The effect of some injurious agents on the biochemistry of brain development and a study of some characteristics of synaptic membrane acetylcholinesterase

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THE EFFECT OF SOME INJURIOUS AGENTS ON THE BIOCHEMISTRY OF BRAIN DEVELOPMENT AND A STUDY OF SOME CHARACTERISTICS OF SYNAPTIC MEMBRANE ACETYLCHOLINESTERASE.

BY

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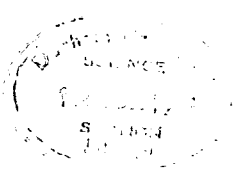

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ABSTRACT

The effects of maternal hyperthermia on the state of development of newborn guinea-pig brain have been studied with particular regard to DNA content and the activities of brain plasma membrane marker enzymes.

Hyperthermia of about $4^{\circ}C$ applied to pregnant guinea-pigs daily between the 18th and 25th days of gestation resulted in newborn exhibiting a high degree of microencephaly, but with no other obvious abnormalities. Microencephalic animals were also found to have reduced brain DNA content. However, the reduction in brain weight and DNA content were localised primarily to the cerebral hemispheres, as no such effects were detected for cerebellar tissue.

The protein specific activities of plasma membrane marker enzymes from synaptic and microsomal membrane fractions from the brains of microencephalic newborn were found to be similar to those from control animals. On the other hand, the activities of these enzymes in homogenates of microencephalic guinea-pig cerebral tissue, when expressed as a function of DNA content, have been shown to be higher than such values for control cerebral tissue.

These results have been discussed in terms of current models of brain development, and it is suggested that heat damage in the earliest stages of brain development can influence the subsequent differentiation of brain cells. In this case it is suggested that hyperthermia indirectly brings about more elaborate dendritic development in surviving brain cells.

The effects of hypothyroidism on the development of the specific activities of synaptic membrane enzymes in the rat brain has also been investigated. In control rats the specific activities of synaptic plasma membrane ATPases and acetylcholinesterase increased in the immediate postnatal period. The rates of increase in the activities of these enzymes in membrane preparations from hypothyroid rat brain were lower than for control preparations after about the 10th or 12th days of gestation, and did not attain normal adult values by 44 days post partum. These lower enzyme activity values have been shown not to be due to an increase in the 'latent' enzyme fraction hidden within membrane vesicