-	-
Read et al, 1982	1/8	-	-	-	-	0/1	-	-	-	-	-	1/1	-	-	-
Brock et al, 1983	-	-	-	-	-	-	-	0/1	-	-	-	-	-	-	-
Goldfine et al, 1983a	-	-	-	-	-	-	-	1/1	-	-	-	-	-	-	-
Goldfine et al, 1983b	10/10	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Holgreve & Golbus, 1983	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2/2
Simpson, 1983	-	-	-	-	-	-	-	-	0/2	-	1/1	-	-	-	-
van Regemorter et al, 1983	0/3	1/3	-	-	-	-	-	-	-	-	-	-	-	-	0/4
Aitken et al, 1984	6/12	5/13	-	-	0/2	-	-	0/1	-	-	-	-	-	-	-
Wyvill et al, 1984	6/7	3/4	-	-	-	-	-	-	-	-	-	-	-	-	-
TOTAL	112/156	29/45	6/6	2/2	0/24	1/2	2/2	2/5	1/11	0/2	2/2	1/1	3/8	1/2	2/6

False positive results are displayed as fraction of the total number of cases e.g. 1/8, 1 positive result from 8 such cases

The advantages of AChE over AFP are outlined in Table 4.5, however at present its widespread use is largely confined to samples with elevated AFP or where there is known blood staining or uncertainty about dates. AFP measurement has been in use for some time and AChE is now accepted as an important adjunct able to reduce the false positive rate. Indeed, in the Lancet editorial on AChE [Editorial, Lancet 1980] the Editor quotes "The ability of AChE to distinguish normal pregnancies from those with fetal NTD has been impressive. The physiological basis of the new test is also convincing .... sensitivity for anencephaly and open spina bifida was least as good as and probably better than amniotic fluid AFP measurement .... AChE appeared to cope particularly well with fetal blood, one of the major problems in the use of AFP measurement. .... AChE is the most effective complimentary test to AFP in the early diagnosis of NTD. It may eventually supplant AFP as the primary biochemical test on all second trimester amniotic fluids".

The molecular forms of AChE present in amniotic fluid have not been satisfactorily identified in accordance with the accepted structural classification outlined in Section 2.2. The present work sets out to identify and quantitate these. If an NTD specific

Table 4.5 AChE and AFP measurement in amniotic fluid in the detection of NTD compared on the basis of several criteria important in the routine use of each assay.

Table 4.5 Relative merits of amniotic fluid AChE and AFP measurement in detection of NTD

Possible source of difficulty		AFP	AChE
Change with gestational age	:	Marked	Slight if at all
Effect of fetal bloodstaining	:	Marked	Little effect unless marked bloodstaining
Discrimination of non-NTD lesions e.g. exomphalos, congenital nephrosis	:	Does not discriminate	Discriminates exomphalos and congenital nephrosis
Simplicity, speed and cheapness of assay	:	Takes 24 hrs complicated & moderately expensive	Rapid [30 min] simple less than 1p per test
False positive rate	:	0.5%	Table to reduce AFP false positive rate by 90%
False negative rate	:	2.0%	0.5%

AChE form could be identified then perhaps a suitable quantitative assay could be devised which would combine the virtues of both the quantitative and qualitative tests presently employed and consequently replace amniotic fluid AFP estimation in detection of NTD.

4.4[iv] Other tests which have been used

The shortcomings of AFP measurement in detection of NTD have long been recognized. In particular false positive results and fetal blood staining have created problems. Prior to AChE measurement, several other markers were examined, with varying degrees of success, these included concanavalin A reactive AFP and "rapidly adhering cells". These together with several other less well publicised parameters are outlined below:

Concanavalin A reactive AFP

Initially this test appeared to be promising. AFP exists in concanavalin A [Con A] -reactive and unreactive forms. In the presence of NTD there is an increase in Con A reactive AFP in amniotic fluid. It was suggested that this could be applied diagnostically [Smith,C.J., et al, 1979]. Unfortunately a lack of independence from AFP measurement itself, together with marked changes in response to gestational age have meant that this test provides little additional information [Brock, 1983].

Consequently, the technique which is both time consuming and difficult has not gained widespread acceptance.

Rapidly adhering cells

When amniotic fluid cells are plated out for in-vitro culture they normally adhere slowly to glass or plastic. In the presence of NTD, the number of cells which adhere rapidly [less than 24 hours] is increased [Sutherland et al, 1975]. The application of this technique in diagnosis of NTD has been difficult to verify and the technique has not gained widespread acceptance, perhaps due to its subjective element in classifying cell types.

Several other parameters such as amniotic fluid IgM, β-trace protein, fibrinogen degradation products, α_2 macroglobulin, β-lipoprotein, ferritin and transferrin have been measured; despite occasional claims to the contrary they appear to have little to offer over AFP measurement [Seller et al, 1981; Legge and Potter, 1983]. Multifactorial approaches have been made such as amniotic fluid amino acid profiles with similarly disappointing results [Pettit and Allen, 1981].

More recently, monoclonal antibodies have been applied in the detection of neurofilaments. It has been proposed that this may be of value in the

detection of NTD and reagents are available [Labsystems Ltd]. Their usefulness remains to be evaluated.

4.4[v] Overall diagnostic plan

The overall success of prenatal detection for NTD depends not only upon the efficiency of individual tests used, such as amniotic fluid AFP, but also upon the way in which these are set into an overall plan [Fig.4.4] and the care with which they are interpreted.

Both amniotic fluid AFP and AChE measurement are usually performed on high risk groups or those patients from which amniotic fluid has already been collected for other reasons. These groups include women with a previous NTD-affected pregnancy, those with an elevated maternal serum AFP and those in whom amniocentesis has been performed to detect chromosomal abnormalities e.g. Downs Syndrome.

Ultrasonography is routinely performed prior to amniocentesis and is used to accurately date the pregnancy and to exclude multiple pregnancies. Following amniocentesis, if AFP or AChE results are positive, ultrasonography may be able to define the fetal abnormality present.

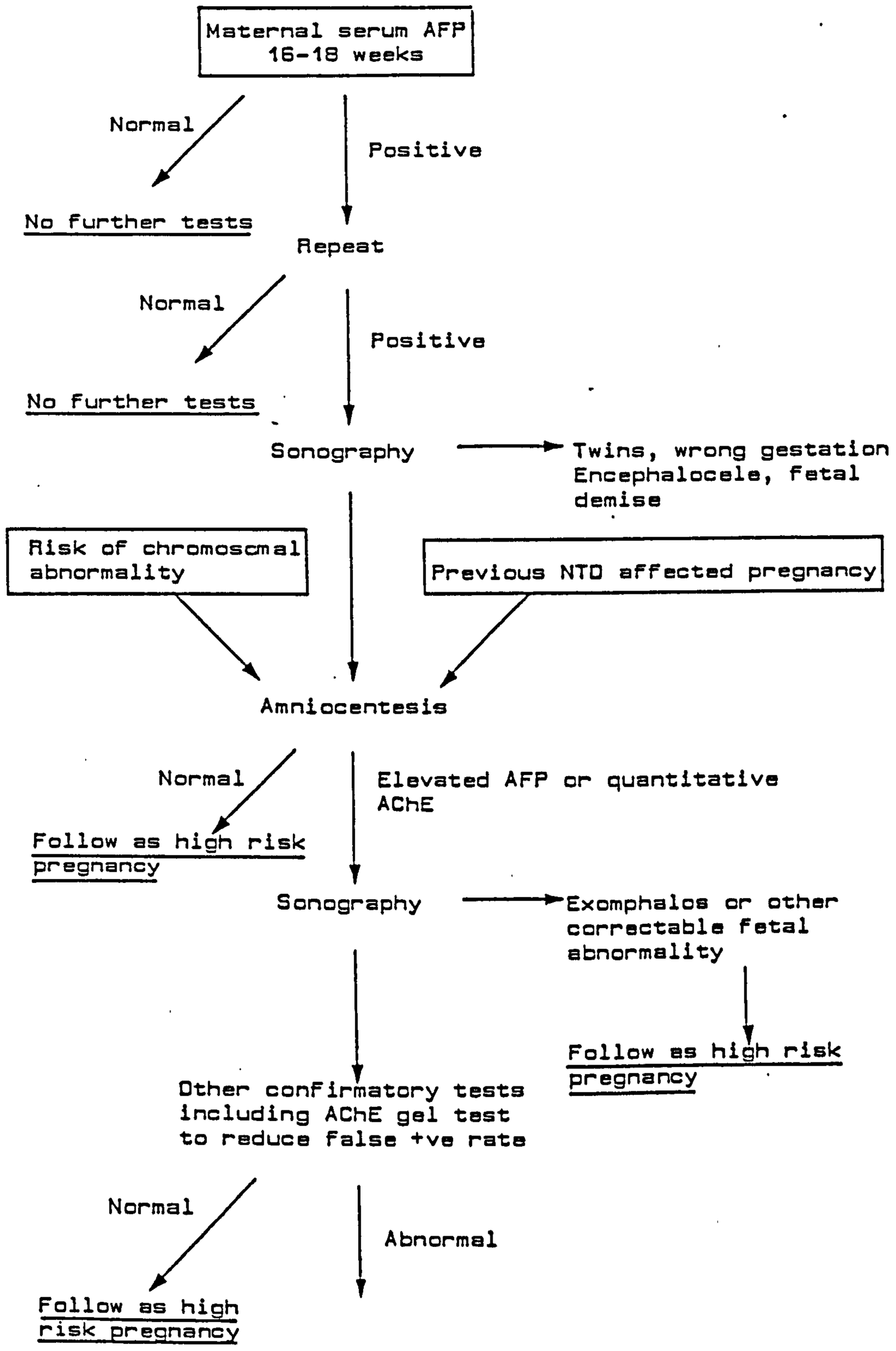
Fig. 4.4 Adapted from Leonard, 1981 - A suggested diagnostic plan for detection of NTD.

Three initial groups are considered:

Those screened for elevated maternal serum AFP.

Those with a previous NTD affected pregnancy.

Those on whom amniocentesis was desirable because of an increased risk of chromosomal abnormality due to advanced maternal age.



Adapted from Leonard, 1981

Fig. 4.4 A diagnostic plan for the prenatal detection of NTD

As each test is found to be positive the risk of NTD increases [Smith, 1982]:-

	Chance of NTD
Positive maternal serum AFP only	1 : 13
Positive maternal serum AFP + positive amniotic fluid AFP	18 : 1
Positive maternal serum AFP + positive amniotic fluid AFP + positive AChE gel test	288 : 1

The classification of individual results, e.g. amniotic fluid AFP, depends upon continual updating of reference ranges on the basis of pregnancy outcome and the analysis of this data. It is possible [Strike and Smith, 1982] to calculate the individual risk for each pregnancy based on the actual level of maternal serum AFP, amniotic fluid AFP and the result of the AChE gel test; this is greater if the levels are higher and less if lower, even when beyond the accepted positive cut-off value.

When dealing with population screening procedures strict adherence to a programmed investigation is vital together with careful interpretation of test results on the basis of continually updated data.

4.5 Ethical difficulties of prenatal diagnosis

The operation of a prenatal programme raises difficult questions. These can be classified as:-

a] The purely pragmatic

Is such a programme cost effective? The consensus opinion is that this is clearly a cost effective procedure when long term care of the affected individuals is considered [Leonard, 1981].

b] The mixed pragmatic/moral

When the incidence of the disorder in the group under study is low,. as in thos undergoing amnio-centesis for detection of chromosomal abnormalities, [where the rate of NTD is about 0.5%] it may be uncomfortably near or even exceeded by the false positive rate of the test [also about 0.5% for amniotic fluid AFP]. This effectively means terminating one perfectly normal pregnancy for every NTD affected fetus detected.

c] The purely moral

It is reported [Leonard, 1981] that with better means of treatment 'many individuals lead happy productive lives'. This must obviously raise doubts about whether anyone has the right to terminate this life. Even when the defect is severe, life may prove just as valuable to the individual concerned and their family, if society implicitly or explicitly counsels otherwise this may set a dangerous precedent.

In practice, the moral justification for prenatal detection, rests upon "properly informed discussion" with the family involved. Alas, it is

probably naive to imagine that this is achieved in all cases, even if attempted. Indeed, the relativistic morality of the present society may not provide a sufficiently strong framework [Schaeffer and Koop, 1982] on which to base the eventual decision which has irrevocable consequences not only for the family, but also for the newborn child. These questions are far too often mutually hedged by all concerned.

4.6 Prevention and treatment

The suggestion of a link between NTD and vitamin deficiency was first suggested by Smithells in 1976 [Smithells et al, 1976]. Further studies in this are summarised by Smithells, [1982] and show that the recurrence rate in 493 mothers not receiving a multivitamin supplement including folic acid, ascorbic acid and riboflavin during pregnancy was 23 of 493 studied. A similar group of 397 who were given this supplement showed only two recurrences. The difference is highly significant $p < 0.0003$.

The fuller investigation of this phenomena has been hampered by the ethical difficulties of witholding, what would appear to be an effective preventative measure, from women at risk [Editorial; Lancet 1982]. It would appear however, that particularly in low income groups, vitamin supplement is useful in preventing NTD.

When an NTD affected child is carried to term the decision to treat may be difficult [Nixon, 1978]. Pure meningocele without neurological disorder is obviously treatable but association with the Arnold Chiari malformation and gross spinal kyphosis gives a poor prognosis. Corrective surgery includes removal of prominant bifid spines and closure, using adjacent skin. During the post-operative period particular attention should be given to the urinary system and longer term management too must be carefully arranged. In particular impairment of skin sensation below the lesion may produce wounds which are difficult to heal [Macri et al, 1981].

The decision whether or not to treat the NTD affected child must be made promptly, it is unfortunate that such a difficult decision must often be made in relative haste, however evidence shows that the best results are obtained by operation within 48 hours of birth [Nixon, 1978].

Section 5: Aims of the Thesis

It is intended to:-

5.1 Further investigate AChE assays currently in use

In particular for:-

a) Diagnosis of Hirschsprung's disease

Studies have been performed on a limited number of cases [Section 3]. A larger retrospective analysis is necessary, comparing biopsy AChE levels with the final diagnosis established.

b) Detection of neural tube defects

The present method in use is performed manually. An automated method would improve precision and allow samples to be processed more rapidly. This is to be investigated.

5.2 Examine AChE and BChE molecular forms in Hirschsprung's disease and in amniotic fluid from NTD affected pregnancies

a) Hirschsprung's disease

It is intended to investigate both AChE and BChE molecular forms in normal and aganglionic rectal tissue. To quantitate the changing levels of these forms and to compare these changes with those seen histochemically and histologically in the transition from normal to aganglionic gut.

b) Neural tube defects

Molecular forms of AChE and BChE are to be examined in amniotic fluid from normal pregnancy and those affected by anencephaly, spina bifida and

exomphalos. The forms are to be quantitated and the increase in the level of each form compared with the increase in overall activity. The aim if possible, is to identify a form of AChE, present in NTD pregnancies alone, which would form the basis of a much improved diagnostic test.

Quantitation of the activity of molecular species in both rectal tissue and amniotic fluid must include information about their stability and the possible effects of native proteases on the molecular form profile. In addition their release from rectal tissue is also to be investigated, this will provide information about their possible cellular location.

5.3 Characterise the molecular forms present in amniotic fluid and rectal tissue

Particular attention will be paid to any form or forms specifically increased in either Hirschsprung's disease or NTD. Further characterisation of these forms will include:

a] Approximate molecular mass determination by comparison of gel filtration and sucrose density data.

b] In the case of amniotic fluid their behaviour on polyacrylamide gel electrophoresis.

c] Thermal stability studies.

d] Response to substrate inhibition.

e] Relative sensitivity to commonly used inhibitors.

f] Interaction with detergents - suggesting possible cellular localisation.

MATERIALS AND METHODS

Section 6 : Assays in current use

Section 7 : Molecular forms - analytical techniques

Section 8 : Materials studied and patients investigated

Section 6: Assays in current use

6.1 Measurement of AChE and BChE in amniotic fluid

The measurement of AChE activity in amniotic fluid has been previously described [Dale et al, 1981]:-

Each amniotic fluid sample was centrifuged at 15,000g for 3 min in an Eppendorf Model 5414S microcentrifuge [Anderman Ltd] to remove cellular debris and contaminating erythrocytes.

The following reagents were added to a 10mm light path cuvette:

A. 2.55ml of 0.1M - disodium hydrogen/potassium dihydrogen phosphate buffer; pH8.0

B. 0.05ml of 9.52×10^{-4}M ethopropazine hydrochloride [May and Baker; Sigma Chemical Co.]

C. 0.1ml of 0.01M DTNB in 0.1M disodium hydrogen/ potassium dihydrogen phosphate buffer; pH7.0

To this mixture was added 0.2ml of centrifuged amniotic fluid which was then mixed by inversion three times and incubated for 5 min at 30^{o}C. The reaction was initiated by the addition of 0.1ml of 0.015M acetylthiocholine iodide. The initial rate of change in absorbance at 412nm was then followed for 5 to 10 min at 30^{o}C. Amniotic fluid was omitted from blank cuvettes set up to correct for non-enzymic hydrolysis. Results are calculated in

International Units per litre [U/l]:-

$$U/l = \frac{abs./min}{E_m} \times \frac{TV}{SV} \times 10^6 = abs/min \times 1103$$

abs./min: change in absorbance [optical density units] per minute

E_m - molar extinction cofficient of the nitrobenzoate ion i.e. 1.36×10^4

TV - total volume in the cuvette.

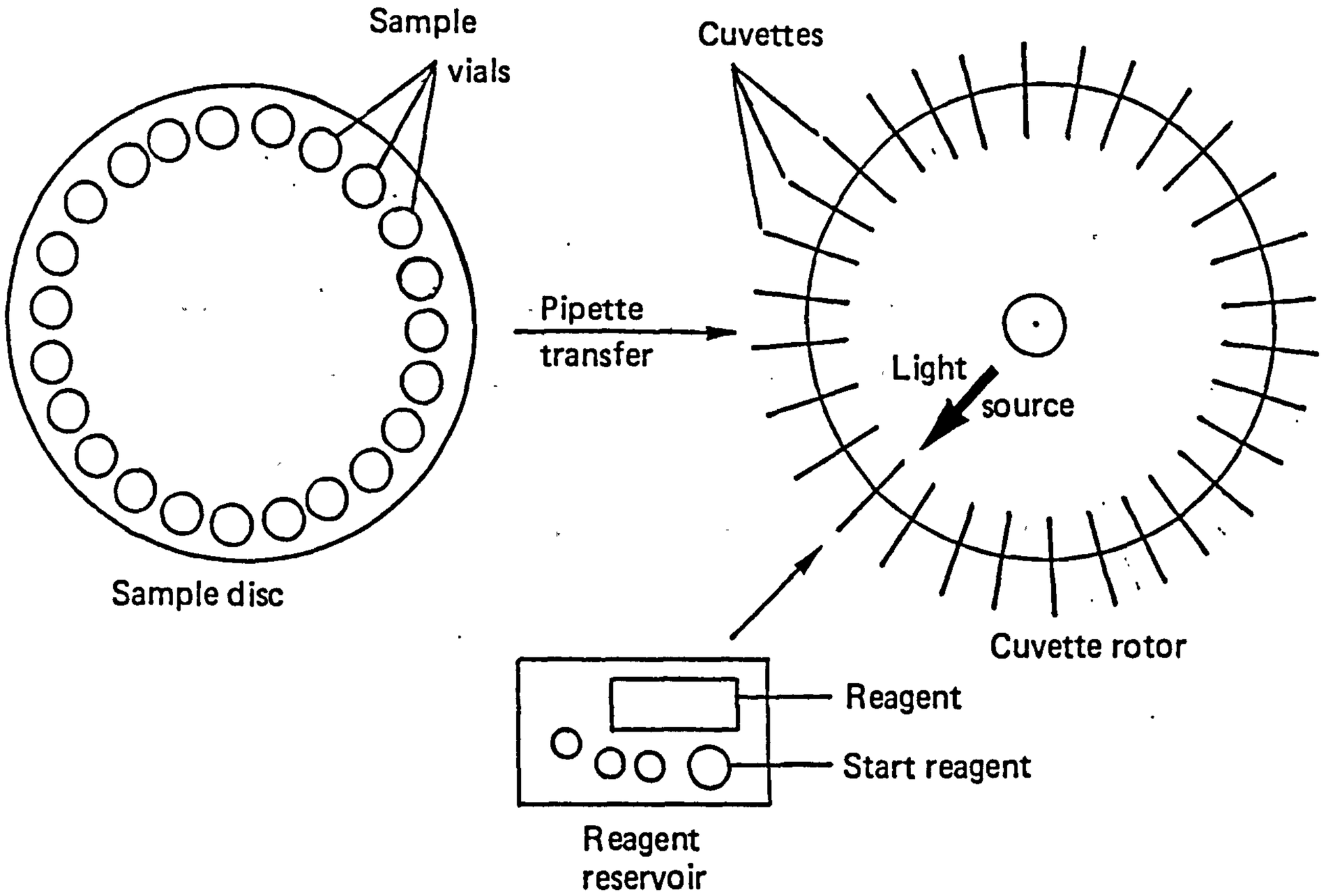
SV - sample volume

Acetylthiocholine [AThCh] iodide may be replaced in the assay by acetyl-β-methylthiocholine iodide [AβMThCh] [0.015M], this improved the specificity of the assay by reducing cross reaction with BChE [Bonham et al, 1981].

It is possible without change in final concentration to adapt these assay procedures for use on a centrifugal fast analyser [CFA] such as the Cobas Bio [Roche Ltd].

The sample volume is reduced to 0.02ml and 28 samples can be analysed simultaneously in an assay time of less than 15 min. The transfer and pipetting of reagents and samples is automated [Fig.6.1] which would be expected to improve overall precision. Other analytical parameters are listed in Table 6.1.

Fig. 6.1 A schematic guide to the operation of a centrifugal fast analyser [Cobas Bio, Roche Ltd]. A cross section of a single cuvette is shown with the position of reagent and sample pockets and the reaction cuvette illustrated.



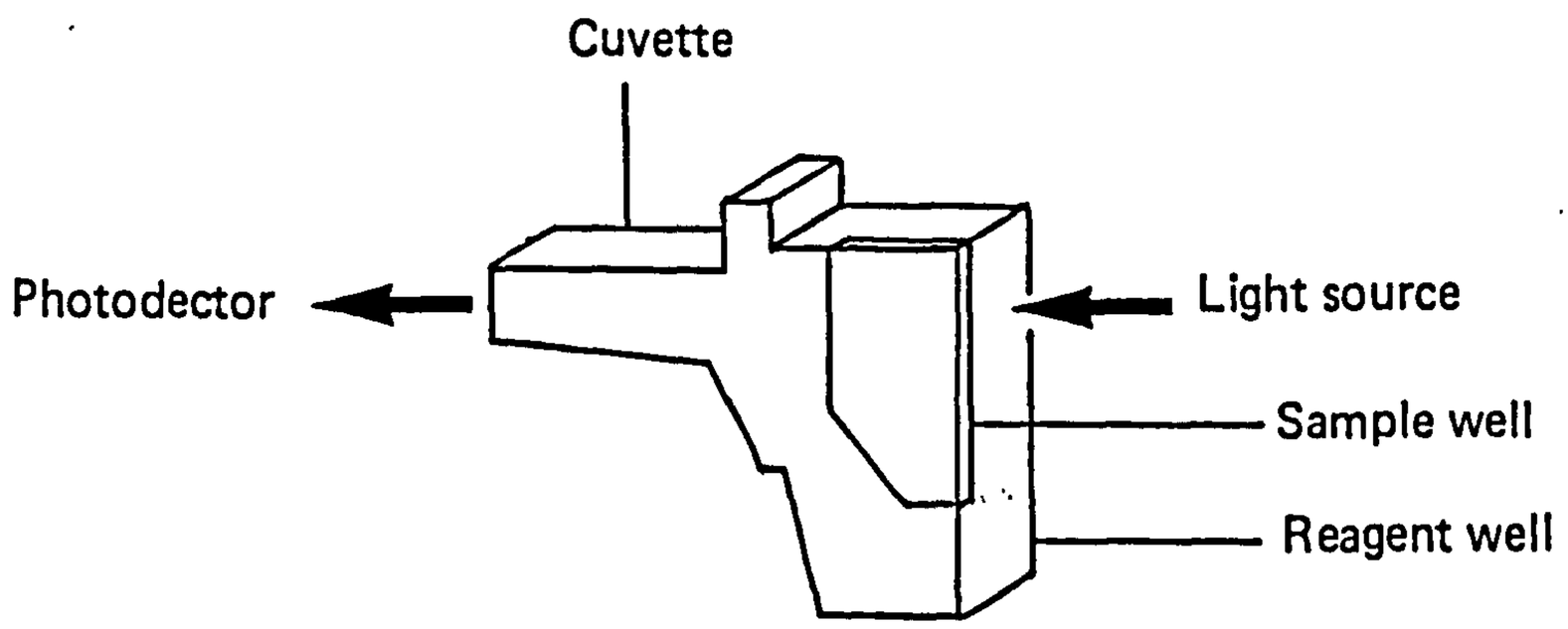


Fig. 6.1 A schematic guide to the operation of the centrifugal fast analyser

Table 6.1 Parameter listing together with reagent concentration used for assay of AChE and BChE in amniotic fluid specimens with the Cobas Bio centrifugal fast analyser.

Table 6.1 Parameter listing and reagent composition used for AChE and BChE assay of amniotic fluid samples on the CFA

Parameter listing

1	Units	U/l
2	Calculation Factor	920
3	STD 1 Conc	0
4	STD 2 Conc	0
5	STD 3 Conc	0
6	Limit	1
7	Temperature [Deg C]	30.0
8	Type of analysis	2
9	Wavelength [nm]	412
10	Sample volume [μl]	20
11	Dilutent volume [μl]	60
12	Reagent volume [μl]	300
13	Incubation time [Sec]	0
14	Start reagent volume [μl]	0
15	Time of first reading	40
16	Time interval [Sec]	30
17	Number of readings	20
18	Blanking mode	1
19	Printout mode	1

Reagent Composition:-

DTNB: 4.3×10^{-4}M

ATCH: 6.4×10^{-4}M

Ethopropazine: 1.8×10^{-5}M

Contained in pH8.0, 0.1M

disodium hydrogen/dihydrogen

potassium phosphate buffer

The results of this automated technique are compared with those of the manual method in Section 9.1.

The normal reference values for the manual technique were:

Mean = 2.6 ± 1.1 [SD]; Range 0.4 - 5.8 U/l [Dale et al, 1981].

6.2 Measurement of rectal suction biopsy AChE

Rectal suction biopsies were made as described in Section 3. The biopsy specimens without added liquid or preservative were placed in tightly capped tubes and transported to the laboratory as quickly as possible. The tubes were then refrigerated at -20°C for at least 30 min in order to allow the samples to freeze. This was carried out on all samples to standardise the technique for those which it was not possible to analyse the same day.

Samples were thawed, dried by contact with filter paper and weighed on a microbalance Microforce Mk 2C [C.I. Electronics]. If the weight was greater than 10mg the sample was divided to produce a specimen of less than 10mg. This was then transferred to a glass homogeniser body [2ml, Potter Elvehjem type - Jencons Ltd] and 1ml of 0.1M, pH8.0 phosphate buffer added. The sample was then homogenised using a motor driven PTFE pestle at 3,000 rpm and the resulting suspension centrifuged at 15,000g in an Eppendorf microcentrifuge [Anderman Ltd].

The method of assay was described by Dale et al, 1977. The following reagents were added to 10 mm light path cuvettes:-

Cuvette 1 [ml]	Cuvette 2 [ml]	Reagents
2.35	2.4	0.1M, disodium hydrogen/potassium dihydrogen phosphate buffer, pH8.0
0.05	-	8.52×10^{-4}M ethopropopazine hydrochloride
0.10	0.10	0.01M DNTB in 0.1M disodium hydrogen/potassium dihydrogen phosphate buffer pH7.0

Ethopropazine hydrochloride was dissolved in 30ml of 2M HCl and made up to 100ml with 0.1M pH7.0 phosphate buffer. 0.4ml of the centrifuged homogenate was then added to each cuvette, mixed by inversion and incubated for 5 min at 25^{o}C. The reaction was initiated by the addition of 0.1ml of 0.015M AThCh made up in 0.01M HCl. The rate of change of absorbance at 412nm was followed for 10 min at 25^{o}C in a Pye Unicam SP8100 spectrophotometer [Pye Unicam Ltd]. 0.4ml of 0.1M, pH8.0 phosphate buffer was substituted for centrifuged homogenate in blank cuvettes set up to correct for non-enzymic hydrolysis.

A suitable control material was prepared from bovine erythrocyte AChE [Sigma Chemical Co Ltd] and

human plasma [as a source of BChE], made up in pH6.0 phosphate buffer to produce a change in absorbance of approximately 0.006/min due to AChE and 0.008/min attributable to BChE. The aliquoted material was stored in liquid nitrogen and removed as required.

Results were calculated in terms of International Units $\times 10^{-1}$ per gram of wet weight tissue [U $\times 10^{-1}$/g]:-

$$U \times 10^{-1}/g = \frac{\text{abs./min}}{E_m} \times \frac{TV}{SV} \times \frac{10^6}{\text{Sample Wt [mg/ml]}} \times 10$$

$$U \times 10^{-1}/g = 5515 \,/\, \text{Sample Wt [mg/ml]}$$

abs/min, TV , SV and E_m are described in Section 6.1.

Cuvette 1: In the presence of ethopropazine, measured only AChE

Cuvette 2: Without ethopropazine, measured AChE + BChE

The ratio:-

$$\frac{\text{Cuvette 1}}{\text{Cuvette 2}} \times 100 = \frac{\text{AChE}}{\text{AChE + BChE}} \times 100,$$

is the percentage of total cholinesterase activity attributable to AChE,[%AChE]. This parameter was found to be particularly useful as it was unaffected by weighing errors or indeed other possible experimental variations common to both cuvettes, such as temperature fluctuation. This was especially important for small biopsy specimens as described in Section 3.

The results obtained over a seven year period using this technique are shown in Section 11 and reference values for AChE and %AChE are described.

6.3 Histological and Histochemical assessment

Suction biopsies were frozen and 8 μM cryostat sections were cut and stained for acetylcholinesterase activity by the method of Karnovsky and Roots, [1964]. Positive and negative control sections were processed simultaneously for each section.

The remaining previously frozen tissue was then fixed in 10% formalin and processed routinely for paraffin embedding and sections were stained with haemotoxylin and eosin. The criterion of normality was taken to be the presence of ganglion cells.

The rectal mucosal sleeve obtained during the surgical procedure for correction of the disorder was similarly treated. Adjacent sections were assessed histochemically and histologically and in addition the profile of AChE molecular forms determined [Section 12.2].

The histochemical and histological studies were performed by Dr D.J. Scott [Department of Histopathology, Newcastle General Hospital] to whom I would accordingly like to record my thanks.

6.4 AFP estimation

AFP was determined in amniotic fluid samples by radial immunodiffusion [Behring AFP-RID plates,

Hoescht Ltd). Patient samples were compared with serially diluted standards which allowed results to be calculated in μg/ml. These estimations were performed by Mr K Creene, Department of Human Genetics, University of Newcastle upon Tyne.

Section 7: Molecular forms - Analytical techniques

7.1 Sucrose density sedimentation

The theoretical basis of this technique is outlined in Section 2.6. Ultracentrifugation was performed in the Neurochemistry and Neuropathology Departments, Newcastle General Hospital, where the staff offered technical assistance for which I would like to record my appreciation.

(i) Gradient characteristics

While several materials are available for performing density gradient centrifugation including: glycerol, metrizamide, Ficoll and Percoll, the majority of reports of the separation of AChE molecular forms utilize sucrose. In accordance with the work of Hall, [1973] and many other authors it was decided to begin by using 5-20% w/v sucrose gradients resting on a 50% w/v sucrose cushion. In common with other workers using this type of gradient, difficulty was encountered in separating monomeric [G_1] and dimeric [G_2] AChE forms. Subsequently the gradients were changed to 10-40% w/w sucrose and run at higher speeds. As expected [Griffiths, 1979] this had the effect of sharpening the peaks and improving the resolution between G_1 and G_2.

In each case gradients were formed in 5.0ml polyallomer Beckman centrifuge tubes. They were constructed as follows:-

5-20% w/v : Equal volumes [1.025ml] of 20%, 15%, 10% and 5% w/v sucrose [Sigma Chemical Co, Grade 1] made up in 0.01M phosphate buffer [pH7.2 containing 0.5% v/v Triton X-100 [BDH Ltd] and 1M NaCl [BDH Analar], were successively overlayed. The layers were allowed to diffuse for approximately three hours at room temperature. 0.5ml of 50% w/v sucrose was then slowly introduced at the bottom of the tube.

10-40% w/w : Equal volumes [1.15ml] of 40%, 30%, 20% and 10% w/w sucrose solution were successively overlayered. The gradients were left to diffuse as for the 5-20% w/v gradients.

In each case the gradients were then chilled for 15 min on ice prior to sample application.

[ii] <u>Centrifugation conditions</u>

As referred to in Section 2.6, Young, [1978] has designed a computer program to allow the sedimentation characteristics of various gradients under different run conditions to be calculated. The program required some modification to be compatible

with PET [Commodore] Basic. This modified program is included in Appendix 1.

It is possible to use the program to:

a) Simulate gradient separation characteristics. Hence with a given gradient the optimum centrifugation conditions including: rotor type, temperature, length and speed of run can be calculated. This was carried out to define suitable conditions for separation of the species with the S-values under study, the results compared well with experimentally obtained data.

b) Calculate S-value. Reversing the above procedure by defining the run conditions, gradient characteristics and the position on the gradient of the substance under study allowed calculation of the S-value. It was found that this was a less accurate method of determination than comparison with markers of known S-value [Fig. 7.6]. The program was therefore used mainly for determination of run conditions. These were then checked experimentally, final run conditions were:

5-20% w/v gradients : 37,500 rpm in a Spinco L or L275B Beckman centrifuge [Beckman Ltd] with an SW65 swing out rotor for 17 hours [140,000 g max].

10-40% w/w gradients : 40,000 rpm in a Spinco L with an SW50.1 rotor for 24 hours g max or 55,000 rpm in an L275B with an SW65 rotor for 17 hours [300,000 g max].

[iii] Sample and marker application

5 μl of each of the regularly used marker enzymes were mixed with 200-400 μl of sample and carefully layered on top of the gradients. The exact amount of sample applied depended upon the activity present. Marker enzymes used included, equine liver alcohol dehydrogenase, 4.8S, [Sigma Chemical Co Ltd; Cal Biochem Ltd] adjusted to 12×10^3 U/l; E.Coli β-galactosidase, 16.0S, [Sigma Chemical Co] 10^5 U/l; bovine liver catalase, 11.3S, [Sigma Chemical Co] 2.5×10^7 U/l and E.Coli alkaline phosphate, 6.3S, 6000 U/l. Enzyme assays are described in Section 7.1[vi].

[iv] Fractionation

Following centrifugation the open end of the gradient tube was covered with "parafilm" and the bottom punctured with a 21 gauge hypodermic needle to allow fractions to be collected drop wise. Between 30 and 40 fractions were collected for each gradient into pre-weighed fraction tubes suitable for direct use on the Cobas Bio centrifugal fast analyser. After fractionation the collecting tubes were reweighed allowing the volume of each fraction to be calculated from the density determined by refractive index. The gradient tubes were washed with 0.5ml of gradient buffer to remove any sedimented activity adhering to the tube walls [this was undetectable or negligible in all cases].

[v] Measurement of cholinesterase activity

The average fraction volume recovered was approximately 140 μl. Using the centrifugal fast analyser [CFA], [Cobas Bio; Roche Diagnostics Ltd] it was possible to measure, AChE, BChE and three marker enzymes on each sample, leaving sufficient for the sucrose concentration to be calculated from refractive index measurement [Pocket refractometer, Bellingham and Stanley Ltd]. Sucrose concentrations were calculated using conversion tables published by Griffiths, [1979].

AChE activity was measured using AβMThCh as substrate in the presence of ethopropazine by a method adapted from Bonham et al, [1981]. BChE was measured using butyrylthiocholine [BThCh] as substrate. In both assays the CFA parameter listing is identical and is shown in Table 7.1.

Linearity and reproducibility were checked using an erythrocyte lysate or tissue homogenate as a source of AChE, and plasma as a source of BChE. The assay was found to be linear up to 430 U/l for AChE and 250 U/l for BChE [Fig.7.1 a]. using a sample volume of 20 μl [as above] and a reduced reagent volume. Reproducibility even at low levels of activity was excellent [Fig. 7.1b].

The fractions obtained from the gradients contained 0.01M phosphate buffer pH7.3, 1M NaCl,

Fig. 7.1a The extent of the linearity of AChE and BChE assays is shown for an increasing concentration of each enzyme. Erythrocyte haemolysate and human plasma was used as a source of AChE and BChE respectively.

Fig. 7.1b The reproducibility of AChE and BChE automated assays [CFA] are shown at two different levels of enzyme activity.

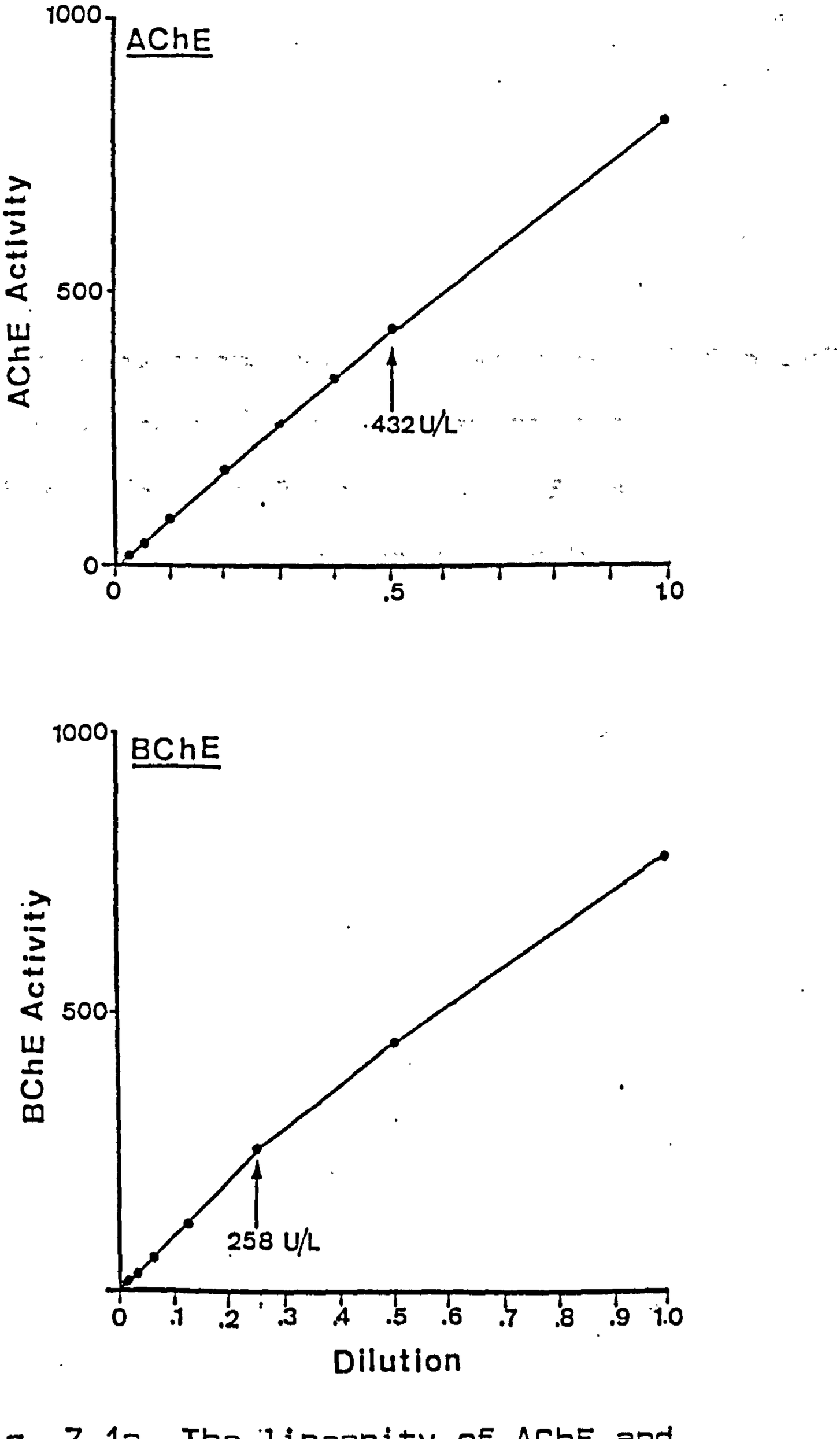


Fig. 7.1a The linearity of AChE and BChE assays

	Mean activity	CV
	U/l	%
AChE	58.0	0.9
	1.9	4.7
BChE	64.0	0.9
	2.2	2.2

Fig. 7.1b The reproducibility of AChE and BChE assays

Table 7.1 Parameter listing together with reagent concentration used for assay of AChE and EChE in gradient fractions obtained from sedimentation analysis.

Table 7.1 Parameter listing and reagent composition used for AChE and BChE assay of gradient fractions on the CFA

Parameter listing

1	Units	U/l
2	Calculation Factor	919.12
3	STD 1 Conc	0
4	STD 2 Conc	0
5	STD 3 Conc	0
6	Limit	0
7	Temperature [^{o}C]	37
8	Type of analysis	2
9	Wavelength [nm]	412
10	Sample volume [μl]	20
11	Diluent volume [μl]	20
12	Reagent volume [μl]	300
13	Incubation time [Sec]	0
14	Start reagent volume [μl]	0
15	Time of first reading [Sec]	40
16	Time interval [Sec]	10
17	Number of readings	20
18	Blanking mode	1
19	Printout mode	1

Reagent Composition:-

DTNB: 3.8×10^{-4}M

AβMThCh or BThCh - 5.7×10^{-4}M

Ethopropazine: 1.6×10^{-5}M

Contained in pH8.0, 0.1M, disodium hydrogen/dihydrogen potassium phosphate buffer.

0.5% v/v Triton X-100 and sucrose of either 5-20% w/v or 10-40% w/w. Each of NaCl, Triton X-100 and sucrose were assessed for their effect upon the assay reagents and upon AChE and BChE activity.

[a] Effect of Triton X-100

0.5% w/v Triton produced a 25% increase in the activity of a red blood cell haemolysate AChE, and a 29% inhibition of plasma BChE. Otherwise Triton was without direct effect upon the assay reagents.

[b] Effect of NaCl

NaCl up to 1 M did not effect either the assay reagents nor AChE activity. However, 1M NaCl was found to inhibit BChE by 10%.

[c] Effect of Sucrose

Sucrose from different sources [BDH AR grade and Sigma Chemical Co, Grade 1], while not affecting AChE or BChE activity directly, did however react with the assay reagents. Although DTNB was stable in the presence of sucrose and alone produced no increase in absorbance in the presence of substrate it did appear to react with substrate [AβMThCh, AThCh or BThCh] in the presence of DTNB to produce a measurable rate. In this way it possessed esterase-like activity. This was more marked at higher pH and sucrose concentrations [Fig. 7.2a], consequently

Fig. 7.2a The effect of changing the pH of the assay buffer, from pH8.0 to 7.0 upon the artefactual activity attributable to interference in the assay by sucrose.

Fig. 7.2b The sensitivity of the AChE assay at different buffer pH.

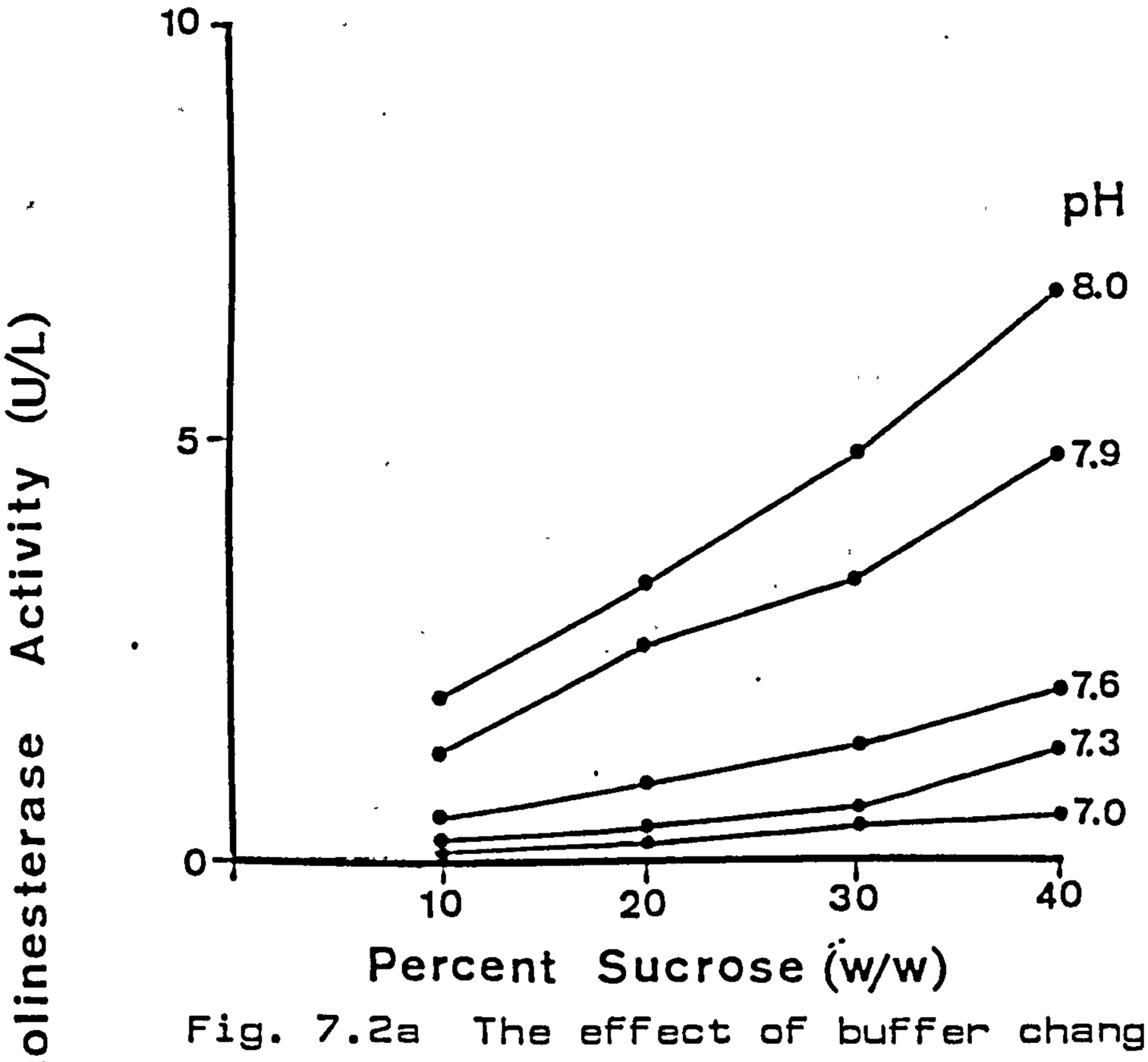


Fig. 7.2a The effect of buffer change on assay interference by sucrose

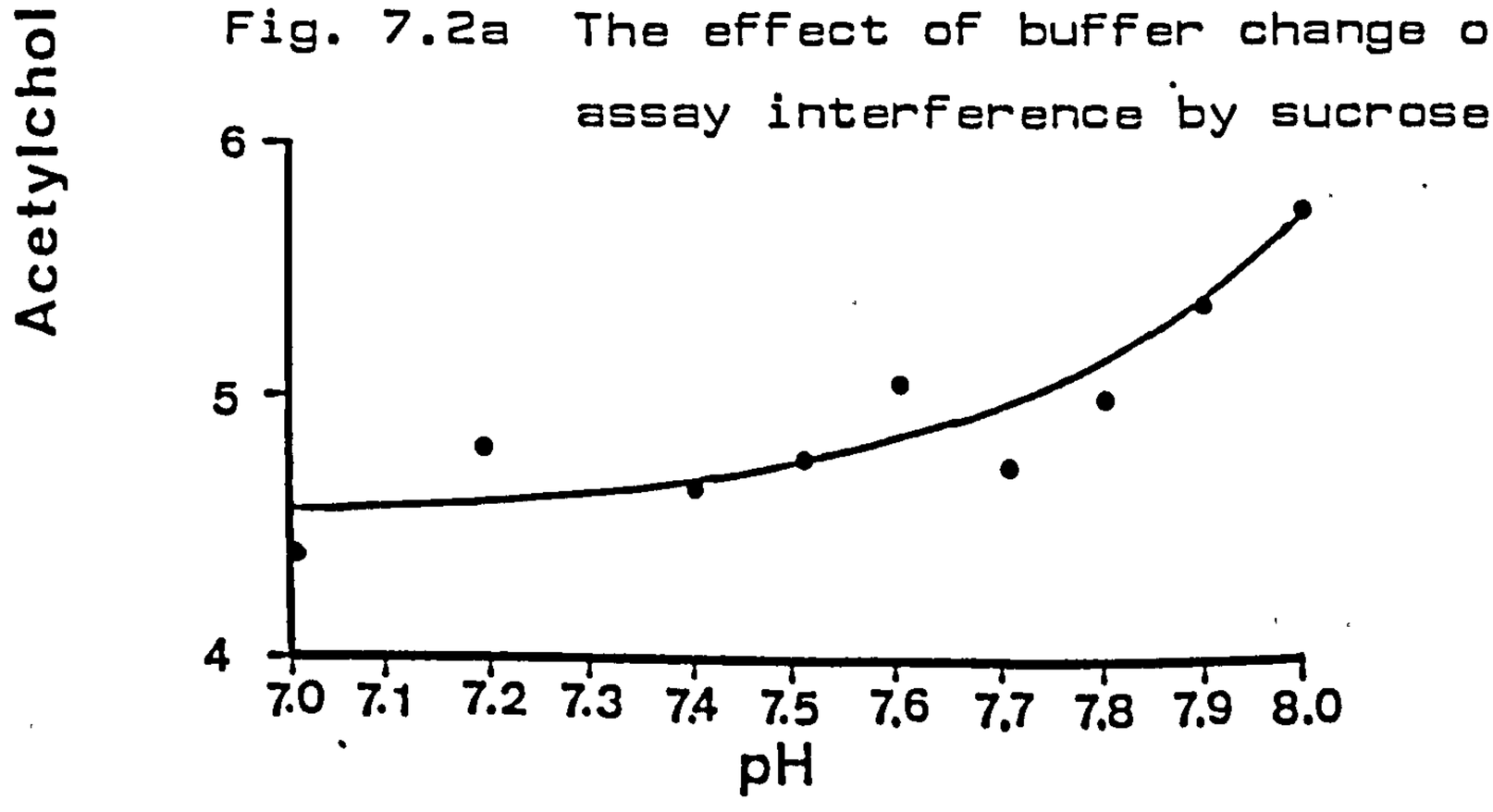


Fig. 7.2b The effect of pH change on assay sensitivity

the pH of the assay was reduced from pH8.0 [Bonham et al, 1981] to pH7.3, this in turn reduced the "artefactual" esterase activity of 40% w/w sucrose by more than 80% but produced only a moderate decrease in the sensitivity of the assay [Fig.7.2b]. The slight remaining artefactual activity was corrected for by the computer program used to calculate the results, this was effectively a blank correction [Section 7.1[vii].

[vi] Measurement of marker enzymes

Four enzymes of known sedimentation coefficient and molecular weight were regularly used as S-value markers.

[a] β- Galactosidase [Sigma Chemical Co Ltd] 16.0S

A kinetic assay procedure described in Data Sheet with Product No G-5635 Sigma Chemical Co and based upon the hydrolysis of σ-nitrophenyl-β-D-galactopyranoside [ONPG] [Craven et al, 1965] was adapted for use on the CFA and required only 10 μl of sample. The reaction was linear up to 1000 U/l and had a CV of 3.5% at 48 U/l.

[b] Alcohol dehydrogenase [Sigma Chemical Co and Cal-Biochem] 4.8S

The original preparation obtained from Sigma Chemical Co contained some interfering esterase activity; a second preperation obtained from Cal-Biochem contained only negligible amounts and this was

subsequently used. The assay based upon the linked oxidation of ethanol to NAD [Valee and Hoch, 1955] was adapted for use on the CFA. The rate of reduction of NAD to NADH was followed at 340nm. A 10 μl sample was used and the reaction was linear up to 400 U/l the CV at 36 U/l was 2.4%.

[c] Alkaline phosphatase [Sigma Chemical Co] 6.3S

The assay based upon that of Gaven and Levinthal, [1960] using p-nitrophenylphosphate as substrate and following its conversion to p-nitrophenol with a consequent increase in absorbance at 405nm was adopted for use on the CFA. The sample volume required was 10 μl.

[d] Catalase [Sigma Chemical Co] 11.3S

It was necessary to measure this enzyme manually in silica cuvettes since the plastic cuvettes of the CFA do not permit measurement at UV wavelengths below 340nm. The method [Beers and Sizer, 1952] followed the catalytic degradation of 0.2% v/v hydrogen peroxide [BDH, AR grade] in phosphate buffer at pH 7.0 by observing the decrease in absorbance at 240nm.

[e] Additional markers

In addition to these regularly used markers, the sedimentation characteristics of the gradients were more closely defined by using additional markers [Fig.7.6], these included:- Cytochrome C, ovalbumin,

yeast alcohol dehydrogenase, rabbit muscle aldolase and thyroglobulin [all obtained from Sigma Chemical Co].

Cytochrome C was measured by its absorbance at 405nm, [the absorption maximum] ovalbumin and thyroglobulin by Lowry assay [Lowry et al, 1951], yeast alcohol dehydrogenase by a modification of the previously described assay for equine liver alcohol dehydrogenase in which the concentration of ethanol was increased ten fold. This increase was necessary because of the higher Km of the yeast enzyme. Aldolase was measured manually by a colorimetric method described in Sigma Technical Bulletin No. 752 [Sigma Chemical Co] using reagents obtained from the same company.

[vii] Calculation and presentation of results

The number of fractions obtained from each gradient varied from 30 to 40 making simple graphical plots of Enzyme activity Vs Fraction number difficult to compare. In order to permit direct comparison between subsequent gradients and accurate calculation of the amounts of each molecular form present it was necessary to calculate the volume of each fraction. The fraction weight and sucrose concentration were measured and used to calculate the volume, so that enzyme activity could be plotted

against increasing volume. In addition, as previously described in Section 7.1[v], there was an artefactual increase in AChE and BChE activity due to interference from sucrose; this could be compensated for by applying a mathematical correction based on the sucrose concentration in that fraction.

The calculations involved were tedious and time-consuming and a computer program was constructed which, given fraction number, weight, AChE activity, BChE activity and sucrose concentration, would correct for sucrose interference and calculate the fraction volume and the enzyme activity [i.e. volume x activity U/l]. By culmulative addition of these data the gradient volume could be calculated and a running culmulative total of enzyme activity and volume produced. The results were then presented in both graphic and tabular form, an example of tabulated results for a theoretical four fraction gradient are shown below:-

Total AChE activity: 2.09 mU
Total volume: 0.644 ml
Total BChE activity: 3.20 mU

Fraction No	Wt [g]	Vol [ml]	%Vol [%]	AChE [U/l]	BChE [U/l]	% suc [g/100g]	%AChE [%]	%BChE [%]
1	0.150	0.131	20.23	3.4	2.8	40	21.2	11.5
2	0.200	0.180	48.12	2.0	2.4	30	38.4	25.1
3	0.174	0.162	73.23	6.0	13.4	20	91.8	92.6
4	0.179	0.173	99.99	1.0	1.4	10	99.9	99.9

Recovery [AChE]= 84% Recovery [BChE] = 80%

Given the activity and volume of the sample originally applied, the program will also calculate the recovery of activity as shown above. Results are displayed in both tabular and graphic form including plots of AChE, BChE and sucrose concentration Vs culmulatively increasing volume. It is therefore possible to calculate the amount of each form present from the areas under the peaks, this is simplified by comparison with the culmulatively increasing % AChE and % BChE at each point on the graph. The actual calculation from these data of the amount of each form present can be done in one of two ways.

METHOD A: Directly by comparing the percentage of activity under each given peak with the total AChE or BChE activity printed at the top of the tabular listing.

METHOD B: Indirectly by comparing the percentage of activity under each peak with the activity of AChE or BChE in the sample originally applied.

Method "A" was suitable for the high activities encountered using biopsy specimen homogenates, whereas method "B" was more suitable for the very low activity found in amniotic fluid, where minor baseline variations could significantly affect the calculated total activities.

The program [Appendix 2], therefore offers several advantages:

1] Saves several hours for each sample in tabulating results and plotting data points.

2] Performs a mathematical correction of interference due to sucrose.

3] Calculates the volume of each fraction from fraction weight and sucrose concentration.

4] Permits direct comparison between subsequent gradients from graphically produced data.

5] Enables the rapid quantitation of each of the molecular forms present.

6] Provides the necessary data, i.e. the position on the gradient for subsequent calculation of S-value.

7] Calculates the recovery of activity for each gradient.

A schematic representation of the whole procedure of density gradient centrifugation and processing of results is shown in Fig. 7.3.

[viii] <u>Analytical separation</u>

The separation of molecular forms produced by the gradients are shown in Fig. 7.4. As anticipated

Fig. 7.3 A schematic guide to the procedure used for density gradient sedimentation is shown. The sample described illustrates the procedure for 10-40% w/w gradients centrifuged for 17 hr in an SW65 rotor at 55,000 rpm.

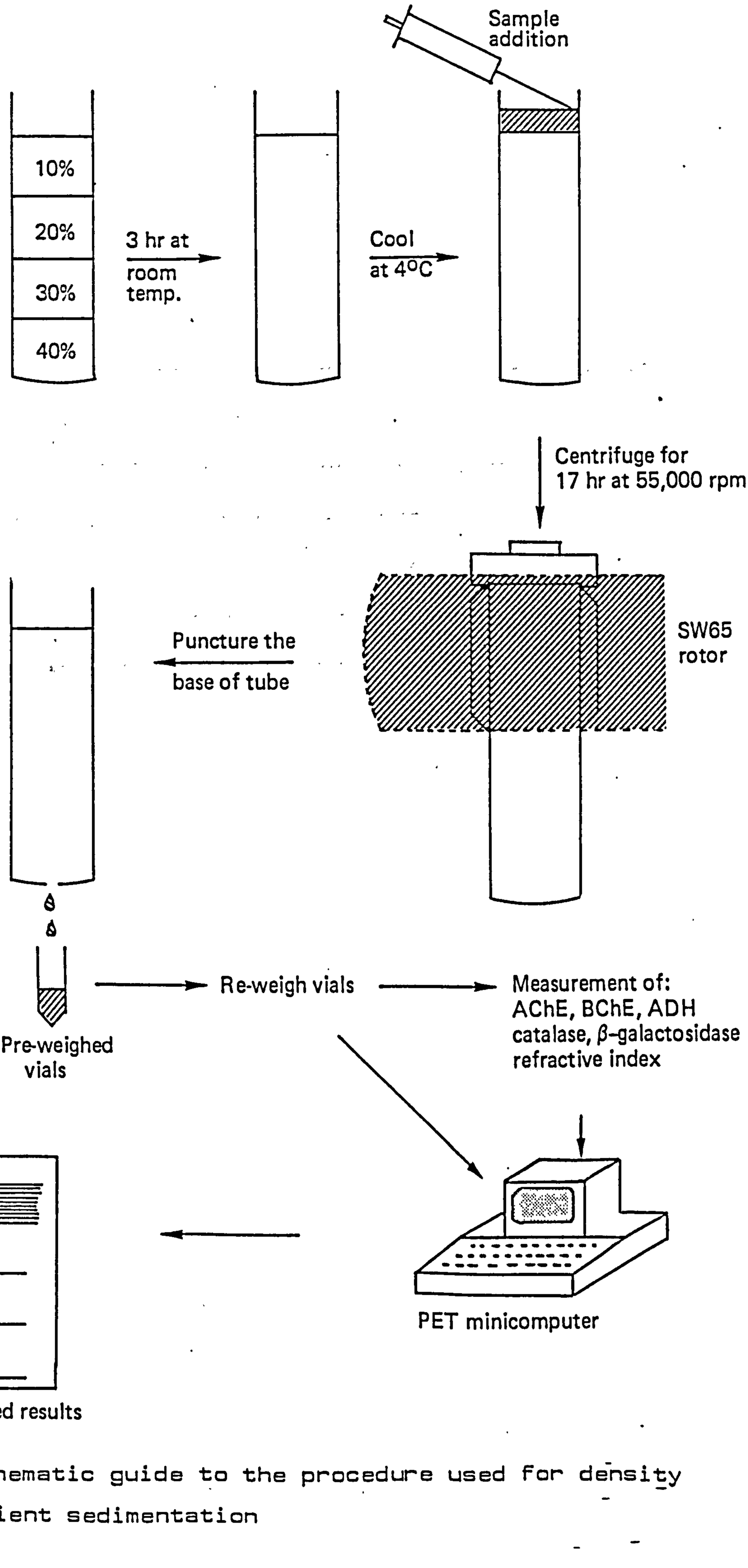
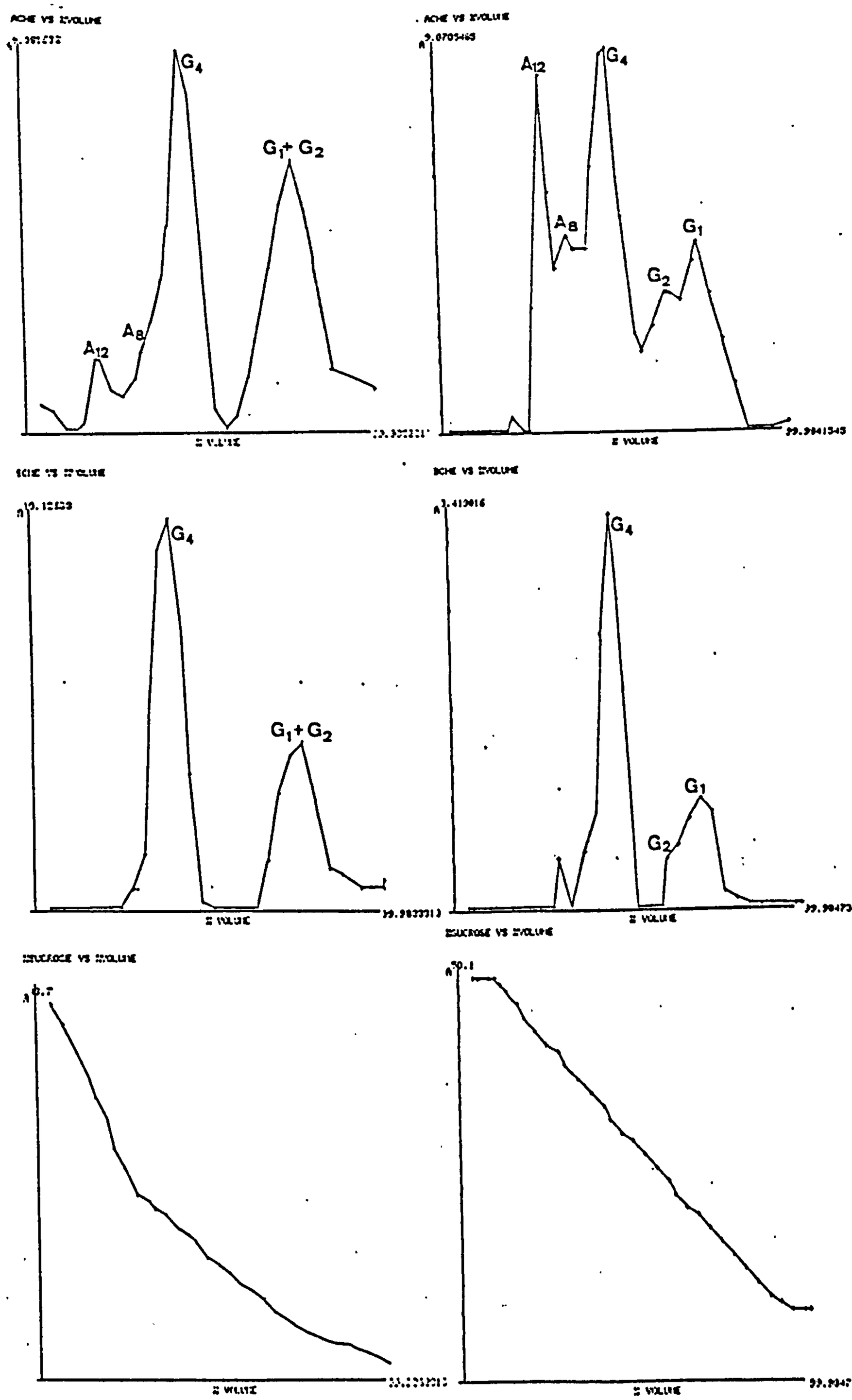


Fig. 7.3 A schematic guide to the procedure used for density gradient sedimentation

Fig. 7.4 Examples of AChE, BChE activity and sucrose concentration are shown as they vary in 5ml gradients of 5-20% w/v sucrose and 10-40% w/w sucrose. The results are plotted in this form by the program [listed in Appendix 2] with the only exception that the points are not joined as they appear opposite.



5–20% w/v Sucrose
+50% w/v cushion

10–40% w/w Sucrose

Fig. 7.4 Graphs of AChE, BChE and sucrose concentration versus percentage gradient volume as produced by the computer program

in Section 7.1[i] the separation of monomeric, G_1, and dimeric G_2 enzyme is improved by employing 10-40% w/w rather than 5-20 w/v gradients. The sucrose concentration plotted against culmulative gradient volume is also shown, there is some distortion of the 5-20% w/v gradients introduced by the 50% w/v cushion. The graphs are shown in eight fold reduction, as they appear from the computer printer except that the points are linked and the molecular forms labelled for ease of interpretation.

[ix] Reproducibility of quantitation

To test the reproducibility with which the molecular forms can be quantitated three samples were analysed each in triplicate. Two were homogenised rectal tissue and the third amniotic fluid. The forms were quantitated as described in Section 7.1[vii] and the results shown in Table 7.2. At levels above 2 U/l the reproducibility was good; below 2U/l, the variation became more marked and consequently too much importance should not be attached to minor quantitative changes in forms which are present at or below this level.

[x] The effect of different inhibitors

The selective BChE inhibitor ethopropazine was used in the assay of the gradient fractions described in Section 7.1[v] to enable specific measurement of AChE activity. In addition to this direct method, an indirect method is often reported

Table 7.2 The reproducibility of quantitating individual AChE molecular forms was assessed by analysing three samples in triplicate.

Table 7.2 Assessment of the reproducibility of molecular form quantitation

	G_1	G_2	G_4	A_{12}	
	U/l				
Sample 1 in triplicate	3.6 [3.38-3.82]	3.81 [3.39-4.23]	4.55 [4.28-4.82]	1.02 [0.92-1.12]	Homogenised tissue
Sample 2 in triplicate	3.43 [3.36-3.50]	3.3 [3.23-3.37]	2.89 [2.9 -3.08]	0.7 [0.59-0.81]	
Sample 3 in triplicate	1.15 [0.80-1.50]	1.65 [1.20-2.1]	8.8 [8.1 -9.5]	-	Amniotic fluid

Results are shown as: Mean [Range] in each case

[Chubb and Smith, 1975]. In this approach the compound BW284c51 is used to selectively inhibit AChE; AChE being measured as the difference between the total and remaining activity after BW284c51 inhibition. To compare these two methods the same gradient was assayed for:

a] total cholinesterase activity

b] ethopropazine-resistant activity

c] BW284c51 sensitive activity

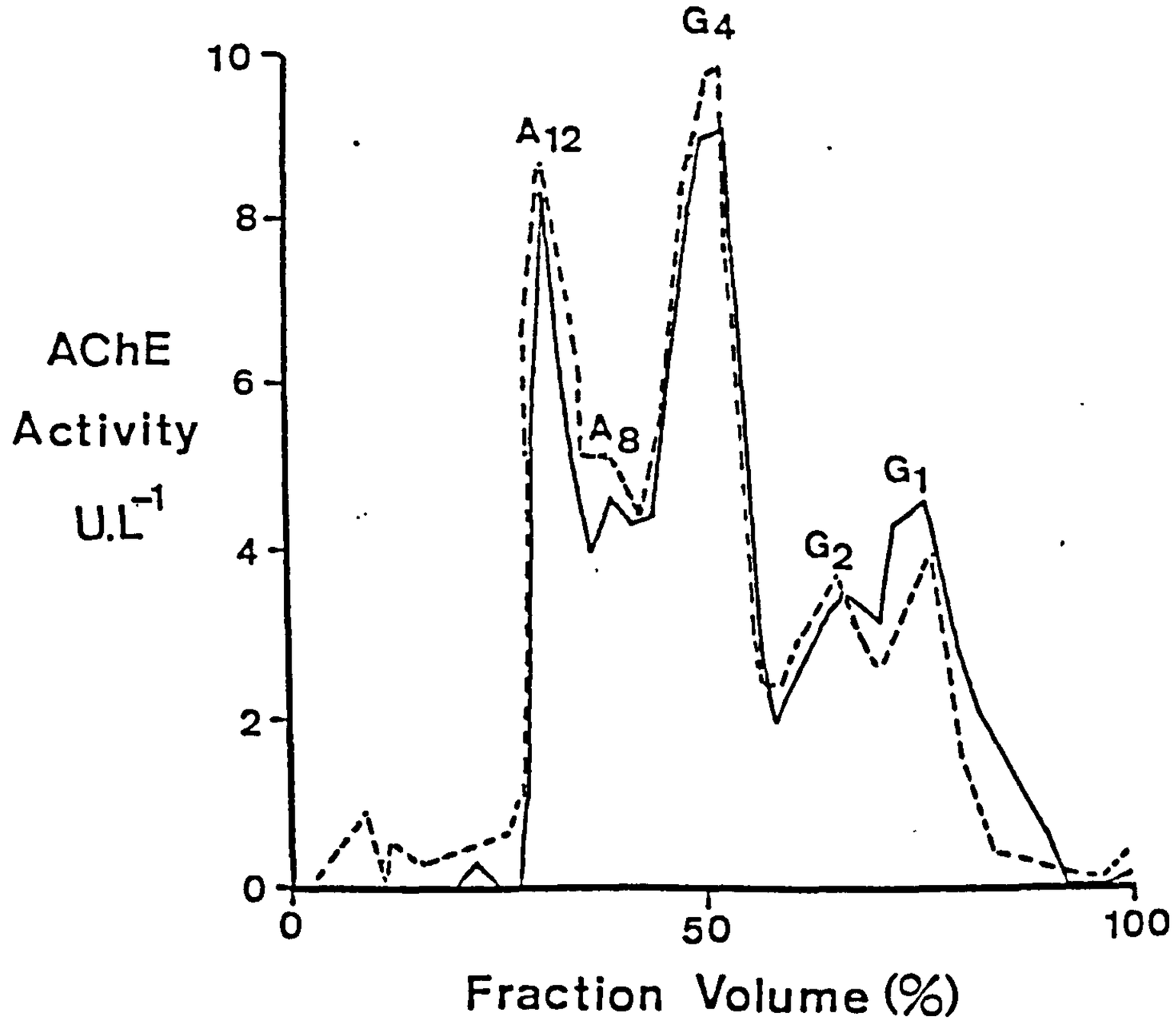
Fig. 7.5 shows that the profiles of ethopropazine-resistant and BW284c51 sensitive activity compare well. It is reassuring that different inhibitors produce such similar results. Quantitative results for the molecular forms are shown:

A_{12}	A_8	G_4	G_2	G_1	
U/l					
2.3	1.1	4.8	1.6	2.2	lysivane resistant
2.6	1.2	4.9	1.8	1.9	BW284c51 sensitive

7.1[xi] Recovery of activity

In order to check the recovery of enzyme activity, a Triton X-100 extract of rectal tissue was layered onto the prepared gradients. The use of Triton X-100 containing extracts rules out any

Fig. 7.5 The effect of using two different methods of selective inhibition on finally calculated AChE activity. BW284c51 sensitive and ethopropazine resistant activity is equivalent in each molecular form illustrated.



----- BW284c51 sensitive

——— Ethopropazine resistent

Fig. 7.5 AChE activity measured using two different selective inhibition methods

variability due to the effect of this detergent in the gradient buffer. Following fractionation each gradient tube was sealed and washed out to detect any cholinesterase activity which may have sedimented onto the bottom of the tube, in all cases this was negligible.

Recoveries obtained were:

AChE : Mean 97.4%; SEM = 1.3 Range: [93.3 - 102.0]

BChE : Mean 94.0%; SEM = 3.6 Range: [80.0 - 106.9]

The recoveries were close to 100%, the slight loss in activity of BChE is probably attributable to inhibition by NaCl contained in the gradient buffer system [Section 7.1[V]].

7.1[xii] Calculation of S-value

To calculate the S-value of the AChE and BChE forms, two methods were compared. The actual S-value gradient was determined by a calibration with nine markers between 1.7 and 19.2S. The two methods were:

1] Calculation from curvilinear interpolation using a computer program [Appendix 3], based upon three markers of established S-value; ADH, 4.8S, catalase, 11.3S and β-galactosidase, 16.0S.

2] Direct calculation of S-value from the relevant parameters i.e. spin-speed, dimensions of the

rotor, duration of the run, temperature, gradient sucrose concentration. The program was referred to in Section 7.1[ii].

Fig.7.6 shows the actual gradient as constructed on the basis of the nine known markers. The gradients predicted by curvilinear interpolation of the three markers [ADH, catalase and β-galactosidase] and by prediction based on centrifugation parameters were compared.

The curvilinear prediction is close to the experimentally determined gradient based on the use of nine markers, this method was therefore adopted for all S-value calculations. The calculation based on centrifugation parameters, while being parallel [Fig.7.6] to the true gradient, consistently underestimated the S-value. However, as referred to in Section 7.1[ii]it was found to be useful in simulating changes in gradient and run conditions, saving many hours of experimental work.

7.2 Preparative-scale density centrifugation

The analytical sucrose gradients described in Section 7.1 did not provide sufficient material to allow further studies following the assay of AChE, BChE, sucrose and marker enzymes. To prepare adequate quantities of these molecular forms a larger scale preparation was necessary.

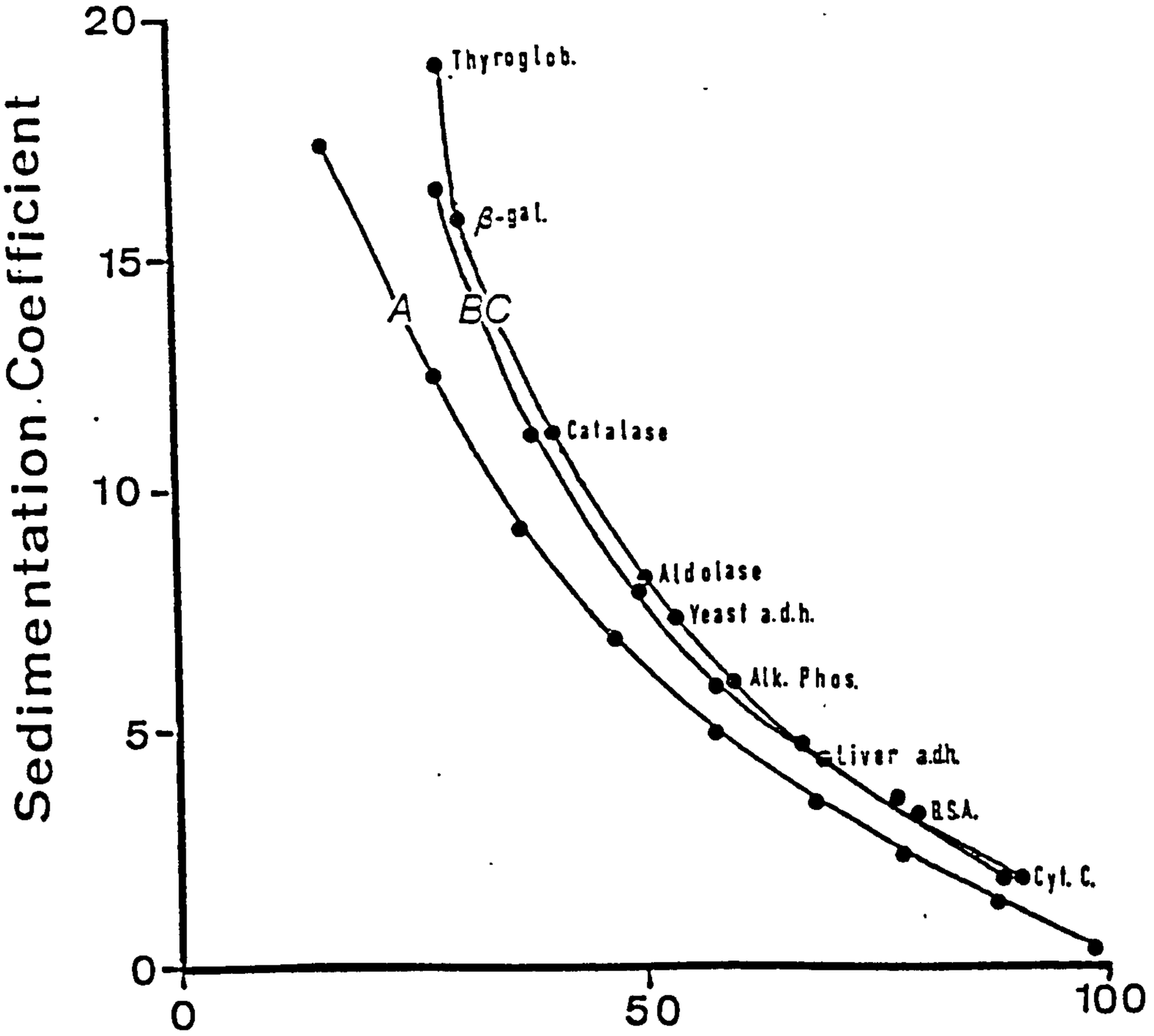
Accordingly, centrifugation was performed in a Beckman L-8 centrifuge with an SW-41, six place

Fig. 7.6 Comparison of different methods of predicting S-value. These include:

A- Calculation by computer program from basic run parameters [Appendix 1]

B- Calculation by curvilinear interpolation [Appendix 3] of three markers of known S-value: β-galactosidase, 16.0S; catalase, 11.3S; alcohol dehydrogenase, 4.8S.

The experimentally determined curve produced by nine markers of known S-value is shown for comparison, *C*.



A — Predicted by the simulation program

B — Predicted by curvilinear interpolation

C — Experimentally determined

Fig. 7.6 Comparison of different methods of predicting S-value

rotor. The gradients, 5-15% w/w sucrose were designed by the computer program outlined in Section 7.1[ii]to give separation of the forms G_1, G_2, G_4 and A_{12} [Fig.7.7]. They were formed by successive overlayering of 3.8ml aliquots of 15, 10 and 5% w/w sucrose bufferred as described in Section 7.1[i].

The sample volume was limited to 600 μl to include the marker enzymes: β-galactosidase, catalase and alcohol dehydrogenase as referred to in Section 7.1[iii].

The gradients were run at 36,000 rpm for 23¼ hr at 4°C and then fractionated immediately by puncturing the tube as outlined in Section 7.1 [iv]. 20-30 fractions of approximately 450 μl were collected into pre-weighed tubes which were subsequently re-weighed to calculate the fraction volume. Fractions in the immediate region [usually one either side] of the peak activity for each molecular form were speedily pooled and placed in liquid nitrogen for storage, or used in further studies on the same day. When necessary the pools were concentrated by vacuum ultrafiltration employing the "Collodion bag" system [Sartorius Instruments Ltd] by this method molecules of greater than 12 kDa are retained and concentrated.

Fig. 7.7 The separation of AChE forms achieved using 13ml 5-15% w/w sucrose gradients. The change in S-value at each point in the gradient is also shown.

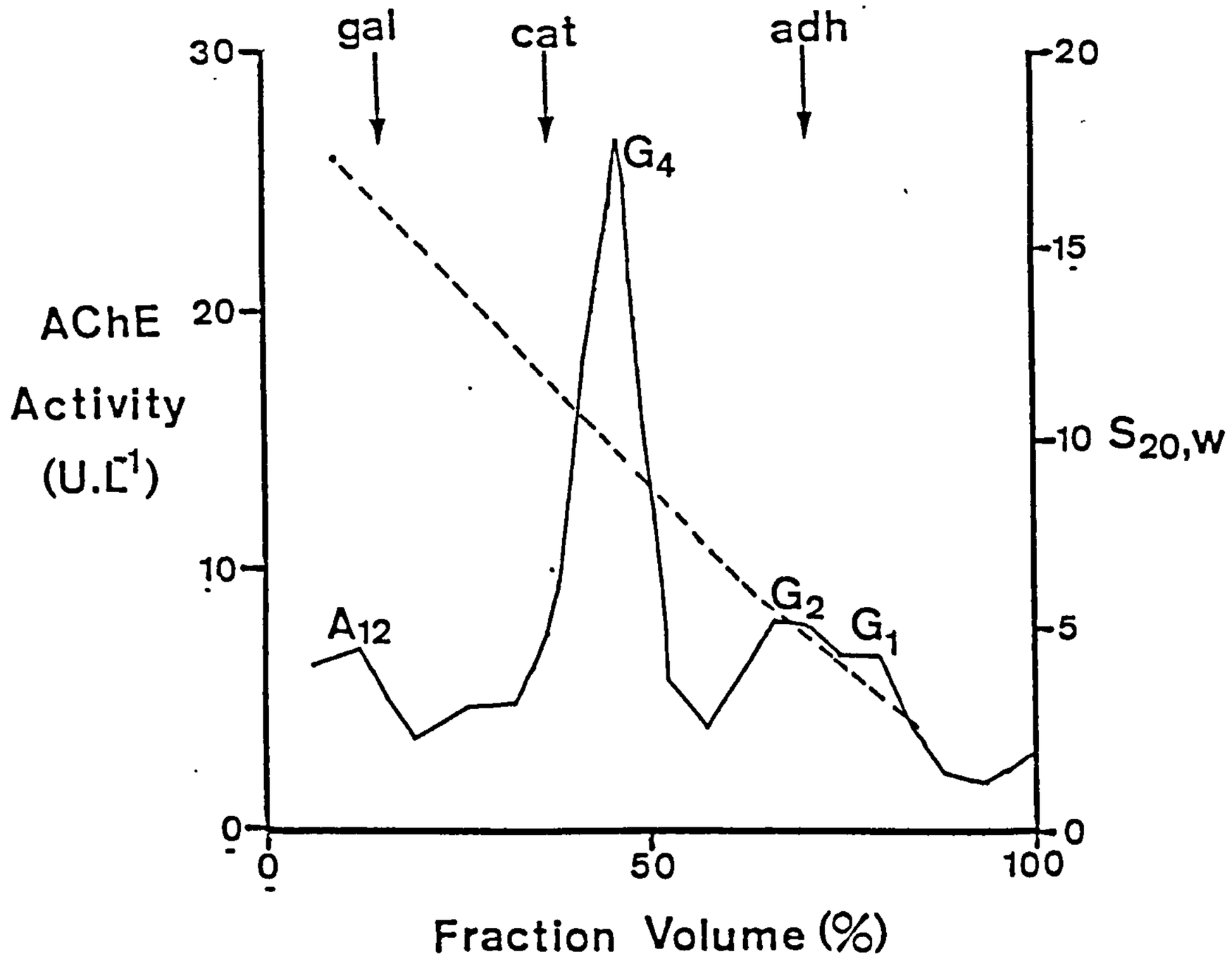


Fig. 7.7 The separation achieved using preparative scale density gradient sedimentation

7.3 Gel filtration

AChE and BChE molecular forms can be separated by gel filtration using suitable media. This technique was used to determine the Stokes radius of individual molecular forms as described in Section 2.6 and hence calculate the approximate, "true" rather than "apparent" molecular mass of these forms using the sedimentation data.

In addition the technique provided a check on the homogeneity of molecular forms separated by sucrose density sedimentation.

The equipment used included:

LKB gel filtration column 95cm long, 1.6cm internal diameter together with a water jacket, and fittings, valves and flow adaptors [LKB Instruments Ltd].

LKB Multitemp Flow Cooler [LKB Instruments Ltd]

P-1 Peristaltic pump [Pharmacia Ltd]

FRAC-100 Fraction collector [Pharmacia Ltd]

The molecular mass of G_1, G_2 and G_4 AChE forms are approximately 80, 160 and 320 kDa respectively. Consequently, Ultrogel AcA34 [LKB Instruments Ltd] a 3% acrylamide, 4% agarose mixed gel with a quoted linear fractionation range of 20-350 kDa [Product Information Sheet No 200908, LKB Instruments Ltd] appeared a suitable medium for their separation.

Unfortunately, because the molecular forms are asymmetric their apparent molecular mass proved to be considerably greater than expected, [Section 15.1] and therefore the use of this gel was abandoned in favour of the larger pore size Ultrogel AcA22 with a linear fractionation range of 100-1,200 kDa [Product Information Sheet No 200908, LKB Instruments Ltd].

The column was packed following the gel manufacturers instructions and equilibrated at 8^{o}C with 0.01M phosphate buffer, pH7.2 containing 0.5% v/v Triton X-100, 1.0M NaCl and 0.02% sodium azide [BDH Chemicals Ltd]. With the exception of the azide, this buffer system was identical to that described in Section 7.1[i] for use in sucrose density sedimentation analysis. The whole procedure is shown schematically in Fig.7.8. The samples including molecular mass markers of known Stokes Radius were applied in gel buffer in a total volume of up to 2.0ml [approximately 1% of the bed volume] using a three way valve system. The flow rate was set at 3.5ml/hr using the peristaltic pump and 1.0ml fractions were collected. The total bed volume Vt, was calculated from the dimensions of the column. The void volume Vo, from calibration with Blue

Fig. 7.8 A schematic guide to the procedure for separation and identification of AChE forms by gel filtration.

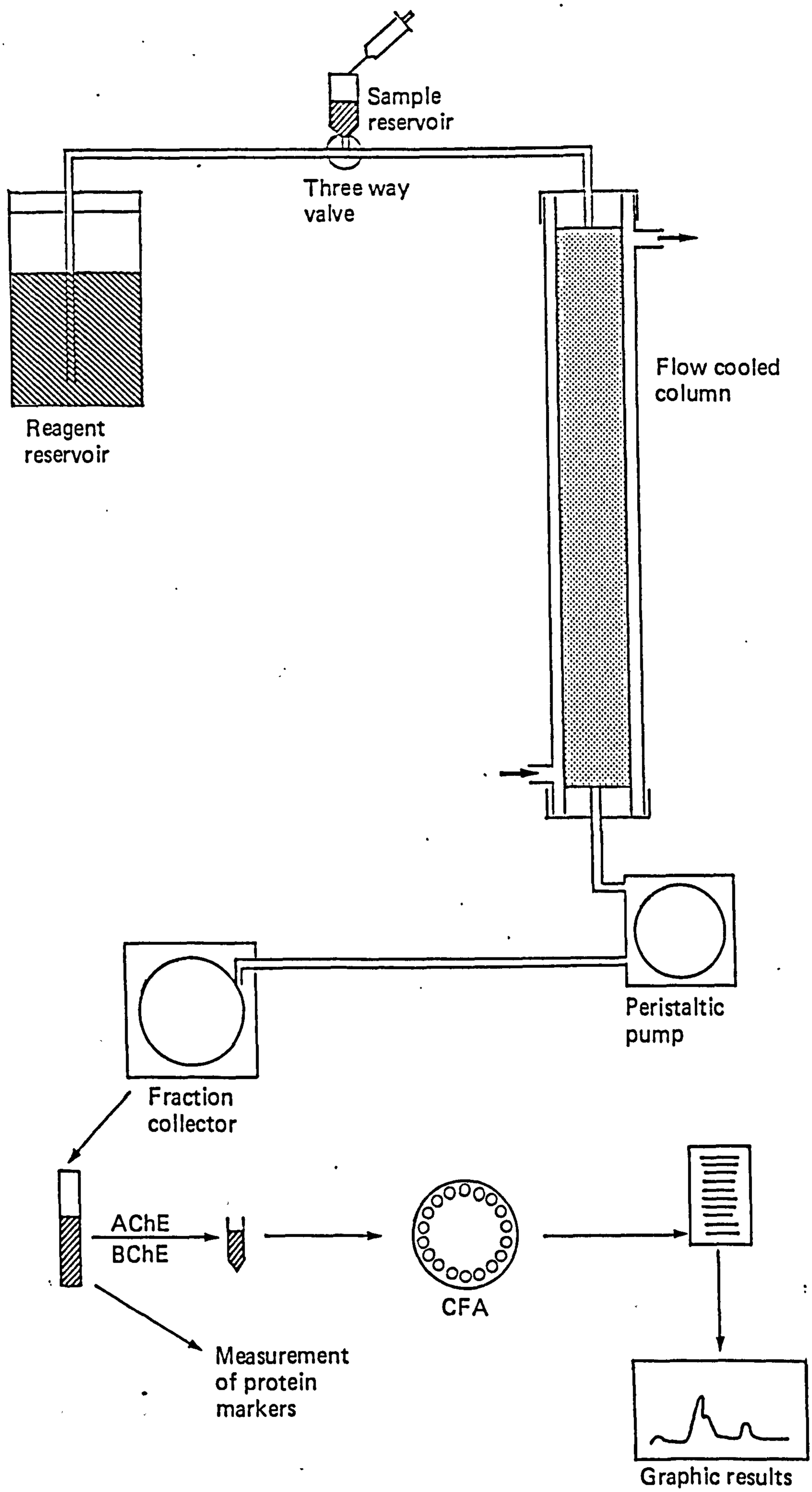


Fig. 7.8 A schematic guide to the procedure used for gel filtration

Dextran 2000 [Pharmacia Ltd] and the volume not accessible to solvent, Vg from the equation:

$$Vg = \frac{Vt}{B.d}$$

Where

Vg - the volume not accessible to the solvent

Vt - total volume of the gel bed

B - bed volume reconsituted per gram of dry gel

d - density of the dry gel

Values of B and d for Ultrogel AcA22 were supplied by LKB Instruments [personal communication] and were 132ml/g and 1.007g/ml respectively.

From these data the volume of the stationary phase, Vs can be calculated:

$$Vs = Vt - Vg - Vo$$

and kd values calculated as described in Section 2.6

$$kd = \frac{Ve - Vo}{Vs}$$

where Vs is the elution volume of the molecule studied.

The protein markers of known molecular mass and Stokes radius were all obtained from Sigma Chemical Co and are listed in Table 7.3.

Cytochrome C, haemoglobin, ferritin and Blue Dextran were measured by the absorbance at their absorption maxima, 415, 408, 400 and 615nm respectively. Lactate dehydrogenase was measured according to the

Table 7.3 Molecular mass and Stokes radius of the protein markers used in calibrating the gel filtration column containing Ultrogel AcA22.

Table 7.3 Molecular mass and Stokes radius of the nine protein markers used in cel filtratien

Protein marker	Molecular mass [KDa]	Stokes radius [nm]
Horse heart cytochrome C[1]	12.4[1]	1.74[1]
Bovine blood haemoglobin *1	32[1]	2.40[1]
Chick egg ovalbumin	43[3]	3.04[3]
E. Coli Alkaline phosphatase	90[5]	-
Rabbit muscle lactate dehydrogenase	140[4]	3.74[2]
Beef heat catalase	232[1]	5.22[2]
Horse spleen ferritin	440[4]	8.0[2]
E. Coli β-galactosidase	540[3]	8.2[3]
Procine thyroglobulin	669[4]	8.5[3]

Data taken from Siegel and Monty, [1966][1]; Ackers, [1964][2]; Rakonczay et al, [1981a][3]; Pharmacia Fine Chemicals, [1980][4]; Taylor and Coleman, [1972][5].

*Andrews, [1962] has shown that at the concentrations used, haemoglobin is highly dissociated upon gel filtration so that the subunits exist as dimers rather than tetramers [$\alpha_2\beta_2$]. The molecular parameters reported here reflect this dissociation.

recommendation of the Scandanavian and Dutch Committee on Enzymes [Scandanavian Society for Clinical Chemistry and Clinical Physiology, 1974] based upon the reduction of pyruvate to lactate linked with production of NAD. The method was performed on the Cobas Bio centrifugal fast analyser [Roche Diagnostics Ltd] using reagents supplied by J.T. Baker Chemicals. Ovalbumin and thryoglobulin were measured by their protein concentration as determined by the Lowry assay [Lowry et al, 1951], centrifugation of the fractions cleared the samples of the gelatenous precipitate produced by Triton. Alkaline phosphatase, catalase and β-galactosidase activities were measured as described in Section 7.1[vi].

Cholinesterase activity was measured with AThCh or BThCh as substrate on the Cobas Bio CFA at 37^{o}C using a pH8.0, 0.1M phosphate buffer system. The method was as described in Section 7.1[v] except that the sample volume was increased to 40 μl and the time interval between readings to 20 sec. These changes produced a fourfold increase in sensitivity, this was important as the enzyme activities being measured were particularly low. The reproducibility of the assay at these low activities was also improved reflected in a CV of only 3.6% at an AChE level of 0.91 U/l.

Calculation of gel pore size

The average Kd values for the molecular mass markers is shown for 12 runs with the same column conditions plotted against log [Molecular mass] in Fig. 7.9.

The combination of experimentally determined Kd with literature values for Stokes Radius, Re, allowed the gel pore size, r, to be calculated according to the equation [Ackers, 1964]:

$$Kd = \left(1-\frac{Re}{r}\right)^2 \left[1 - 2.104 \cdot \frac{Re}{r} + 2.09\left(\frac{Re}{r}\right)^3 - 0.95\left(\frac{Re}{r}\right)^5\right]$$

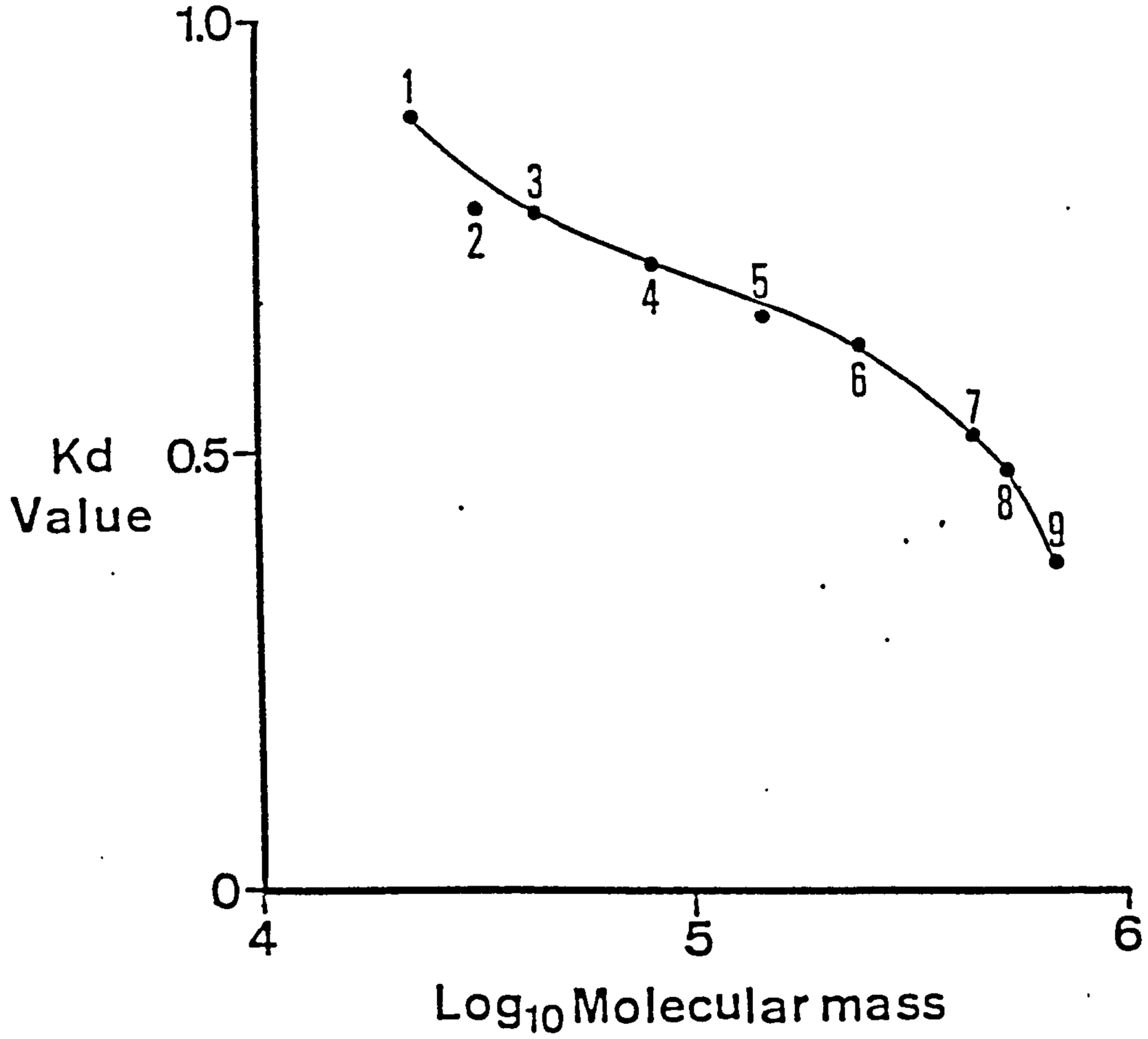
When averaged for the seven markers, haemoglobin, ovalbumin, LDH, catalase, ferritin, β-galactosidase and thyroglobulin, the mean was 46.36 ± 5.62 [SD]nm. Cytochrome C was disregarded because of its reported anomalous behaviour, Siegel and Monty, [1966].

True molecular weight calculation

Kd values for the molecular forms of AChE from rectal tissue homogenates and amniotic fluid were determined as described for the marker proteins. Having already determined the effective gel pore size, r, to be 46.36nm, the equation of Ackers, [1964] shown above could then be applied in reverse to determine the Stokes Radius, Re, of these forms.

These data were used in combination with the sedimentation data to give an approximate measurement

Fig. 7.9 . Average Kd values of marker proteins obtained by gel filtration using Ultrogel AcA22 plotted against $\log_{10}$ [molecular mass].



1 — Cytochrome C
2 — Haemoglobin
3 — Ovalbumin
4 — Alkaline phosphatase
5 — Lactate dehydrogenase
6 — Catalase
7 — Ferritin
8 — β-galactosidase
9 — Thyroglobulin

Fig. 7.9 Average Kd values of protein markers on Ultrogel AcA 22 versus $\log_{10}$ [molecular mass]

of the true molecular mass, according to the equation:

$$Re.S_{20},W = f.\ [Molecular\ mass]$$

[Rakonczay et al, 1981],

Where f is a constant.

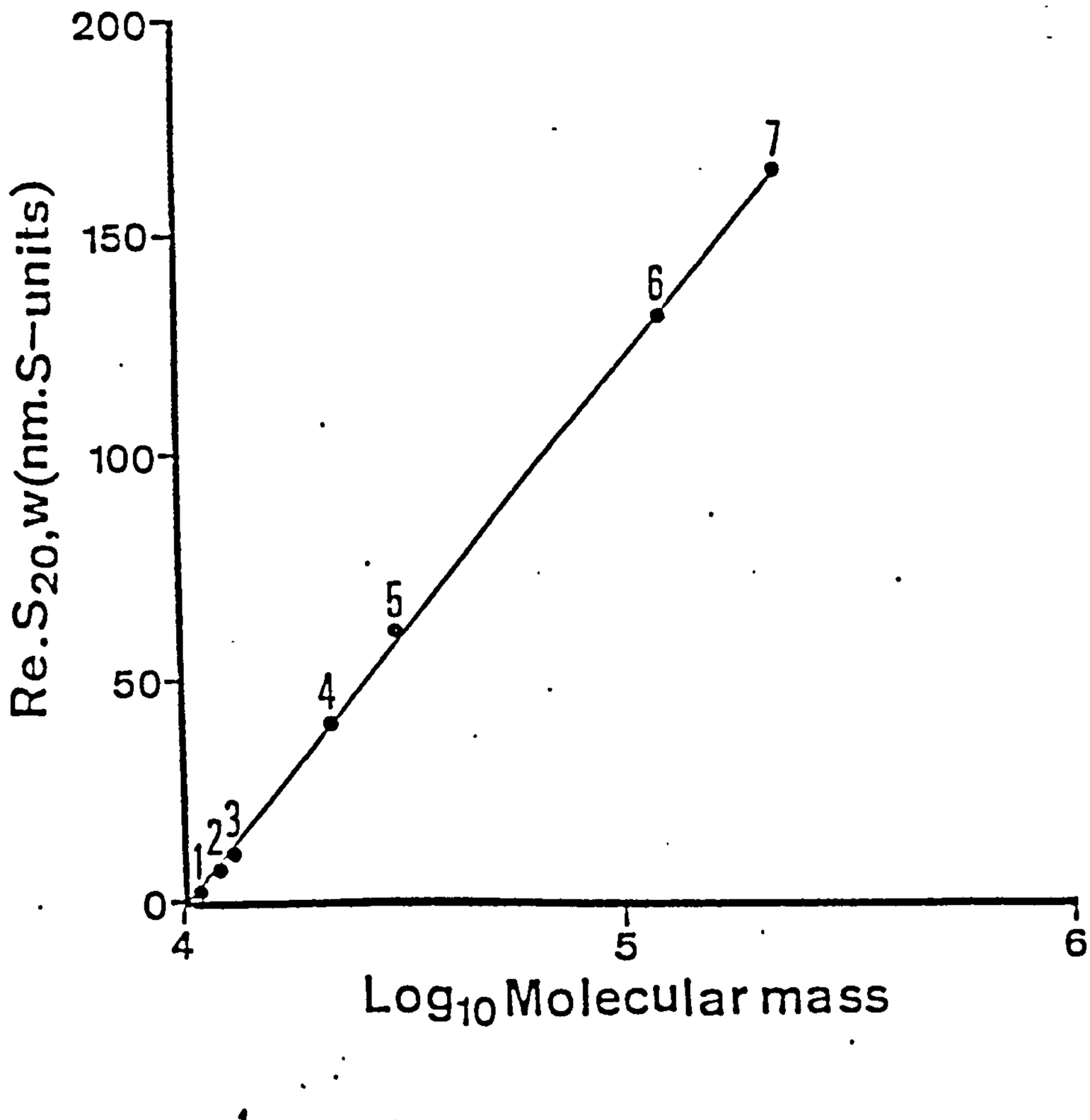
A plot of $Re.S_{20},W$ versus molecular mass is shown in Fig. 7.10 for markers of known Re and S_{20},W value. This is remarkably linear and consequently the value of f could be determined and used in the subsequent calculation of molecular mass, given Re and S_{20},W values. Based upon the markers shown in Fig. 7.10, f = 3958 ± 124 [SD].

The separation of AChE molecular forms achieved by the column as well as the determination of "apparent" and approximate "true" molecular mass are described in Sections 15.1 and 2.

7.4 Polyacrylamide gel electrophoresis [PAGE]

Electrophoresis in continuous buffer systems using constant concentration slab or tube gels has been used to separate AChE and BChE molecular forms, Section 2.6. The separation obtained is dependent on charge as well as molecular mass and differs from those obtained by mass-only dependent methods such as, sucrose density sedimentation and gel filtration. Nevertheless, the technique has been

Fig. 7.10 A plot of Stokes radius [nm] x Sedimentation coefficient versus molecular mass for protein markers. The slope of the graph is 3958 ± 124 nm.S-value.log molecular mass^{-1}.



1— Cytochrome C

2— Haemoglobin

3— Ovalbumin

4— Aldolase

5— Catalase

6— β-galactosidase

7— Thyroglobulin

Fig. 7.10 Stokes radius x sedimentation coefficient versus molecular mass for seven protein markers

widely applied, particularly in the diagnosis of NTD [Section 4.4]. Consequently, it was decided to investigate the results of this technique with amniotic fluid samples. Recent reports have suggested that densitometric scanning of the gels improved the usefulness of the method [Goldfine et al, 1983a,b] and this was investigated. The equipment used consisted of:

Flow cooled gel electrophoresis tank suitable for gel slabs, GE-2/4 LS [Pharmacia Ltd]

LKB Multitemp flow cooler [LKB Instruments Ltd]

LKB 2103 high voltage power supply [LKB Instruments Ltd]

LKB scanning laser densitometer [LKB Instruments Ltd]

The 5% gels were prepared with 5% cross linking by mixing equal quantities of:

1. Acrylamide 19g + N,N-methylene-bis-acrylamide 1g [Electran grade, BDH Chemicals Ltd] dissolved in 100ml water.
2. N,N,N′,N′, tetramethylethylenediamine [TEMED] [Sigma Chemical Co] 100 μl in 100ml TRIS/ glycine buffer, pH8.1, 6g TRIS +29g glycine per litre [Sigma Chemical Co]
3. Water [distilled]
4. Ammonium persulphate [AR grade, BDH Chemicals Ltd] 1.2g/100ml water.

The solutions were degassed with helium and mixed in the order 1, 2, 3, +4 and quickly, but evenly poured, between two 8 x 8cm clean glass plates separated by 3mm plastic or glass spacers and sealed at the bottom and sides. Sample wells were formed by the insertion of a plastic toothed former before the gel had set. The gels were left to harden at $4^{o}C$ overnight.

They were then placed in the electrophoresis tanks containing TRIS/glycine buffer, pH8.1 [1.5g TRIS/7.25g glycine per litre] and initially electrophoresed for one hour at 100V. Samples [up to 30 μl] containing 13% w/w sucrose and 1 μl of 0.5 w/v bromophenol blue were then pipetted into the sample wells. The electrophoresis buffer was maintained at $10^{o}C$ by the flow cooler throughout. The gels were then run at 100V for 20 min followed by 1½ hr at 200V until the bromophenol blue ran from the bottom of the gel.

The gels were removed and carefully stained by a method based on that adopted by Buamah, [1980]:-

To harden gel and partially fix proteins:

Incubation for 30 min in 0.07M sodium maelate buffer pH6.5 [BDH Chemicals Ltd] saturated with sodium sulphate [BDH Chemicals Ltd] and containing 10^{-4}_{M} ethopropazine.

To stain for cholinesterase activity:

Overnight incubation at room temperature
in 0.07M sodium maelate buffer, pH6.5
saturated with sodium sulphate
containing 4mM copper sulphate, 20mM
glycine, 3mM magnesium chloride, [all
obtainable from BDH Chemical Co]
and 4mM acetylthiocholine iodide
together with 5×10^{-5}M ethopropazine.

The white bands of copper thiocholine precipitated as a result of the reaction were then counterstained by immersion for a few minutes in a saturated solution of dithioxamide; this copper-complexing reagent produces a green colour with copper thiocholine and helps visualisation. The gels were then scanned using a laser densitometer.

The results of the traces produced by this high resolution scanning densitometer are shown in Section 10.2. The whole procedure is represented schematically in Fig. 7.11.

Fig. 7.11 A schematic guide to the procedure used for polyacrylamide gel electrophoresis of amniotic fluid samples and the subsequent identification of AChE activity by the method of Buamah, 1981.

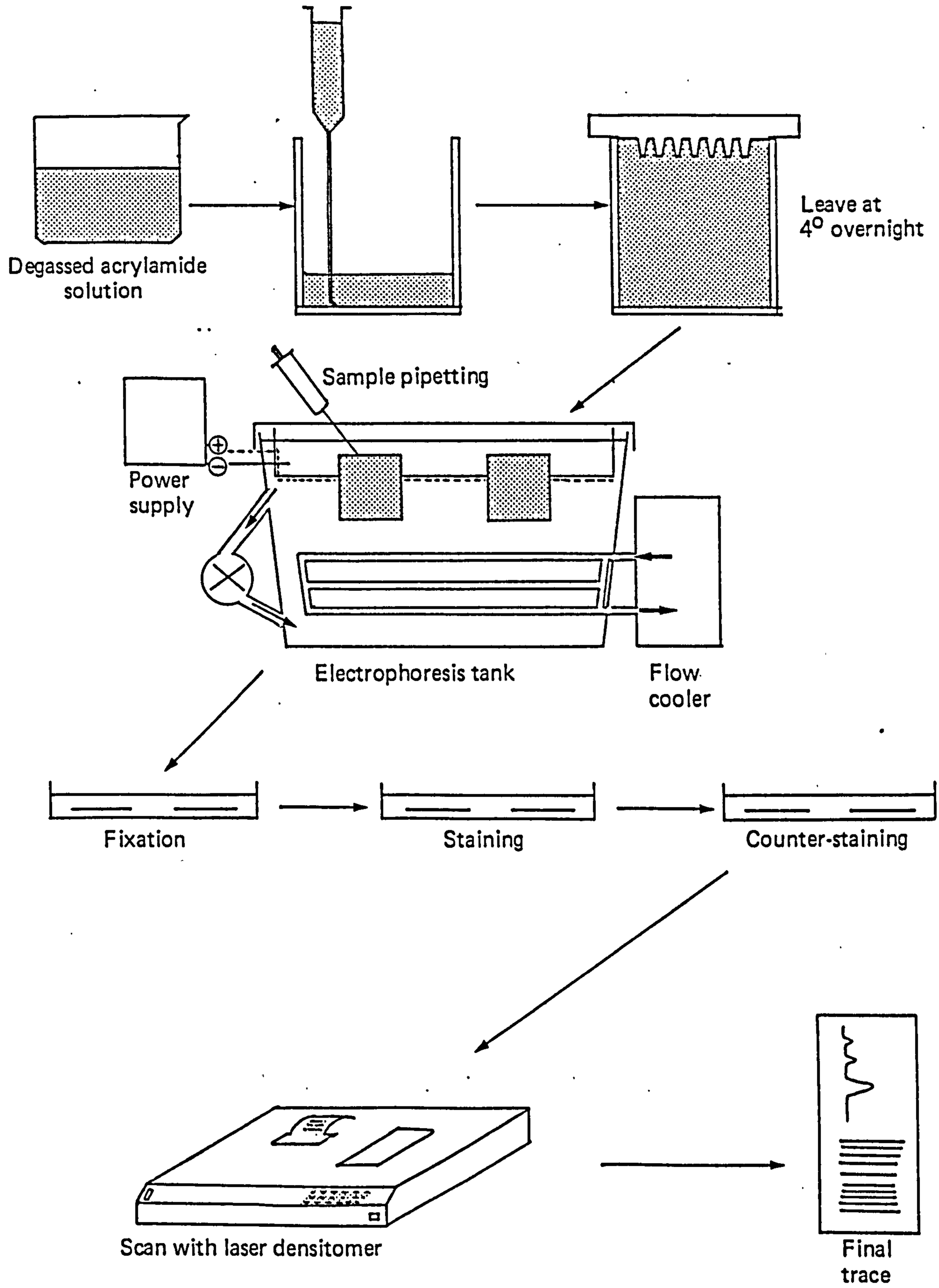


Fig. 7.11 A schematic guide to the procedure used for polyacrylamide gel electrophoresis

Section 8: Materials studied and patients investigated

8.1 Amniotic Fluids

This study group included 39 patients in whom transabdominal amniocentesis was considered desirable on clinical grounds [increased risk of NTD or advancing maternal age enhancing the likelihood of Downs Syndrome].

The gestational age of the pregnancies concerned ranged from 15 - 25 weeks and included 17 with normal outcome, nine with an anencephalic fetus, seven with spina bifida, five with exomphalos and one pregnancy with subsequently confirmed intra-uterine death.

AFP levels were measured as described in Section 6.4 and aliquots were then stored at -20^{o}C. The average length of time of storage was six months, sufficient to allow the outcome to be established. The effect of this type of storage on the activity of individual molecular forms of AChE is reported in Section 10.1.

Prior to measurement of AChE or analysis by other techniques including sucrose density sedimentation, the specimens were thawed and spun at 15,000g for 3 min in an Eppendorf centrifuge.

Frequently the amount of specimen available

was limited and the techniques for separation of the molecular forms were designed in a way to accommodate this, e.g. for sucrose density sedimentation only 200-300 μl of amniotic fluid was required. Gel filtration, because of the high dilution of the original sample required that amniotic fluids were concentrated before use. This was performed as described for the pooled fractions from preparative density sedimentation [Section 7.2].

Amniotic fluids could be used for PAGE studies without prior concentration. Pooled fractions obtained from sucrose density sedimentation runs were used for various studies and are outlined in the experiments concerned [Section 15.1-4].

8.2 Biopsy specimens

Over a seven year period 213 children were investigated for chronic constipation or failure to pass meconium. Suction rectal biopsies were made in each of these cases both for histological/ histochemical assessment [Section 6.3] and for quantitative measurement of AChE activity [Section 6.2].

On receipt, the specimens were dealt with as outlined in Section 6.2. On the basis of histological classification 45 of these 213 were subsequently confirmed to have Hirschsprung's disease.

The results of quantitative AChE measurement are reported in Section 11.

8.3 Rectal "pull-through" specimens

During corrective surgery using the Soave operative technique, tissue was removed and sent to the Pathology Department for histological confirmation of the disease.

The tissue included rectal sleeve [dissected out during operation and consisting of mucosa only], frequently together with more proximal upper rectum and sigmoid colon [including both muscle and mucosal layers]. The tubular specimen was opened using dissecting scissors and pinned out onto a cork board. Three strips were then cut longitudinally; one was used for histological investigation, one for histochemical assessment and one for biochemical measurement of AChE and BChE molecular forms. The laterally adjacent strips were then divided transversely into between five and nine sections of 2-3cm length dependent upon the overall length of the specimen. In this way histochemistry, histology and biochemistry could all be directly compared in transversely adjacent segments. The samples for histology were fixed in formalin and embedded in

paraffin wax; those for histochemistry were immediately frozen and 8 μm sections cut for cholinesterase staining and those for biochemical investigation individually placed in screw capped tubes for storage in liquid nitrogen. The whole procedure from surgical removal to liquid nitrogen storage took less than three hours.

Material was obtained and processed in this way from seven patients:

Case A: Rectal sleeve measuring 10cm consisting of mucosal layer only from a male infant aged ten months.

Case B: A 15cm length of bowel including upper rectum and sigmoid colon involving both muscle and mucosal layers obtained from a male infant aged eleven months.

Case C: A 24cm length of bowel including distal colon and rectum comprising both muscle and mucosal layers from a male infant aged eleven months.

Case D: A 20cm length of descending colon containing both muscle and mucosal layers from a male infant aged eleven months.

Case E: Rectal sleeve measuring 14cm consisting of mucosal layer only from a male infant aged one year.

Case F: Control tissue; a 30cm segment of bowel extending from upper rectum into sigmoid colon and containing both muscle and mucosal layers, excised from a 14 year old girl on clinical grounds other than Hirschsprung's disease.

Case G: Rectal sleeve and upper rectum measuring 20cm and including both muscle and mucosal layers taken from a female infant aged one year. Used for gel filtration studies only.

Case A - E and G had histologically confirmed Hirschsprung's disease.

When both muscle and mucosa were present these were separated under a binocular microscope after removal from liquid nitrogen storage and immediately before molecular form analysis. When only one layer was analysed the remaining dissected tissue was replaced into liquid nitrogen storage.

These samples were homogenised in 0.1M phosphate buffer, pH8.0 as outlined in Section 6.2, unlike the biopsy specimens however, the amount of buffer used was adjusted to give a final homogenate of 5% w/v. In one case, E, the initial homogenisation was performed by only 20 passes of the homogeniser using 0.1M phosphate buffer, pH7.3, this was followed

by resuspension of the pellet in 0.01M phosphate buffer, pH7.2 containing 1M NaCl, 1mM EDTA and 0.5% w/v Triton X-100 all obtainable from BDH Chemicals Ltd. These two methods differentiated Triton releasable [possibly membrane-bound] forms from easily solubilised species. In all cases sucrose density sedimentation was carried out on the same day that the homogenates were made. 200-300 μl of 5% w/v homogenate was used for each gradient, in total 65 samples were processed for sedimentation analysis and the results are shown in Section 12.2-6.

The preparation of tissue samples as explained above is shown schematically in Fig. 8.1.

Gel filtration studies were performed on homogenates made from Cases C and G. The extraction buffer was 0.1M phosphate, pH8.0.

Tissue from cases C and G was used in the preparative scale sucrose density sedimentation described in Section 7.2.

Where tissue from other sources is used in various experiments this is detailed within the description of these experiments.

Fig. 8.1 A schematic guide to the tissue preparation procedure employed in "rectal pull through" specimens. The procedure includes storage in liquid nitrogen prior to further microscopic dissection of the samples and the measurement of AChE and BChE molecular forms by sedimentation or gel filtration.

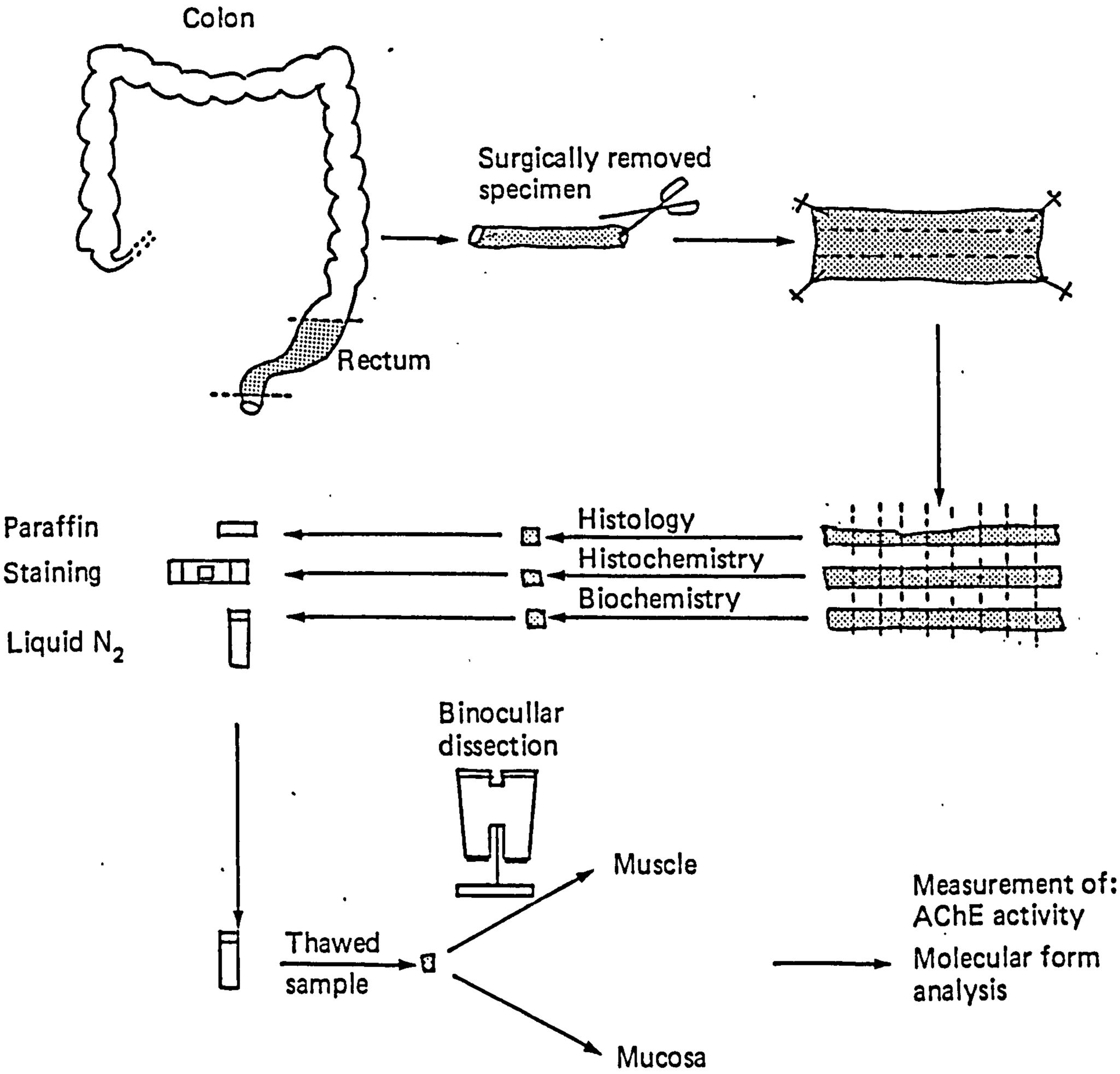


Fig. 8.1 A schematic guide to the tissue preparation used for "rectal pull through" specimens

R E S U L T S

Section 9 : AChE measurement in amniotic fluid

Section 10: Molecular forms of cholinesterases in amniotic fluid

Section 11: Measurement of total AChE activity in rectal biopsy specimens

Section 12: Molecular forms of AChE in ganglionic and aganglionic bowel

Section 13: Molecular forms of BChE in ganglionic and aganglionic bowel

Section 14: Summary of the results from amniotic fluid and resected bowel segments

Section 15: Characterisation of AChE and BChE molecular forms.

Section 9: AChE measurement in amniotic fluid

9.1 Comparison of results by "manual" and "CFA" assay

Dale et al, [1981] reported the range of AChE in amniotic fluid for 101 pregnancies with a normal outcome as 0.4-5.8 U/l [Mean 2.56 ± 1.1 [SD] U/l], these values compare well with others reported in the literature, they go on to report a coefficient of variation from ten replicate samples of 3.7%.

Automation of this assay would be expected to have several advantages including: increased speed of assay when large numbers are being analysed, improved reproducibility and smaller sample volume, [Section 6.1]. However, good comparison with the manual assay would be important if the automated technique were to replace it without affecting interpretation. An assay was designed to comply with the requirements of the laboratory centrifugal fast analyser [CFA] [Cobas Bio, Roche Ltd, UK] without any change in the final reagent concentration. The assay parameters and reagent composition are described in Section 6.1.

Twenty four amniotic fluid samples including 15 with normal outcome and nine affected by NTD were assayed by both methods and the results compared, Fig. 9.1. The correlation was excellent with a coefficient, r = 0.99, a linear regression of y, [CFA assay] Vs x, [manual assay] gave a straight

Fig. 9.1 The correlation between results derived using: automated [centrifugal fast analyser] and manual methods for measuring AChE activity in amniotic fluid.

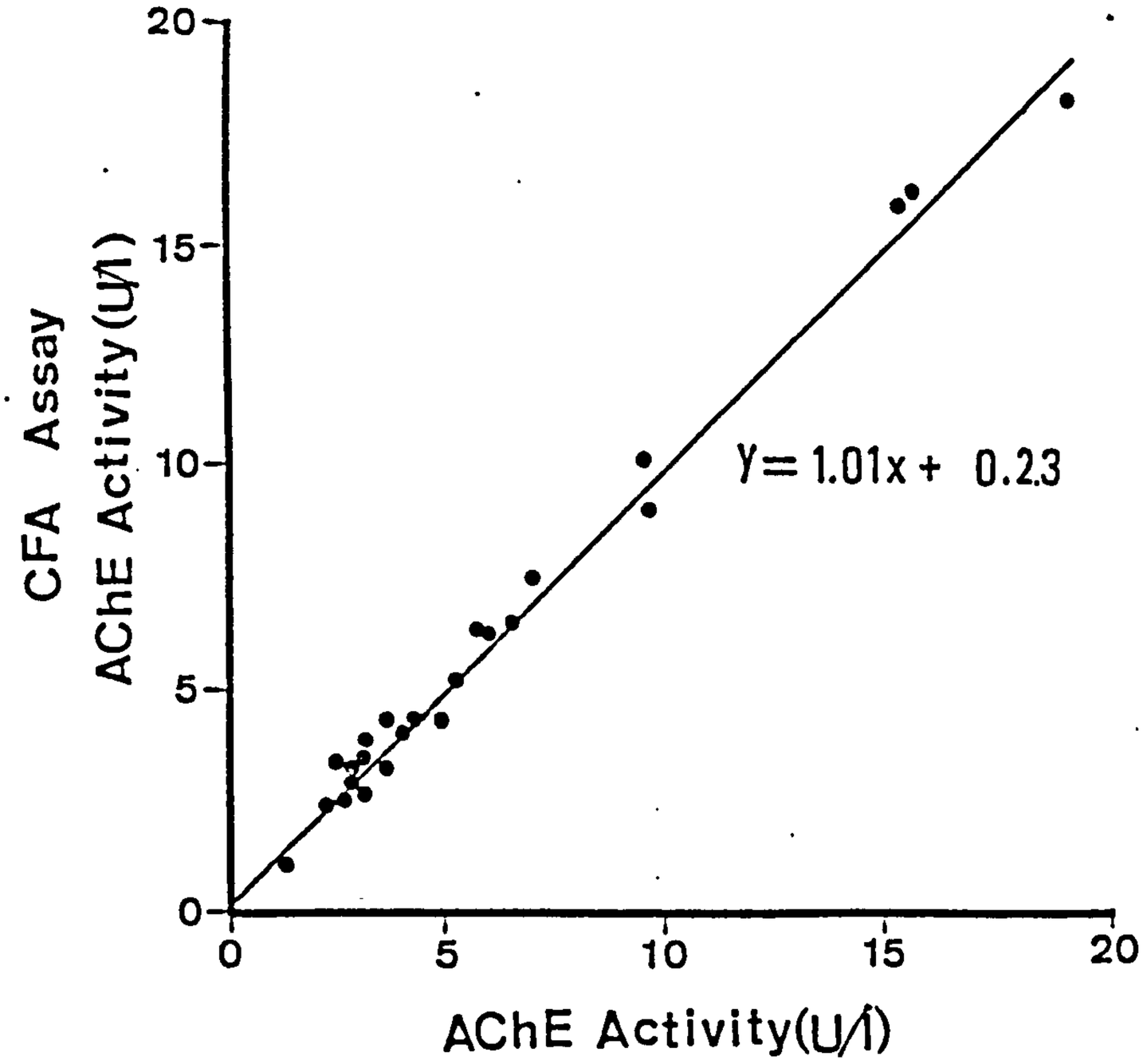


Fig. 9.1 Automated and manual assays for measurement of AChE in amniotic fluid compared

line of the equation y = 1.01x + 0.23. The coefficient of variation was 2.1% for 13 replicates at a mean activity of 2.9 U/l.

The good correlation between "manual" and "CFA" assays meant that these could be used interchangeably, using the manual assay for single samples and the faster automated assay when larger batches were being analysed. This flexibility was useful when considering a routine service for diagnosis of NTD.

9.2 AChE and AFP results from the amniotic fluid samples for molecular form analysis

The AChE and AFP results from the 39 amniotic fluid samples subsequently studied by density gradient sedimentation analysis are shown in Table 9.1.

All normal pregnancies gave results within the normal reference range 0.4 - 5.8 U/l quoted by Dale et al, [1981], and while some of the NTD pregnancies were close to the upper limit of this this range [N 4; AChE = 5.1 U/l], all were correctly classified by the test. Two of the NTD-affected cases [SB1 and SB6] were reported to have "border-line" AFP levels of 54 and 48 μg/ml respectively. This is important when comparing the sensitivity with which lesions may be detected, large lesions with markedly elevated AFP results are relatively easy to classify correctly by most techniques.

Table 9.1 Gestational age, AFP level and AChE activity in the amniotic fluid samples under study. The results are grouped on the basis of pregnancy outcome.

Table 9.1 Gestational age, AFP and AChE levels of the amniotic fluid specimens under study

Pregnancies with normal outcome				NTD affected pregnancies				Other fetal malformations			
Case Number	GA (wks)	AFP (μg/ml)	AChE (U/l)	Case Number	GA (wks)	AFP (μg/ml)	AChE (U/l)	Case Number	GA (wks)	AFP (μg/ml)	AChE (U/l)
N 1	16	16	4.5	An1	16	369	17.1	IUD 1	16	275	15.1
N 2	15	23	3.2	An2	17	350	14.8				
N 3	18	40	3.0	An3	*	374	8.4	Ex1	17	87	3.4
N 4	16	46	5.1	An4	19	365	19.0	Ex2	19	376	5.0
N 5	16	41	2.8	An5	16	402	18.7	Ex3	25	123	4.6
N 6	16	41	3.5	An6	16	425	17.9	Ex4	25	75	4.6
N 7	16	36	2.8	An7	23	226	6.1	Ex5	17	177	4.0
N 8	16	32	5.2	An8	*	362	18.7				
N 9	16	26	3.7	An9	16	284	16.8				
N10	18	18	2.7								
N11	17	16	3.5	SB1	17	54	7.1				
N12	16	20	2.4	SB2	18	120	10.9				
N13	17	22	3.8	SB3	16	78	6.3				
N14	17	28	3.9	SB4	17	99	6.8				
N15	16	26	3.0	SB5	16	69	9.1				
N16	15	36	2.5	SB6	18	48	6.7				
N17	19	20	4.2	SB7	15	150	13.6				

N - Normal An - Anencephalic SB - Spina Bifida IUD - Intrauterine death Ex - Exomphalos

* - Not recorded GA - Gestational Age

Cases with borderline AFP levels allow some assessment of the practical additional value and sensitivity of a new assay.

The five cases of exomphalos all gave AChE results within the normal range whereas the single case of intrauterine death had a distinctly elevated AChE activity.

Section 10: Molecular forms of cholinesterases in amniotic fluid

10.1 Sucrose density sedimentation results

(i) Quantitation of AChE molecular forms

Samples of amniotic fluid from the 39 cases listed in Table 9.1 were carefully layered onto preformed sucrose gradients and the centrifugation, fractionation, measurement and calculation of results carried out as outlined in Section 7.1(vii).

The results from these gradients are shown in Figs. 10.1-5. In all cases of NTD and the single case of intrauterine death there was a prominent peak migrating in the region of the catalase marker. This peak had sedimentation coefficient of 10.3 ± 0.5S, the position characteristic of G_4 AChE [Section 2.2]. This form of the enzyme was either entirely absent or present only in trace amounts in pregnancies with normal outcome. In exomphalos cases where there is leakage of fetal plasma, with exposure of neural tissue, G_4 was present at a low activity [Fig. 10.5]

The actual amounts of the three forms G_1, G_2 and G_4 were quantitated as described in Section 7.1. and the results are shown in Table 10.1. It can be seen from these results that the increase in AChE activity in amniotic fluids from NTD affected pregnancies is due largely to the presence of G_4 AChE.

Figs. 10.1-5 The heterogeneity of AChE in amniotic fluid demonstrated by sucrose density gradient sedimentation. Samples include those from: pregnancies with normal outcome [N1 - N17] and those affected by anencephaly [An1-9] spina bifida [SB 1-7] exomphalos [Ex 1-5] and intrauterine death [IUD]. Markers with known sedimentation are shown: βgalactosidase [gal,S_{20},W = 16.0S]; catalase [cat,S_{20},W = 11.3S] and alcohol dehydrogenase [adh,S_{20},W - 4.8S]. The molecular forms of AChE identified on the basis of their sedimentation coefficient are labelled G_1, G_2 and G_4.

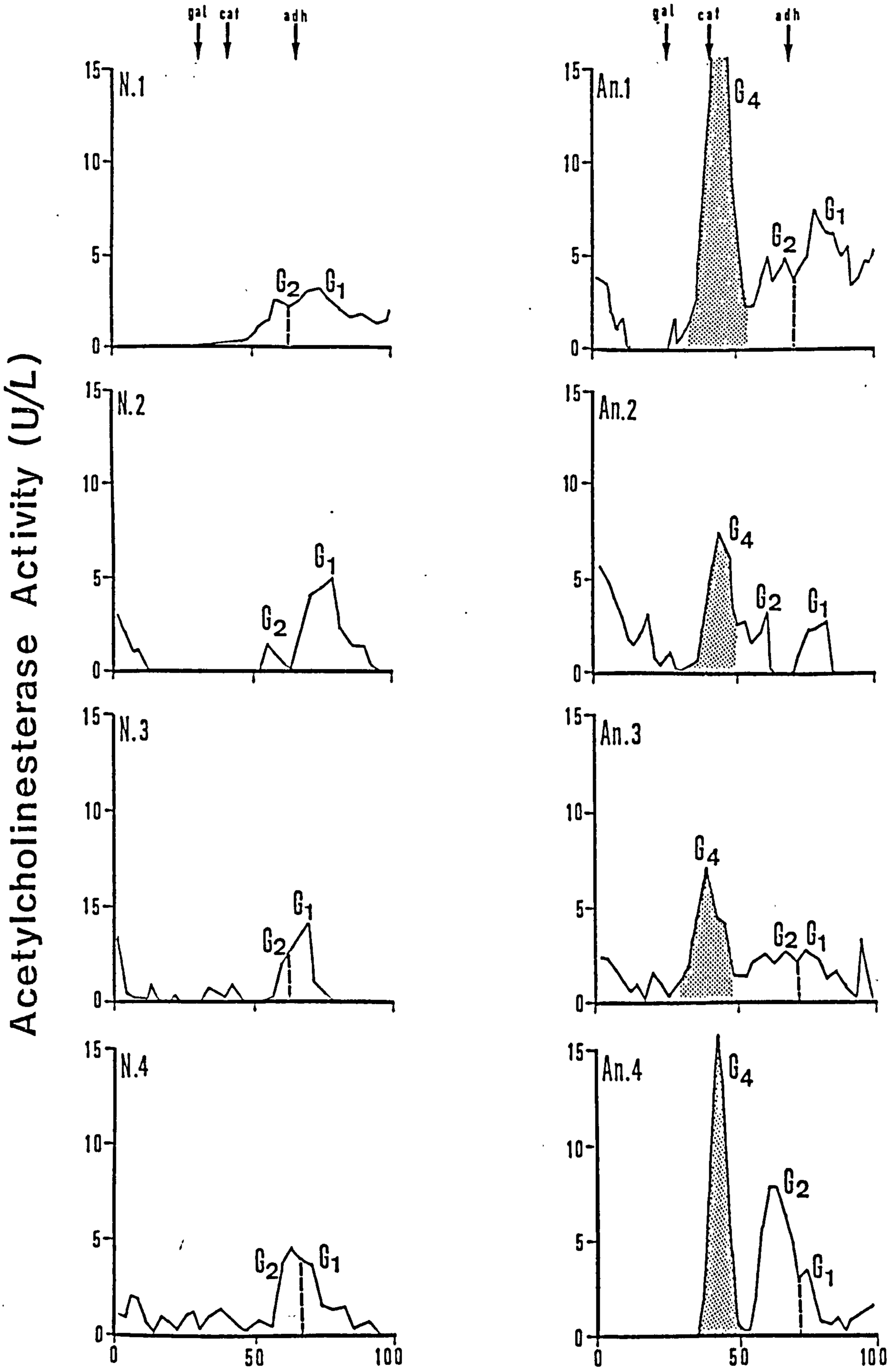


Fig. 10.1 Molecular forms of AChE in cases N1-4 and An1-4

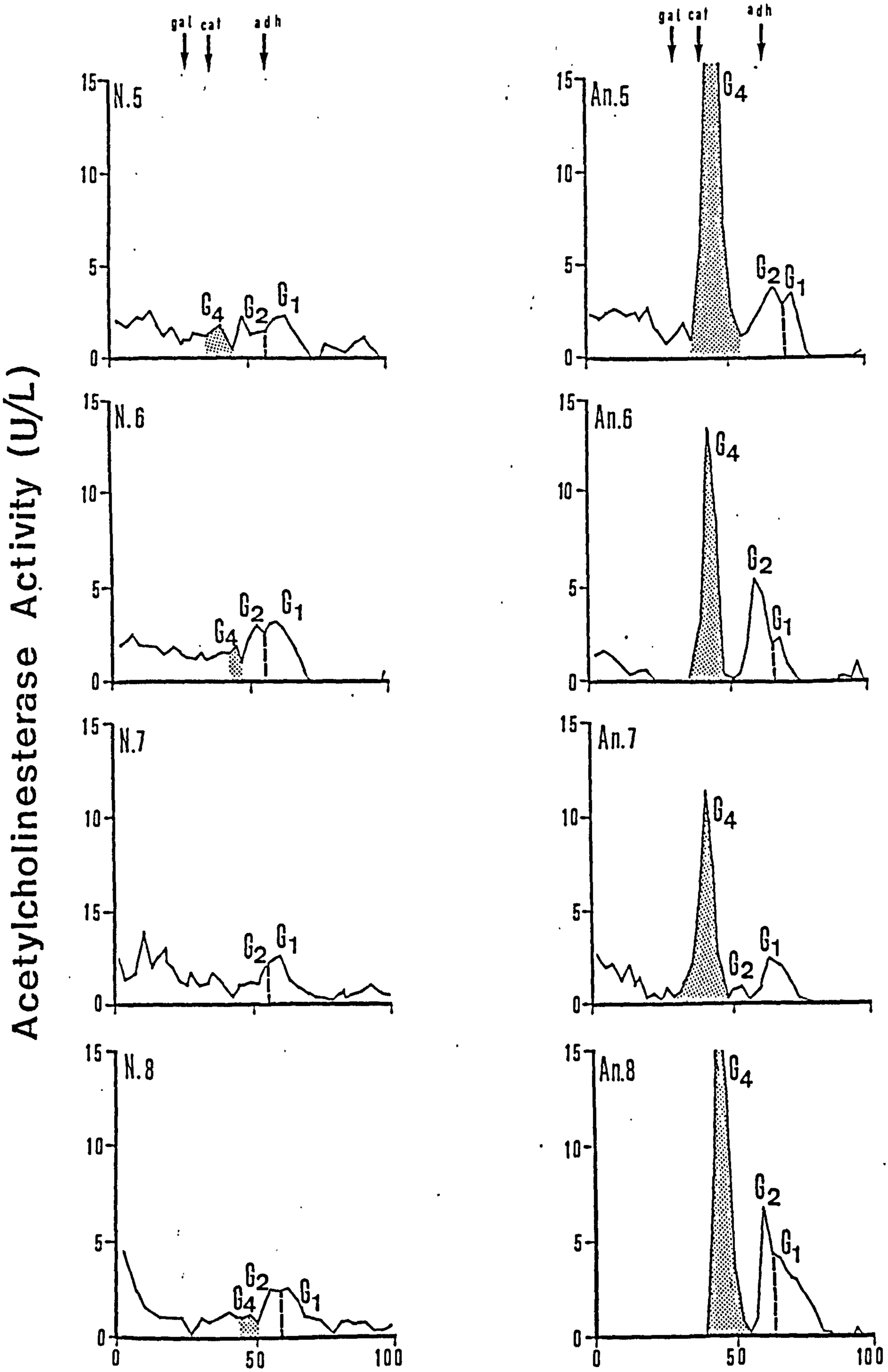


Fig. 10.2 Molecular forms of AChE in Cases N5-8 and An5-8

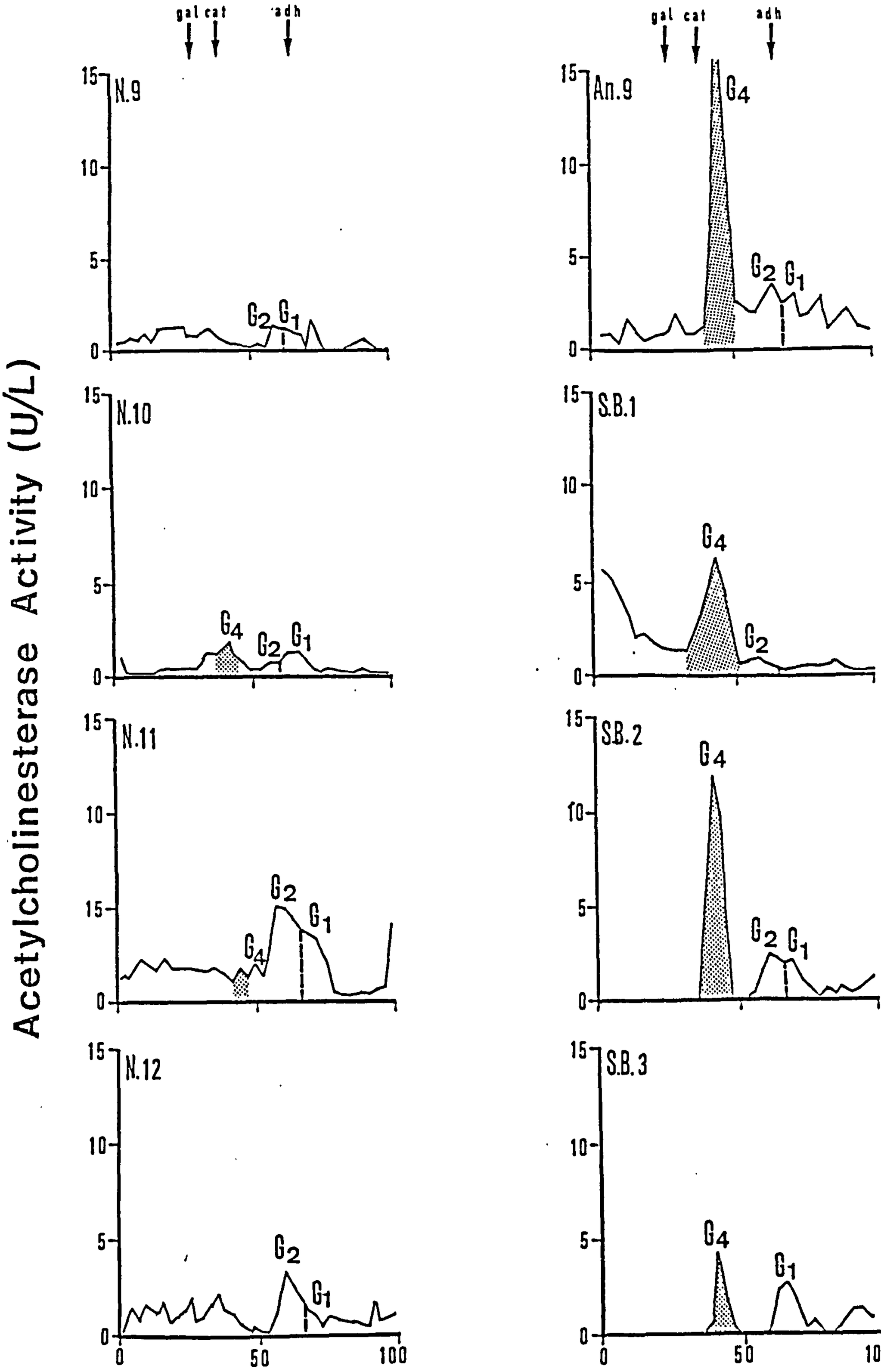


Fig. 10.3 Molecular forms of AChE in Cases N9-12, An9 and SB1-3

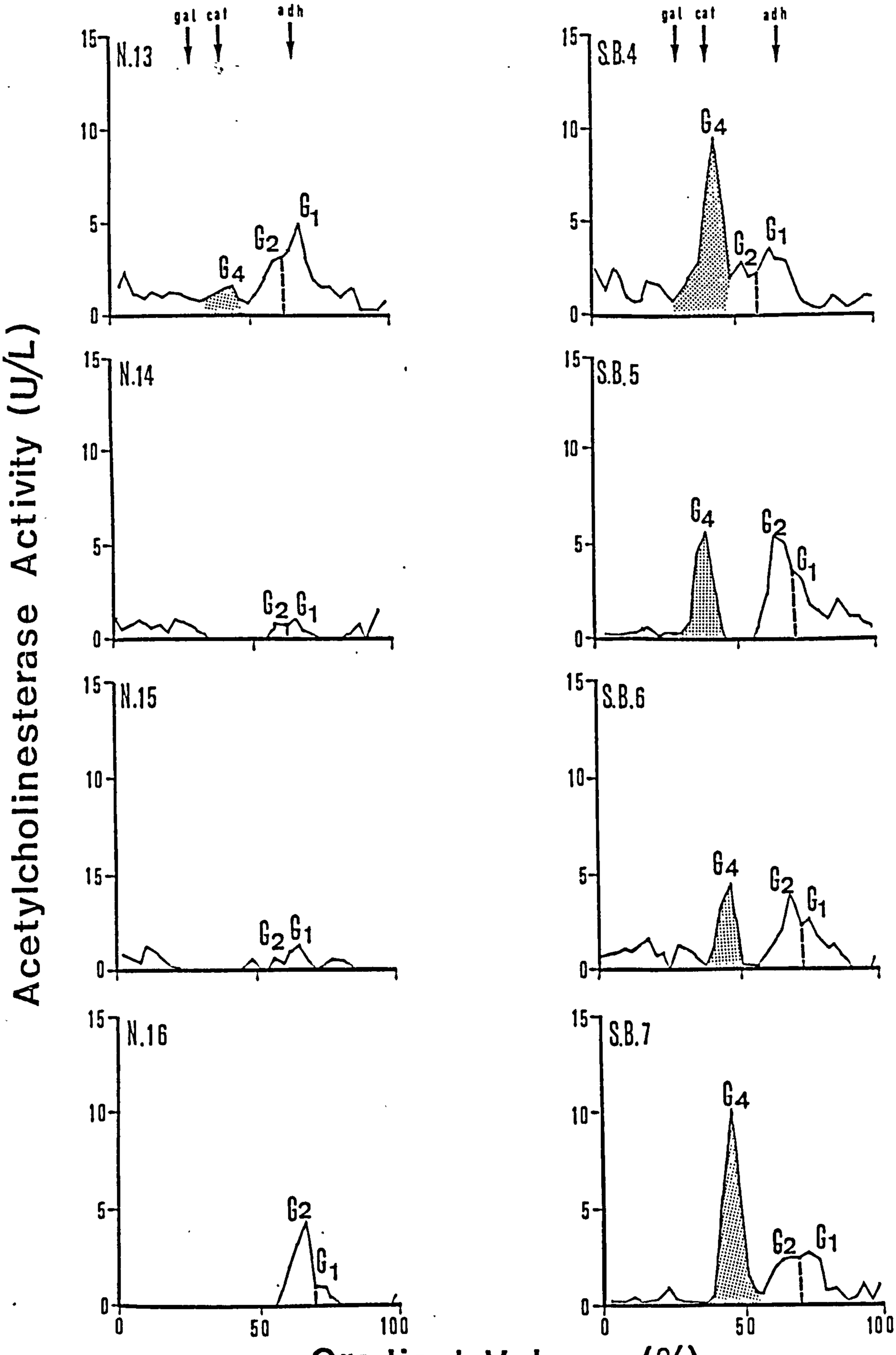


Fig. 10.4 Molecular Forms of AChE in Cases N13-16 and SB 4-7

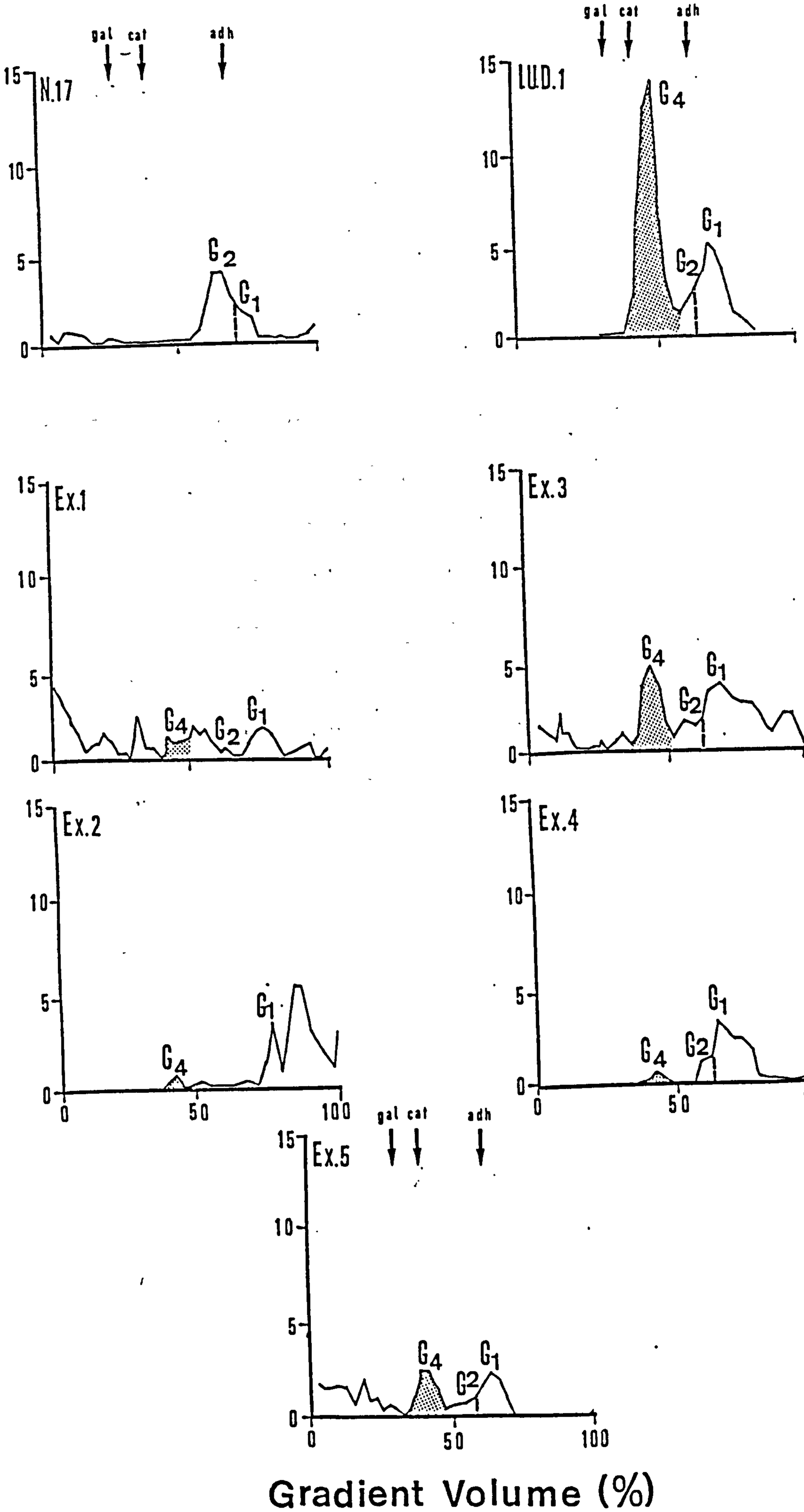


Fig. 10.5 Molecular forms of AChE in Cases N17, IUD1 and Ex1-5

Table 10.1 Quantitive measurement of AChE molecular forms (G_1, G_2 and G_4). The activity of each form present was calculated from the area of the corresponding peak of the sedimentation profile. The mean and standard deviation [SD] of each form is shown for amniotic fluid from normal pregnancies and those affected by neural tube defects [NTD].

Table 10.1 Quantitation of AChE molecular forms in amniotic fluid samples

Pregnancies with normal outcome				NTD affected pregnancies				Other fetal malformations			
Case Number	G_4	G_2	G_1	Case Number	G_4	G_2	G_1	Case Number	G_4	G_2	G_1
	[U/l]				[U/l]				[U/l]		
N 1	nil	0.7	2.3	An1	7.8	1.1	2.6	IUD 1	9.4	0.9	4.2
N 2	nil	0.7	2.2	An2	5.5	1.3	1.7				
N 3	nil	0.7	1.4	An3	3.4	0.9	0.7	Ex1	0.6	0.3	0.8
N 4	nil	2.0	1.3	An4	8.1	7.7	1.5	Ex2	0.1	nil	0.9
N 5	0.4	0.2	0.5	An5	10.9	2.1	1.1	Ex3	1.2	0.3	1.4
N 6	0.3	0.7	1.2	An6	9.2	4.4	1.0	Ex4	0.3	0.6	2.1
N 7	nil	0.3	0.4	An7	4.3	0.3	1.4	Ex5	0.9	0.2	1.0
N 8	0.4	1.3	1.5	An8	11.3	3.2	3.9				
N 9	nil	0.6	0.6	An9	8.8	1.4	1.2				
N10	0.6	0.3	0.6								
N11	0.2	1.1	0.5	SB1	4.4	0.5	nil				
N12	nil	1.2	0.1	SB2	7.1	2.0	1.1				
N13	0.2	0.7	1.0	SB3	1.8	nil	2.5				
N14	nil	0.3	0.4	SB4	2.8	0.5	1.4				
N15	nil	0.2	0.8	SB5	2.5	3.3	1.6				
N16	nil	1.8	0.3	SB6	1.6	1.9	0.7				
N17	nil	2.5	0.3	SB7	7.0	2.9	2.0				
Mean ± SD:	0.1±0.2	0.9±0.7	0.9±0.7		6.0±3.2	2.1±1.9	1.5±0.9				
Range :	[0-0.6]	[0.3-2.0]	[0-2.3]		[1.6-11.3]	[0-7.7]	[0-3.9]				

N - Normal An - Anencephalic SB - Spina Bifida IUD - Intrauterine death
Ex - Exomphalos

In Fig. 10.6 the levels of each form are compared. The increase in total AChE activity reflected the increase in G_4 [p.<0.0005], whereas increases in G_1 and G_2 were less significant [$p < 0.05$, $p < 0.02$ respectively]. Measurement of G_4 offers the advantage that in normals the activity of this form is clustered around zero. Consequently assessment of G_4 AChE provides the potential for a simple "yes or no" test for detection of NTD.

[ii] <u>Determination of sedimentation coefficient of AChE forms</u>

The sedimentation coefficient for each of the AChE species was determined by curvilinear interpolation between protein markers as outlined in Section 7.1.

The results are shown below:-

Species	Mean	SD	n
G_4	10.3	0.5	26
G_2	5.5	0.7	32
G_1	4.0	0.6	32

[iii] <u>Stability of molecular forms on storage</u>

Under certain conditions AChE molecular may interconvert [Section 2.2]. In particular, larger forms, such as G_4 may dissociate to the monomeric

Fig. 10.6 A graphic representation of the activities of AChE molecular forms G_1, G_2 and G_4 in amniotic fluid obtained from pregnancies with various outcomes including:

Intrauterine death —— □

Anencephaly ———— △

Spina bifida ———— ○

Exomphalos ———— ▲

Normal ———————— ●

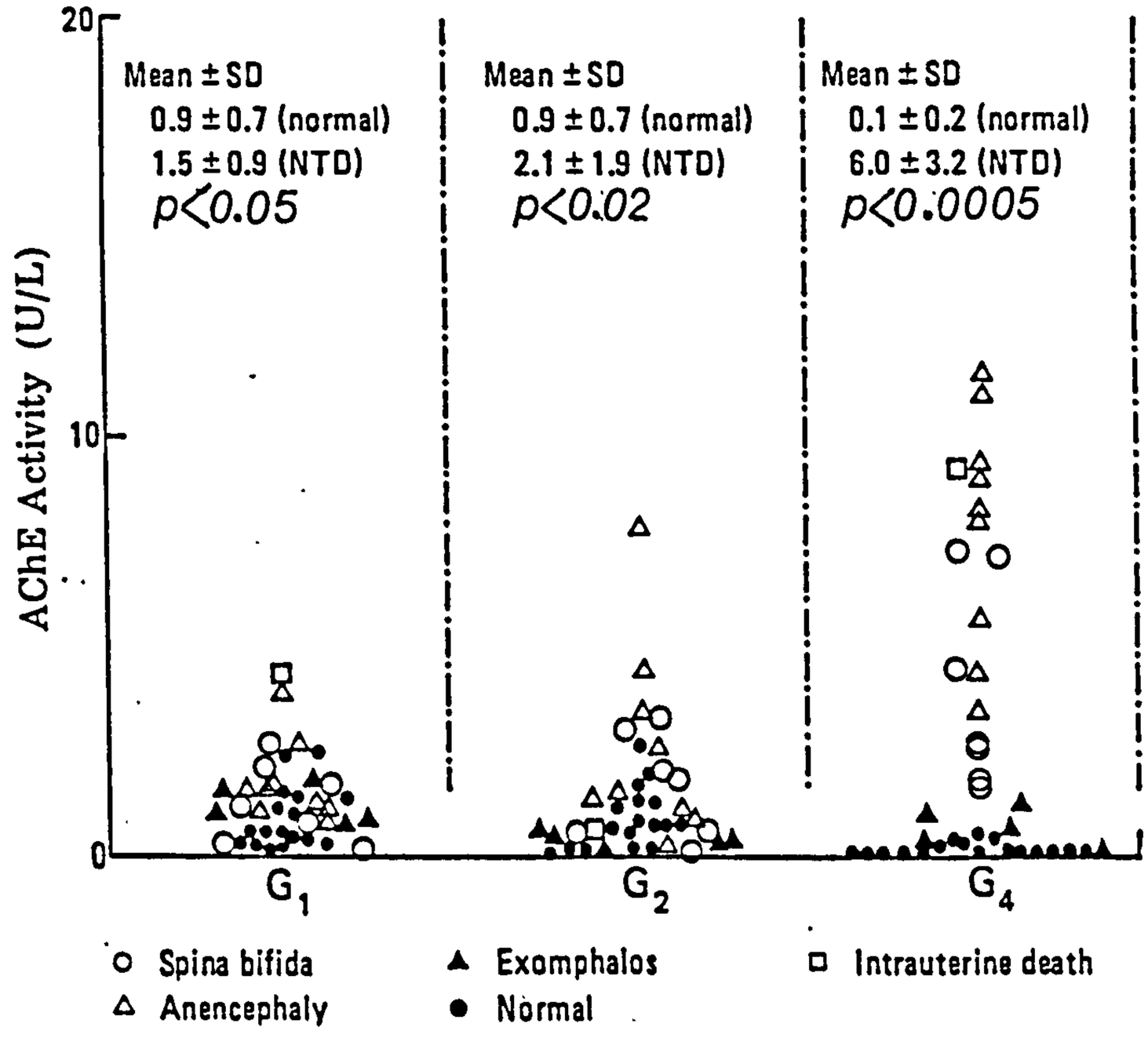


Fig. 10.6 The activity of G_1, G_2 and G_4 AChE compared in amniotic fluid samples from pregnancies with varying outcome

species G_1. To measure the extent of these storage-induced changes freshly obtained amniotic fluid [Case A5] was divided into three aliquots:- one was analysed immediately, one was stored at -20°C for four months and one stored at 4°C for six weeks.

AChE species	Analysed Immediately		Stored at -20°C for four months		Stored at 4°C for six weeks	
	Activity [U/l]	% of each form	Activity [U/l]	% of each form	Activity [U/l]	% of each form
G_4	10.9	77%	9.2	83%	2.2	8%
G_2	2.1	15%	1.3	12%	1.2	5%
G_1	1.1	6%	0.6	5%	10.2	75%

Prolonged storage at -20°C produced little change in the molecular form profile whereas following six weeks storage at 4°C much of the G_4 had dissociated with a resultant increase in G_1 activity. Patient specimens were all stored at -20°C.

10.2 Polyacrylamide gel electrophoresis [PAGE]

The identification of a prominent AChE band on PAGE of amniotic fluid specimens from NTD affected pregnancies has been widely reported [see Section 4.4].

More recently densitometric scanning of gels has been applied to improve both the objectivity and sensitivity of the technique [Section 4.4].

To verify these findings and compare the migration of G_4 AChE recovered from sedimentation gradients with the abnormal peak observed on electrophoresis,several samples including: two from anencephalic, two from spina bifida, two from exomphalos and three from normal pregnancies were subjected to electrophoresis in 5% PAG slab gels by the method of Buamah, [1980] as described in Section 7.4. In addition, fractions from sedimentation analyses of amniotic fluid from pregnancies complicated by anencephaly were pooled to provide a source of G_4 and concentrated by vacumn ultrafiltration.

These samples were electrophoresed and the gels stained and scanned as outlined in Section 7.4. The results are shown in Fig. 10.7. A prominent peak was observed in all cases of NTD but not in normal or exomphalos affected cases. G_4 AChE recovered from the gradient migrated in a manner identical with the peak seen in the NTD pregnancies.

These findings confirm that,not only is G_4 largely responsible for the increase in AChE activity measured quantitatively, but also gives

Fig. 10.7 Densitometric scans of amniotic fluid samples subjected to polyacrylamide gel electrophoresis and stained for AChE activity. G_4 obtained by density sedimentation co-migrates with the peak characteristic of neural tube defect affected pregnancies. Pregnancies with normal outcome or affected by exomphalos show no corresponding peak.

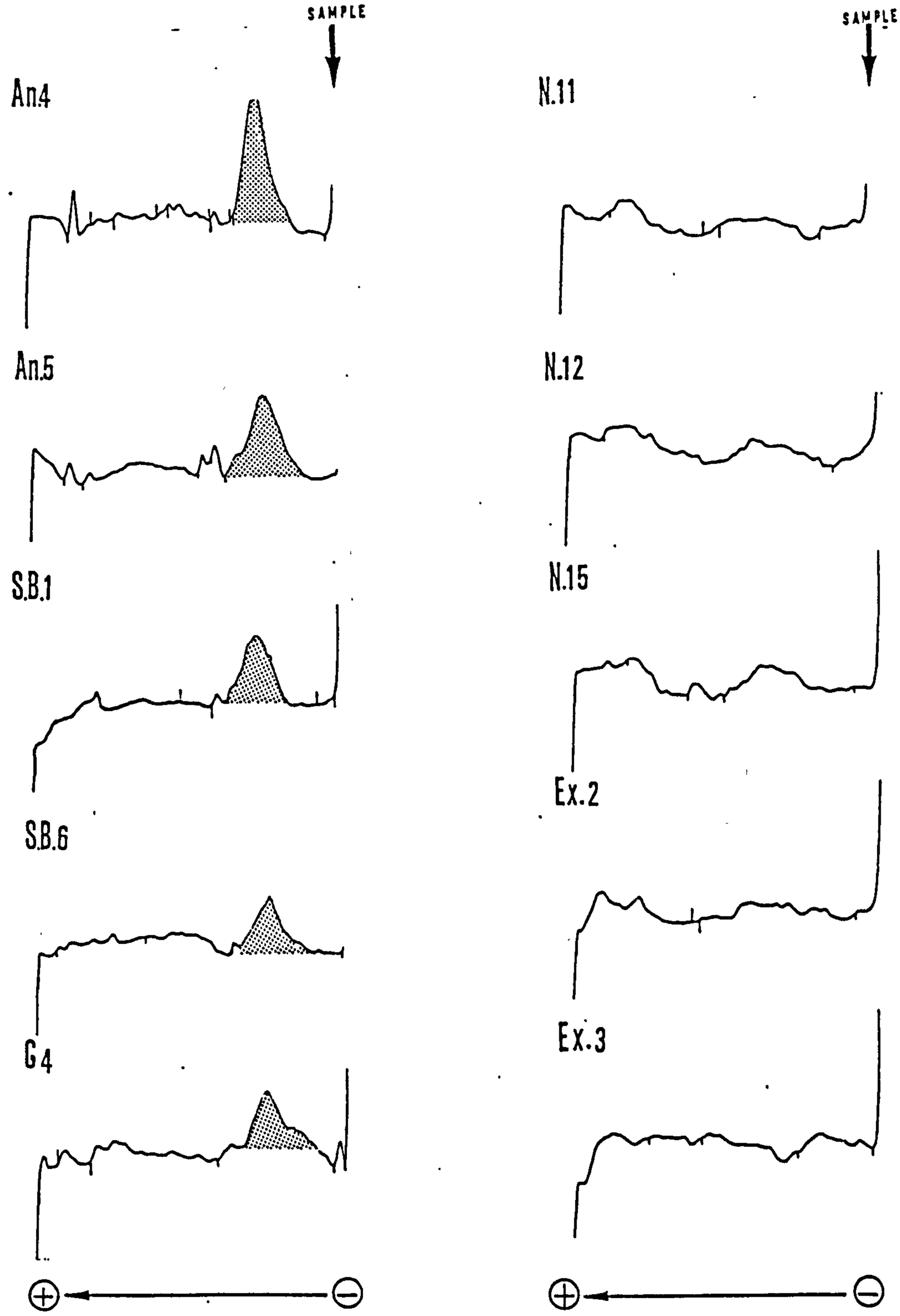


Fig. 10.7 Polyacrylamide gel electrophoresis of AChE in amniotic fluid from pregnancies with varying outcome.

rise to the abnormal peak seen electrophoretically in NTD cases. It therefore appears that this form of AChE is a very useful marker for exposed nervous tissue in utero and for practical purposes may be considered "NTD-specific".

10.3 BChE molecular forms in amniotic fluid

BChE exists in a series of molecular forms similar to those described for AChE i.e. G_1, G_2, G_4, A_8 and A_{12} [Section 2.1]. The predominant BChE species in serum is the tetrameric G_4 form sedimenting at approximately 11.0S [Section 2.3].

The 39 amniotic fluid samples in which AChE forms were determined were also analysed to determine the profile of BChE species present. As in serum, the predominant form in all cases was G_4 sedimenting at 11.6 ± 0.4S. In addition less rapidly sedimenting species in the region of 4.5 ± 0.5S and 6.3 ± 0.7S were identified. These were believed to correspond to G_1 and G_2 forms respectively.

In pregnancies affected by NTD there was a significant increase in G_4 BChE activity [$p < 0.001$; Fig. 10.8]. The actual levels are shown below:-

Pregnancy outcome	G_4-BChE activity [U/l]	Number in group
Normal	4.1 ± 3.3	[n = 17]
NTD group	19.2 ± 10.6	[n = 16]
Exomphalos group	19.5 ± 12.6	[n = 5]
Intrauterine death	47.7	[n = 1]

Fig. 10.8 The heterogeneity of BChE in amniotic fluid demonstrated by sucrose density gradient sedimentation. Samples include those from pregnancies with normal outcome [N1-4] and those affected by anencephaly [An1], spina bifida [SB1], exomphalœs [EX1] and intrauterine death [IUD]. Markers of known sedimentation coefficients are shown: β galactosidase [gal,S_{20},W = 16.0S]; catalase [cat,S_{20},W = 11.3S] and alcohol dehydrogenase [adh,S_{20},W = 4.8S]. The molecular forms of AChE identified on the basis of their sedimentation coefficient are labelled G_1, G_2 and G_4.

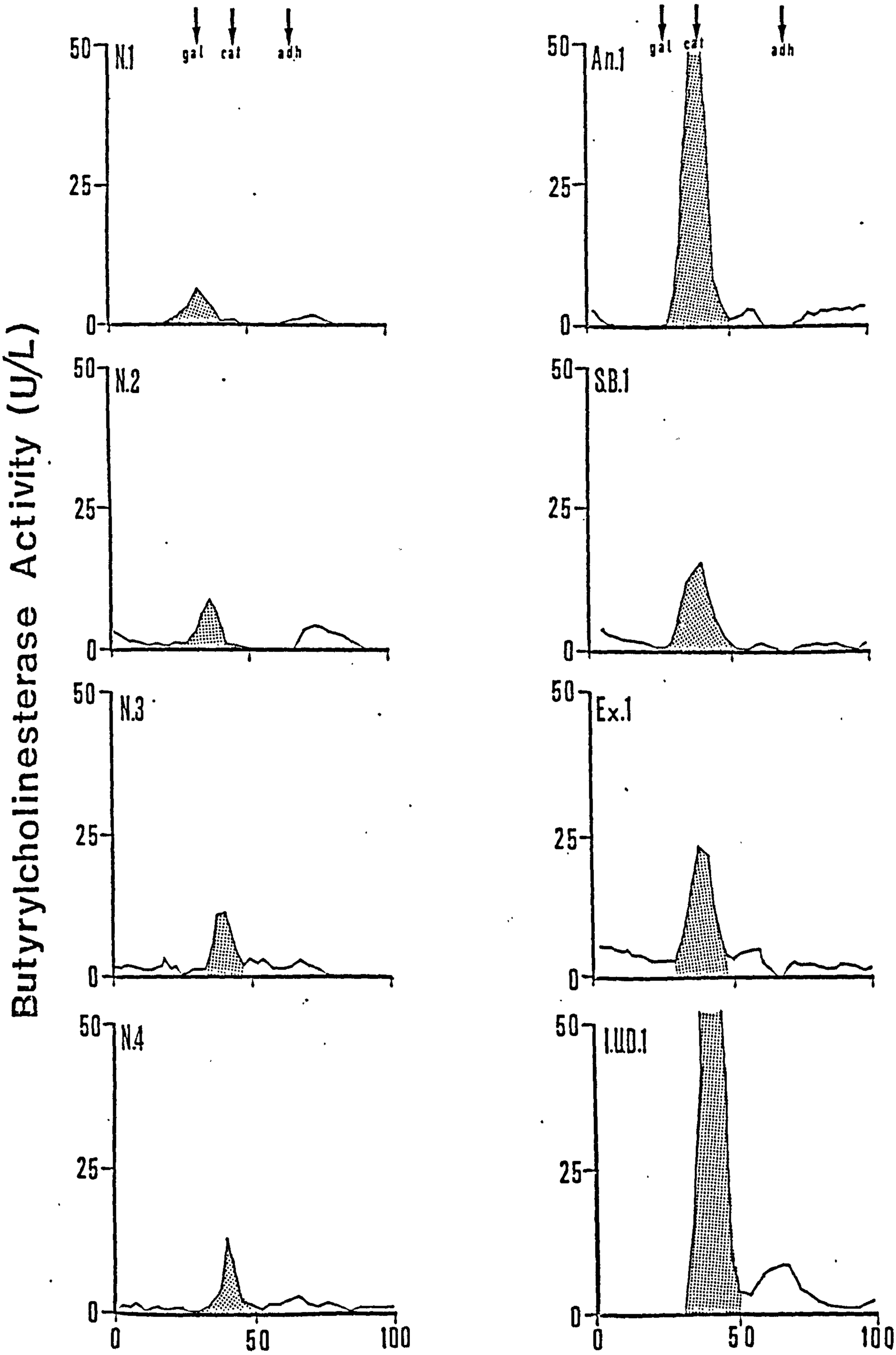


Fig. 10.8 Molecular forms of BChE from Cases N1-3, An1, SB1, Ex1 and IUD1

Unlike the situation with respect to G_4 AChE, the BChE G_4 form is present even in normals and is simply increased in pregnancies affected by NTD. In non-NTD fetal malformations, including exomphalos and intrauterine death, there was also a distinct elevation in G_4 BChE.

It would appear that the most likely explanation is that the increased activity of G_4 BChE in fetal malformation reflects fetal serum leakage and is therefore not as "NTD-specific" as the increase in G_4 AChE.

Section 11: Measurement of total AChE activity in rectal biopsy specimens

11.1 Results of seven year study

It is well established that Hirschsprung's disease is accompanied by a local increase in AChE activity in the aganglionic zone [Section 3.6[v]]. The largest series so far described includes a total of 110 cases, of which only nine had proven Hirschsprung's disease [Patrick et al, 1980].

Over the period of preparation of this thesis further data were collected from routine biopsy samples in which AChE was measured for diagnosis of Hirschsprung's disease. The results were compared with the eventual outcome in each case as verified by histology.

The study group included 213 children aged between 2 days and 14 years in whom failure to pass meconium or chronic constipation suggested a diagnosis of Hirschsprung's disease. Duplicate unfixed biopsy samples were obtained as described in Section 3.6[iii]. One sample was examined histochemically and histologically and in the other AChE activity was measured as outlined in Section 3.6[v].

The non-Hirschsprung's group consisted of 164 children in whom ganglia were clearly identified. The disease was confirmed by histological and clinical criteria in 45 cases and corrective surgery

undertaken by the Soave operative procedure; these patients showed a significant increase [$p < 0.001$] in AChE activity [Table 11.1]. In four cases unexplained discordant results were obtained, two of these proved to be very short segment disease, and sampling beyond the affected region was the probable cause of the inappropriately low results. In one case it is believed that sampling was too superficial. The remaining case showed an unusual histochemical appearance with only a marginal increase in the number of nerve fibres in the aganglionic zone, AChE activity was similarly borderline, this may reflect an atypical pathology.

While AChE is markedly increased in the disease, BChE activity is only marginally affected. Thus the percentage of cholinesterase activity attributable to AChE [%AChE] is also increased. This parameter was diagnostically useful as it was free from variations introduced as a result of dehydration, weighing or pipetting errors. AChE and %AChE results for both groups are shown in Table 11.1.

When AChE is plotted against %AChE [Fig. 11.1], four categories can be defined on the basis of the 2 SD "cut-off" limits for each parameter [AChE, 14.2 U/l; %AChE, 72.7%]: AChE and %AChE, increased;

Table 11.1 AChE and % AChE activity in rectal suction biopsy specimens in cases with Hirschsprung's disease and patients with evidence of constipation but who are histologically normal.

Table 11.1 Acetylcholinesterase activity and percentage original activity in rectal suction biopsy samples

Group	Number of Cases	AChE mean ± SD[a]	% AChE mean ± SD[b]
Hirschsprung's disease	45	34.2 ± 22.7	77.5 ± 6.3
Non-Hirschsprung's disease cases	164	6.6 ± 3.8	57.5 ± 10.6
	p-value	<0.001	<0.001

a Enzyme activity expressed as μmoles x 10^{-1} acetylthiocholine hydrolysed per minute per gram of wet weight tissue, measured at 25°C.

b The proportion of enzyme activity attributable to AChE expressed as a percentage of the total cholinesterase activity i.e. [AChE/AChE + BChE] x 100.

Fig. 11.1 %AChE Versus AChE activity in patients with Hirschsprung's disease and those with no histological evidence of the disease. Four groups are created on the basis of AChE and %AChE activity:

AChE and %AChE increased

AChE increased, %AChE normal

%AChE increased, AChE normal

AChE normal, %AChE normal

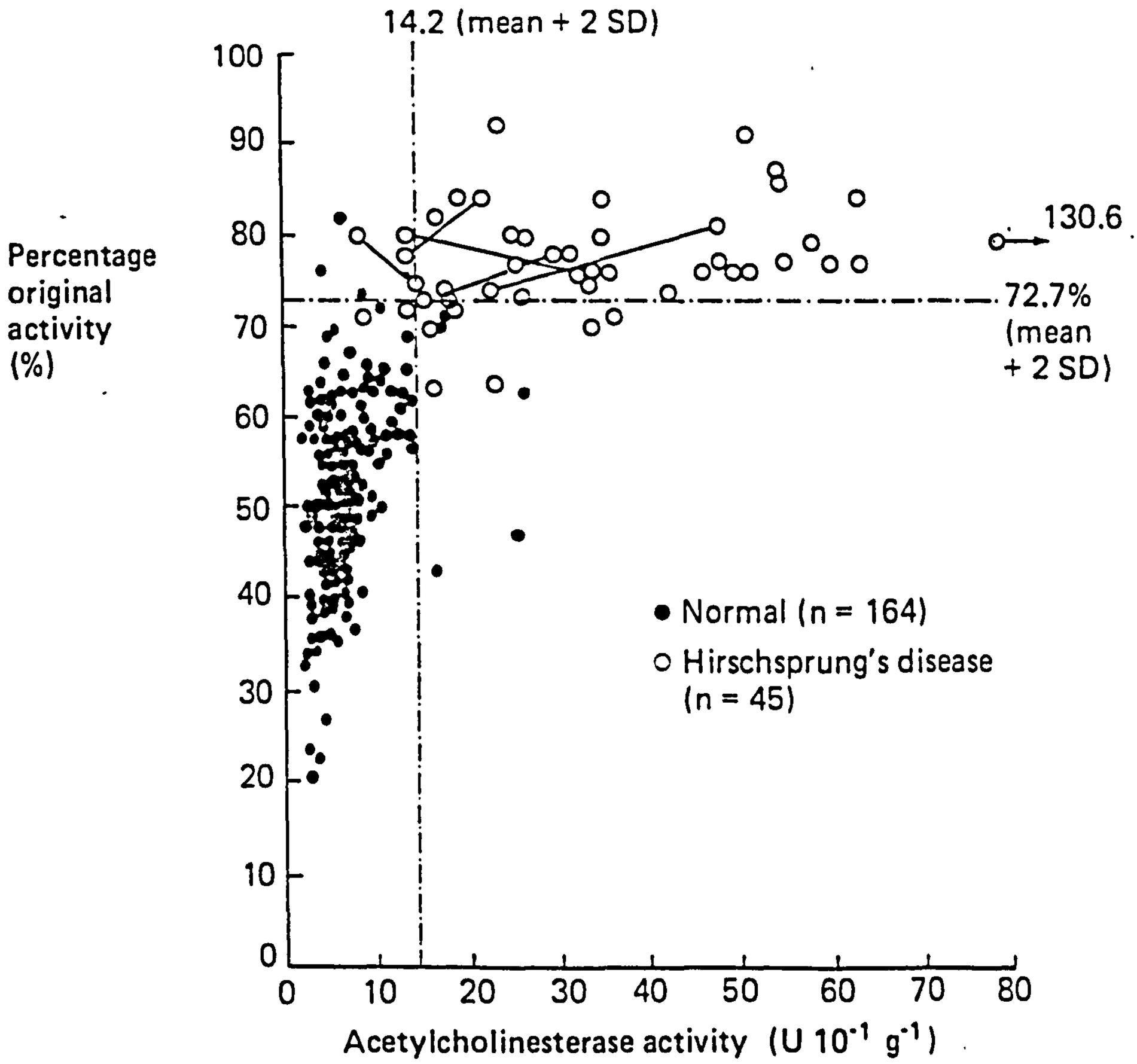


Fig. 11.1 AChE and %AChE in rectal biopsy specimens

AChE and %AChE, normal; AChE increased, %AChE normal; AChE normal, %AChE, increased. If it is assumed that both AChE and %AChE must be increased before a diagnosis of Hirschsprung's disease is considered likely then the false positive rate is zero. Eight per cent of results were regarded as equivocal with one parameter raised and one normal.

The sensitivity of the test in the diagnosis of the disease is 73% and the specificity is 100%.

Where:-

$$\text{Sensitivity} = \frac{\text{True positives}}{\text{True positives + false negatives}} \times 100$$

$$\text{and Specificity} = \frac{\text{True negatives}}{\text{False positives + true negatives}} \times 100$$

In practice this means that measurement of AChE is a simple and effective way of excluding Hirschsprung's disease in chronically constipated children.

The increase in AChE activity in Hirschsprung's disease was confirmed to be diagnostically useful. It was decided to investigate the change in molecular forms which gave rise to this increase. Since the biopsy samples were too small [<5.0mg] for adequate assessment of individual molecular forms, material resected as a result of surgical correction of the disorder was used. These "pull-through" specimens

of bowel included the aganglionic zone and the transition into normal ganglionic bowel. Direct comparison could be made by sampling along the length of these specimens and performing molecular form analysis and histology on transversely adjacent samples. The results are described in the following section.

Section 12: Molecular forms of AChE in ganglionic and aganglionic bowel

12.1 Patients included in the Study

In five cases of Hirschsprung's disease, segments of bowel were resected during corrective surgery. Details of these specimens and the patients from which they were obtained are described in Section 8.3.

In four of these cases, specimens contained both aganglionic and ganglionic [normal] tissue. In one case of long segment disease while the tissue was entirely aganglionic, nerve trunk hypertrophy present in the distal region of resected bowel was not evident in the proximal region. This is in accordance with the view that sacral innervation gives rise to the nerve trunk hypertrophy characteristic of the disease and that such innervation is confined to the distal region of the gut [Garret et al, 1969].

A single patient whose sigmoid colon was resected for a cause other than Hirschsprung's disease was also sampled as a control specimen. Further controls were considered unnecessary as each specimen included its own control, that is the normal [ganglionic] region with which changes in molecular forms could be compared.

In all cases the specimens were sampled for histological and histochemical investigation. In

three instances the routine biopsy total AChE assay was also performed on samples taken along the length of the specimen.

As described in Section 8.3, some segments of bowel contained both muscle and mucosal layers; where this occurred the layers were separated by microscopic dissection and the molecular forms of AChE examined independently in each layer.

After sampling, individual specimens were stored in liquid nitrogen until required. They were then removed, thawed and homogenised in pH8.0, 0.1M phosphate buffer, this is described in greater detail in Section 8.3. In one case the samples were homogenised in two different buffers: Firstly in a mild aqueous medium of 0.1M phosphate buffer at pH7.3. The pellet from this was then resuspended in a second phosphate buffer containing 1M NaCl and 0.5% w/v Triton X-100. These buffers would be expected to release soluble and membrane bound forms respectively.

12.2 Variation in activity of AChE molecular forms in relation to the changing histological and histochemical appearance

The changes in molecular forms of AChE shown in Figs. 12.1-6 are compared with the changing histological and histochemical appearance. Each

Fig. 12.1-6 The changing level of activity of AChE molecular forms [G_1, G_2, G_4 and A_{12}] as they vary along the length of resected bowel segments. Cases A, B, C and E show a clear transition from a region of aganglionosis to a normal [ganglionic] region. Case D is an example of long segment disease in which the segment of bowel analysed was entirely aganglionic. Case F, a child with no evidence of Hirschsprung's disease acts as an independent control. In each case the changing histological and histochemical appearance is shown for comparison. The actual AChE molecular form profile from extreme ends, proximal and distal, is illustrated. Inclusion of the profile at each point would have made the figure unnecessarily complex.

In Cases B, C and E the total AChE activity was measured at intervals by the biopsy assay outlined in Section 11. The changing total AChE activity reflects the increase in G_4-AChE and the changing histochemical appearance.

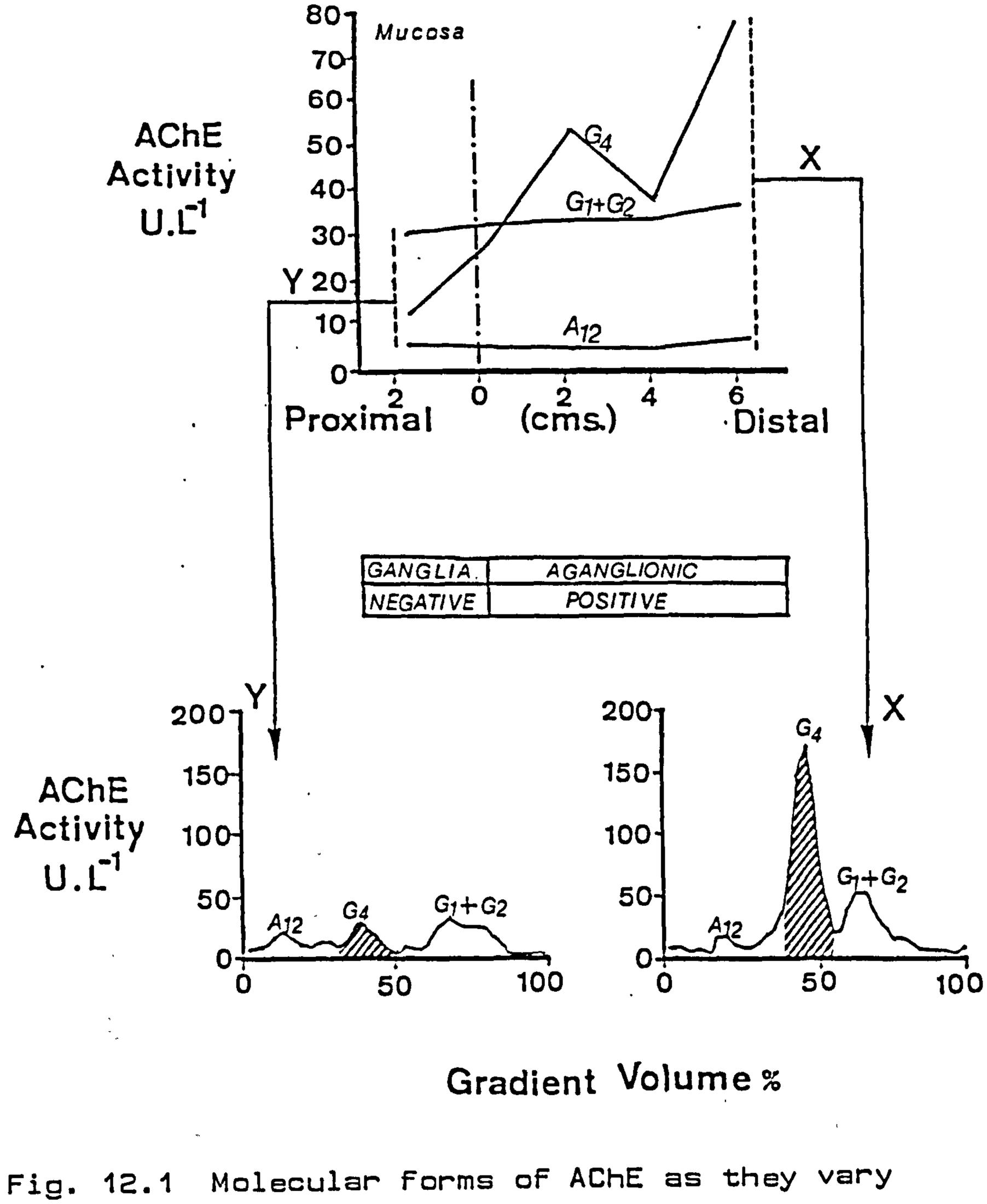


Fig. 12.1 Molecular forms of AChE as they vary in Case A

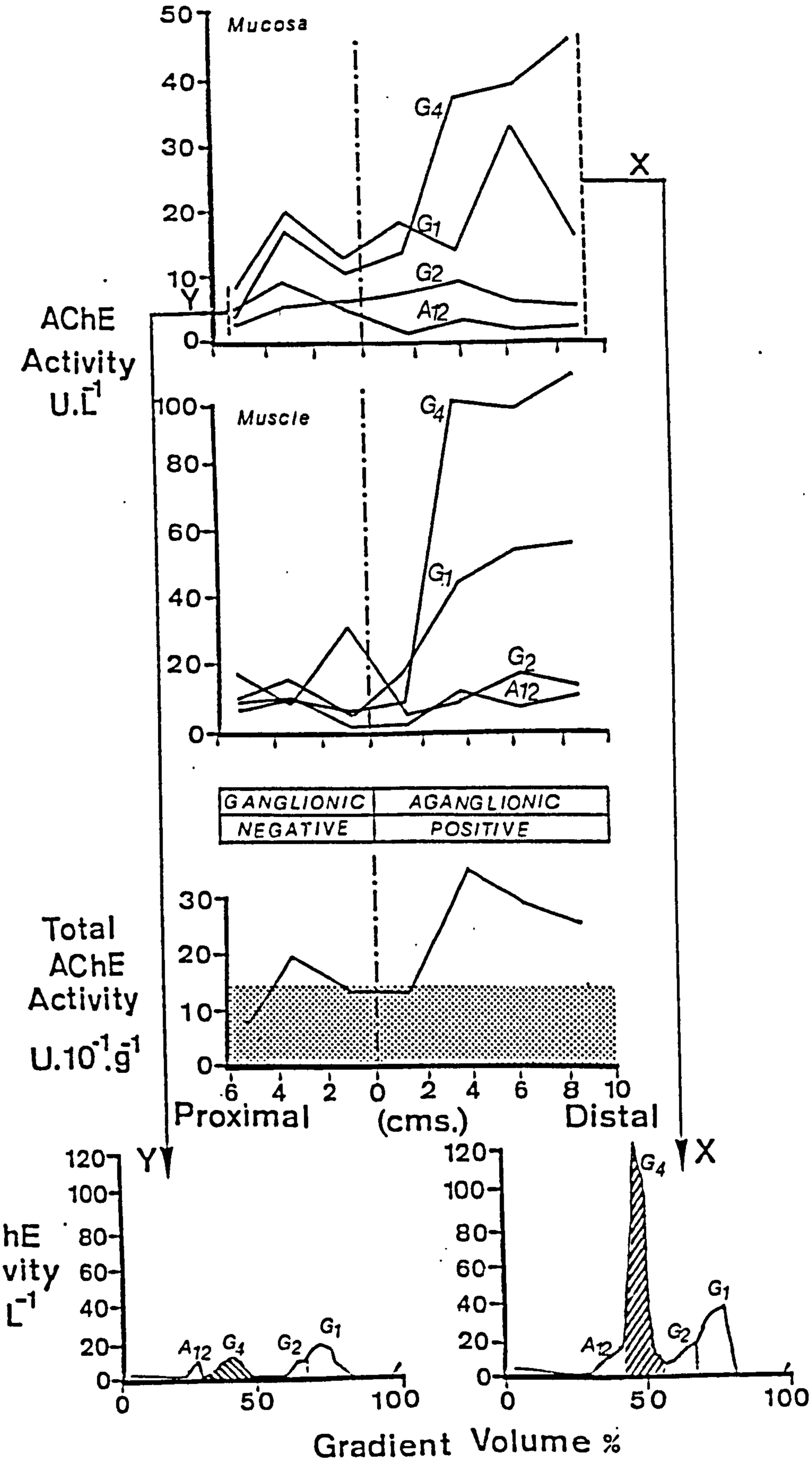


Fig. 12.2 Molecular Forms of AChE as they vary in Case B

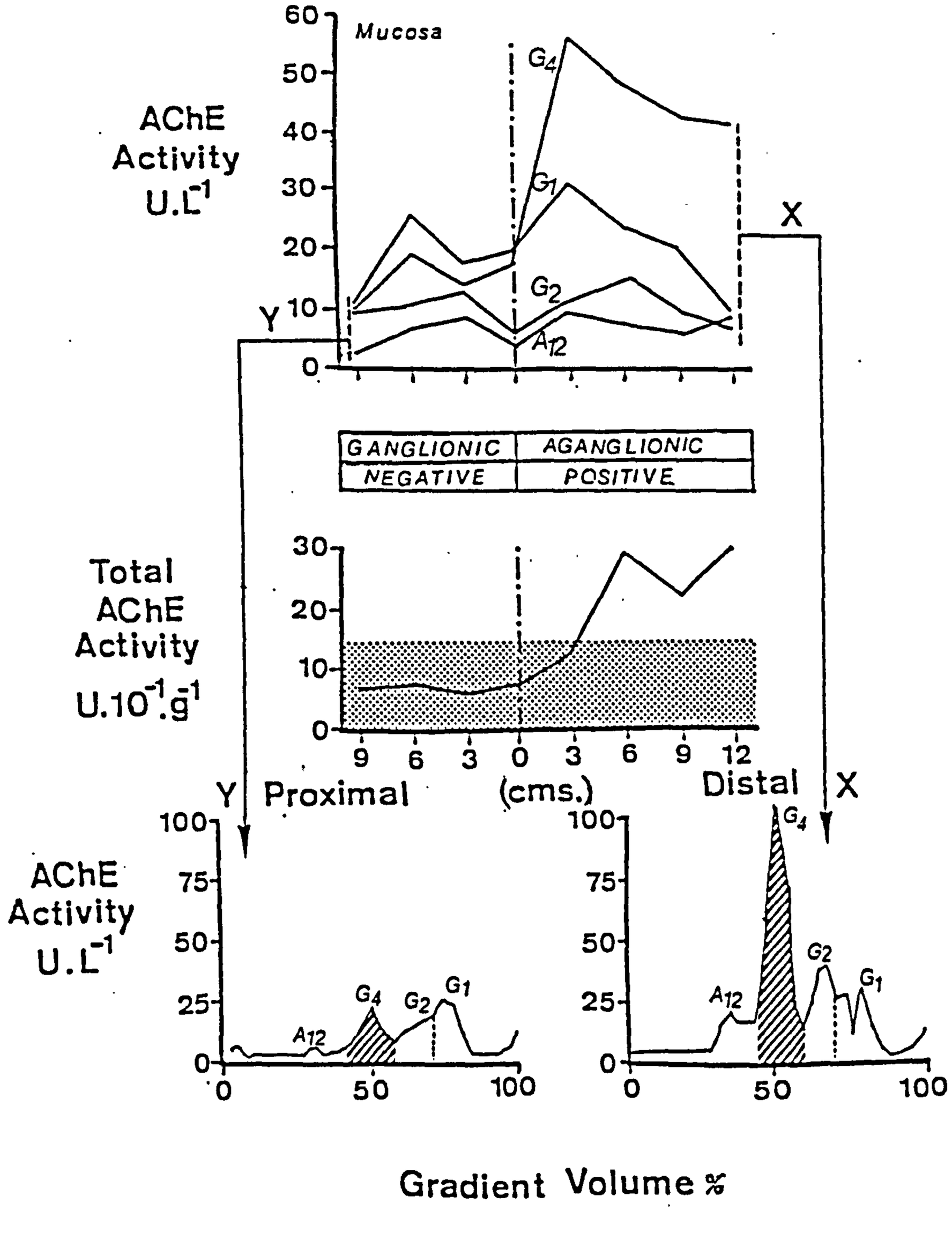


Fig. 12.3 Molecular forms of AChE as they vary in Case C

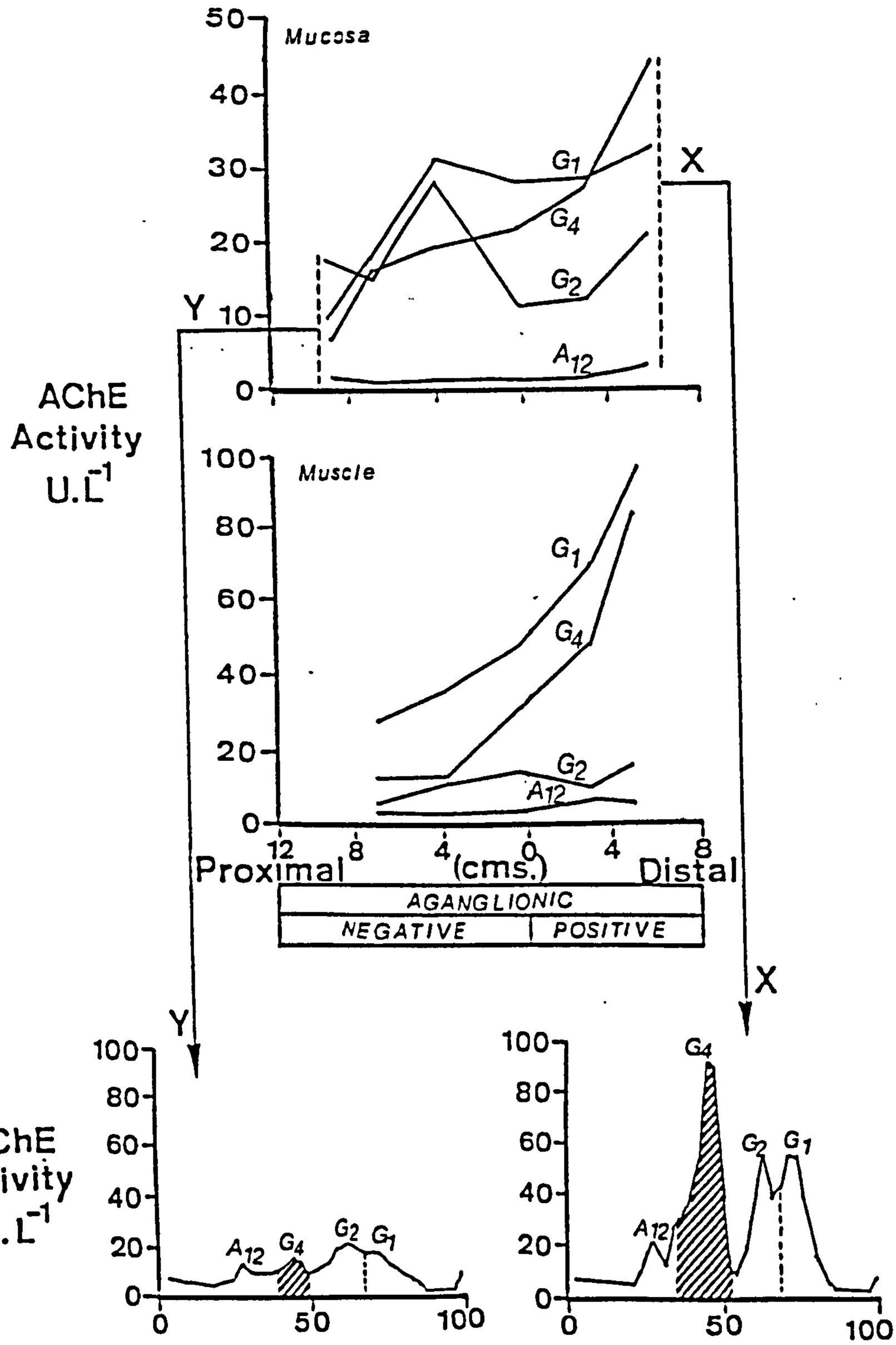


Fig. 12.4 Molecular forms of AChE as they vary in Case D

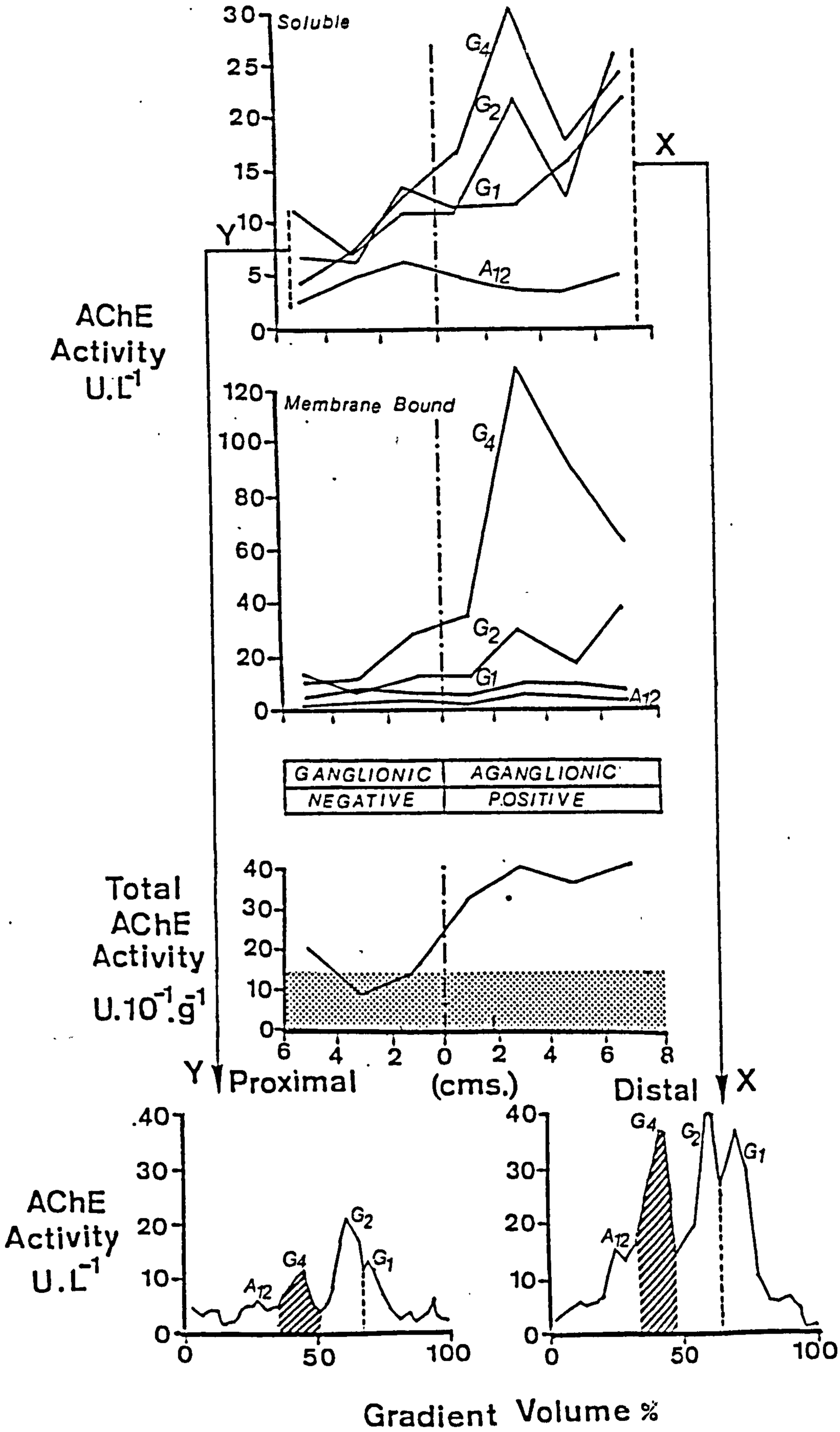


Fig. 12.5 Molecular forms of AChE as they vary in Case E

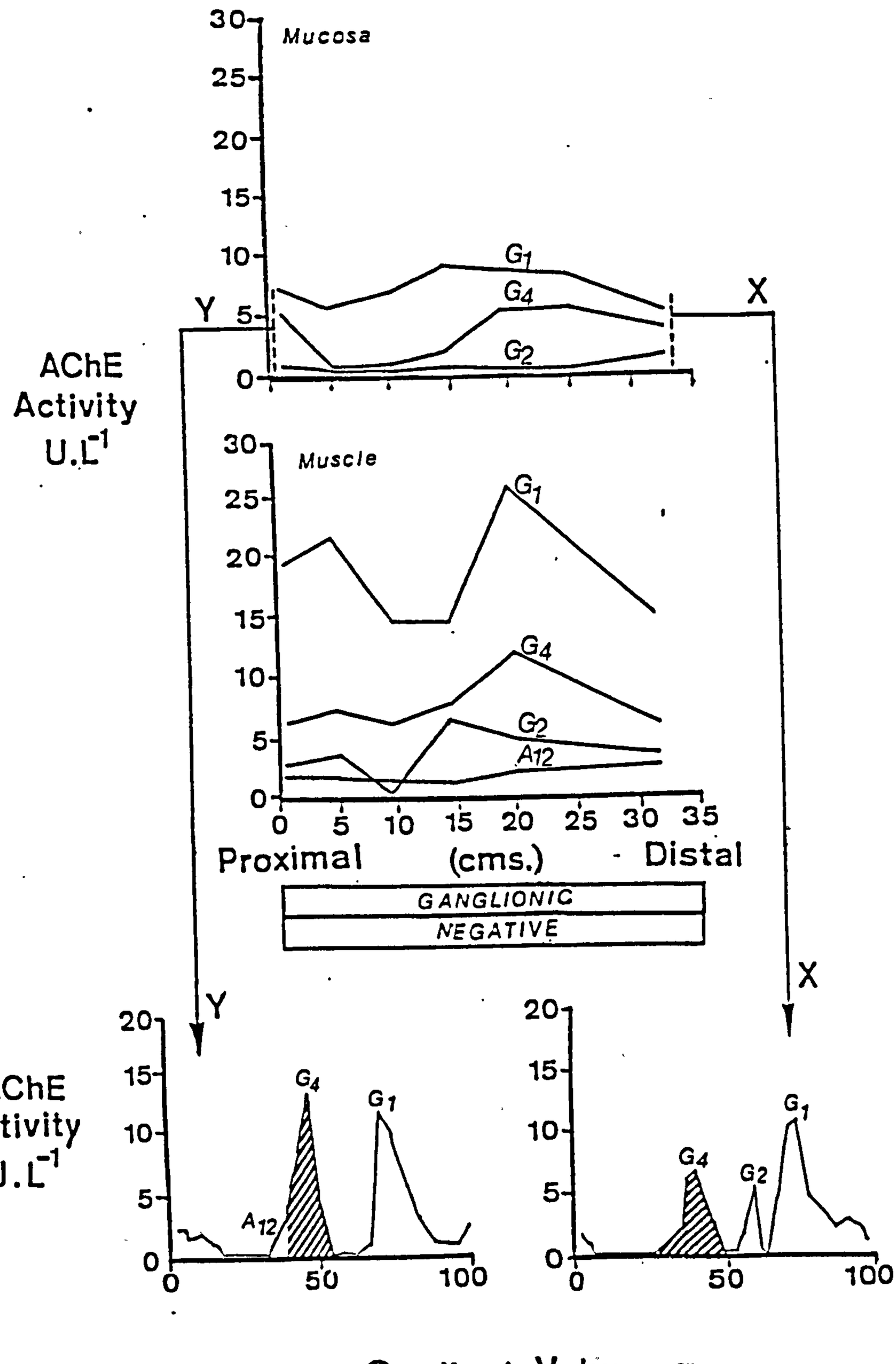


Fig. 12.6 Molecular forms of AChE as they vary in Case F

figure also shows the actual AChE sedimentation profile from the extreme ends of the resected bowel specimen. The inclusion of the actual AChE sedimentation profile results at each point would make the figure unnecessarily complicated, these are therefore summarised in the display of the changing level of each form.

In all cases an increase in the activity of G_4 AChE accompanied the transition into the region where nerve trunk hypertrophy was demonstrable histochemically. This increase in G_4 ranged from 4-to 14-fold. Where both muscle and mucosal layers were present, Cases B and D, the changes in G_4 were more prominent in the muscle than mucosal layers. The actual level of these forms are shown in Table 12.1.

Forms G_2 and A_{12} showed no consistent variation when compared with the histological and histochemical appearance. However, the activity of G_1 was higher in the aganglionic and histochemically positive region of cases B, D and E than in the normal ganglionic zone. In the muscle layers of cases B and D this increase in G_1 was particularly well defined [Figs. 12.2 and 12.4].

In the control case F, Fig. 12.6, there was no discernable pattern of change in any of the AChE molecular forms from either muscle or mucosal layers.

Table 12.1 Quantitative activity of AChE molecular forms at intervals along resected bowel segments from cases A-F. The presence or absence of ganglia at each point is also shown for comparison.

Table 12.1 The activity of AChE molecular forms in Cases A - F

Section No.		G_1	G_2	G_4	A_{12}	Ganglia
		U/l				
Case A						
	(mucosa)					
Proximal	1	8.5	20.8	12.9	5.4	Present
	2	11.4	19.9	27.4	5.6	Absent
	3	32.1		53.0	6.1	Absent
	4	12.5	19.0	37.0	3.3	Absent
Distal	5	35.2		76.5	6.8	Absent
Case B						
	(mucosa)					
Proximal	1	6.7	3.0	6.0	0.5	Present
	2	20.0	5.5	17.0	8.5	Present
	3	12.5	5.0	10.5	5.1	Present
	4	18.0	7.0	14.0	1.5	Absent
	5	13.0	8.5	37.5	2.4	Absent
	6	33.0	5.5	39.0	1.6	Absent
Distal	7	17.0	5.0	47.0	2.1	Absent
	(muscle)					
Proximal	1	9.0	17.5	7.5	7.5	Present
	2	14.5	4.5	9.5	10.0	Present
	3	6.0	31.0	5.0	1.0	Present
	4	11.0	6.0	7.5	0.5	Absent
	5	45.0	9.5	98.0	11.5	Absent
	6	53.0	17.0	95.0	7.0	Absent
Distal	7	56.5	14.0	106.5	10.5	Absent
Case C						
	(mucosa)					
Proximal	1	10.9	9.8	10.6	2.9	Present
	2	25.2	10.3	18.6	6.7	Present
	3	17.2	12.5	14.0	8.5	Present
	4	19.5	6.4	17.1	3.8	Present
	5	31.0	12.0	55.8	10.0	Absent
	6	23.2	15.1	48.4	7.6	Absent
	7	20.1	10.5	42.8	5.5	Absent
Distal	8	9.5	17.5	41.7	9.4	Absent
Case D						
	(mucosa)					
Proximal	1	9.0	17.5	6.5	1.1	Absent
	2	17.0	14.5	15.0	0.7	Absent
	3	30.5	12.5	19.5	0.7	Absent
	4	27.5	11.0	22.0	1.5	Absent
	5	28.5	12.5	26.5	0.9	Absent
Distal	6	32.5	21.5	44.5	3.5	Absent

Section No		G_1	G_2	G_4	A_{12}	Ganglia
		(U/l)				
Case D (Contd)						
	(muscle)					
Proximal	1	26.5	6.0	16.0	2.9	Absent
	2	33.5	10.5	13.0	1.6	Absent
	3	47.5	14.5	32.0	2.7	Absent
	4	68.0	10.5	47.5	6.6	Absent
Distal	5	95.0	16.0	82.5	5.0	Absent
Case E						
	(soluble)					
Proximal	1	7.0	11.5	4.5	2.5	Present
	2	6.0	7.5	7.5	4.5	Present
	3	13.5	11.5	13.0	6.5	Present
	4	11.0	11.5	16.0	5.0	Absent
	5	11.5	22.5	30.1	3.4	Absent
	6	15.5	13.5	17.5	3.3	Absent
Distal	7	21.5	16.5	24.2	5.3	Absent
	(membrane bound)					
Proximal	1	4.3	13.0	10.0	2.3	Present
	2	6.0	7.3	11.0	1.6	Present
	3	5.0	10.5	27.0	3.3	Present
	4	3.2	11.0	33.0	2.1	Absent
	5	8.2	30.5	126.0	6.2	Absent
	6	8.9	17.8	87.6	2.2	Absent
Distal	7	5.7	37.0	60.5	1.6	Absent
Case F						
	(mucosal)					
Proximal	1	7.2	nil	5.5	nil	Present
	2	6.0	nil	1.7	nil	Present
	3	7.7	nil	0.9	0.4	Present
	4	9.2	nil	2.4	0.1	Present
	5	-	nil	5.6	nil	Present
	6	8.5	nil	2.5	nil	Present
Distal	7	5.5	1.3	3.8	nil	Present
	(muscle)					
Proximal	1	18.8	2.3	6.1	2.0	Present
	2	20.6	3.3	7.3	1.5	Present
	3	14.5	nil	5.7	0.9	Present
	4	14.1	6.5	7.4	1.0	Present
	5	25.1	5.0	11.9	2.2	Present
Distal	6	14.2	2.6	5.9	2.8	Present

This supports the view that the variation is linked to the transition from normal to aganglionic bowel.

Assay of total AChE performed at intervals along the segments of Cases B, C and E showed changes which paralleled those of G_4 and compared well with the histochemical and histological findings.

12.3 AChE activity in a single case of long segment Hirschsprung's disease

In a patient with total colonic aganglionosis a segment of descending colon measuring 24 cm was removed, Case D. The specimen was entirely aganglionic, however, nerve trunk hypertrophy was observed only in the distal 8 cm. This case provided a useful model for independent study of the effects of nerve trunk hypertrophy and aganglionosis on molecular forms of AChE. The increase in G_4-AChE was linked [Fig.12.4] to the transition to the region of nerve trunk hypertrophy and aganglionosis. The significance of these findings are discussed in Section 19.4.

12.4 Variation in membrane bound and soluble AChE in Hirschsprung's disease

In Case E, Fig. 12.5, different extraction techniques allowed membrane bound and soluble forms to be studied separately. The change in G_4 activity

was more pronounced in the membrane bound fraction than in the soluble component. The activity of the other AChE forms were similar in both instances.

12.5 Summary of AChE molecular form changes in Hirschsprung's disease

In summary, the changes in AChE molecular forms in resected bowel segments, cases A-F, suggest that variation in AChE activity largely reflect changes in the G_4 form. The most prominent changes in G_4 occur in the membrane bound fraction and this is directly associated with the nerve trunk hypertrophy seen in the disease.

Fig. 12.7, summarises these findings by superimposing results from the musocal layers of Cases A, B, C and E. The transition from normal to aganglionic bowel was used as a marker point from which direct comparison could be made. Case D which was entirely aganglionic and the control Case F were ommitted from this figure as neither included the transition from ganglionic to aganglionic bowel.

12.6 Determination of sedimentation coefficient

The sedimentation coefficient of each molecular form was determined by curvilinear interpolation between protein markers as outlined in Section 7.1.

Fig. 12.7 A summarised representation of the variation in the activities of G_1, G_2, G_4 and A_{12} molecular forms in resected bowel segments from Cases A, B, C and E. The transition from the aganglionic [distal] region to the ganglionic [proximal] zone is indicated, ·—·—·—·.

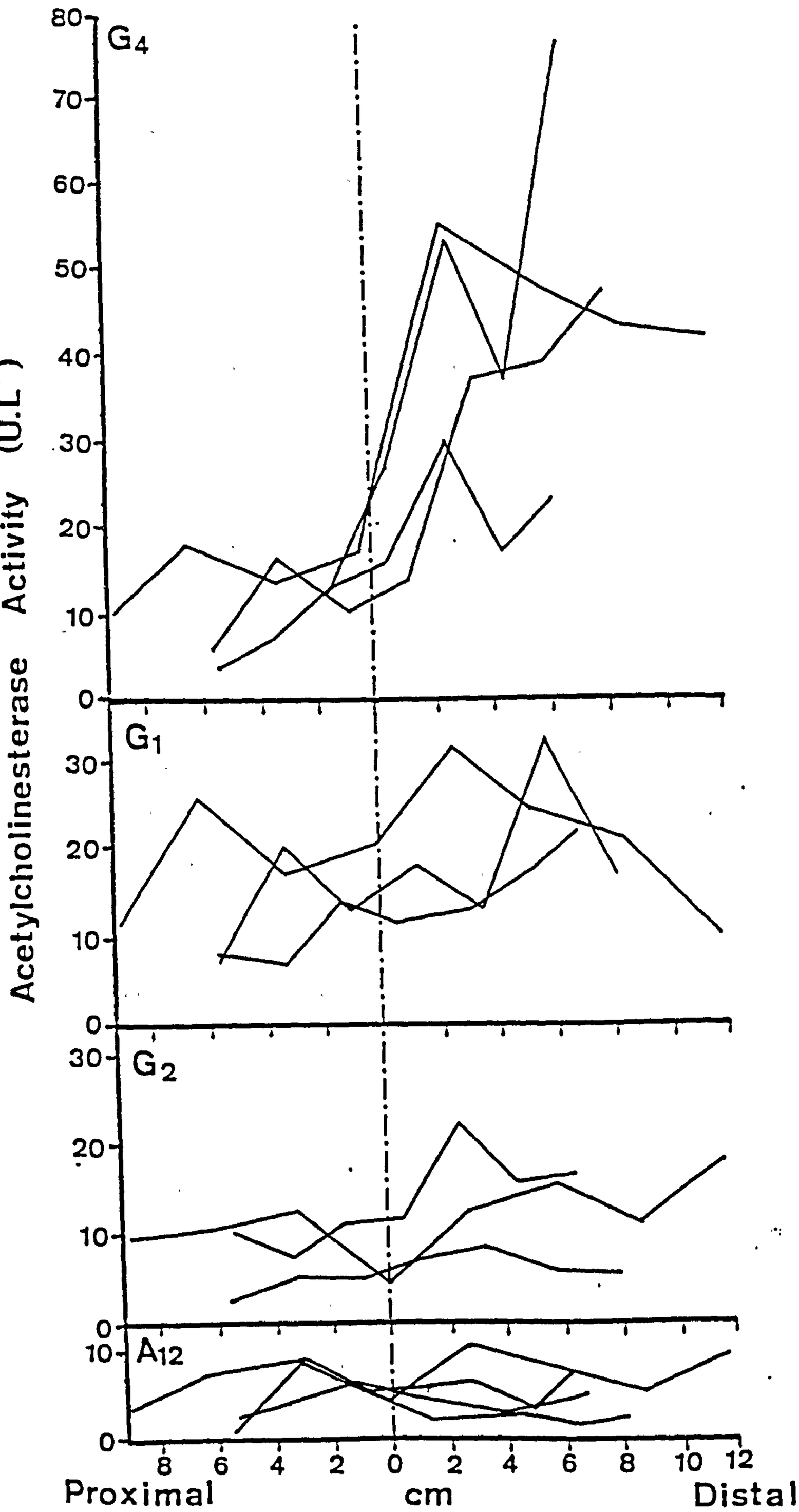


Fig. 12.7 The variation in individual molecular forms of AChE in Cases A, B, C and E

Results were collected from all the profiles obtained from Cases A-F and the Mean and SD calculated for each form.

Sedimentation Values

Species	Mean	SD	n
G_1	3.50	0.51	57
G_2	4.99	0.53	58
G_4	9.22	0.75	63
A_{12}	16.78	1.01	52

12.7 Differential extraction of AChE molecular forms from rectal tissue

Three different extraction media were used successively in the homogenisation of a 5% w/v suspension of rectal tissue using a Potter Elevehjem type homogeniser. The molecular forms released by each media in turn were determined by velocity sedimentation analysis. The experimental details are shown schematically in Fig. 12.8. The three different buffer systems included:

Buffer A : A physiological buffer; pH7.4 based on Hank's medium.

Fig. 12.8 A schematic representation of the experimental assessment of the effect of different extraction media on AChE release from rectal tissue.

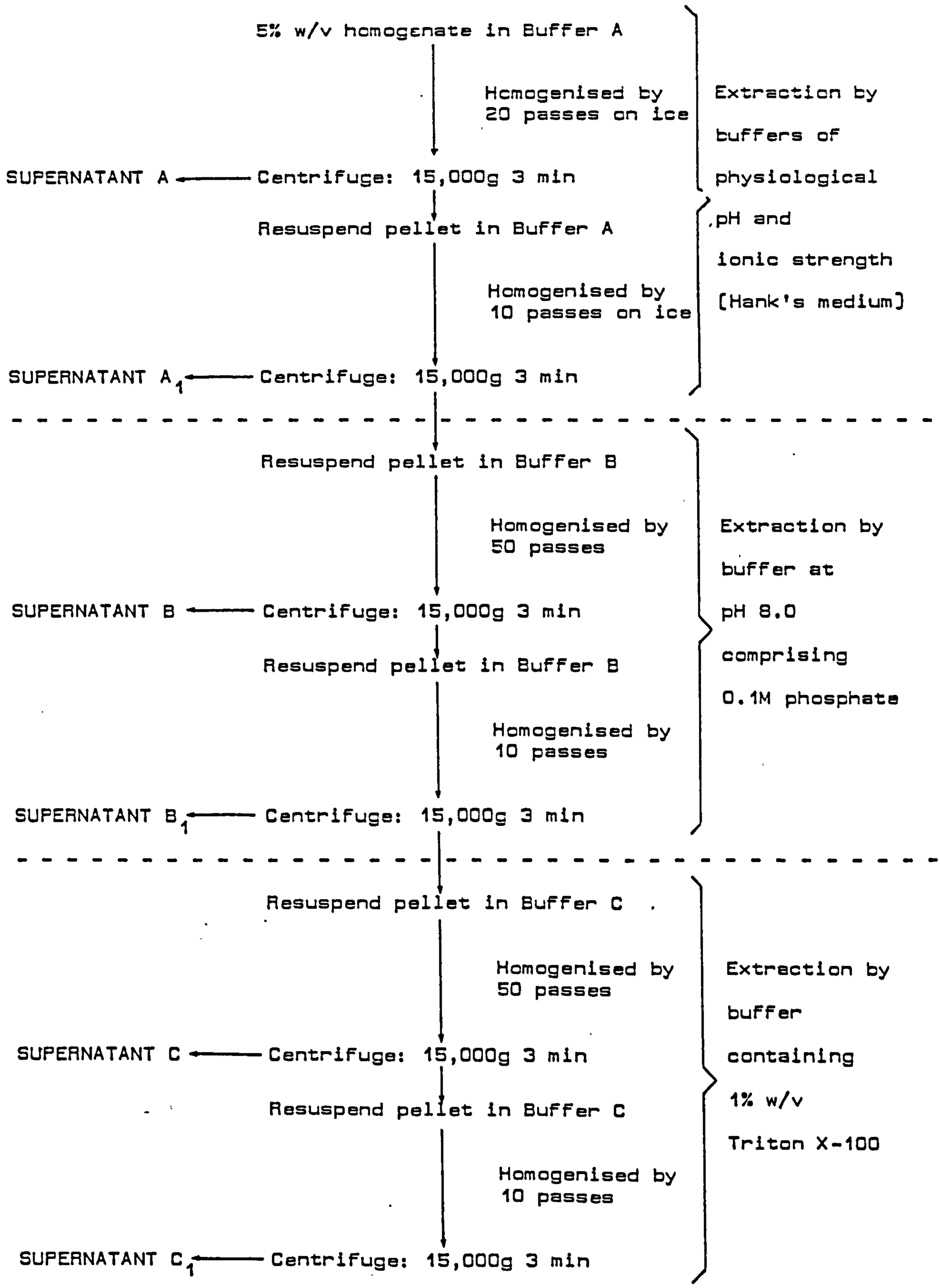


Fig. 12.8 The effect of various buffers on AChE release

Buffer B : The buffer used in the routine biopsy AChE assay; pH8.0, 0.1M Na/K phosphate.

Buffer C : A buffer containing the detergent Triton X-100 capable of releasing membrane bound components; pH7.3, 0.1M Na/K phosphate containing 1% w/v Triton X-100.

The tissue was homogenised twice in each buffer system to ensure complete release of those molecular forms involved at each stage. The profile of AChE species was determined for the first of the two extractions in each case. Supernatants A_1, B_1 and C_1 were assumed to contain the same distribution of forms as their parent supernatants A, B and C respectively.

The results obtained using tissue from Case B were as follows:

Supernatant		G_1	G_2	G_4	A_{12}
		U/l			
Buffer A	A	9.0	7.8	11.7	-
	A_1	2.8	2.4	3.6	-
Buffer B	B	2.6	1.4	3.0	1.5
	B_1	0.4	0.2	0.4	0.2
Buffer C	C	1.3	1.8	5.9	0.6
	C_1	0.2	0.3	0.9	0.1
Total activity		16.3	13.9	25.5	2.4

From these results it can be seen that a substantial proportion of the globular forms G_1, G_2 and G_4 were released by a single homogenisation in physiological buffer, 55% of G_1, 56% of G_2 and 46% of G_4 respectively. Further amounts of each form were released by buffer B used in the routine biopsy assay including the majority of the A_{12} activity. Buffer C containing Triton, released the remaining membrane bound activity of which G_4 was the major component.

These experiments suggest that the majority of AChE activity, 80%, is released by buffer B used in the routine biopsy AChE assay, however, a proportion of AChE remains on the membrane and can only be released by Triton. G_4 in particular has almost one third of its activity in this bound form.

12.8 The effect of native proteases on AChE molecular forms in rectal tissue

Some reports in the literature suggest that native proteases present in muscle may enhance the release of, or even interconvert AChE molecular forms [Section 2.4].

In order to test the effect of native proteases on AChE in rectal tissue, mucosa from the aganglionic

region of resected bowel was finely minced using a scalpel and then allocated to three separate aliquots: A, B and C.

A - was immediately homogenised at a concentration of 5% w/v on ice in 0.1M, pH8.0 phosphate buffer containing a mixture of protease inhibitors: Aprotinin, 0.2mg/ml, Benzamidine 2mM, EGTA 5mM, Leupeptin 0.02mg/ml, Ovomucoid 0.02mg/ml, Pepstatin 0.02mg/ml, Soya bean trypsin inhibitor 0.1mg/ml and Lima bean trypsin inhibitor 0.2mg/ml [Barnard et al, 1984]. All were obtained from Sigma Chemical Co Ltd.

B - was immediately homogenised at a concentration of 5% w/v in pH8.0, 0.1M phosphate buffer without protease inhibitors.

C - was stored at room temperature for four hours in a moist environment and then homogenised at a concentration of 5% w/v in pH8.0, 0.2M phosphate buffer without protease inhibitors.

The resulting homogenates were then centrifuged at 15,000g for 3 minutes and the molecular form

profile assessed by velocity sedimentation analysis.

The results are shown below:

Aliquot	G_1	G_2 U/l	G_4
A [homogenised on ice with protease inhibitors]	22.0	12.0	32.0
B [homogenised without protease inhibitors]	23.5	13.5	37.5
C [Four hours at room temperature followed by homogenisation without protease inhibitors]	13.0	11.5	35.5

Homogenisation in the presence or absence of protease inhibitors did not substantially affect the activity of any of the molecular forms.

The molecular forms also appeared quite stable to incubation for four hours at room temperature although some decrease in G_1 activity was noticeable.

Section 13: Molecular forms of BChE in ganglionic and aganglionic bowel

13.1 Variation in the level of BChE molecular forms

BChE exists in molecular forms similar to those of AChE [Section 2.3]. In rectal tissue three major forms are identifiable, Fig. 13.1, these include G_1, G_2 and G_4.

Molecular form profiles of BChE were determined at intervals along the length of the resected bowel segments of Cases A-F as previously described for AChE forms [Section 12.1].

The results for those cases showing a clear transition from ganglionic to aganglionic bowel are summarised in Fig. 13.2. BChE activity does not appear to vary in a consistent way in any of its forms between ganglionic and aganglionic tissue. This is in direct contrast to AChE which shows a distinct increase in G_4 in aganglionic tissue [Fig. 12.7].

13.2 Determination of sedimentation coefficient

The sedimentation coefficient of each molecular form was determined by curvilinear interpolation between protein markers as outlined in Section 7.1.

Fig. 13.1 A density gradient sedimentation profile of BChE molecular forms present in rectal tissue homogenates.

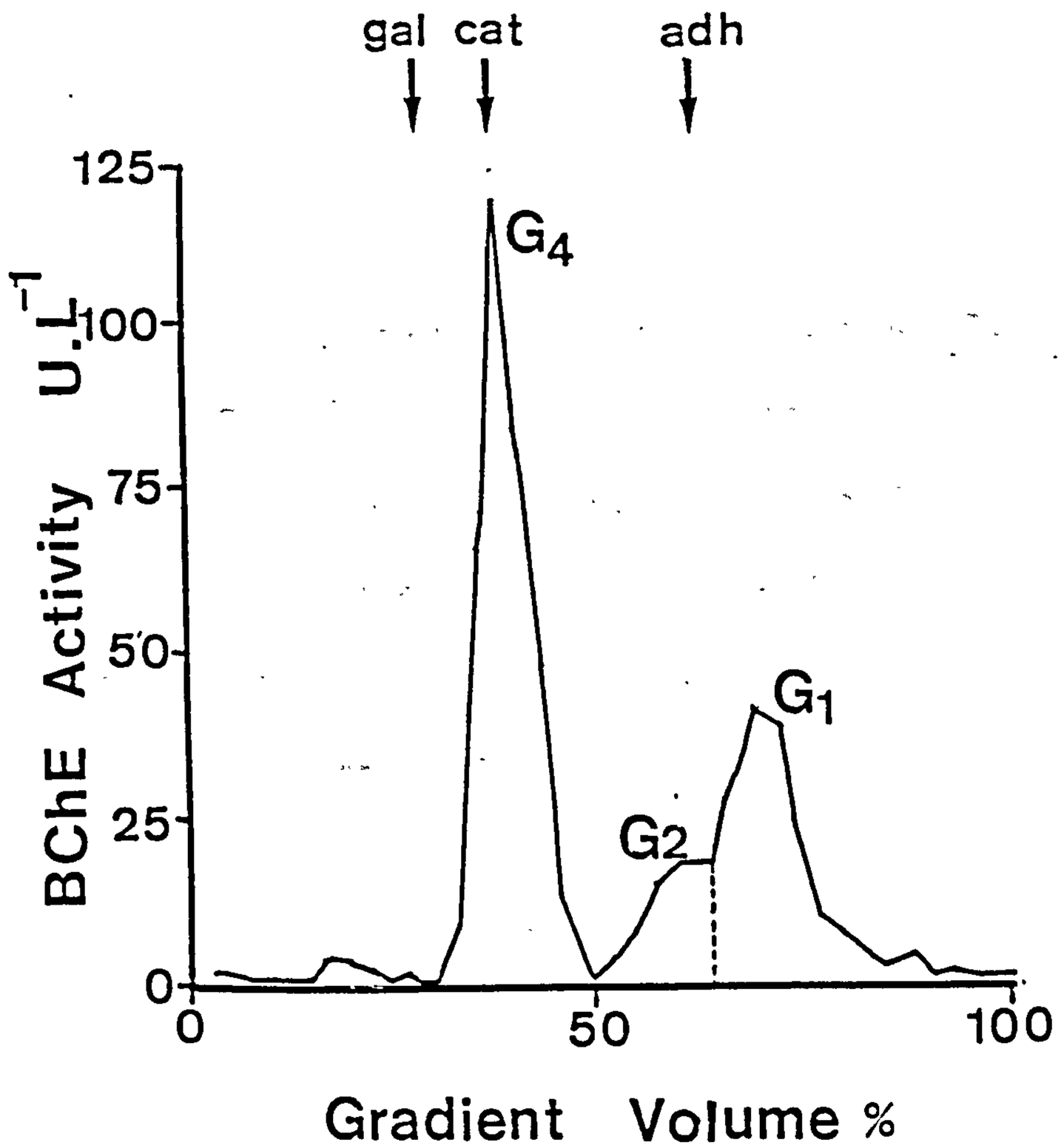


Fig. 13.1 Molecular forms of BChE identifiable in rectal tissue

Fig. 13.2 A summarised representation of the variation in activity of G_1, G_2 and G_4, BChE molecular forms in resected bowel segments from Cases A, B, C and E. The transition from aganglionic [distal] region to the ganglionic [proximal] zone is indicated, ·—·—·—·.

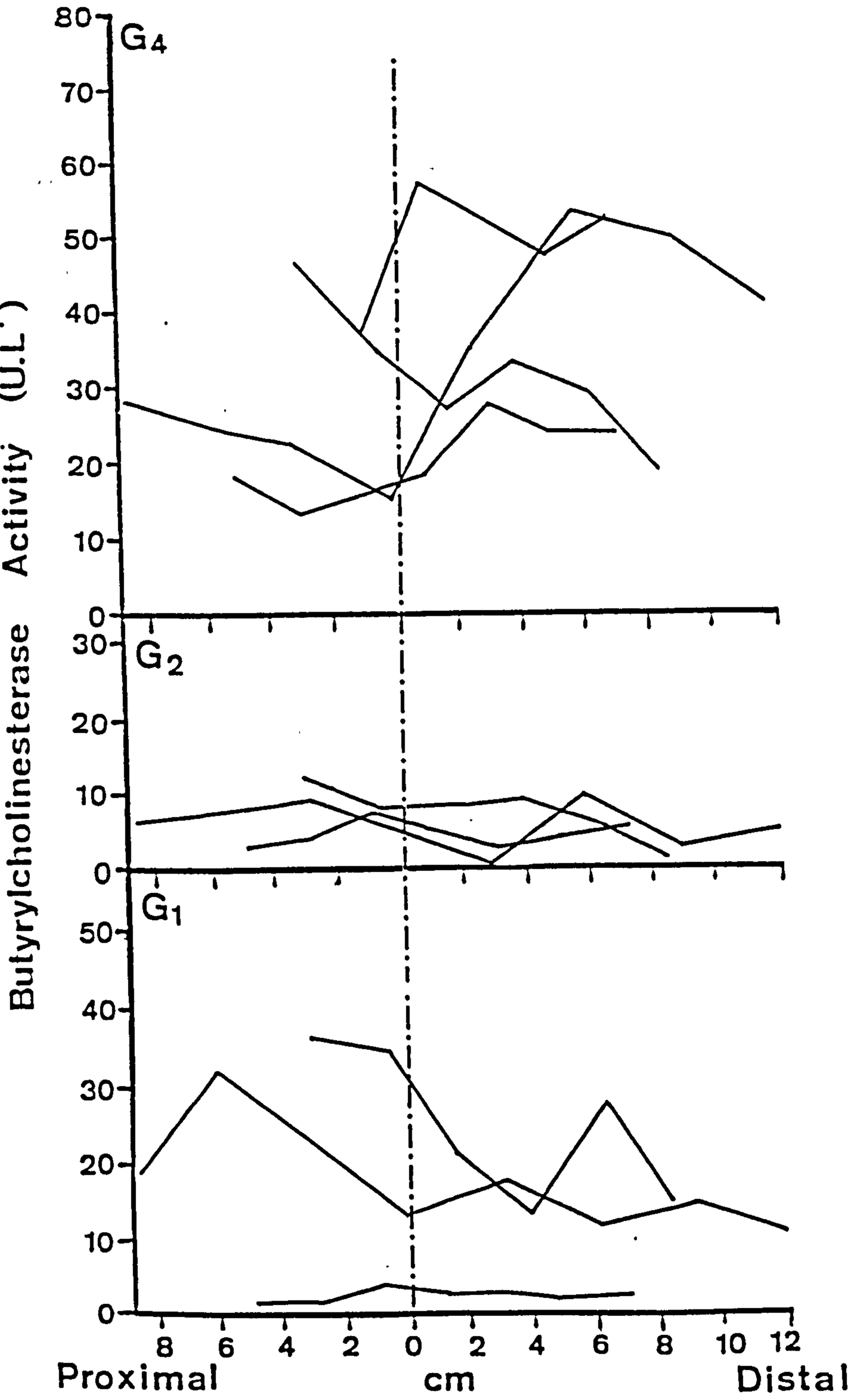


Fig. 13.2 The variation in individual molecular forms of BChE in Cases A, B, C and E

The results were collected for all of the profiles obtained from Cases A-F and the Mean and SD calculated for each form.

Sedimentation Values

Species	Mean	SD	n
G_1	4.1	0.33	57
G_2	5.2	0.57	56
G_4	11.4	0.65	61

The sedimentation coefficients of these BChE species are slightly higher than their AChE counterparts, [Section 12.6]. These values are in accord with the reported values for mammalian cholinesterases, [Section 2.2] and suggests a somewhat higher molecular mass for BChE forms.

Section 14: Summary of the results from amniotic fluid and resected bowel segments

14.1 Molecular forms of AChE and BChE in amniotic fluid

Three forms of both AChE and BChE were identifiable in amniotic fluid by sucrose density sedimentation analysis. The sedimentation coefficients of these forms are shown in Table 14.1. Both AChE and BChE activity was increased in amniotic fluid from NTD affected pregnancies when compared with those with a normal outcome. This increase was particularly marked in a form sedimenting at approximately 10S and believed to correspond to the tetrameric, G_4 form of the enzyme.

G_4 BChE, while increased in the presence of NTD and other fetal malformations, was also present in normal pregnancies. It is probable that the increase in G_4 BChE is due to fetal serum leakage.

G_4 AChE was virtually absent in amniotic fluid from pregnancies with normal outcome and appears to be NTD-specific. It is probable that this marker is released from the nervous tissue exposed as a result of the lesion.

14.2 Molecular forms of AChE and BChE in rectal tissue

Four forms of AChE and three of BChE were identifiable in tissue homogenates made from resected

Table 14.1 The sedimentation coefficients of AChE and BChE molecular forms present in amniotic fluid and rectal tissue.

Table 14.1 Sedimentation coefficients of AChE and BChE molecular forms from amniotic fluid and rectal tissue

Source	G_1	G_2	G_4	A_{12}
Amniotic fluid:				
AChE	4.0 ± 0.6	5.5 ± 0.7	10.3 ± 0.5	-
BChE	4.5 ± 0.5	6.3 ± 0.7	11.6 ± 0.4	-
Rectal tissue:				
AChE	3.5 ± 0.5	5.0 ± 0.5	9.2 ± 0.8	16.8 ± 1.0
BChE	4.1 ± 0.3	5.2 ± 0.6	11.4 ± 0.7	-

bowel segments taken from five cases of Hirschsprung's disease and one control case.

There was an increase in overall AChE activity in the aganglionic region of bowel in each case. One particular form of AChE sedimenting at approximately 10S and believed to represent the tetrameric form, G_4, of the enzyme, comprised the major component of this increase.

The increase in G_4-AChE followed the nerve trunk hypertrophy seen in the disease and was not directly related to aganglionosis. It would seem probable that this G_4-AChE is a product of the nerve trunk hyperplasia.

BChE molecular forms showed no consistent changes when aganglionic and ganglionic regions were compared.

Section 15: Characterisation of AChE and BChE molecular forms

In the previous Sections, 10-13, the variation in molecular forms of AChE and BChE was described in amniotic fluid and rectal tissue. These findings are summarised in Section 14 and in particular an increase in G_4-AChE is described in amniotic fluid in the presence of NTD and in rectal tissue in regions of aganglionosis.

In this section the individual characteristics of AChE and BChE molecular forms are studied and compared with particular reference given to G_4-AChE.

15.1 Gel filtration

It is possible to separate AChE molecular forms by gel filtration using media with a suitable fractionation range, (Section 2.6). The experimental details are outlined in Section 7.3. This technique not only provides independent evidence confirming the existence and homogeneity of molecular forms identified by sucrose density sedimentation, but also, when combined with sedimentation data, allows the true molecular mass to be calculated.

Ultrogel AcA 22 proved to be a suitable medium for separation. The void volume, Vo, of the column

was estimated from the elution volume of Blue Dextran. The total bed volume, Vt, by the dimensions of the column and the volume not accessible to solvent, Vg, from the equation:

$$Vg = Vt / B.d$$

Where B is the bed volume per gram of dry gel, 132 ml/g and d is the density of the dry gel, 1.007 g/ml.

From this data Vs, the volume of the stationary phase could be calculated:

$$Vs = Vt - Vg - Vo$$

Hence, Kd values were calculated:

$$Kd = \frac{Ve - Vo}{Vs}$$

Employing this method, Kd values of the nine protein markers were estimated and averaged from the results of 12 runs. These average values are shown in Fig. 7.9 plotted against log [molecular mass].

In the same way samples including markers were applied tothe top of the column in a volume of less than 2 ml and 1 ml fractions collected. From these data Kd values of AChE molecular forms were calculated.

The elution profiles obtained using rectal tissue homogenates are shown in Fig. 15.1. These included samples obtained by homogenisation in 0.1M,

Fig. 15.1 AChE molecular forms from rectal tissue separated by gel filtration on Ultrogel AcA22. The void volume is shown as Vo and individual molecular forms are labelled: G_1, G_4 and A_{12}.

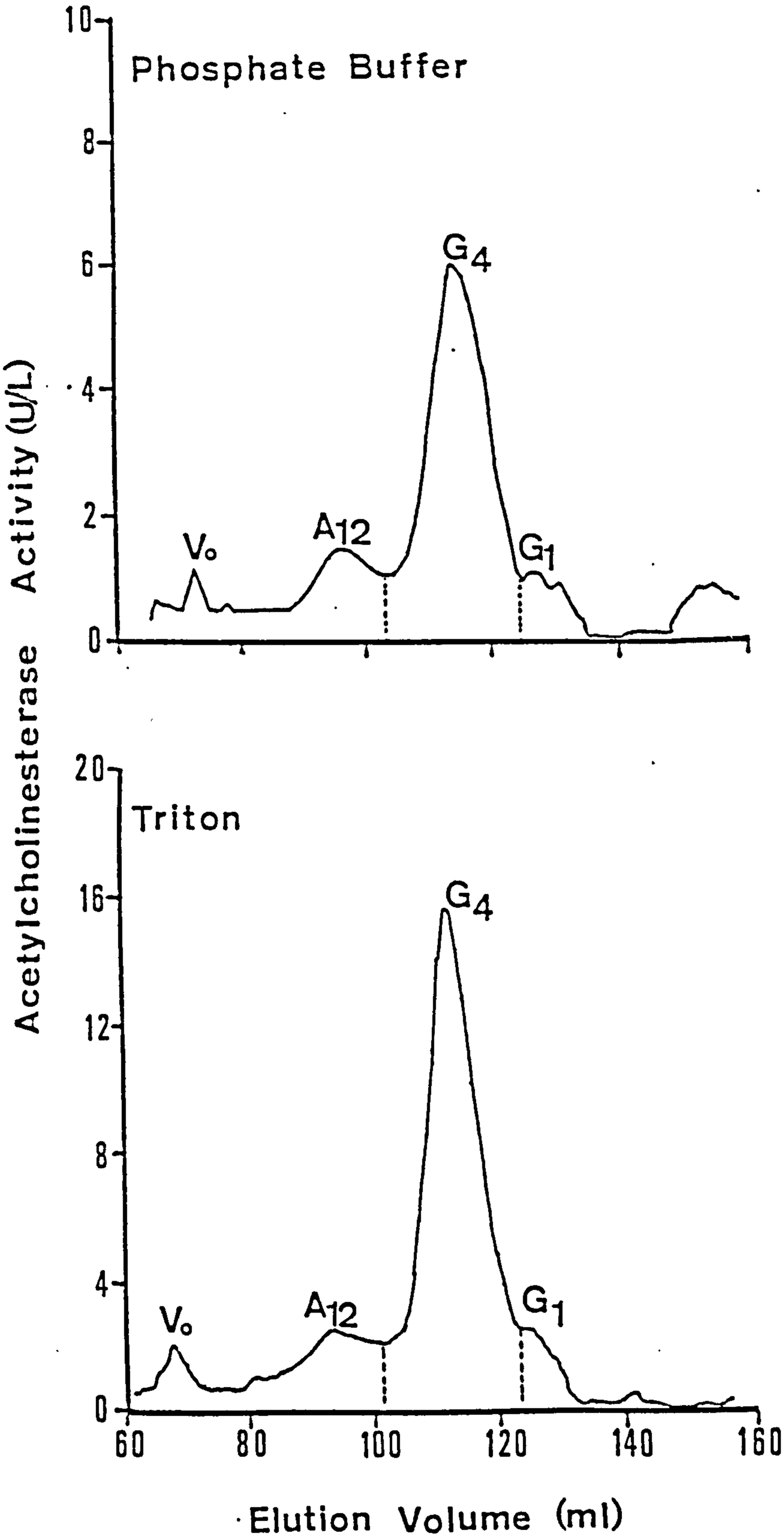


Fig. 15.1 Separation of molecular forms of AChE from rectal tissue by gel filtration

pH8.0 phosphate buffer and also by further extraction of the tissue with buffer containing 1M NaCl and 0.5% w/v Triton. In both cases the results show the presence of G_1, G_4 and A_{12} AChE molecular forms.

Fractions containing distinct molecular forms G_1, G_2 and G_4 obtained by preparative sucrose density gradient sedimentation [Section 7.2], were pooled, concentrated and chromatographed as for rectal tissue homogenates. The results are shown in Fig. 15.2.

Average Kd values obtained for each AChE molecular form present in rectal tissue were calculated and when compared with the protein marker calibration curve, Fig. 15.3, apparent molecular mass could be estimated. The results are shown below:

Molecular mass	Kd	Apparent molecular mass [KDa]
A_{12}	0.28	>670
G_4	0.47	550
G_2	0.52	470
G_1	0.60	285

The results are in excess of the true molecular weight and reflect the asymmetry present in the molecules. Compensation can be made on the basis

Fig. 15.2 Gel filtration using Ultrogel AcA22 of individual AChE molecular forms [G_1, G_2 and G_4] obtained by density sedimentation, the elution volume in each case is shown as Vo.

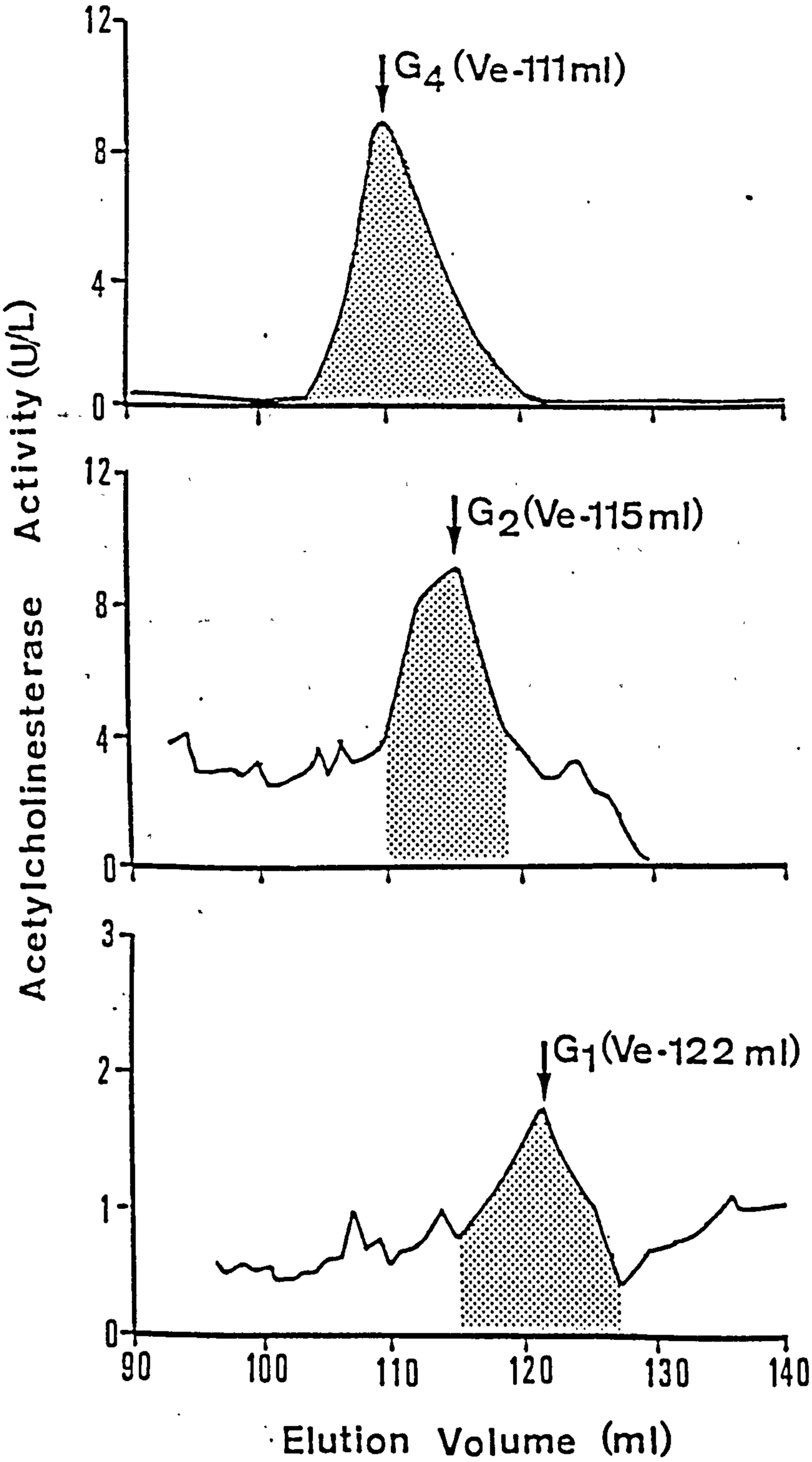
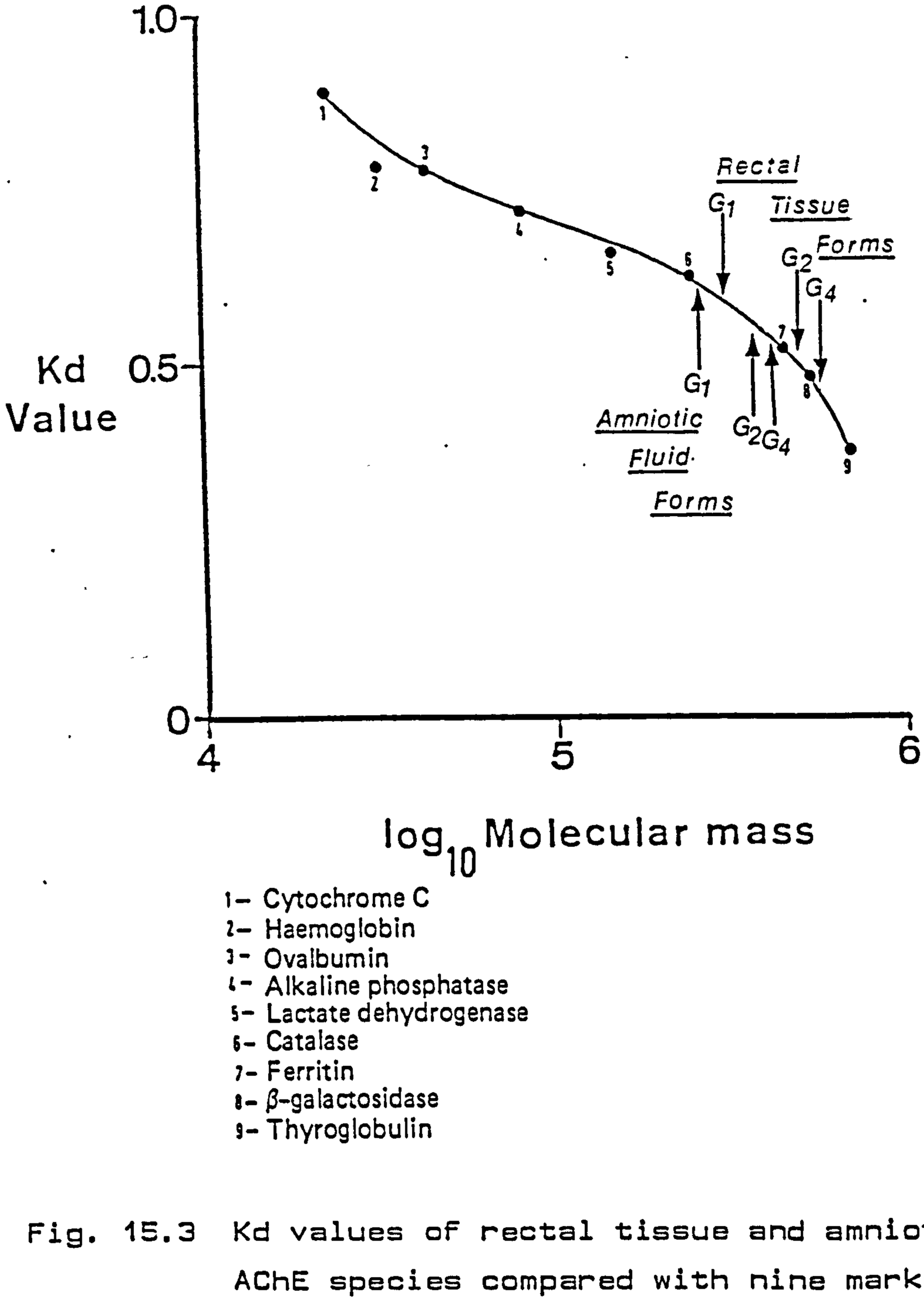


Fig. 15.2 Gel filtration of individual AChE molecular forms

Fig. 15.3 The distribution coefficient, Kd versus $\log_{10}$ molecular mass for nine proteins of known molecular mass. The Kd values of AChE molecular forms from rectal tissue and amniotic fluid are also indicated. The apparent molecular mass of these AChE species can be calculated by comparison with the protein marker curve.



1- Cytochrome C
2- Haemoglobin
3- Ovalbumin
4- Alkaline phosphatase
5- Lactate dehydrogenase
6- Catalase
7- Ferritin
8- β-galactosidase
9- Thyroglobulin

Fig. 15.3 Kd values of rectal tissue and amniotic fluid AChE species compared with nine marker proteins

of S value to give true molecular weights and this is described in the following section. The Kd values for A_{12} AChE are less than the largest protein marker and consequently apparent molecular mass for this form cannot be estimated accurately and is reported as >670 KDa.

Pooled and concentrated amniotic fluid from pregnancies affected by anencephaly and spina bifida were also chromatographed; results are shown in Fig. 15.4. Kd values were calculated and the apparent molecular mass of these amniotic fluid forms estimated from comparison with Fig. 15.3. These are shown below:

Molecular form	Kd	Apparent molecular mass (KDa)
G_4	0.54	405
G_2	0.57	335
G_1	0.62	230

Forms present in amniotic fluid have a greater Kd value and correspondingly lower apparent molecular mass than their counterparts obtained from rectal tissue. This difference in hydrodynamic behaviour is also reflected in the difference in S-value of the forms from different sources as shown in Table 14.1. Taken together these results suggest that the

Fig. 15.4 Gel filtration of AChE molecular forms present in amniotic fluid from pregnancies affected by anencephaly and spina bifida. The AChE species are labelled and the void volume, Vo, is indicated.

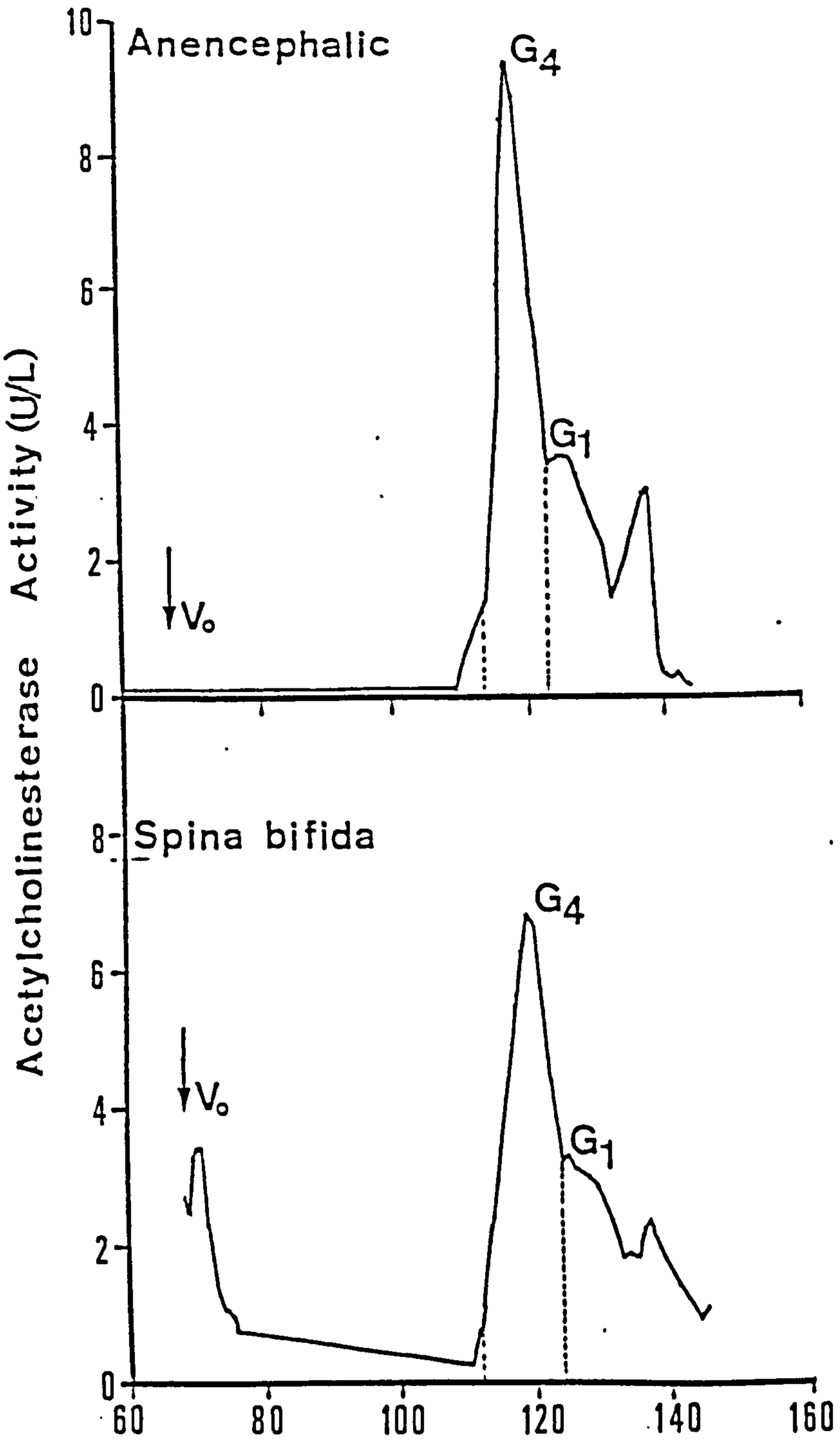


Fig. 15.4 Gel filtration of AChE molecular forms from amniotic fluid

species present in rectal tissue are more expanded in structure, with an increased molecular asymmetry, giving rise to lower Kd and S values. The increased asymmetry slows down sedimentation and artefactually increases the apparent molecular mass toward gel filtration. This effect can be circumvented by combining S-value and gel filtration data to give an estimate of true molecular mass for each form as outlined in the following section.

15.2 Estimation of molecular mass of AChE forms

The combination of experimentally determined Kd for the protein markers with literature values for Stokes radius, Re, allowed the effective gel pore size, r, to be calculated according to the equation [1] [Ackers, 1964]:

$$Kd = \left(1-\frac{Re}{r}\right)^2 \left[1-2.104.\frac{Re}{r} + 2.09\left(\frac{Re}{r}\right)^3 - 0.95\left(\frac{Re}{r}\right)^5\right]$$

When this was averaged for all the markers as described in Section 7.3, the pore radius, r, could be calculated:-

Mean effective pore radius = 46.36 ± 5.62 [SD]. nm

By applying equation [1] with known pore size, r and known Kd value for AChE molecular forms, the Stokes radius, Re for each form could be calculated.

These are shown below:

Molecular form	Kd value	Stokes radius (nm)
Rectal tissue		
A_{12}	0.28	12.19
G_4	0.47	7.54
G_2	0.52	6.60
G_1	0.60	5.33
Amniotic fluid		
G_4	0.55	6.27
G_2	0.57	5.75
G_1	0.62	4.92

Because, $Re.S_{20},w = f$ (molecular mass) (Section 7.3). When f is determined from protein markers with known Re, S_{20},w and molecular mass, this can be applied to determine the molecular mass of any substance of known Re and S_{20},w value.

The value of f determined, as described in Section 7.3, was

$$f = 3958 \pm 124$$

The estimation of molecular mass using this method,

for each AChE form in turn is shown below:

Molecular form	Re [nm]	S20,w [S-units]	f	Calculated molecular mass [KDa]
Rectal tissue				
A_{12}	12.19	16.8	3958	811
G_4	7.54	9.2	3958	275
G_2	6.60	5.0	3958	131
G_1	5.33	3.5	3958	74
Amniotic fluid				
G_4	6.27	10.3	3958	256
G_2	5.75	5.5	3958	126
G_1	4.92	4.0	3958	78

Due to the experimental variability in determining components Re and S_{20},w upon which further calculation is based, the molecular mass is only approximate. However, the results are sufficiently accurate to support the identification of the AChE forms as monomer, dimer, tetramer and in the case of A_{12}, three linked tetramers.

Assuming the tetramer, G_4,to have an arbitrary molecular mass of four, the other molecular forms

then have molecular masses in the ratios shown below:

	A_{12}	G_4	G_2	. G_1
Rectal tissue	11.81	4.00	1.90	1.08
Amniotic fluid	-	4.00	1.96	1.22

These ratios confirm the subscript ratios by which the molecular forms are denoted i.e. G_1, G_2, G_4 and A_{12}.

The results of gel filtration therefore provides further evidence for the existence of AChE forms as distinct molecular entities. These are identifiable by density gradient sedimentation as well as filtration chromotography.

The estimation of molecular mass of these forms supports the assumption that they exist as a series: monomer, dimer, tetramer, i.e. G_1, G_2, and G_4 respectively.

15.3 Thermal Stability

There is evidence [Section 2.8] that molecular forms of AChE differ in their sensitivity towards inactivation by heat. Edwards and Brimijoin, [1983],

describe how G_1-AChE is inactivated by heating for 10-15 min at 51°C, whereas G_4 is relatively resistant to this treatment.

In order to investigate this, molecular forms of AChE from rectal tissue were prepared by sucrose density centrifugation. The appropriate fractions corresponding to distinct species were pooled and made up to a volume of 1ml in each case. Both the separation of these forms by density centrifugation and the experiments determining their temperature sensitivity were performed on the same day to avoid possible interconversion on storage.

1ml pools of each form G_1, G_2 and G_4 obtained in this way were incubated in thin walled pre-heated glass tubes in a 49°C water bath. 60 μl aliquots were removed into pre-cooled tubes in an ice bath at the times indicated. Finally, after 30 min at 49°C, the temperature of the water bath was increased to 80°C and the remaining samples incubated for a further period of 60 min at 80°C. The activity of the samples heated in this way provided a "blank" result which was then substracted from the other measured activities.

Time interval [min]	Enzyme activity [U/l]			Percentage remaining activity [%]		
	G_1	G_2	G_4	G_1	G_2	G_4
0	10.3	9.4	26.4	100.0	100.0	100.0
1	10.5	9.9	27.7	102.0	105.3	104.9
2	10.1	9.9	27.5	98.1	105.3	104.2
3	9.6	9.3	27.8	93.2	98.9	105.3
4	9.6	9.3	27.6	93.2	98.9	104.5
5	8.9	9.3	27.3	86.4	98.9	103.4
6	8.5	9.2	27.3	82.5	97.9	103.4
7	7.9	9.1	25.9	76.7	96.8	98.1
10	7.1	8.5	26.6	68.9	90.4	100.8
15	6.2	-	25.5	60.2	-	96.5
20	5.7	8.0	24.9	55.3	85.1	94.3
25	5.8	7.0	24.6	56.3	74.5	93.2
30	4.7	6.7	23.8	45.6	71.3	90.2

The results, Fig. 15.5, expressed both in terms of activity and percentage change, show that in accordance with the findings of Edwards and Brimijoin, [1983], G_1 is the most thermally labile species and G_4 is relatively resistant to inactivation.

In all cases G_1, G_2 and G_4, increase in activity is observed following brief incubation at $49^{o}C$, this is limited to 5% of the original activity at time zero.

These findings are important to two ways. Firstly, they substantiate the results of another group, Edwards and Brimijoin, [1983] and also

Fig. 15.5 The effect of incubation at 49°C on individual AChE molecular forms recovered from density gradient sedimentation of rectal tissue homogenates. The activity of each form [G_1, G_2 and G_4] is shown as both: the actual measured activity and as the change relative to the initial activty, taken as 100%.

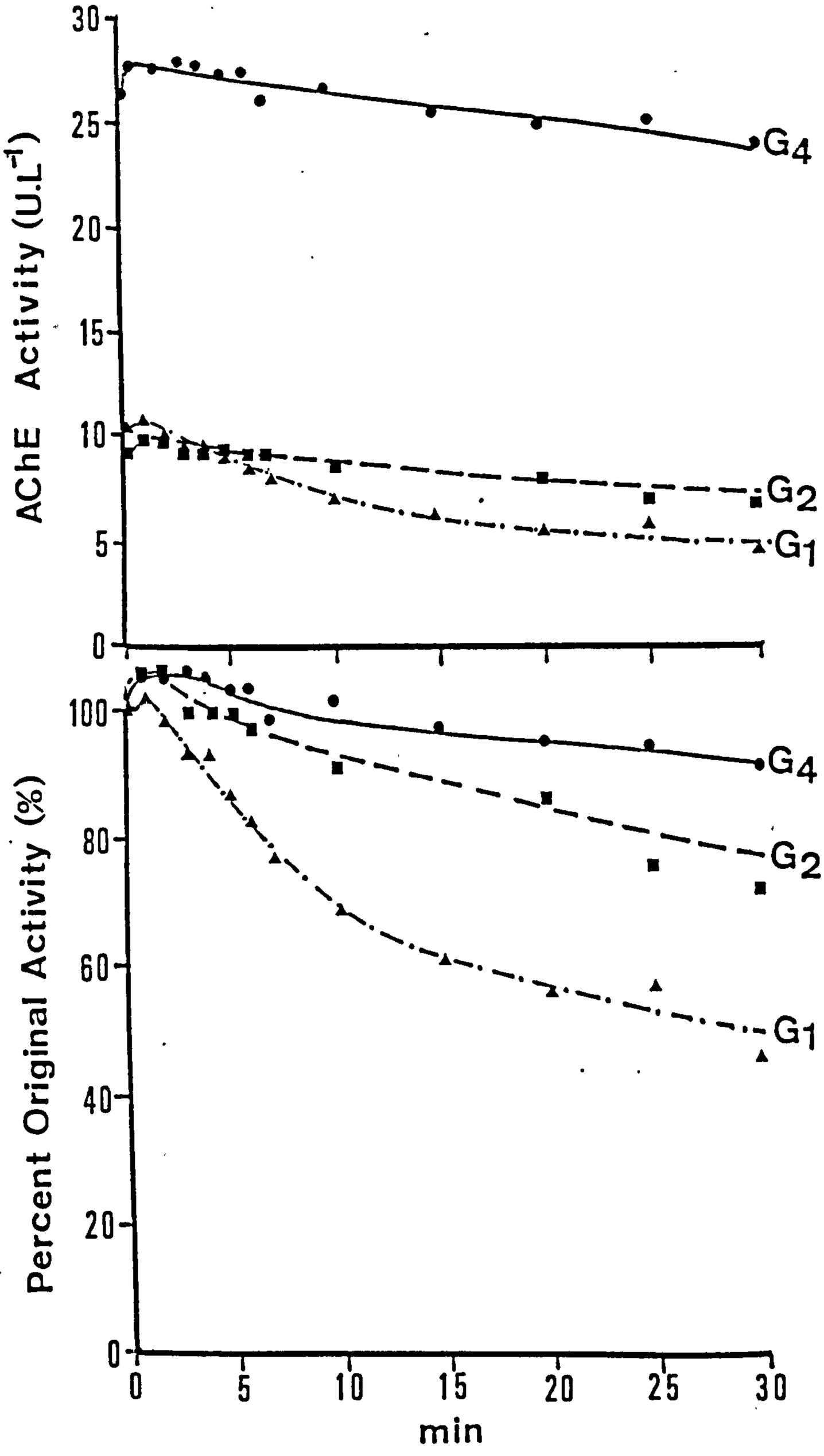


Fig. 15.5 Temperature lability of individual AChE molecular forms

indicate the similarity between AChE forms from different sources, human rectal tissue and rat heart muscle respectively. Secondly, they confirm that distinct molecular entities are being studied and that the peaks obtained by density centrifugation represent behaviourally and structurally differing forms of the same enzyme. Lastly, the relative resistance of G_4-AChE toward heat inactivation may prove valuable in the design of a specific assay directed toward this form.

15.4 Substrate inhibition

It is well attested that AChE is subject to substrate inhibition at concentrations in excess of 2-3 mM [Fig. 1.2].

In order to assess the effect of varying substrate concentration on individual molecular forms, these were prepared by sucrose density sedimentation of rectal tissue homogenates. Appropriate fractions were pooled representing G_1, G_2 and G_4 respectively. The activity of these pools was measured, on the same day to avoid storage effects, using a final concentration of acetylthiocholine ranging from 0.07 to 35 mM. The measurements were performed on a centrifugal fast analyser [Cobas Bio] immediately after addition of the substrate to reagent containing pH7.3 phosphate buffer, DTNB and the selective inhibitor ethopropazine. The analyses, on duplicate

samples for each of the three forms were complete within four minutes. It was important to perform the assays quickly due to the rate of increase of optical density caused by the non-enzymic break-down of AThCh at high concentration. A reagent blank containing substrate of equivalent concentration to that in the test was used in every case.

The results are listed below:

Substrate concentration [mM]	AChE activity [U/l]		
	G_1	G_2	G_4
0.07	5.6	8.1	29.7
0.18	6.8	11.3	46.8
0.35	7.6	13.8	59.0
0.44	7.9	14.7	60.5
0.88	9.8	16.0	67.3
1.20	9.3	18.6	72.9
1.77	10.9	18.4	73.3
3.54	9.0	18.0	71.8
8.84	8.4	16.3	63.8
17.70	6.0	14.2	56.5
35.40	-	-	54.1

The results, Fig. 15.6, are similar for each molecular form with peak activity in the region of 2.0 mM. This confirms the widely held view, expressed by Brimijoin, [1983], that the molecular forms are catalytically similar.

Fig. 15.6 The effect of increasing substrate [acetylthiocholine] concentration on the activity of individual AChE molecular forms recovered from density gradient sedimentation of rectal tissue homogenates. The optimum activity in each case is approximately 2.0mM, at higher concentrations there is inhibition due to excess substrate.

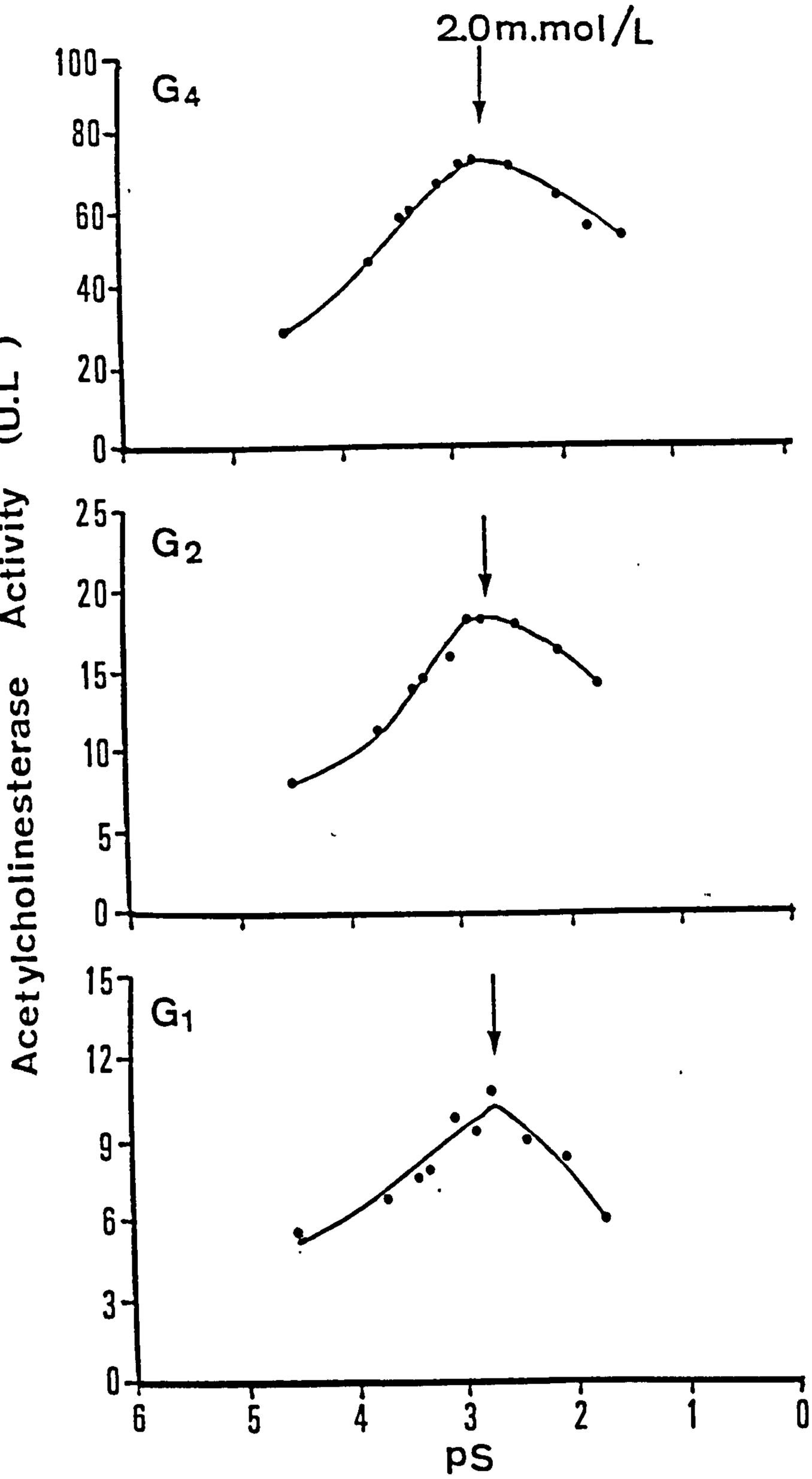


Fig. 15.6 Substrate inhibition of individual AChE molecular forms

15.5 Triton reactivity

Each of the globular series of AChE, G_1, G_2 and G_4 occur in two forms:

[i] Soluble species, which may be secreted or cytoplasmic, and are easily released by aqueous buffers at physiological pH

[ii] Membrane bound species, which are attached either internally or externally to cell membrane components.

The soluble forms may or may not react with detergents such as Triton X-100, [Section 2.8] whereas membrane bound forms will react with Triton in such a way that their sedimentation coefficient is altered, [Lazar and Vigny, 1980]. Therefore, Lazar and Vigny reported that when sucrose density sedimentation analysis of AChE derived from a T_{28} neuroblastoma x sympathetic neuron hybrid is performed in media with and without Triton, the measured S-value was altered. G_4 gave an S-value of 10.2S in the absence of Triton and 9.7S in its presence, while G_1 gave S-values of 4.5S and 3.2 in the absence and presence of Triton respectively.

To test the reactivity toward Triton of AChE molecular forms from rectal tissue and amniotic fluid, sucrose density sedimentation was performed

with and without 0.5% v/v Triton X-100 in the centrifugation media of 0.01M phosphate buffer, pH7.2 containing 1M NaCl.

Two different homogenisation media were used in turn with the rectal tissue: a) without Triton, b) with 1% v/v Triton. The detergent was subsequently removed from homogenate by dialysis against three changes of 600 volumes of 0.1M phosphate buffer pH8.0.

In all, three types of sample were analysed, to compare the results of density centrifugation in the presence and absence of Triton. Namely, amniotic fluid, rectal tissue homogenised in the presence of 1% v/v Triton, and rectal tissue homogenised in a phosphate buffer that contained no detergent.

The S-values obtained by curvilinear interpolation between protein markers in each case are shown below and the profiles obtained illustrated in Fig. 15.7.

AChE species	Rectal tissue [Triton Extract]		Rectal tissue [Phosphate buffer extract]		Amniotic fluid	
G_4	9.4	11.3	9.8	10.9	10.9	10.7
G_2	*	*	5.3	7.1	*	*
G_1	3.9	4.4	3.9	5.1	4.6	4.6
	+T	-T	+T	-T	+T	-T

+T, with Triton [0.5% v/v]; -T, without Triton

* - present only in trace amounts, S value not accurately measurable.

Fig. 15.7 Sucrose density sedimentation performed in the absence, -T and presence, +T of 0.5% v/v Triton X-100 on:

a] A phosphate buffer [pH8.0,0.1M] extract of rectal tissue

b] A Triton extract of rectal tissue

c] Amniotic fluid

Individual AChE molecular forms are identified and the AChE profile obtained in the absence of Triton X-100, -T is shaded.

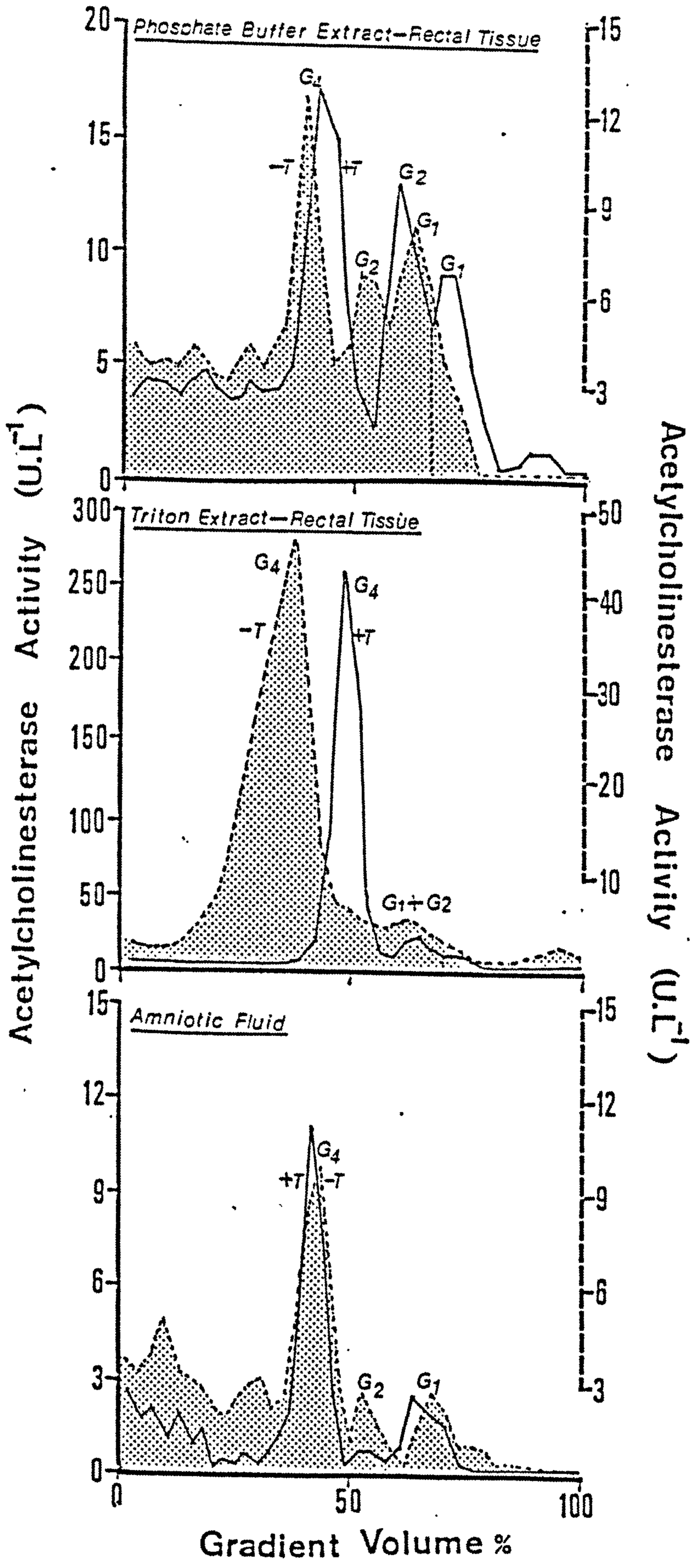


Fig. 15.7 The effect of Triton X-100 on the sedimentation of AChE molecular forms from rectal tissue and amniotic fluid

From these results it can be seen that the most marked differences occur in the Triton extract of rectal tissue, suggesting that these forms derive from cell membranes. Changes in S-value are also seen in the samples produced by homogenisation in phosphate buffer, this suggests that at least some membrane-bound enzyme is released by this buffer system. The changes in S value of rectal tissue forms are of the same order as those reported by Lazar and Vigny, [1980] and point to a behavioural similarity between AChE species from different sources.

In addition to the changes in S-value, a loss of activity is also noticeable in the absence of Triton, [Fig. 15.7] particularly in the Triton extractable species.

Removal of Triton from the centrifugation media does not affect the sedimentation of amniotic fluid AChE forms indicating that these are soluble species which may derive from exposed nerve trunks by secretion into the amniotic fluid.

D I S C U S S I O N

Section 16 : Acetylcholinesterase measurement in the detection of neural tube defects

Section 17 : Molecular forms of AChE and BChE in amniotic fluid

Section 18 : Measurement of rectal biopsy AChE in the diagnosis of Hirschsprung's disease

Section 19 : Molecular forms of AChE and BChE in rectal tissue in Hirschsprung's disease

Section 20 : Characterisation of AChE molecular forms present in rectal tissue and amniotic fluid

Section 16: Acetylcholinesterase measurement in the detection of neural tube defects

16.1 The value of acetylcholinesterase measurement.

A recent review article [Turnbull and MacKenzie, 1983] describes how, following the legalisation of pregnancy termination less than 20 years ago, amniocentesis has become increasingly important as a means of investigating the unborn fetus. This technique has gained particularly widespread application in the prenatal detection of NTD.

Prior to 1980 the identification of NTD affected pregnancies rested almost exclusively on the demonstration of an increased level of AFP [Editorial,Lancet 1980] in the amniotic fluid from such individuals [first described by Brock and Sutcliffe, 1972]. However, in Brock's own words: "The effectiveness of AFP assay is not as complete as might be desired in a form of diagnosis on which critical decisions about termination of pregnancy must be made", [Brock and Hayward, 1980]. Therefore despite the additional contribution made by ultrasound the need for a further biochemical test to complement or replace AFP was apparent [Editorial, Lancet, 1980].

It was against this background that in 1979 Smith, A.D. et al, Chubb et al and Dale et al,b] first described the close association between raised amniotic fluid AChE and NTD. Initial studies suggested that AChE was more specific and possibly more sensitive than AFP in the detection of NTD [Smith, 1982]. In the same review [Smith, 1982] the author asserts, the now commonly held view, that this improvement in specificity derives from a neural origin for AChE in the presence of NTD, probably due to leakage of CSF [the species identified in amniotic fluid showed the same electrophoretic mobility as the CSF enzyme]. Research using experimental animals with an NTD lesion have since confirmed this hypothesis [Pilowsky et al, 1982] and have provided further support for the use of AChE in the detection of NTD in man.

16.2 <u>Comparison of qualitative and quantitative techniques</u>

Following these initial findings there was an increase in the number of reports confirming and at times qualifying these results. Only recently has this begun to "tail-off" [Fig. 16.1]. The later reports are fewer but tend to be on larger populations, with two of the more recent: Aitken et al, [1984] and Wyvill et al, [1984] including 3785

Fig. 16.1 The culmulative total of reports from 1979 to 1985 concerning the use of cholinesterase measurement in the detection of neural tube defects. Data for1985 is still incomplete.

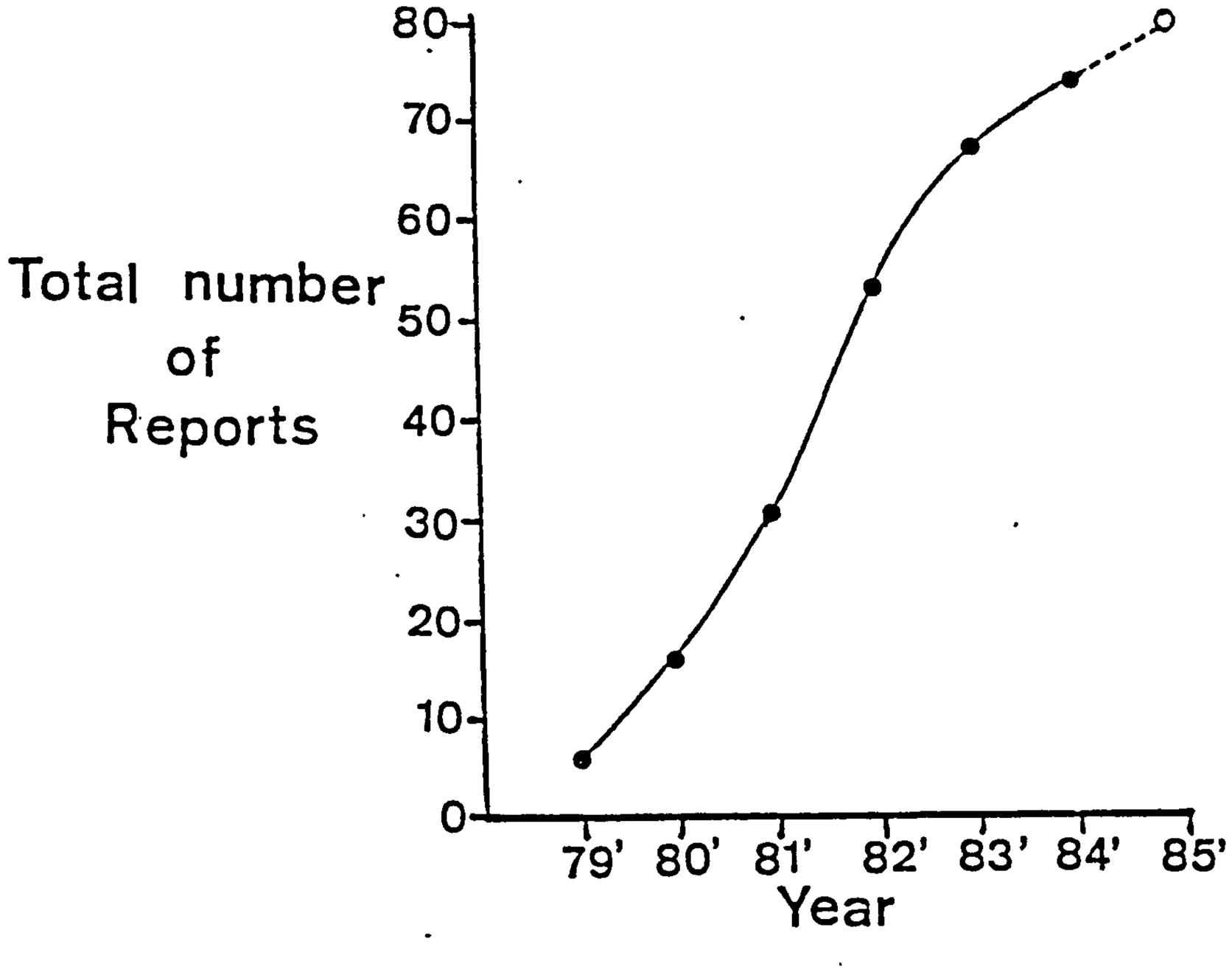


Fig. 16.1 The culmulative total of reports describing cholinesterase measurement in amniotic fluid

and 1495 samples respectively.

In their original article, Smith, A.D. et al, [1979] described two distinct approaches to amniotic fluid AChE measurement:

[i] Quantitative assay, in which the rate of hydrolysis of acetylthiocholine and the subsequent detection of the evolved thiol is determined in the presence and absence of the selective AChE inhibitor, BW284c51.

[ii] Qualitative AChE assay, demonstrating a fast migrating band of AChE activity on electrophoresis in polyacrylamide gels in the presence of NTD, which was absent in pregnancies with normal outcome. This is often simply referred to as the "gel-test".

Since then there has remained controversy over the relative merits of these two approaches to the detection of NTD. The points are detailed in Section 4.4 [iii].

In favour of the qualitative assay are several reports which suggest that it is less affected by contamination with blood than the quantitative test [Buamah et al, 1980; Voigtlander et al, 1981; Brock and Hayward, 1980]. This is important as it has been demonstrated that approximately 10% of all

samples contain blood [Smith, 1982]. In addition to its benefits with respect to blood staining, several reports suggest that the gel test shows better discrimination between NTD affected and normal pregnancies than the quantitative assay [Seller and Cole, 1980; Aitken et al, 1984; Wyvill, 1984]. At least some of this improvement in specificity may result from the assertion that this test measures one specific secretory form of AChE [Hodgson et al, 1981]. It is certainly true that the "gel-test" has gained more widespread acceptance than the quantitative assay [Brock et al, 1985] and its usefulness has been clearly demonstrated in practice [Collaborative AChE Study, 1981].

Despite these advantages, even advocates of the qualitative approach allow that it has certain drawbacks. Gels can be prone to cross contamination via the cathode buffer. There are occasional problems in the interpretation of faint bands [Barlow et al, 1982a] and, despite the fact that densitometry has been used to make the test more objective [Milunsky and Sapirstein, 1982], it remains at best semi-quantitative, requiring a considerable degree of operator time and experience to produce reliable results.

In contrast, the quantitative measurement of AChE is rapid, simple to perform and easy to interpret [Smith, 1982] it is entirely objective and not operator-dependent. In addition, several reports support the view that it discriminates well between NTD and normal pregnancies [Dale et al, 1981; Bonham et al, 1981; Legge and Potter, 1983] and consequently its proponents have described this approach as "the method of choice" [Legge and Potter, 1983].

It would appear a sensible view not to consider these techniques as competing alternatives but as complementary [Buamah et al, 1980]. The objectivity, speed and simplicity of the quantitative assay coupled with the ease of automation allow this method to be used in ways in which the gel test could not be. For instance, it has been suggested that quantitative amniotic fluid AChE measurement could be completed and reported during an out patient visit, consequently allaying anxiety in cases where a favourable outcome could be assured [Hullin et al, 1981]. It has also been anticipated that AChE might in the future altogether replace AFP in the detection of NTD [Milunsky and Sapirstein, 1982]. In this situation the quantitative assay with its ease of automation would be a more

suitable procedure for large numbers of samples than gel electrophoresis.

On the other hand, the qualitative test has certain advantages in specificity, particularly in the face of blood contamination. It would appear a reasonable approach to measure all samples quantitatively and then proceed to electrophoresis only in cases where the results were borderline or there was visible evidence of blood contamination.

It has been one of the aims of this thesis to examine the diagnostic potential of the molecular heterogeneity of both AChE and BChE in amniotic fluid demonstrated by sucrose density sedimentation, which prior to this work had not been used in this context. It was anticipated that this would create a better understanding of both the "gel-test" and the quantitative assay by identifying the molecular species present in each case. If one particular form of the enzyme were demonstrated to be responsible for the increased activity of the quantitative assay and the additional band seen on the "gel-test" this would form the basis for a new assay i.e. quantitative measurement of that particular species. This would combine the advantages of both approaches.

16.3 AChE results from the samples under study

The study group consisted of 39 samples from subjects with known pregnancy outcome: 17 were normal, nine were affected by affected by anencephaly, seven by spina bifida, five by exomphalos and there was a single case of intrauterine death. The samples were chosen to include some with borderline AFP results: SB1 AFP = 54 μg/ml and SB6 AFP = 48 μg/ml. It was in these cases that the identification of any new marker would be most rigorously tested.

The results obtained in this group are shown in Table 9.1. It can be seen that there is a clear separation between groups with no overlap. These results are consistent with those of Dale et al, [1981]; Bonham et al, [1981] and Legge and Potter, [1983] but are at variance with the recent reports of Aitken et al, [1984] and Wyvill, [1984] and other workers [Seller et al, 1980]. It has been suggested that these differences may be attributable to "subtle method changes from operator to operator" [Legge and Potter, 1983]. Alternatively, they may simply reflect a larger collection of samples with small lesions producing more borderline results.

The AChE activity determined in normal, spina bifida and anencephalic groups are shown in Table 9.1.

The means in each case are 3.5, 8.7 and 15.2 U/l respectively. The activity was significantly higher in the NTD cases, $p < 0.0005$. Taking into account minor methodological variations, these results compare well with the corresponding overall means obtained by averaging results from the literature shown in Table 4.2, these are 2.9, 6.1 and 11.6 U/l respectively for the corresponding groups. The 39 samples in which molecular forms of AChE and BChE are to be identified can therefore be assumed to be representative of the population of samples normally encountered in routine detection of NTD.

16.4 Automation of AChE assay in amniotic fluid

It was stated in Section 16.2 that one advantage of the quantitative assay was its suitability for automation. This has been capitalised on by several groups [Lamedica et al, 1983; Moreau et al, 1982; Wyvill et al, 1984; Hay et al, 1983] who have designed assays based upon different types of CFA, an instrument widely used as a means of routine enzyme analysis in clinical chemistry laboratories.

In addition to the saving in time when large numbers of specimens are to be analysed, automation will also improve the precision of an assay. This is particularly important as early reports suggested that poor precision, possibly attributable to lack of familiarity with the assay [Seller et al, 1980], was a problem. The results outlined in Section 9.1 obtained by the automated procedure and described in Section 6.1 showed excellent precision. The CV = 2.1% at 2.9 U/l, was close to the CV of 1.9% at 3.1 U/l reported by Hay et al, [1983], using a very similar procedure and better than the CV of 9.6% at 4.7 U/l quoted by Wyvill et al, [1984] using a Multistat III CFA [IL Instruments Ltd]. The correlation between the manual and automated techniques was also excellent [r = 0.99].

The flexibility of this type of procedure enables large and small batches of samples to be coped with. Up to 28 samples can be processed in less than 30 min and once reagents have been prepared, the analyser is capable of measuring up to 150 samples per hour. This type of adaptability would permit even the highest workloads from regionally based prenatal detection programmes to be accommodated.

16.5 Comparison between AFP and AChE in the detection of NTD

The availability of a simple, reproducible, automated procedure for measuring amniotic fluid AChE brings with it the possibility of abandoning amniotic fluid AFP completely in favour of AChE. Consequently, it is worth considering the relative merits of these two approaches and how they might each be applied.

Interpretation of amniotic fluid AFP measurement has three main problem areas, [Brock and Hayward, 1980]: Samples contaminated with fetal blood which give spuriously elevated AFP levels; pregnancies in which gestational dating is uncertain making interpretation of AFP [itself related to gestational age] dubious; the observation that other fetal malformations, some of which are surgically correctable, are also associated with a clearly raised AFP concentration [Table 4.1, Section 4.4]. Despite these difficulties AFP has been extensively used for the past 13 years and several large studies testify to its effectiveness in practice, notably the Collaborative AFP Study [1979] where 13,940 fluids were analysed and that of Milunsky, [1980] who described experience

with samples from 20,000 pregnancies. The detection rates quoted were 98% and 95% respectively and the false positive rate for each corresponded to 0.5% and 1%. While these data appear acceptable, nevertheless, as the incidence of NTD in the population is low [0.2-0.3%; Alberman, 1978] this false positive rate could lead to as many incorrect as correct diagnosis [Editorial, Lancet, 1980].

It is in this area of reducing the false positive rate of the AFP assay that the measurement of AChE has found considerable support. The report of the Collaborative AChE Study, [1981] confirmed that AChE is capable of reducing the number of false positives by 94% while only decreasing the detection rate by 1%. It is true that ultrasound too, may have a contribution in this respect, however, it reduces the true positive rate to a greater extent than is the case for AChE [Collaborative AChE Study, 1981]. As a result, and despite the range of alternative secondary tests to AFP which have been proposed, Read et al, [1982] was led to conclude that, "the gel-test is the only suitable adjunct to AFP" and Haddow and Miller, [1982] arrived at similar conclusions.

Quantitative measurement of AChE is simple, cheap, rapid, reproducible [Dale et al, 1979b] and

easily automated. The possibility was raised by Smith, A.D. et al [1979] that it may eventually supplant AFP altogether, over which it displays several potential advantages:-

Unlike AFP, AChE is derived predominantly from the nervous system and is likely to be a better marker for a fetal CNS abnormality. In this respect the actual results are somewhat disappointing [Brock, 1983], nevertheless, it is capable of differentiating abdominal wall defects such as exompahlos from NTD [Bonham et al, 1981] which AFP is not. This is an important group of disorders in which the incidence is 12% of that shown by NTD itself [Collaborative AChE Study, 1981], if left to term they are amenable to surgical correction, but if AFP alone is used as a guide these cases are prematurely aborted.

Cholinesterase measurement is less affected by contamination with blood than AFP [Milunsky et al, 1979], the "gel-test" is particularly resistant to interference from this source [Brock and Hayward, 1980].

The major advantage of quantitative AChE measurement compared with AFP is its independence of change with gestational age during the second

trimester [Bonham et al, 1981; Hodgson et al, 1981]. This enables one reference range to be used and avoids a major cause of interpretative difficulty with the AFP assay.

The large amount of experience amassed with AFP measurement has sustained its position so far, as the primary marker for NTD. In addition, the use of maternal serum AFP screening programmes first suggested by Brock et al, [1973] also maintained the inertia of continuing with AFP measurement. There are no equivalently large studies using AChE and the most comprehensive reports that do exist concentrate on the "gel-test" [Aitken et al, 1984; Wyvill et al, 1984]. Such studies establish the use of the "gel-test" as an adjunct to AFP. The eventual replacement of AFP by quantitative AChE, probably awaits the development of an assay directed toward that fraction of AChE activity derives from nerve tissue exposed as a result of the lesion. Before such an assay can be designed an "NTD-specific" form of AChE must be identified.

Section 17: Molecular forms of AChE and BChE in amniotic fluid

17.1 The importance of understanding the AChE forms present in amniotic fluid

In order to gain a clear understanding of the interpretation of AChE results it is important to answer some basic questions relating to the nature and origin of the increased levels of the enzyme in the presence of NTD. It has been known for sometime that both AChE and BChE exist in a number of electrophoretically distinct forms [Section 2.1]. More recently, polymorphism based upon molecular size and distinguishable by sedimentation coefficient has been described, firstly in electric fish [Massoulié and Rieger, 1969] and subsequently in mammals [Hall, 1973]. Distinct quaternary structures have been proposed for each of these differently sedimenting forms., [Section 2.2].

AChE is found to exist in at least six molecular forms including three globular species; G_1, G_2 and G_4 and three asymmetric species; A_4, A_8 and A_{12}. In man, CSF is found to contain soluble AChE corresponding mainly to the G_4 form [Massoulié

and Bon, 1982], which is probably derived by direct secretion from nerve tissue [Scarsella et al, 1979]. By identifying the forms of AChE present in amniotic fluid in NTD affected pregnancy and comparing these with those known to occur in fetal serum and CSF, it may be possible to indicate the likely origin of the increased AChE activity. The potential effects of other interpretative difficulties could also be more accurately assessed, for example contamination by fetal or maternal blood. In addition a modified specific assay may be able to be designed leading to an eventual definitive test for NTD.

17.2 Molecular forms of AChE identified by sucrose density sedimentation

The results of the sucrose density sedimentation analysis performed on 39 samples of amniotic fluid are described in Section 10.1. The samples included 17 from pregnancies with normal outcome, nine affected by anencephaly, seven by spina bifida, five cases of exomphalos and a single case of intrauterine death.

In all samples from cases of NTD, but not normals, there was a prominent peak of AChE activity migrating with a sedimentation coefficient of 10.3S.

Comparing this value with those described for G_4-AChE in the literature [Section 2.2, Table 2.1] it was assumed that this peak corresponded to the tetrameric, G_4 form of AChE. In addition to this peak, there were also AChE species present sedimenting at 5.5 and 4.0S. These were believed to represent G_2 and G_1 respectively. Confirmation that these molecular forms did indeed correspond to monomeric, dimeric and tetrameric AChE is described in Section 15.2, where it is shown that their molecular masses are approximately in the ratios 1:2:4. The five samples from pregnancies affected by exompahlos showed small amounts of G_4, which in all cases was less than that seen in the presence of NTD. The single case of intrauterine death, however, showed a markedly elevated level of G_4, possibly due to increased membrane permeability secondary to cell death.

These results taken together, indicate the existence of an "NTD-specific" form of AChE, G_4, which is responsible for 80% of the AChE activity in the amniotic fluid of NTD affected pregnancies. The increase in G_4 is statistically highly significant, $p < 0.001$. There are also slight increases in G_1 and G_2 in the presence of NTD but

these are less significant with $p<0.05$ and $p<0.02$ respectively.

It is not possible to directly compare these results with those obtained elsewhere as sedimentation techniques have not previously been employed in this context. However, Biagioni et al, [1984] comment upon the qualitative demonstration in amniotic fluid of a distinct band [corresponding to the "secretory form" present in human CSF] separable by PAGE and characterised by the use of specific substrates and inhibitors.

The demonstration by sedimentation of an NTD-specific form of AChE is important for several reasons which will be discussed separately:

i] The existence of such a form could lead to the design of a more specific and sensitive assay.

ii] Because 80% of the AChE activity accompanying NTD is attributable to one particular species this can be used to indicate the origin of the enzyme in amniotic fluid.

iii] It enables direct comparison between the basis of the "gel-test" and quantitative techniques. This is discussed more fully in Section 17.3.

i] An NTD specific assay based on measurement of G_4-AChE

The potential advantages of such an improved assay for detection of NTD include:

Increased Specificity

When the close association between amniotic fluid AChE and NTD was first noted it was anticipated that because AChE is derived from the nervous system it would prove a more specific marker for a fetal neural abnormality [Chubb et al, 1979 a]. This was later confirmed and there have been a number of reports suggesting that measurement of AChE, unlike AFP, will not give false positive results in the presence of some fetal abnormalities such as congenital nephrosis [Brock and Hayward, 1980; Collaborative AChE Study., 1981; Aitken et al, 1984; Macri et al, 1981] and can differentiate abdominal wall defects from NTD lesions [Bonham et al, 1981; Dale et al, 1981; Wald et al, 1984].

Despite this, measurement of AChE was proved somewhat disappointing with respect to its specificity toward NTD and this led Brock [1983] to comment "AChE does not contribute a great deal to the differential diagnosis of fetal malformations".

In addition, fetal blood staining in amniotic fluid can give rise to misleading results [Brock, 1983].

In this respect the "gel-test" is less affected than the quantitative assay, Section 16.2, probably because it measures a specific secretory form of the enzyme [Hodgson et al, 1981; Biagioni et al, 1984].

In Section 10.1 it can be seen that if one particular form, G_4, associated with NTD alone, were measured, the specificity of the quantitative assay would be enhanced. Indeed, it is evident from the results shown in Section 10.2 that amniotic fluid from exomphalos-affected pregnancies contains only traces of this form of AChE.

In addition to increased specificity towards NTD malformations, assay of G_4 would also exclude interference from AChE derived from other sources, such as the G_2 found on erythrocyte membranes. Measurement of G_4-AChE would therefore be less affected by blood staining than measurement of total AChE activity. Nevertheless, problems may still be encountered by contamination with fetal plasma [Barlow et al, 1982b] known to contain G_4-AChE.

In summary, quantitative assay of G_4-AChE would be expected to combine the specificity of the "gel-test" with the objectivity of total AChE measurement.

Improved sensitivity

While measurement of total AChE is already a sensitive means of detecting NTD, even in cases with borderline AFP results, measurement of G_4-AChE would further improve this sensitivity. The mean AChE level in NTD pregnancies in the 39 samples studied was 3.6 x the mean level with normal outcome. The mean G_4-AChE in NTD affected pregnancies was 60 x that in normals. This increase in sensitivity is particularly marked in borderline cases, e.g. SB1 and SB6, with AFP results of 54 and 48 μg/ml respectively. The total AChE in both these cases was approximately 2 x the normal mean, whereas G_4-AChE showed an increase of 44 and 16 fold respectively.

From the foregoing discussion it is clear that measurement of G_4-AChE improves both the specificity and the sensitivity of detection of NTD and should be accompanied by a decrease in both false positive and false negative results in practice.

Possible means of selective G_4-AChE measurement

While sucrose density gradient sedimentation allows identification and quantitation of G_4-AChE, this technique has limited practical value in routine

use. The centrifugation [approximately 16 hours], preparation of the gradients, fractionation and subsequent assay of gradient fractions are all lengthy procedures. It takes two full days to analyse only three amniotic fluid specimens and this precludes its use in the routine clinical chemistry laboratory.

It is therefore necessary to examine alternative means of designing a suitable assay. Catalytically, the differences between G_1, G_2, G_4 are minor [Massoulié, 1980]. Consequently, it is unlikely that a sufficiently specific assay could be created by this approach. These species also show similar response to substrate concentration [Section 15.4], and there is little in the literature to suggest any other kinetic differences e.g. inhibitor sensitivity.

One interesting distinction between different forms is their sensitivity to heat denaturation [Edwards and Brimijoin, 1983]. This is investigated in Section 15.3 and discussed more fully in Section 20.3, results indicate that following incubation at $49^{o}C$ for 30 min, G_1 activity is reduced by 50%, whereas G_4 is reduced by less than 10%. While far from entirely selective, this will partially differentiate G_1 and G_4 AChE.

From the evidence so far available neither minor catalytic differences nor differential behaviour toward heat denaturation, are sufficiently selective to allow the design of a specific assay for G_4-AChE.

Two alternative approaches to this problem are density gradient PAGE and the preparation of monoclonal antibodies directed against individual AChE species.

Density gradient PAGE will separate AChE molecular forms. However, the qualitative nature of this technique and the difficulty in automation make this method only marginally more desirable than conventional PAGE electrophoresis for routine use.

The use of specific antisera to identify and differentiate AChE and BChE has been reported in the detection of NTD [Norgaard-Pederson, 1982 and Brock and Bader, 1983]. More recently, Brock et al, [1985] has described a quantitative AChE technique using monoclonal antibodies: in this report the antibody was raised against erythrocyte AChE [G_2] but cross reacted with the enzyme in amniotic fluid [predominantly G_4 in the presence of NTD] and therefore does not offer any obvious advantage over enzymic determination of total AChE activity.

There have,however, been several attempts [Abe et al, 1983; Taylor et al, 1984; Brimijoin and Mintz, 1984] to produce monoclonal antibodies against individual molecular species of AChE. Taylor et al, [1984] report antibodies with enhanced specificity towad G_2 from the electric organ of Torpedo. Brimijoin and Mintz, [1984] are currently endeavouring to raise specific antibodies against mammalian AChE. It is probable that selective measurement of AChE species will increasingly be approached in this way. Such assays are adaptable for routine diagnostic use and would offer specific quantitation of G_4-AChE for use with large numbers of sample.

ii) The origin of AChE in amniotic fluid

It has been suggested that in normal amniotic fluid, AChE activity is largely of placental origin, whereas in NTD the activity is enhanced by direct secretion from nerve trunks or by CSF leakage [Pilowsky et al, 1982; Smith, 1982]. The results of this study support this hypothesis.

It is known that the major form of AChE present in human CSF is a soluble G_4, with a sedimentation coefficient of approximately 11.0S [Massoulié and Bon, 1982]. This species also comprises the major form produced by nerve trunks

[Brimijoin, 1983] and it is generally accepted that secretion by nerve cells is the means by which AChE activity is maintained in the CSF [Biagioni et al, 1984; Scarsella et al, 1979].

Identification of G_4-AChE in the amniotic fluid of NTD affected pregnancies but not in normals indicates that the ultimate origin of the increased activity produced in NTD is nerve trunks.

Not only is the enzyme of the same type as that secreted by nerves, but there is further evidence that the species present in amniotic fluid is indeed a secretory product. All membrane bound forms of AChE have their hydrodynamic properties altered, e.g. sedimentation coefficient, by the presence of Triton X-100, that is, they are "detergent sensitive" [Brodbeck and Ott, 1984]. However, the secreted soluble forms can include both detergent sensitive and insensitive components [Massoulié et al, 1984].

The results in Section 15.6 show that there is no difference between the S-value of G_4 from amniotic fluid whether it is measured in the absence or presence of 0.5% v/v Triton X-100. That is, this form is detergent insensitive. These results are in accord with those of Ralston et al, [1985] who have purified a soluble G_4 form from fetal bovine serum and report that 1% Triton does not affect its sedimentation.

The available data therefore supports the hypothesis [Pilowsky et al, 1982; Smith, 1982] that G_4 in amniotic fluid is a soluble enzyme secreted from nerve trunks.

These findings provide a firmer theoretical basis upon which to understand and interpret AChE results in the prenatal detection of the disease.

17.3 Polyacrylamide gel electrophoresis of amniotic fluid

The most widely used method of measuring AChE activity in amniotic fluid for the detection of NTD is "gel-test" [Section 4.4].

In order to compare the sedimentation data, [Section 10.1], with this method of demonstrating AChE heterogeneity, G_4 prepared by density gradient sedimentation was subjected to electrophoresis on 5% PAG by the method of Buamah, [1980] used in the detection of NTD. In addition amniotic fluid samples from two cases of exomphalos, spina bifida and anencephaly and three samples from normal pregnancies were analysed in the same way.

The gels were stained and subsequently scanned in a laser densitometer, [Section 7.4] and the results are shown in Fig. 10.7.

The amniotic fluid specimens from NTD affected pregnancies revealed a distinct band of AChE which

was absent in those with normal outcome or those affected by exomphalos. It can also be seen that this NTD-band occupies the same position on the gel as G_4-AChE prepared by density sedimentation [Fig.10.7]. It is therefore probable that the basis of the success of the "gel-test" is measurement of G_4-AChE.

Larger membrane bound forms of AChE would be excluded from the gel [Webb et al, 1981]. However, other globular forms G_1 and G_2 enter the gel. Because separation is on the basis of net charge and as these forms have similar charge characteristics, they may occupy the same position on the gel as G_4.

It is probable that AChE activity in the "gel test" in cases of NTD results from G_4, but the possibility of interference from other globular forms cannot be excluded. The test therefore may be less specific than measurement of G_4 alone.

17.4 Butyrylcholinesterase in amniotic fluid

There have been several studies reporting the usefulness of total cholinesterase [AChE + BChE] measurement in the detection of NTD [Milunsky et al, 1979; Dale et al, 1981; Zeisel et al, 1980; Lawton, 1981; Hodgson et al, 1981; Coombes et al, 1982]. Among the advantages claimed are simplicity [Milunsky et al, 1979] and the avoidance of toxic inhibitors [Zeisel et al, 1980]. There is also some

evidence that the assay improves the sensitivity of detection of NTD [Lawton, 1981].

BChE exists in a series of molecular forms homologous with those of AChE [Brimijoin, 1983]. The heterogeneity of BChE in amniotic fluid of normal pregnancies and those affected by NTD and exomphalos is shown in Fig. 10.8. The results indicate that the increased activity seen accompanying NTD is largely attributable to the tetrameric, G_4 form of the enzyme. Unlike AChE this species is also increased in the presence of exomphalos, [Fig. 10.8]. G_4-BChE is also present in high activity in both maternal and fetal sera [Lockridge et al, 1979]. It would be anticipated that measurement of BChE or total cholinesterase activity [AChE + BChE] would be less specific than AChE alone in the identification of NTD and the exclusion of other fetal malformations. In addition it would be more liable to false positive results arising due to contamination with fetal or maternal blood.

These views are supported by Davies et al, [1979] who concluded that, if cholinesterase measurement is to be of any value in the detection of NTD it must be carried out using specific inhibition of the BChE fraction. The findings of

Hodgson et al, [1981] reflect the validity of this statement, they report that while BChE activity is increased in the presence of NTD, nevertheless it is a less useful marker than either AChE or AFP.

Despite the evidence that BChE alone provides only poor detection of NTD and its theoretical lack of specificity in the presence of other fetal malformations or blood contamination, nevertheless its measurement may still occasionally prove valuable. For instance, a marked increase in BChE activity accompanied by a marginal increase in AChE, is very suggestive of contamination with either fetal or maternal plasma. Bonham et al, [1981] suggest that in cases of exomphalos [which has an associated leakage of fetal plasma] the quantitative assay of BChE and AChE produces a ratio of greater than 10:1. Similarly, quantitative densitometry has been used in the "gel-test" to calculate the ratio of the densities of BChE and AChE bands. Goldfine et al, [1983b] reports this to be greater than 7:1 in cases of exomphalos and gastroschisis. This was further substantiated by Wald et al, [1984] who reported ratios of grater than 10:1 in these cases. Contamination with serum can also be detected by this means, where similarly high BChE:AChE ratios are produced [Peat and Brock, 1983; Barlow et al, 1984].

17.5 Summary of the findings in the study of amniotic fluid

In summary, the results [Section 10.1] represent the first reported description of AChE molecular forms in amniotic fluid distinguished on the basis of their sedimentation coefficients. Three globular forms were identified corresponding to G_1, G_2 and G_4-AChE, asymmetric forms of the enzymes were not apparent.

G_4-AChE was shown to be closely linked with the presence of NTD and can be regarded as "NTD-specific" in this context [Bonham et al, 1983; Bonham and Dale, 1985b]. It was found that the basis of both existing approaches to the detection of NTD using AChE, namely, the "gel-test" and the quantitative measurement of the enzyme, relied upon the increase in this particular species. The identification of this marker may well form the basis of a much improved diagnostic assay for NTD, when appropriate methodology becomes available.

The G_4-AChE has the same characteristics as the major form of AChE found in CSF and also the predominant species secreted by nerve tissue. The data therefore support the widely held view that the exposed nerve tissue gives rise to the increased AChE activity in NTD.

EChE, on the other hand, is shown to be a less specific indicator of NTD as the particular form of the enzyme associated with NTD is also present in amniotic fluid from normal pregnancies and in fetal and maternal plasma.

Section 18. Measurement of rectal biopsy AChE in the diagnosis of Hirschsprung's Disease

18.1 Diagnosis of Hirschsprung's disease

Hirschsprung's disease is said to be the commonest single cause of admission to hospital for obstruction in the newborn period [Jones, 1970]. The disease which occurs in approximately 1:5000 live births shows a male preponderance of 4:1 [Lavery, 1983; Section 3.3]. Once diagnosed its surgical correction is both safe and effective, [Section 3.7]. However, if the condition remains untreated during childhood, there is a gradual increase in the degree of constipation and a progressive distention of the abdomen. In addition, entercolitis, which develops in 15-29% of patients by the time of diagnosis [Kleinhaus et al, 1979; Ikeda and Goto, 1984] is a particularly dangerous complication with a 30% mortality [Kleinhaus et al, 1979]. If the incidence of entercolitis and its complications are to be effectively diminished, the diagnosis of Hirschsprung's disease must be established during the first month of life [Kleinhaus et al, 1979]. It is for this reason, and to avoid a potential period of chronic ill health, that interest has been concentrated on establishing a safe and reliable

means of diagnosis that can be used in the newborn period.

The techniques employed, which already have been described [Sections 3.6[i]-[vi] have included

a] Radiology, rarely diagnostic by itself, is particularly difficult to interpret early in life [Lavery, 1983]. It is nevertheless a valuable additional investigation which can often give a measure of the length of the affected segment.

b] Rectal manometry, introduced by Schuster et al, [1965] depends upon the demonstration of an abnormal response of the internal anal sphincter towards rectal distention produced by inflation of a balloon in situ. This widely used technique [Ikeda and Goto, 1984] has an established place as a diagnostic test in assessing rectal function [Davies et al, 1981]. It may however, be unreliable in the newborn period and requires a high level of operator skill to produce acceptable results. Recent technical developments in manometric detection equipment may avoid some of these problems [Tamate et al, 1984] but at present this method cannot be depended upon as the sole means of diagnosis.

c] Histological demonstration of the absence of ganglion cells in the affected segment is the

definitive technique. However, it requires full thickness biopsy and therefore is accompanied by the operative risks of an anaesthesia and tissue scarring which may prejudice further corrective surgery [Lake et al, 1978]. It is therefore not a suitable screening test in the constipated neonate.

d] The hazards encountered in obtaining a full thickness biopsy led Bodian, [1960] to suggest the use of mucosal biopsies to establish a histological diagnosis, this was accompanied by the disadvantage that such small samples, often produced equivocal results. Consequently, when Meier-Ruge, [1972] reported the demonstration of hypertrophied [cholinesterase-staining] nerve trunks as a recognizable feature in suction biopsy specimens from the affected segment, this quickly became a widely adopted diagnostic procedure [Chow et al, 1977; Lake et al, 1978; Patrick et al, 1980; Ardrassy et al, 1981; Nezlof et al, 1981; Kawekami, 1981; Hamoudi et al, 1982; Veregas et al, 1982; Plenat et al, 1982; Lake and Claireaux, 1983; Ziegler et al, 1984]. False positive and false negative results do occur but these rates are acceptably low, 4.2% and 6.3% respectively [Table 3.1].

The major problems with the histochemical approach are, its subjective interpretation, the number of equivocal results due to poor biopsy specimens [6.9%, Table 3.1] and the occasional need to resort to full thickness biopsy in some atypical cases [Coker-Huntley et al, 1982].

e] It was in an attempt to overcome some of these drawbacks that Boston et al, [1975] used a direct method of quantitating the level of cholinesterase activity present in homogenates of rectal biopsy specimens. This has proved to be a simple, reliable and objective means of diagnosis. In particular the test is not dependent on the subjective interpretation required by qualitative histochemistry. A criticism of the method, levelled by Lake et al, [1979], has been that the nature of the sample [i.e. whether it contains mucosa, submucosa, lymphoid follicles, etc] remains unknown. In the studies so far reported [Dale et al, 1977; Dale et al, 1979a; Bajgar and Hak, 1979; Patrick et al, 1980; Yangihara et al, 1983; Rakonczay and Nemeth, 1984; Bonham et al, 1985c] this does not appear to have affected the usefulness of the test and it is doubtful whether this theoretical difficulty is a significant practical problem. Nevertheless, it is part of the work of this thesis to include a larger series than any previously reported and comment further on this and other aspects of the assay.

f] In order to determine whether this increase in rectal tissue AChE is reflected by an increase in plasma and erythrocyte AChE activity, several groups have measured the enzyme in these locations. The results have proven inconclusive; some groups have reported an increase in both plasma and erythrocyte AChE activity [Boston et al, 1978], whereas others could demonstrate an increase in only one of these [Okasara et al, 1983; Ya-xiong, 1984], Yanagihara et al, [1993] found no increase in either. The most recent report [Bamforth, 1985] could demonstrate no increase in erythrocyte AChE. At present there is no known link between ertythrocyte AChE and any local alteration in a disorder clearly limited to the distal gastrointestinal tract. It is unlikely that these results will have anything concrete to add to the diagnosis of the disease.

The most promising diagnostic procedures are those using rectal suction biopsy specimens combined with both histochemical and quantitative AChE assays. These methods rely upon a less invasive technique than those requiring a full thickness biopsy and at the same time provide more accurate diagnostic information than either radiology

or manometry. They can be performed on an outpatient basis and give rapid and reliable results. It would be beneficial - firstly, to assess the benefits and limitations of tests of this kind in practice and secondly, to improve their existing performance.

18.2 Measurement of rectal suction biopsy AChE

There have been several large studies reporting the usefulness of the histochemical assessment of AChE activity in rectal biopsy specimens, notably a recent report by Lake and Claireaux,[1983] included results from 800 patients. However, while quantitative AChE measurement is becoming widespread, [Section 3.6[v]] the largest series so far recorded comprised only 110 patients of which only nine had proven Hirschsprung's disease [Patrick et al, 1980].

Therefore, it was decided to collect the data from the routine measurement of AChE in rectal biopsy specimens in this laboratory and compare these results with the eventual diagnosis arrived at in each case.

18.3 Analysis of results from a series of 213 patients

The study group, collected over a seven year period [1976-1983] consisted of 213 children aged

between two days and 13 years presenting with chronic constipation or failure to pass meconium. Of these, 164 cases were subsequently found not to have the disease and showed histological evidence of ganglion cells. In 45 cases, Hirschsprung's disease was confirmed by histological criteria and corrective surgery carried out in each case.

The results of AChE measurement in these patients are shown in Section 11. There was a five-fold increase in AChE activity in the Hirschsprung's disease group when compared with the non-Hirschsprung's mean. This increase in AChE activity can also be reported as % AChE, i.e. AChE activity expressed as a percentage of the total cholinesterase activity. As BChE remains relatively constant, % AChE increases when the AChE level is raised. An advantage of this method of reporting AChE activity is its relative insensitivity to evaporative water loss from the biopsy specimen. A probable disadvantage is its expected imprecision compared with AChE alone, resulting from incorporating both the variation in both AChE and total cholinesterase estimation used in its calculation. AChE is therefore more reliable when evaporative losses can be minimised and % AChE when this is difficult to avoid [for instance with very small biopsy specimens]. The mean AChE,

6.6 U/l, obtained from the present study group compared well with those reported earlier by Dale et al, [1977 and 1979a] of 6.3 U/l and 5.0 U/l respectively.

Combining AChE and % AChE, in the interpretation of biopsy results improves the overall diagnostic accuracy of the test [Fig. 11.1]. In this way four groups are generated:

Group 1 : AChE and % AChE elevated [greater than 2SD above the mean]

Group 2 : AChE elevated, % AChE normal

Group 3 : AChE normal, % AChE elevated

Group 4 : Both AChE and % AChE within normal limits

Group 1 is regarded as suggestive of Hirschsprung's disease, groups 2 and 3 as equivocal and group 4 as normal. On this basis the diagnostic sensitivity of the test is 73% and its specificity 100% [Section 11]. The assay is therefore useful in identifying the disease in a group of chronically constipated children. On this evidence it will not wrongly classify normal children as having the disease, that is the false positive rate is zero. Equivocal results account for approximately 10% of specimens analysed and in these cases the test should be repeated or additional evidence sought.

Sampling problems can produce misleading results in both histochemical and quantitative AChE assays; among these are included superficial biopsy and specimens taken proximal to the affected region in short segment disease [Coker-Huntley et al, 1982]. In the present series four subjects gave discordant results. Of these two proved to have a very short affected segment and it is likely in these cases that the normal ganglionic region was biopsied. This is an acknowledged difficulty and frequently leads to the disease being missed in such patients [Orr and Scobie, 1983].

In a further case in which a false negative result was recorded this was probably due to a superficial mucosal biopsy in a patient where the densely staining cholinesterase positive nerve fibres, characteristic of the disease did not extend into the lamina propria.

The remaining biopsy sample with unexpected results was obtained from a patient in whom only a marginal increase in the number of nerve fibres was visible despite clear aganglionosis. This may reflect an atypical underlying pathology and emphasises the requirement advocated by Morikawa et al, [1979] to perform conventional full thickness biopsy where unusual pathology is suspected. There are several

such conditions including, hypoganglionosis, chronic adynamic bowel, colonic neuronal dysplasia, immature ganglia, abncrmality of the argyrophil plexus and acquired aganglionosis secondary to ischaemia; these are reviewed by Nixon and Lake, 1982.

The series reported here, the largest so far recorded, confirms the value of quantitative AChE measurement in the diagnosis of Hirschsprung's disease. It is simple and rapid [taking less than 30 minutes from receipt of the sample] and produces quantitative results not open to subjective interpretation [Dale et al, 1977]. In this hospital it is used in conjunction with histochemistry and our findings agree with those of Patrick et al, [1980] that the two techniques are useful complimentary procedures. Satisfactory results are obtainable on specimens as small as 1 mg and such samples can be provided by suction biopsy performed without the need for anaesthetic. In addition the results are found to be reliable in the neonatal period. On the basis of these results this test will continue to be of great value in the diagnosis of a potentially lethal disease among a relatively large population of chronically constipated children. The safety of the procedure permits its increasing use in neonates

as a means of excluding the possibility of Hirschsprung's disease, this in turn should avoid the development of enterocolitis with its attendent high risk of mortality.

Section 19: Molecular forms of AChE and BChE in rectal tissue in Hirschsprung's disease

19.1 The importance of determining the heterogeneity of AChE forms present in rectal tissue

The measurement of AChE in rectal biopsy specimens has proved to be invaluable in the diagnosis of Hirschsprung's disease. The histochemical demonstration of enzyme activity has been in wide-spread use for almost fifteen years and the quantitative assay, using the methodology as outlined in the present research project has gained increasing popularity.

Despite the established importance of these assays and the well known heterogeneity of AChE, few reports have addressed themselves to the problem of identifying the particular forms of AChE increased in Hirschsprung's disease. Bajgar and Hak, [1979] described the presence of an extra-band of AChE activity in aganglionic rectal tissue homogenates when subjected to polyacrylamide gel electrophoresis; they went further to suggest that this finding might be "diagnostically useful". However, at the commence-ment of the present work, no reports existed describing the structural heterogeneity of AChE by density sedimentation or related techniques.

Knowledge of the forms of AChE responsible for the increase in AChE activity in Hirschsprung's disease would be expected to improve the understanding of the assay currently used, leading to a further improvement in specificity based upon the measurement of species selectively augmented in the disease. There already exists strong histochemical evidence that the increase in AChE activity is due to nerve trunk hypertrophy. In addition G_4-AChE is known to be the major form of the enzyme associated with nerve trunks [Brimijoin, 1983]. It was therefore hardly surprising that during the period of this study Rakonczay and Németh, [1984] were able to demonstrate an increase in 10S [G_4] AChE accompanying aganglionosis and nerve trunk hypertrophy in rectal biopsy specimens from patients with Hirschsprung's disease.

The results discussed here confirm and extend these findings by documenting the variation in the activity of AChE and BChE species in resected segments of bowel from aganglionic and normal [ganglionic] regions. The ease of extraction and the detergent-sensitivity of these forms are also discussed and this provides evidence indicating their likely cellular location.

19.2 Patients involved in the study

Segments of terminal bowel were obtained at operation from six patients, four showed a clear transition from normal (ganglionic) to aganglionic regions. In one of the remaining two cases the entire colon was uniformly aganglionic (long segment disease) and the other contained ganglia throughout its length (an independent control specimen).

In each case where the proximal region was ganglionic this provided a normal control tissue and was preferable to comparison with bowel taken from patients without the disease, as it more closely defines the changes arising secondarily to aganglionosis and excludes individual variation. The single case without evidence of Hirschsprung's disease acted as an independent control.

The specimens were divided transversely into a varying number of segments and longitudinally into three strips, which were used for histological and histochemical assessment and for measurement of AChE and BChE molecular forms. Therefore direct comparison between these three methods at each point along the sample was obtained. Further dissection was also necessary in some cases to separate muscle and mucosal layers. In all, 65 sections

were analysed by density gradient sedimentation, a considerable task for which automated AChE and BChE assay was clearly desirable.

19.3 The variation in AChE molecular forms in resected bowel segments

In all cases, four forms of AChE were identifiable with sedimentation coefficient of 16.8, 9.2, 5.0 and 3.5S. These were believed to correspond to A_{12}, G_4, G_2 and G_1 respectively. It was demonstrated that the molecular mass of these forms was in keeping with this assumption, i.e. 11.8 : 4.0 : 1.9 : 1.1 respectively [Section 15.2].

There was a 4 to 14-fold increase in the activity of G_4-AChE in both the muscle and mucosal layers, in the presence of aganglionosis when compared with the proximal ganglionic region. G_1, G_2 and A_{12} showed no consistent changes.

The magnitude of the change of G_4 in the muscle layer, was greater than that in the mucosal layer [Fig. 12.2]. In each case the variation was related to the transition from ganglionic to aganglionic region and corresponded to the change from the presence of normal nerve trunks into a region of distinct nerve trunk hypertrophy, typical of Hirschsprung's disease. The independent control case showed no evidence of consistent variation in any of the molecular forms.

These findings agree with those of Rakonczay and Németh, [1984] who reported a relative 2.5 fold increase in 10S [G_4] AChE in biopsy specimens from the aganglionic region compared with normal controls. They attributed this increase to the proliferation of nerve fibres in the affected region. The technique which they employed did not however, adequately separate G_1 and G_2 forms nor identify A_{12} AChE. Consequently, the results cannot be compared directly with those presented here. Their report is also unclear about the total number of patients included in the study but there were not more than three with the disease.

In the present study, it was not possible from the four cases in which the disappearance of nerve trunk hypertrophy coincided with the end of the aganglionic region to establish whether the observed increase in G_4 AChE was due directly to aganglionosis or produced as a consequence of the nerve trunk hypertrophy. The variation in G_4 AChE activity in the segment of bowel resected from the patient with long segment disease [Fig. 12.4] helped resolve this uncertainty.

The extrinsic sacral innervation which gives rise to the hypertrophied nerve trunks seen in Hirschsprung's disease [Okamoto et al, 1982] is

confined to the distal colon and rectum. Consequently, while distally, aganglionosis is accompanied by nerve trunk hypertrophy, in more proximal regions this is not the case. The transition in innervation occurs distal to the splenic flexure [Lake and Claireaux, 1983]. It can be seen from Fig. 12.4 that the level of G_4-AChE activity closely follows the transition from the region where thickened nerve trunks are visible to that, where, despite continued aganglionosis, they are no longer evident indicating that nerve trunks are indeed the source of G_4-AChE.

There is reliable histochemical evidence that in some cases of long segment disease, distinct nerve trunk hypertrophy may even be limited to rectum alone. Consequently AChE measurement in the diagnosis of Hirschsprung's disease from biopsy specimens obtained proximal to the recto-sigmoid junction should be interpreted with caution. Nor should AChE measurement be used to assess the limit of aganglionosis in long segment cases for the purposes of corrective surgery. These limitations apply to both quantitative and histochemical assays of AChE activity.

The gradual decline in the number of AChE-positive nerve fibres occurring between the rectum

and splenic flexure in Hirschsprung's disease [Meier-Ruge et al, 1972] is associated with the gradual decline in G_4-AChE [Fig. 12.4] - offering support for the proposed caudal-cranial direction of extrinsic sacral parasympathetic outflow postulated by Garret et al, [1969].

In two of the cases examined there was evidence that G_1 activity was slightly increased in the aganglionic region [Figs. 12.4 and 12.5] these changes were however less consistent than those seen in G_4. It is probable that the changes in G_1 were secondary to those of G_4 and simply represented an accumulation of a precursor or dissociation product of G_4-AChE.

It is suprising that the level of A_{12}-AChE, present at synaptic junctions, was not altered in the aganglionic region, where ganglionic synapses are absent. The maintenance of A_{12} activity might well be attributable to the existence of synaptic junctions between the extrinsic parasympathetic nerve fibres and the smooth muscle of the gut wall. The level of A_{12} AChE was however less than 2 U/l [the minimum recommended for reproducible quantitation, Section 7.1[ix],so that a more sensitive means of detection must be employed before any firm conclusions can be drawn.

19.4 The significance of increased G_4-AChE in diagnosis of Hirschsprung's disease

The distal enteric nerve plexuses are largely self regulating but their action is modified by extrinsic efferent nerves of sacral origin. In Hirschsprung's disease the normal plexus structure degenerates and intermediary neurones and ganglia are absent [Fig. 3.3] and the extrinsic nerve fibres ramify throughout both muscle and mucosal layers and even extend into the lamina propria.

The increase in G_4-AChE acts as a specific marker for this extrinsic nerve trunk hypertrophy and is largely responsible for the increase in total AChE activity which can be assessed both quantitatively and histochemically.

This information provides a clearer insight into the basis and limitations of AChE measurement in the diagnosis of Hirschsprung's disease. The detection of the disease can only be made using biopsies obtained from regions of nerve trunk hypertrophy where G_4-AChE is consequently increased. This excludes the use of AChE measurement in diagnosis of long segment Hirschsprung's disease from biopsies made proximal to the sigmoid colon. In addition, in a substantial proportion of cases [Chow et al, 1977; Coker-Huntley et al, 1982] the

thickened nerve trunks do not extend into the lamina propria, in these cases, superficial biopsy may result in a false negative result.

The means of designing an assay to determine G_4-AChE selectively is discussed in Section 17.2. This would improve the specificity of the existing total AChE assay in identifying Hirschsprung's disease cases. Erythrocytes, inevitably contained within the biopsy specimen must contribute to the total AChE activity, despite centrifugation. Measurement of G_4-AChE would, however, be unaffected by this interference, as erythrocytes contain mainly G_2-AChE. Structures such as lymphoid follicles known to contain some AChE activity [Lake et al, 1979] would be less likely to affect the determination of one specific form of the enzyme. This might answer a theoretical criticism of the quantitative assay that, in contrast to the histochemical assay, the source of the AChE being measured cannot be visualised [Lake et al, 1979].

The relative increase in G_4-AChE activity in the aganglionic region is greater than the increase in total AChE. Consequently, measurement of G_4 alone will improve the sensitivity of detection of the disease and reduce the false negative rate [currently, 4.4%].

The clinical presentation of Hirschsprung's disease is very variable and it is not possible to correlate the degree of intestinal obstruction simply with the length of affected bowel [Bodian et al, 1949]. There have been several reports to suggest that the severity of obstruction correlates with the number of nerve fibres visible in the aganglionic region [Garret et al, 1969]. The greatest number of AChE positive nerves are found in the circular muscle of distal aganglionic bowel from those children who had required a colostomy for severe intestinal obstruction in the neonatal period [Garret et al, 1969]. Measurement of G_4-AChE provides a direct quantitative means of assessing the degree of nerve trunk hypertrophy and the results can therefore be used not only to detect the disorder but also to indicate its severity and the urgency of surgical intervention.

In addition to these benefits G_4-AChE determination will also be of value in helping to classify a variety of conditions which present in a similar way to Hirschsprung's disease. These include, neuronal colonic dyplasia,chronic adynamic bowel, hypoganglionosis, immature ganglia, abnormality of the argyrophil plexus, acquired aganglionosis and segmental aganglionosis [Lake and Nixon, 1982]. Some of these disorders, notably acquired- and segmental-aganglionosis are poorly understood.

Having established a direct quantitative marker for nerve trunk hypertrophy distinct from aganglionosis, this can now be used to objectively assess changes in these disorders and to improve the understanding of the underlying pathophysiological changes in these cases.

19.5 Differential extraction of AChE forms

While it is clear that an increase in G_4-AChE activity is closely linked to the development of nerve trunk hypertrophy, it is not obvious whether this form of the enzyme is bound to the nerve fibres or is a soluble, secretory product. The sections from a segment of bowel containing both ganglionic and aganglionic tissue were subjected to mild homogenisation in pH7.3 phosphate buffer, followed by a more rigorous extraction with a buffer containing Triton X-100 [Fig. 12.5]. There was a substantial amount of activity which was only released by the presence of detergent. This could be attributable to two distinct, but not mutually exclusive causes:-

1. Triton, might simply help the dissociation of tissue fragments during homogenisation and therefore enhance the release of already soluble enzyme.
2. Triton may actually be responsible for the release of membrane bound enzyme.

To investigate this further, repeated homogenisation was performed using three buffer systems, [Section 12.7]. This included successive homogenisation of tissue in Hanks medium, pH8.0 phosphate buffer and finally buffer containing 1% w/v Triton. These experiments indicated that approximately 50% of the activity of all AChE forms are released by thorough homogenisation in a physiological buffer such as Hanks medium, and can therefore be regarded as soluble. However, there remains a proportion of AChE, which despite homogenisation twice in this media, followed by repeated homogenisation in pH8.0 phopshate buffer, can only be released by the presence of Triton and can therefore be presumed to be membrane bound. In the case of G_4-AChE this accounts for approximately 30% of the total G_4 activity. These findings are in accord with the proposal of Kása and Rakonczay, [1982] that 10S AChE is associated with the external surface of the axolemma and has a substantial membrane bound component.

The results indicate that the determination of AChE in the diagnosis of Hirschsprung's disease would be improved by inclusion of a detergent such as Triton to release membrane bound activity.

19.6 Triton reactivity of AChE forms in rectal tissue

AChE exists in both Triton-reactive and non-reactive forms [Massoullié, 1980]. This can be readily demonstrated by the shift in S-value which occurs during sedimentation performed in the absence and presence of Triton [Lazar and Vigny, 1980].

While all forms requiring Triton for their release are Triton-reactive, soluble species may or may not be [Massoullié et al, 1984].

In amniotic fluid the soluble AChE molecular forms are Triton-insensitive [Section 17.2]. AChE released from rectal tissue behaves somewhat differently. Those forms, which are clearly membrane bound and released only by Triton, show a marked Triton reactivity [Section 15.5], the S-value of G_4 changing from 9.4 - 11.3S when the detergent is omitted [Fig. 15.7].

The enzyme released by 0.1M pH8.0 phosphate buffer is also Triton-reactive, although in this case the shift in S-value of G_4 9.8 to 10.9S is less than that of the Triton extractable species. This may reflect a mixed population of G_4, including Triton-reactive and non-reactive forms. In this respect the G_4 of rectal tissue is different from that found in amniotic fluid. This difference might indicate post-release modification of AChE

while in the amniotic flud, including proteolytic removal of the Triton binding site [Dutta-Choundhury and Rosenberry, 1984]. Alternatively, G_4 in rectal tissue may be intrinsically different from that which is being secreted into the amniotic fluid. It is important to remember that the aganglionosis of Hirschsprung's disease represents a pathological state. The structure of the neural matrix is completely disrupted and extrinsic nerves present in the tissue are hypertrophied. Consequently, the release of AChE may itself be disturbed, resulting in the secretion of immature molecular forms of the enzyme. In order to resolve this, comparison between the Triton sensitivity of AChE species in normal and aganglionic rectal tissue would be required.

19.7 The effect of endogenous proteases on AChE molecular forms

The distribution of molecular forms of AChE obtained from skeletal muscle extracts can vary as a result of endogenous protease digestion [Silman et al, 1978; Lyles et al, 1982; Barnard et al, 1984]. In particular larger species can be converted into forms of smaller molecular mass. In

order to test the effect of protease digestion in rectal tissue, minced tissue was homogenised:

a] Immediately in the presence of a cocktail of protease inhibitors [Barnard et al, 1984].

b] Following a four hour incubation at room temperature in a moist environment without such inhibitors.

The results, Section 12.8, show a similar distribution of molecular forms, with no loss of G_4 activity in favour of G_1. Endogenous protease digestion of larger AChE species does not occur to any great extent in rectal tissue. Minor effects occurring between surgical removal and storage in liquid nitrogen cannot be excluded, but this evidence indicates that such changes would be small and not materially alter the interpretation of the present data.

19.8 BChE forms in rectal tissue

BChE is present in rectal tissue, where normally it accounts for almost half of the total cholinesterase activity [Dale et al, 1977, 1979a]. In the presence of Hirschsprung's disease there is a five fold increase in AChE with only a two fold increase in the BChE fraction.

Just as in the case of amniotic fluid, the BChE present in rectal tissue exists in a set of

molecular forms similar to those of AChE. Three major BChE forms are identifiable, with sedimentation coefficients of 4.1, 5.2, 11.4S respectively. These are slightly higher than those of the corresponding AChE species; this is consistent with the results of other studies [Lockridge et al, 1979].

In order to determine how these BChE species change in the transition from ganglionic to aganglionic bowel, BChE was measured in each of the gradients in addition to AChE [Fig. 13.2]. Unlike AChE there are no consistent changes in any of the BChE species in the aganglionic compared with ganglionic regions.

These results indicate that the hypertrophied nerve trunks, typical of Hirschsprung's disease do not produce or release BChE to the same extent as AChE. Nor is any particular form of BChE closely identifiable with these thickened nerve trunks. This is the first study in which the change in BChE molecular forms in Hirschsprung's disease is reported, the earlier studies reporting AChE heterogeneity in Hirschsprung's disease, Bajgar and Hak, [1979] and Rakonczay and Németh, [1984] do not comment on BChE and therefore these results cannot be compared.

The moderate increase in overall BChE activity which occurs in Hirschsprung's disease cannot be

utilised diagnostically, nor does the change in any EChE form correlate well with the changing histochemical appearance.

19.9 Summary of AChE and BChE molecular form changes in Hirschsprung's disease

In summary, the data set out in Section 11, represent the largest study so far reported, in which AChE is measured quantitatively in the diagnosis of Hirschsprung's disease. The assay is found to be reliable in routine diagnostic use. Over a seven year study period no patient was falsely classified as having Hirschsprung's disease [Bonham et al, 1985c].

From sedimentation analysis performed on tissue obtained from ganglionic and aganglionic regions, it is evident that the increase in AChE activity, upon which both the histochemical and quantitative techniques rely is due to an increase in one particular AChE form, G_4. The level of G_4 is shown to reflect the degree of nerve trunk hypertrophy and not aganglionosis directly.

These investigations more closely define the nature and the source of the increased AChE activity seen in the disease and suggest a means of further refining the existing assay, by specific measurement of G_4-AChE [Bonham et al, 1985c].

BChE, while moderately increased in the presence of Hirschsprung's disease shows no consistent change in any of its molecular forms.

Section 20: Characterisation of AChE molecular forms present in rectal tissue and amniotic fluid

20.1 Introduction

The molecular forms of AChE identified [Sections 10 and 12] in rectal tissue and amniotic fluid, have depended for their demonstration upon sucrose density sedimentation. While this is currently the most widely used method of differentiating AChE species, other techniques have been used to demonstrate the heterogeneity of AChE. These have included gel electrophoresis, gel filtration, equilibrium sedimentation, isoelectric focusing and density gradient gel electrophoresis. The use of gel filtration in particular provides valuable extra information.

a] There is always the possibility that results obtained are merely artefacts of that method of separation rather than those of the native enzyme. If consistent results are produced by an alternative means this offers confirmation that the molecular species observed reflect a genuine diversity of forms.

b] The use of an auxilliary method serves to establish the homogeneity of the peaks identified by the major technique. In this case, the homogeneity of peaks obtained from sucrose density sedimentation was tested by pooling the fractions corresponding to individual AChE species and examining these by gel filtration.

c] Gel filtration permits determination of the apparent molecular mass and by combining these data with the results of sedimentation experiments the true molecular mass can be calculated [Ackers, 1964; Siegel and Monty, 1966].

Gel filtration chromatography of AChE molecular forms has been described using several media including: Sepharose 6B [Chang and Blume, 1976], Biogel A [Bon et al, 1976] and Ultrogel AcA22 [Rakonczay et al, 1981a]. In this study Ultrogel AcA22 with a linear fractionation range of 100-1, 200 kDa, was employed. Samples included homogenates of aganglionic rectal tissue as well as amniotic fluid specimens from NTD affected pregnancies. The profiles obtained [Figs. 15.1 and 15.4], closely followed those obtained by sucrose density sedimentation analysis of the corresponding tissue [Figs. 10.1-5 and 12.1-5].

When individual molecular species of AChE obtained by sedimentation were subjected to gel filtration chromotography they yielded single peaks with distinguishable elution volumes [Fig. 15.2], confirming their homogeneity.

The resolution of AChE species by gel filtration was less complete than obtained with density sedimentation so that G_1 and G_4 did not entirely resolve. Nevertheless, the technique confirms the validity of the sedimentation data and also the homogeneity of the peaks obtained.

20.2 Calculation of molecular mass

The apparent molecular mass of the AChE forms studied was estimated by comparison with protein markers. The apparent mass of the forms obtained from rectal tissue exceeded those of amniotic fluid species. G_4-AChE behaved as if its mass were 550 KDa and 405 kDa in rectal tissue and amniotic fluid respectively. These results are in accord with those obtained by Rakonczay et al, [1981a], who reported a Triton extractable G_4 and a soluble G_4 in mammalian brain, with apparent molecular masses of 490 kDa and 342 kDa respectively.

Applying the equation of Ackers, [1964], enabled the effective gel pore size, r, to be calculated from the Kd values obtained with protein

markers of known Stokes radius, Re. Subsequently, using the values of r, and Kd of individual AChE molecular forms, their Stokes radius was calculated.

The results [Section 15.2] indicate that A_{12} from rectal tissue has an Re = 12.19nm, consistent with the definition of asymmetric AChE species whose Re$>$12nm [Massoulié et al, 1984].

In the remaining species G_1, G_2 and G_4, there were differences between rectal tissue derived and amniotic fluid derived AChE. The Stokes radius of the enzyme from rectal tissue was 15% greater, on average, than its counterpart obtained from amniotic fluid [7.54nm and 6.27nm respectively]. Rakonczay et al, [1981a], using a similar technique with enzyme derived from rat brain, identified Triton extractable and soluble G_4 forms with Stokes radius of 8.05 and 6.75nm respectively. Bon et al, [1976] reported the same molecular form [G_4] from Electrophorus to have a Stokes radius of 8.75nm. Allowing for species differences the results compare well. The results of Rakonczay et al, [1981a] in particular paralleled the findings of this thesis, in that the soluble enzyme [from amniotic fluid] has a smaller Stokes radius than the equivalent form from rectal tissue which may include a membrane bound component.

Having ascertained the S-value and Stokes radius of each of the AChE molecular forms, their approximate true molecular mass was then calculated according to the equation:

$$Re.S_{20},W = f\ [Molecular\ mass]$$

The forms from rectal tissue, G_1, G_2, G_4 and A_{12}, identifiable on the basis of their sedimentation coefficient were found to have molecular masses in the ratio of 1.1:1.9:4.0:11.8 while those of amniotic fluid, G_1, G_2 and G_4 were in the ratio of 1.2:2.0:4.0.

The molecular mass of the monomer, G_1 was 74 and 78 kDa in rectal tissue and amniotic fluid respectively, these results were similar to the 80kDa accepted for the monomeric catalytic subunit from Electrophorus [Massoulié and Bon, 1982]. The mass determined for the dimer, G_2 from rectal tissue and amniotic fluid of 126 and 131 kDa respectively, was less than the 151 kDa reported by Ott et al, [1982] for G_2 from erythrocyte membrane. The discrepancy might be due to innacuracy in the determination of Kd for G_2 in the current study, which was present in particularly low activity.

The results obtained for G_4 AChE are compared with those obtained for this form from other sources

Table 20.1. The true molecular masses of G_4 AChE form amniotic fluid and rectal tissue showed closer agreement [within 7%] than their corresponding apparent molecular masses. This indicates that there are differences arising due to teriary or quaternary structural change. This would be expected to affect the degree of asymmetry and consequently their hydrodynamic properties without affecting their molecular mass. Rakonczay et al, [1981a], Table 20.1, have reported similar findings for the soluble and membrane bound species of rat brain. The Stokes radius of the soluble species were smaller and the S-value greater than the corresponding membrane derived forms. As a consequence, the apparent molecular masses of these forms differed by almost 30% whereas their true molecular masses differed by less than 8%. It has already been demonstrated [Section 15.5] that amniotic fluid AChE does not react with Triton and is probably a soluble secreted enzyme. The hydrodynamic behaviour of amniotic fluid AChE also supports this view, and in accord with the behaviour of the soluble G_4-AChE investigated by Rakonczay et al, [1981a], it shows a smaller Stokes radius and higher sedimentation coefficient than the rectal tissue derived enzyme which contains a membrane bound component. More recently,

Table 20.1 Molecular mass, Stokes radius and S-value of G_4 from several different species. Soluble and membrane bound forms are also differentiated.

Table 20.1 The molecular mass. Stokes radius and S-value of G$_4$AChE from different sources

	Re [nm]	$S_{20,W}$ [S-Units]	Apparent molecular mass [KDa]	True molecular mass [KDa]
Bon et al, 1976				
Electrophorus	8.75	11.8	-	331
Massoulié et al, 1980				
Ox	8.1	10.5	-	341
Rakonczay et al, 1981a				
Rat: Soluble	6.75	10.7	342	304
Triton extractable	8.05	9.8	488	328
Ralston et al, 1985				
Fetal bovine serum	7.6	10.8	-	340
Gennari and Brodbeck, 1985				
Human caudate necleus:				
Salt soluble	-	10.6	-	300
Detergent soluble	-	10.0	-	280
Present Study				
Amniotic fluid	6.27	10.3	405	256
Rectal tissue	7.54	9.2	550	275

Ralston et al, [1985] have purified soluble G_4-AChE from fetal bovine serum and report that this enzyme sediments at 10.8S [close to the 10.3S determined for the G_4 of the amniotic fluid] and is also insensitive to Triton X-100.

20.3 Thermal inactivation of AChE molecular forms

In a recent report describing the thermal inactivation of AChE molecular forms derived from rat tissue, Edwards and Brimijoin, [1983], outlined the relevance of studying the thermolability of proteins. They describe how this technique is "perhaps the most sensitive test for genetic variants that differ in primary structure" and that it is capable of detecting changes of only one amino-acid in certain instances. In addition to detecting primary sequence variation, differences in thermolability can also be attributable to tertiary or quaternary structural diversity. In particular the disulphide bond known to link the monomers of AChE within the G_2 dimer is capable of conferring heat stability on the molecule. Also the non-covalent, hydrophobic forces, which are responsible for maintaining the association of the two dimers of the tetrameric G_4 form contribute to the thermal stability of this structure.

For these reasons it is not surprising that Edwards and Brimijoin, [1983], found monomeric AChE to be the most thermally labile AChE species and that Lockridge et al, [1979], found the same to be true of the BChE monomer.

In the present study it is valuable to determine the thermolability of the forms described for the following reasons:

a] It allows direct comparison with the results of other workers gained using AChE from different sources i.e. rat heart and diaphragm, Edwards and Brimijoin, [1983].

b] By comparing the relative behaviour of molecular forms identified in this study with that seen in other reports may offer yet further confirmation of a monomer, dimer, tetramer series.

c] If the differential behaviour of AChE species is marked, it might be used as a means of selective measurement of individual species.

These points are discussed in turn.

The results [Section 15.3] show a marked loss of G_1 AChE activity at $49^{o}C$ compared with G_2 and G_4

forms. These findings are consistent with those of Edwards and Brimijoin, [1983], who describe a much more rapid loss in G_1 activity at 48°C than found in G_4 or A_{12}. In that report the fall in G_1 activity was even more marked than our results suggest. This may reflect species variation in primary sequence, as mentioned earlier, single amino acid substitutions can result in temperature sensitivity differences. Nevertheless, the same trend is evident in both studies i.e. G_1 is the most labile species and larger forms e.g. G_4 are increasingly resistant to inactivation.

In both AChE and BChE, the monomers are linked by disulphide bonds to form the dimer, these are then joined by non-covalent hydrophobic interactions in the tetramer. It would be anticipated that the covalent disulphide bond would have relatively greater effect in stabilising the G_2 structure than the non-covalent forces involved in the further formation of G_4. Consequently, G_1 would be the least resistant to heat inactivation and a considerable increase in stability would be conferred by the dimeric structure, a further, relatively less pronounced, increase in stability would result from the formation of the tetramer, G_4. This is exactly the behaviour observed, the greatest difference was noticable

between G_1 and G_2 species. These findings support the sedimentation and gel filtration data, all of which suggest that 4S, 5S and 10S AChE forms correspond to a monomer, dimer, tetramer series.

The differential behaviour toward heat inactivation indicates a means of designing a selective assay directed to individual forms. G_4 is the least thermally labile globular form and it may therefore be possible to measure this species alone following inactivation of G_1 and G_2 by incubation at a suitable temperature.

The present results suggest that such an assay for G_4-AChE would not be entirely selective, however, by altering the experimental conditions more specificity may be attainable. It is particuarly fortuitous that G_4, the species of diagnostic interest in Hirschsprung's disease and NTD, is the most heat stable of the globular forms.

20.4 Substrate inhibition of AChE molecular forms

It is well established that AChE is inhibited by the presence of excess substrate [Section 1.5, Silver, [1974]]. The bell shaped curve of Activity versus log [Substrate concentration] [pS] is characteristic of this behaviour, this is shown in Fig. 1.2 for bovine erythrocytes. In this instance optimal activity is achieved with an acetylcholine concentration of approximately 3.0mM.

In order to ascertain whether or not individual AChE species differed in this respect, rectal tissue derived forms were compared. In each case the optimal substrate concentration was in the region of 2.0mM. These results are identical with those of 2.0mM obtained by Fernandez et al, [1981] using A_{12} from rat muscle *and nerve, and Chubb and Smith, [1975] investigating* the soluble and membrane found forms of *the enzyme* in bovine splanchnic nerve [predominantly G_4].

It would appear, as previously suggested by Massoulié, [1980] that the catalytic differences between AChE multimers are minor and that even between species similar characteristics are evident. This indicates the importance of this aspect of the catalytic behaviour of the enzyme in differing locations and species.

CONCLUSIONS

CONCLUSIONS

The conclusions made from the present study can be summarised in three overlapping areas.

a] Measurement of AChE and BChE in amniotic fluid

The results presented in Section 9, confirm the value of amniotic fluid AChE determination in the pre-natal detection of neural tube defects. This assay offers several advantages over the use of alphafetoprotein in the same context. An automated assay for AChE is described which shows excellent correlation with the manual technique and is ideally suited to routine diagnostic use.

The structural heterogeneity of AChE and BChE in amniotic fluid from normal and NTD affected pregnancies was investigated by sucrose density sedimentation. Three forms of AChE were identifiable, with sedimentation coefficients of 4.0, 5.5 and 10.3S; it was assumed that these corresponded to a monomer, dimer, tetramer series: G_1, G_2, and G_4 respectively. They were shown to have molecular masses of 78, 126 and 256 kDa respectively. Polyacrylamide gel electrophoresis of amniotic fluid samples from normal and NTD affected pregnancies showed an additional band

of AChE activity which co-migrated with G_4-AChE obtained by pooling fractions from density gradient sedimentation analyses.

The results, which represent the first reported attempt to identify and quantitate the structural diversity of AChE forms present in amniotic fluid, indicate that one form of the enzyme i.e. the tetramer G_4, is largely responsible for the quantitative increase in AChE activity and the additional band of AChE activity seen on gel electrophoresis in the presence of neural tube defects. This particular form is shown to be virtually neural tube defect specific. It did not react with the detergent Triton and is therefore probably a soluble enzyme secreted into the amniotic fluid by nervous tissue exposed as a result of the lesion.

Determination of G_4-AChE would improve both the sensitivity and specificity of the existing AChE assay. The means of designing such an improved assay are discussed.

BChE activity, the likely source of which is fetal serum, despite being increased in the presence of neural tube defects is shown to be a less specific indicator of the lesion than AChE.

b) Measurement of AChE in rectal tissue in the diagnosis of Hirschsprung's disease

The results described in Section 11 represent the largest study so far reported in which AChE has been measured quantitatively in rectal biopsy specimens in the diagnosis of Hirschsprung's disease. The test is shown to be a reliable and sensitive indicator of the disease in routine diagnostic use.

Sedimentation analysis of AChE from rectal tissue of patients with the disease identifies four distinct molecular forms with sedimentation coefficients of: 3.5, 5.0, 9.2 and 16.8S. It was assumed that these corresponded to G_1, G_2, G_4 and A_{12} respectively. The molecular masses of these species was 74, 131, 275 and 811 kDa respectively. These forms showed differential thermolability, with G_1 proving the most thermally labile. The substrate optima of each form was almost identical.

In the aganglionic region of bowel from patients with Hirschsprung's disease, one particular AChE form, G_4 showed consistently increased [4-14 fold] activity. This form is predominantly responsible for the increased AChE activity upon which both the quantitative and histochemical assays are based.

In contrast to the G_4-AChE of amniotic fluid the enzyme from rectal tissue did react with Triton resulting in a descrease in S-value. It is therefore probable that the enzyme possesses a membrane associating component. The increased activity of G_4 in the disease was concomittant with histochemically demonstrated nerve trunk hypertrophy suggesting that it was directly associated with nerve trunks, a view substantiated by histochemistry.

The selective measurement of G_4-AChE, a specific marker for nerve trunk hypertrophy in this context, would improve both the sensitivity and specificity of the existing assays available for the diagnosis of Hirschsprung's disease. BChE molecular forms show no consistent change in activity in the disease.

c) <u>Characterisation of AChE molecular forms in rectal tissue and amniotic fluid</u>

In addition to sucrose density sedimentation, gel filtration was used to separate AChE species from rectal tissue and amniotic fluid. The results were in accordance with those obtained by density sedimentation and confirmed the homogeneity of the peaks obtained.

Combining the Stokes radius, determined by gel filtration, and the sedimentation coefficient of each of the AChE species identified enabled their approximate true molecular mass to be calculated. The results were consistent with a monomer, dimer, tetramer series based upon a subunit weight of approximately 70 kDa. The asymmetric form A_{12} had a molecular mass consistent with 12 such subunits. The results were in accord with those reported using AChE derived from other sources.

The thermolability of AChE species from rectal tissue indicated that G_1 was the most thermally labile, all three globular forms behaved similarly with respect to substrate inhibition, with a maxima at 2.0mM, acetylthiocholine. These results too, were consistent with those obtained by other workers using AChE from different sources.

A P P E N D I C E S

Appendix 1 : S-value calculation program

Appendix 2 : Density sedimentation analysis program

Appendix 3 : Curvilinear interpolation program

Appendix 4 : Instrument and reagent suppliers

Appendix 1 : S-value calculation program

This program will calculate the S-value for sucrose gradients in swing out rotors from basic run parameters and rotor dimensions. Adapted from Young, [1980]

```
100 OPEN1,4
110 REM THIS PROGRAM CALCULATES S-VALUES FOR SUCROSE GRADIENTS IN SWING OUT
120 REM ROTORS.ANY ROTOR CAN BE USED , PROVIDED THE DISTANCE(CM)FROM THE CENTR
130 REM OF ROTATION TO THE BOTTOM
140 FORI=1TOP
150 DIM P(40),M(40),X(100),Y(100)
160 DIM L(40),A$(10),O(100)
170 PRINT
180 PRINT#1,"                                    *****S-VALUE PROGAM*****"
190 PRINT#1
200 PRINT,"RUN NO.?"
210 INPUTJJ
220 PRINT#1,"                                          RUN NO.";JJ
230 PRINT#1
240 Z1=0
250 DATA 0.0528,0.4,0.0682,0.345
260 PRINT"IS SAMPLE DNA?(Y/N)"
270 INPUTC$
280 IFC$="N"THEN360
290 PRINT"ALKALINE GRADIENT?"
300 INPUTC$
310 RESTORE
320 IFC$="Y"THEN350
330 READX,X,Z1,Z2
340 GO TO 360
350 READZ1,Z2,X,X
360 N0=2
370 REM THE FOLLOWING DATA STATEMENTS
380 REM CONTAIN THE BASIC INFORMATION
390 REM FOR EACH ROTOR,I.E.DISTANCE
400 REM FROM THE CENTRE OF ROTATION TO THE TUBE BOTTOM AND THE INTERNAL TUBE
410 REM RADIUS (CM) A MAXIMUM OF TEN
420 REM ROTORS CAN BE STORED IN THIS PROGRAM.
430 DATA 12.87,1.30
440 DATA3.86,0.625
450 DATA2,2,2,2,2
460 REM DATA STATEMENTS ABOVE
470 A$(1)="MSE 3X25 ML"
480 A$(2)="MSE 3X6.5 ML"
490 FOR N=1 TO N0
500 PRINT N,A$(N)
510 NEXT N
520 PRINT"WHICH ROTOR DO YOU REQUIRE?"
530 INPUT N1
540 IF N1>N0 THEN 530
550 RESTORE
560 READ X,X
570 FOR N=1TO N1
580 READX,X
590 NEXTN
600 READR1,R2
610 PRINT"DO YOU WANT AUTO MODE?"
620 GETZZ$:IFZZ$=""THEN620
630 IFZZ$="N"THEN650
640 IFZZ$="Y"THEN350
650 PRINT "TOTAL VOLUME (ML)?"
660 INPUTU1
670 PRINT "SAMPLE VOLUME?"
680 INPUT U2
690 PRINT "FRACTION VOLUME?"
700 INPUTU3
```

```
710 N3=U1/U3
720 PRINT"ACCELERATION TIME(MINUTES)?"
730 INPUTT1
740 PRINT "RUN TIME(HOURS)?"
750 INPUTT4
760 T2=T4*60
770 PRINT"DECELERATION TIME (MINUTES)?"
780 INPUTT3
790 PRINT"AVERAGE SPEED (R P M)?"0
800 INPUTQ
810 PRINT"TEMPRATURE (DEG C)?"
820 INPUTT5
830 PRINT"PARTICLE DENSITY?"
840 INPUTP5:GOTO880
850 PRINT"SAMPLE VOL.?"
860 INPUTU2
870 U1=5.0:U3=.25:N3=U1/U3:T1=20:T4=17:T2=T4*60:T3=20:Q=37500:T5=4:P5=1.3
880 P9=3.1415
890 I=15/((T2+(T1+T3)/3)*(P9*Q)↑2)
900 PRINT"? %SUCROSE OF THE ";N3;"FRACTIONS"
910 FORJ=1TON3
920 INPUTP(J)
930 NEXTJ
940 J=1
950 L(J)=LOG(R1-R2+(J*U3-U1+2*P9*(R2↑3)/3)/(P9*R2↑2))
960 IFEXP(L(J))>R1-R2THEN990
970 J=J+1
980 GOTO950
990 L(J)=0
1000 J=J-1
1010 M(1)=L(1)-LOG(R1-R2+(U2/2-U1+2*P9*(R2↑3)/3)/(P9*R2↑2))
1020 FORJ1=2 TOJ
1030 M(J1)=L(J1)-L(J1-1)
1040 NEXTJ1
1050 PRINT
1060 PRINT
1070 PRINT
1080 PRINTA$(N1),"ROTOR"
1090 PRINT#1,A$(N1)
1100 PRINT#1,"USED AT";Q;"RPM FOR";T4;"HRS AT";T5"DEG C"
1110 PRINT#1,"PARTICLE DENSITY";P5;"TOTAL VOLUME";U1;"ML  SAMPLE VOLUME";U2"ML"
1120 IF Z1=0 THEN 1150
1130 PRINT#1, "FRACTION NO   %SUCROSE   S-VALUE   MOL. WT."
1140 GO TO 1170
1150 PRINT#1
1160 PRINT#1,SPC(20);"FRACTION NO   %SUCROSE        S-VALUE"
1170 S0=0
1180 FOR J1=1 TO J
1190 AD=J1
1200 GOSUB 1790
1210 S0=S0+M(J1)*E
1220 Q8=S0*I*1E13
1230 IF Z1=0THEN1270
1240 Q9=EXP((LOG(S0*I*1E13/Z1))/Z2)
1250 PRINT#1,J1;P(J1);Q8;Q9
1260 GOTO1290
1270 PRINT#1,SPC(20);J1,;P(J1),;S0*I*1E13
1280 O(J1)=S0*I*1E13
1290 NEXTJ1
1300 GOTO2020
1310 REMSUBROUTINE TO CALC DENSITY (D),VISCOSITY (V) FROM TEMP (T) AND
1320 REM SUCROSE PERCENTAGE (P)
1330 DATA1.0003698,3.9680504E-5,-5.8513271E-6
1340 DATA .36982371,-1.0578919E-3,1.2392833E-5
1350 DATA 0.17097594,4.7530081E-4,-8.9239737E-6
1360 DATA18.027525,4.8318329E-4,7.783086557E-5
1370 DATA 342.3,18.032
1380 DATA 212.57059,0.13371672,-2.9276449E-4
1390 DATA 146.06635,25.251728,0.070674842
1400 DATA-1.5018327,9.4112153,-1.1435741E3
```

```
1410 DATA 1.050413TE5,-4.6327102E6,1.0323349E8
1420 DATA -1.1023981E9,4.4532191IE9,-1.0603314
1430 DATA-2.0003464E1,4.6066896E2,-5.9517023E3
1440 DATA 3.5627216E4,-7.6542145E4,0,0
1450 DATA 2.1169307E2,1.6077073E3,1.691161E5
1460 DATA -1.41843TE7,6.0654775E8,-1.2985834E10
1470 DATA 1.3532907E11,-5.4970416E11,1.3975568E2
1480 DATA 6.6747329E3,-7.0716105E4,9.0367578E5
1490 DATA -5.5360830E6,1.2451219E7,0,0
1500 R9=1
1510 GOSUB1960
1520 READB1,B2,B3,B4,B5,B6,B7,B8,B9
1530 READA1,A2,A3,M1,M2,C1,C2,C3
1540 READG1,G2,G3
1550 Y1=P/100
1560 Y=(Y1*M1)/(Y1*M1+(1-Y1)*M2)
1570 IFT>30 THEN 1600
1580 D=B1+B2*T+B3*T↑2+(B4+B5*T+B6*T↑2)*Y1+(B7+B8*T+B9*T↑2)*Y1↑2
1590 GOTO1620
1600 D0=Y*M1+(1-Y)*M2
1610 D=D0/(Y*(C1+C2*T+C3*T↑2)+(1-Y)*(A1+A2*T+A3*T↑2))
1620 R9=21
1630 GOSUB 1960
1640 IFP<=48 THEN 1670
1650 R9=23
1660 GOSUB 1960
1670 READ D0,D1,D2,D3,D4,D5,D6,D7
1680 H=D0+D1*Y+D2*Y↑2+D3*Y↑3+D4*Y↑4+D5*Y↑5+D6*Y↑6+D7*Y↑7
1690 R9=37
1700 GOSUB 1960
1710 IF P<=48 THEN1740
1720 R9=45
1730 GOSUB 1960
1740 READD0,D1,D2,D3,D4,D5,D6,D7
1750 B=D0+D1*Y+D2*Y↑2+D3*Y↑3+D4*Y↑4+D5*Y↑5+D6*Y↑6+D7*Y↑7
1760 C=G1-G2*SQR(1+(Y/G3)↑2)
1770 V=10↑(A+B/(T+C))
1780 RETURN
1790 REM SUBROUTINE TO CALC. SEDIM(E) FROM PARTICLE DENSITY(H1),TEMP(T1) AND
1800 REM SUCROSE%(P1)
1810 T1=T5
1820 P1=P(J1)
1830 H1=P5
1840 T=20
1850 P=0
1860 GOSUB1310
1870 H2=D
1880 V2=V
1890 T=T1
1900 P=P1
1910 GOSUB1310
1920 H3=D
1930 V3=V
1940 E=((H1-H2)/(H1-H3))*(V3/V2)
1950 RETURN
1960 RESTORE
1970 FOR N=1 TO 13+R9
1980 READX
1990 NEXTN
2000 RETURN
2010 OPEN6,4,6:OPEN1,4
2020 P=AD
2030 J1=1
2040 FORI=1TOAD:X(I)=P(J1):Y(I)=O(J1):J1=J1+1:NEXTI
2050 PRINT"HOW MANY PEAKS?"
2060 INPUTAB
2070 PRINT"AT WHAT %SUC. ARE THE PEAKS?"
2080 FORS=1TOAB
2090 INPUTAC(S)
2100 NEXTS
```

```
2110 PRINT#1:PRINT#1,SPC(25);"**INTERPOLATED VALUES**":PRINT#1
2120 PRINT#1,SPC(25);"%SUC";TAB(13);"S-VALUE"
2130 FORS=1TOAB
2140 FORI=1TOP
2150 IFX(I)>AC(S)THEN2170
2160 NEXTI
2170 R=(AC(S)-X(I-1))/(X(I)-X(I-1))
2180 B=R*(Y(I)-Y(I-1))+Y(I-1)
2190 PRINT#1,SPC(25);AC(S);TAB(10);B
2200 NEXTS
READY.
```

Appendix 2 : Sucrose density sedimentation analysis program

This program will correct AChE and BChE activity for interference by sucrose and tabulate the results of culmulatively increasing AChE and BChE activity from the gradient. The results are also given in graphic form.

```
100 REM"THIS PROGRAM CALCULTES AND PLOTS FINAL,CORRECTED:ACHE,BCHE,%SUCROSE IF"
110 REM"GIVEN ACHE,BCHE,%SUCROSE IN THAT FRACTION"
120 OPEN1,4:CLOSE6:OPEN6,4,6:PRINT#6,CHR$(24)
130 PRINT"DATE?"
140 INPUTNN$
150 PRINT"RUN NO.?"
160 INPUTN1
170 PRINT#1,SPC(28)"***SEDIMENTATION PROFILE PROGRAM***"
180 PRINT#1,"                "
190 PRINT#1,SPC(33)"RUN NO.";N1;";DATE";NN$
200 DIMP(110),R(110),S(110),T(110),U(110),W(110),Y(110),C(110),D(110),E(110)
210 PRINT#1,"                "
220 DIMX(110),PI(110),X1(110),Y1(110),X2(110),Y2(110),F(110),G(110),K(40)
230 DIMM(110),QP(110),QR(110),QS(110),IH(40),IV(40),AT(40),I(40),YY(40),XX(40)
240 DIMAA(40),CC(40),DD(40),EE(40),FF(40),II(40),GG(40),HH(40),JJ(40),KK(40)
250 DIMZZ(40),AB(40),AC(40),AD(40),V(40)
260 PRINT"HOW MANY AT START OF GRADIENT"
270 INPUTA
280 PRINT"FRACTION NOS.?"
290 FORB=1TOA
300 INPUTC
310 C(B)=C
320 NEXTB
330 PRINT"% OF SUCROSE?"
340 FORB=1TOA
350 INPUTD
360 D(B)=D
370 NEXTB
380 V7=1:GOSUB3240
390 PRINT"NTS. OF FRACTIONS(MG)?"
400 FORB=1TOA
410 INPUTE
420 E(B)=E/1000
430 E(B)=INT(E(B)*1000)/1000
440 NEXTB
450 V7=2:GOSUB3240
460 FORB=1TOA
470 F(B)=0.00376*D(B)+1.000
480 G(B)=E(B)/F(B)
490 G(B)=INT(G(B)*100000)/100000
500 IH=IH+G(B)
510 NEXTB
520 PRINT"ACHE ACTIVITIES?"
530 FORB=1TOA
540 INPUTI
550 I(B)=I
560 REM"ACHE INTERFERENCE DUE TO SUCROSE=0.041U/L PER %(W/V)SUC AT PH7.3"
570 I(B)=I(B)-0.041*D(B)
580 IFI(B)<.02THENI(B)=.01
590 NEXTB
600 V7=3:GOSUB3240
610 PRINT"BCHE ACTIVITIES?"
620 FORB=1TOA
630 INPUTK
640 K(B)=K
650 REM"BCHE INTERFERENCE DUE TO SUCROSE=0.0165U/L PER %(W/V)SUC AT PH7.3"
660 K(B)=K(B)-0.0165*D(B)
670 IFK(B)<.02THENK(B)=.01
680 NEXTB
690 V7=4:GOSUB3240
700 FORB=1TOA
```

```
710 X4=G(B)*I(B)
720 J=J+X4
730 X5=G(B)*K(B)
740 L=L+X5
750 NEXTB
760 PRINT"NO. OF FRACTION WHEN %SUC.=0?"
770 INPUTM
780 PRINT"% OF SUCROSE WHEN FRACTIONS=0?"
790 INPUTM1
800 N=-M1/M
810 PRINT"HOW MANY FRACTIONS ON ST. LINE?"
820 INPUTO
830 PRINT"FRACTION NOS.?"
840 FORB=1TOO
850 INPUTP
860 P( B)=P
870 NEXTB
880 V7=5:GOSUB3240
890 FORB=1TOO
900 Q=N*P(B)+M1
910 R(B)=Q
920 R(B)=INT(R(B)*1000)/1000
930 NEXTB
940 PRINT"WTS. OF FRACTIONS(MG)?"
950 FORB=1TOO
960 INPUTS
970 S(B)=S/1000
980 S(B)=INT(S(B)*1000)/1000
990 NEXTB
1000 V7=6:GOSUB3240
1010 FOR B=1TOO
1020 T(B)=0.00376*R(B)+1.000
1030 U(B)=S(B)/T(B)
1040 U(B)=INT(U(B)*100000)/100000
1050 IV=IV+U(B)
1060 NEXTB
1070 PRINT"ACHE ACTIVITIES?"
1080 FORB=1TOO
1090 INPUTW
1100 W(B)=W
1110 REM"ACHE INTERFERENCE DUE TO SUCROSE=0.041U/L PER %(W/V)SUC AT PH7.3"
1120 W(B)=W(B)-0.041*R(B)
1130 IFW(B)<.02THENW(B)=.01
1140 NEXTB
1150 V7=7:GOSUB3240
1160 PRINT"BCHE ACTIVITIES?"
1170 FOR B=1TOO
1180 INPUTMM
1190 MM(B)=MM
1200 REM"BCHE INTERFERENCE DUE TO SUCROSE=0.0165U/L PER %(W/V)SUC AT PH7.3"
1210 MM(B)=MM(B)-0.0165*R(B)
1220 IFMM(B)<.02THENMM(B)=.01
1230 NEXTB
1240 V7=8:GOSUB3240
1250 FORB=1TOO
1260 X6=U(B)*W(B)
1270 AT=AT+X6
1280 X7=U(B)*MM(B)
1290 Z=Z+X7
1300 NEXTB
1310 PRINT"HOW MANY AT END OF RUN?"
1320 INPUTAA
1330 PRINT"FRACTION NOS.?"
1340 FORB=1TOAA
1350 INPUTCC
1360 CC(B)=CC
1370 NEXTB
1380 V7=9:GOSUB3240
1390 PRINT"%OF SUROSE?"
1400 FORB=1TOAA
```

```
1410 INPUTDD
1420 DD(B)=DD
1430 NEXTB
1440 V7=10:GOSUB3240
1450 PRINT"WTS. OF FRACTIONS(MG)?"
1460 FORB=1TOAA
1470 INPUTEE
1480 EE(B)=EE/1000
1490 EE(B)=INT(EE(B)*1000)/1000
1500 NEXTB
1510 V7=11:GOSUB3240
1520 FORB=1TOAA
1530 FF(B)=0.00376*DD(B)+1.000
1540 GG(B)=EE(B)/FF(B)
1550 GG(B)=INT(GG(B)*100000)/100000
1560 HH=HH+GG(B)
1570 NEXTB
1580 PRINT"ACHE ACTIVITIES?"
1590 FORB=1TOAA
1600 INPUTII
1610 II(B)=II
1620 REM"ACHE INTERFERENCE DUE TO SUCROSE=0.041U/L PER %(W/V)SUC AT PH7.3"
1630 II(B)=II(B)-0.041*DD(B)
1640 IFII(B)<.02THENII(B)=.01
1650 NEXTB
1660 V7=12:GOSUB3240
1670 PRINT"BCHE ACTIVITIES?"
1680 FORB=1TOAA
1690 INPUTKK
1700 KK(B)=KK
1710 REM"BCHE INTERFERENCE DUE TO SUCROSE=0.0165U/L PER %(W/V)SUC AT PH7.3"
1720 KK(B)=KK(B)-0.0165*DD(B)
1730 IFKK(B)<.02THENKK(B)=.01
1740 RR=0
1750 NEXTB
1760 V7=13:GOSUB3240
1770 FORB=1TOAA
1780 XS=GG(B)*II(B)
1790 JJ=JJ+XS
1800 XS=GG(B)*KK(B)
1810 LL=LL+XS
1820 NEXTB
1830 QP=IH+IV+HH
1840 FORUU=1TO3:PRINT#1:NEXT
1850 PRINT#1,"TOTAL VOLUME=";QP;"MLS."
1860 QR=J+AT+JJ
1870 PRINT#1,"TOTAL ACHE ACTIVITY=";QR;"MILLI. U"
1880 QS=L+Z+LL
1890 PRINT#1,"TOTAL BCHE ACTIVITY=";QS;"MILLI. U."
1900 PRINT#1,"NO.WT(GMS) VOLUME  %VOLUME ACHEBCHE %SUC    %ACHE   %BCHE
1910 FORB=1TOA
1920 Z4=(G(B)/QP)*100
1930 Z5=Z5+Z4
1940 YY(B)=Z5
1950 Z5=INT(Z5*1000)/1000
1960 AB(B)=Z5
1970 Z6=(I(B)*G(B)/QR)*100
1980 Z7=Z7+Z6
1990 Z7=INT(Z7*1000)/1000
2000 Z8=(K(B)*G(B)/QS)*100
2010 Z9=Z9+Z8
2020 Z9=INT(Z9*1000)/1000
2030 PRINT#1,;C(B);E(B);G(B);Z5;I(B);K(B);D(B);Z7;Z9
2040 NEXTB
2050 A5=Z5
2060 A7=Z7
2070 A9=Z9
2080 FORB=1TOO
2090 A4=(U(B)/QP)*100
2100 A5=A5+A4
```

```
2110 XX(B)=A5
2120 A5=INT(A5*1000)/1000
2130 AC(B)=A5
2140 A6 =(N(B)*U(B)/QR)*100
2150 A7 =A7 +A6
2160 A7=INT(A7*1000)/1000
2170 A8 =(MM(B)*U(B)/QS)*100
2180 A9 =A9 +A8
2190 A9=INT(A9*1000)/1000
2200 PRINT#1,;P(B);S(B);U(B);A5;N(B);MM(B);R(B);A7;A9
2210 NEXTB
2220 J5 =A5
2230 J7=A7
2240 J9 =A9
2250 FORB=1TOAA
2260 J4 =(GG(B)/QP)*100
2270 J5=J5+J4
2280 ZZ(B)=J5
2290 J5=INT(J5*1000)/1000
2300 AD(B)=J5
2310 J6 =(II(B)*GG(B)/QR)*100
2320 J7 =J7 +J6
2330 J7=INT(J7*1000)/1000
2340 J8=(KK(B)*GG(B)/QS)*100
2350 J9 =J9 +J8
2360 J9=INT(J9*1000)/1000
2370 PRINT#1,;CC(B);EE(B);GG(B);J5 ;II(B);KK(B);DD(B);J7 ; J9
2380 NEXTB
2390 PRINT"VOLUME OF HOMOG. APPLIED TO GRADIENT(MLS.)?"
2400 INPUTAB
2410 PRINT"ACHE ACTIVITY OF HOMOG.?"
2420 INPUTBC
2430 PRINT"BCHE ACTIVITY OF HOMOG.?"
2440 INPUTCD
2450 DE=(QR/(AB*BC))*100
2460 EF=(QS/(AB*CD))*100
2470 PRINT#1,"RECOVERY(ACHE)=";DE;"%  RECOVERY(BCHE)=";EF;"%"
2480 PP=A+O+AA
2490 B=1
2500 FORI=1TOA:X(I)=I(B):Y(I)=YY(B):B=B+1:NEXT:B=1
2510 FORI=(A+1)TO(A+O):X(I)=W(B):Y(I)=XX(B):B=B+1:NEXT:B=1
2520 FORI=(A+O+1)TO(A+O+AA):X(I)=II(B):Y(I)=ZZ(B):B=B+1:NEXT:B=0
2530 FORU=1TO3:PRINT#1:NEXT
2540 PRINT#1,"ACHE VS %VOLUME"
2550 CLOSE6:OPEN6,4,6:PRINT#6,CHR$(16):CLOSE4:OPEN4,4:YL=0:YH=1:PP=(O+A+AA)
2560 GOSUB2820:PP=PP-1
2570 FORI=1TOPP:IFXT<X1(I)THENXT=X1(I)
2580 NEXTI
2590 XN=XT/60
2600 FORI=1TOPP:FORX=1TO60
2610 IFX1(I)<=XN*XTHENX2(I)=X:GOTO2630
2620 NEXTX:X2(I)=60
2630 NEXTI
2640 YT=YH-YL:YN=YT/60
2650 FORI=1TOPP:FORY=1TO60
2660 IFY1(I)<=YN*YTHENY2(I)=Y:GOTO2680
2670 NEXTY:Y2(I)=60
2680 NEXTI:W=61:FORU=1TO2:PRINT#4:NEXT:PRINT#4,N$:PRINT#4
2690 PRINT#4,X1(PP):PRINT#4,"A I"
2700 W=W-1:ZX=0:IFW=0THEN2720
2710 GOSUB2780:GOTO2700
2720 PRINT#4,"  ____________________________________________________________";YH
2730 PRINT#4,"                                % VOLUME
2740 PRINT#1
2750 PRINT#1
2760 PRINT#1
2770 GOTO3020
2780 FORT=60TO0STEP-1
2790 IFX2(T)=WTHENPRINT#4,"  I"SPC(Y2(T)-1);"+"CHR$(141);:ZX=ZX+1
2800 NEXTT:IFZX=0THENPRINT#4,"  I":RETURN
```

```
2810 PRINT#4,:RETURN
2820 FORI=1TOPP
2830 IFYL>Y(I)THENYL=Y(I)
2840 IFYH<Y(I)THENYH=Y(I)
2850 NEXT:X(I)=0:Y(I)=0:PP=PP+1
2860 B=0:H=PP-1:FORQ=0TOH:PI(Q)=Q:NEXTQ
2870 FORQ=0TOPP+2-3STEP2
2880 H=H+1:H1=PI(Q):H2=PI(Q+1)
2890 GOSUB3000
2900 PI(H)=PI:NEXTQ
2910 H4=H4-1:H3=PI(H):IFH3<0THENRETURN
2920 X1(B)=X(H3):Y1(B)=Y(H3):B=B+1
2930 PI(H3)=H4
2940 H3%=H3/2:Q=H3%*2:H3=PP+H3%:IFH3>HTHEN2910
2950 H1=PI(Q):H2=PI(Q+1)
2960 IFH1<0THENPI=H2:GOTO2990
2970 IFH2<0THENPI=H1:GOTO2930
2980 GOSUB3000
2990 PI(H3)=PI:GOTO2940
3000 PI=H1:IFX(H2)<X(H1)THENPI=H2
3010 RETURN
3020 XN=0:XT=0:YN=0:Y2=0:A5=0:A7=0:A9=0:J5=0:J7=0:Z5=0:Z7=0:Z9=0:LL=0
3030 X2=0:Y1=0:W=0:V=0:ZX=0:T=0:H=0:Q=0:PI=0:H4=0:H3=0:H1=0:H2=0:X1=0:JJ=0
3040 RR=RR+1
3050 PRINT#6,CHR$(24)
3060 IFRR=2THENGOTO3170
3070 IFRR=3THENGOTO1770
3080 IFRR=5THENGOTO3170
3090 IFRR=6THENGOTO3700
3100 B=1
3110 FORI=1TOA:X(I)=K(B):Y(I)=YY(B):B=B+1:NEXT:B=1
3120 FORI=(A+1)TO(A+O):X(I)=MM(B):Y(I)=XX(B):B=B+1:NEXT:B=1
3130 FORI=(A+O+1)TO(A+O+AA):X(I)=KK(B):Y(I)=ZZ(B):B=B+1:NEXT:B=0
3140 FORU=1TO3:PRINT#1:NEXT
3150 PRINT#1,"BCHE VS %VOLUME"
3160 GOTO2550
3170 B=1
3180 FORI=1TOA:X(I)=D(B):Y(I)=YY(B):B=B+1:NEXT:B=1
3190 FORI=(A+1)TO(A+O):X(I)=R(B):Y(I)=XX(B):B=B+1:NEXT:B=1
3200 FORI=(A+O+1)TO(A+O+AA):X(I)=DD(B):Y(I)=ZZ(B):B=B+1:NEXT:B=1
3210 FORU=1TO3:PRINT#1:NEXT
3220 PRINT#1,"%SUCROSE VS %VOLUME"
3230 GOTO2550
3240 PRINT"DO YOU WISH TO ALTER?-(@ TO CHANGE GROUP)"
3250 GETZ$:IFZ$=""THEN3250
3260 IFZ$="N"THEN RETURN
3270 IFZ$<>"@"THEN3290
3280 INPUT"GROUP NO.";V7
3290 PRINT"NO. TO ALTER?":INPUTV8
3300 ONV7GOTO3310,3320,3360,3400,3440,3450,3490,3530,3570,3580,3590,3630,3670
3310 PRINT;D(V8):INPUT"NEW VALUE?";D(V8):GOTO3240
3320 PRINT;E(V8):INPUT"NEW VALUE?";E(V8)
3330 E(V8)=E/1000
3340 E(V8)=INT(E(V8)*1000)/1000
3350 GOTO3240
3360 PRINT;I(V8):INPUT"NEW VALUE?";I(V8)
3370 I(V8)=I(V8)-0.041*D(V8)
3380 IFI(V8)<.02THENI(V8)=.01
3390 GOTO3240
3400 PRINT;K(V8):INPUT"NEW VALUE?";C(V8)
3410 K(V8)=K(V8)-0.0165*D(V8)
3420 IFK(V8)<.02THENK(V8)=.01
3430 GOTO3240
3440 PRINT;P(V8):INPUT"NEW VALUE?";P(V8):GOTO3240
3450 PRINT;S(V8):INPUT"NEW VALUE?";S(V8)
3460 S(V8)=S/1000
3470 S(V8)=INT(S(V8)*1000)/1000
3480 GOTO3240
3490 PRINT;W(V8):INPUT"NEW VALUE?";W(V8)
3500 W(V8)=W(V8)-0.041*R(V8)
```

```
3510 IFW(V8)<.02THENW(V8)=.01
3520 GOTO3240
3530 PRINT;MM(V8):INPUT"NEW VALUE?";MM(V8)
3540 MM(V8)=MM(V8)-0.0185*R(V8)
3550 IFMM(V8)<.02THENMM(V8)=.01
3560 GOTO3240
3570 PRINT;CC(V8):INPUT"NEW VALUE?";CC(V8):GOTO3240
3580 PRINT;DD(V8):INPUT"NEW VALUE?";DD(V8):GOTO3240
3590 PRINT;EE(V8):INPUT"NEW VALUE?";EE(V8)
3600 EE(V8)=EE/1000
3610 EE(V8)=INT(EE(V8)*1000)/1000
3620 GOTO3240
3630 PRINT;II(V8):INPUT"NEW VALUE?";II(V8)
3640 II(V8)=II(V8)-0.041*DD(V8)
3650 IFII(V8)<.02THENII(V8)=.01
3660 GOTO3240
3670 PRINT;KK(V8):INPUT"NEW VALUE?";KK(V8)
3680 KK(V8)=KK(V8)-0.0185*KK(V8)
3690 IFKK(V8)<.02THENKK(V8)=.01
3700 PRINT"WHAT IS %SUC. AT 0 FRACTION?"
3710 INPUTRE
3720 Y(1)=RE:X(1)=0
3730 B=1
3740 FORI=2TO(A+1):Y(I)=D(B):X(I)=RB(B):B=B+1:NEXT:B=1
3750 FORI=(A+2)TO(A+O+1):Y(I)=R(B):X(I)=RC(B):B=B+1:NEXT:B=1
3760 FORI=(A+O+2)TO(A+O+AA+1):Y(I)=DD(B):X(I)=RD(B):B=B+1:NEXT:B=1
3770 H=2.5
3780 FORTT=1TO10:PRINT#1:NEXT
3790 PRINT#1
3800 PRINT#1
3810 PRINT#1,SPC(30);"RUN NO.=";N1
3820 PRINT#1
3830 PRINT#1,SPC(20);"%VOL                    %SUCROSE"
3840 P=A+AA+O+1
3850 FORH=2.5TO97.5STEP5
3860 FORI=1TOP
3870 IFX(I)>HTHEN3890
3880 NEXTI
3890 R=(H-X(I-1))/(X(I)-X(I-1))
3900 E=R*(Y(I)-Y(I-1))+Y(I-1)
3910 V(1)=2:U(1)=2:V(2)=5:U(2)=4.3
3920 V(3)=8:U(3)=7.8:V(4)=11:U(4)=10.7
3930 V(5)=14:U(5)=13.3:V(6)=17:U(6)=16
3940 V(7)=20:U(7)=18.7:V(8)=23:U(8)=21.3
3950 V(9)=26:U(9)=23.8:V(10)=29:U(10)=25.1
3960 V(11)=32:U(11)=28.6:V(12)=35:U(12)=31
3970 V(13)=38:U(13)=33.3:V(14)=41:U(14)=35.5
3980 V(15)=44:U(15)=37.8:V(16)=47:U(16)=40
3990 V(17)=50:U(17)=42.1:V(18)=53:U(18)=44.2
4000 V(19)=56:U(19)=46.3:V(20)=59:U(20)=48.4
4010 FORT=1TO20:IFV(T)>ETHEN4030
4020 NEXTT
4030 W=(E-V(T-1))/(V(T)-V(T-1))
4040 Z=W*(U(T)-U(T-1))+U(T-1)
4050 PRINT#1,SPC(20);H;TAB(10)   ;Z
4060 NEXTH
READY.
```

Appendix 3 : Curvilinear interpolation program

This program will estimate the S-value of unknown peaks obtained on density gradient sedimentation by comparing their position [% fraction volume] with markers of known sedimentation coefficient.

```
100 PRINT "CURVILINEAR INTERPOLATION"
110 PRINT
120 DIMX(50),Y(50)
130 PRINT "NUMBER OF KNOWN POINTS";
140 INPUT P
150 FORI=1 TO P
160 PRINT "X,Y OF POINT";I
170 INPUT X(I),Y(I)
180 NEXT I
190 PRINT
200 PRINT "INTERPOLATE: X= ";
210 INPUT A
220 B=0
230 FOR J=1 TO P
240 T=1
250 FOR I=1 TO P
260 IF I=J THEN 280
270 T=T*(A-X(I))/(X(J)-X(I))
280 NEXT I
290 B=B+T*Y(J)
300 NEXT J
310 PRINT "                Y=    ";B
320 PRINT
330 PRINT  "MORE POINTS HERE?";
340 PRINT"(1=YES,0=NO)";
350 INPUT C
360 IF C=1 THEN 190
370 PRINT"ANOTHER CURVE?(1=YES,0=NO)";
380 INPUT C
390 IF C=1 THEN 130
400 END
READY.
```

Appendix 4 : Instrument and reagent suppliers

Anderman and Company Ltd,
Central Avenue,
East Molesey,
Surrey.
KT8 0QZ Tel: 01 979 8112/3

BDH Chemicals Ltd,
Broom Road,
Poole,
Dorset.
BH12 4NN Tel: 0202 737737

Beckman - RIIC Ltd,
Turnpike Road,
Cressex Industrial Estate,
High Wycombe,
Bucks.
HP12 3NR Tel: 0494 41181

Bellingham and Stanley Ltd,
Longfield Road,
Tunbridge Wells,
Kent.
TN2 3EY Tel: 0892 36444

Calbiochem - Behring Ltd,
P O Box 22,
Bishops Stortford,
Herts.
CM22 7RQ Tel: 0279 56081

C I Electronics Ltd,
Brunel Road,
Churchfields,
Salisbury,
Wilts. Tel: 0722 6938

Commodore Business Machines Ltd,
1 Hunters Road,
Wedon,
Corby,
Northampton.
NN17 1QX Tel: 0536 205252

Hoechst AG,
Post F 80 03 20,
D-6230 Frankfurt /M-80,
Federal Republic of Germany.

Instrumentation Laboratory (IL) Ltd,
Kelun Close,
Birchwood Science Park,
Warrington,
Cheshire. Tel: 0925 810141

Jencons (Scientific) Ltd,
Cherrycourt Way Industrial Estate,
Stanbridge Road,
Leighton Buzzard, Beds.
LU7 8UA Tel: 0525 372010

J T Baker Chemicals Ltd,
Distributed by: Diamed Diagnostics Ltd,
Mast House,
Derby Road,
Bootle,
Merseyside. L20 1AE

LKB Instruments Ltd,
232 Addington Road,
South Croydon,
Surrey.
CR2 8YD Tel: 01 657 8822

Pharmacia Ltd,
Pharmacia House,
Midsummer Blvd,
Milton Keynes,
Bucks.
MK9 3HP Tel: 0908 661101

Pye Unicam Ltd,
York Street,
Cambridge,
CB1 2PX Tel: 0223 58866

Roche Ltd,
Diagnostics Division,
P O Box 8,
Welwyn Garden City,
Herts.
AL7 3AY Tel: 07073 28128

Sartorius Instruments Ltd,
18 Avenue Road,
Belmont/Sutton,
Surrey. Tel: 01 642 8691

Sigma Chemical Co Ltd,
Fancy Road,
Poole,
Dorset.
BH17 7NH Tel: 0202 733114

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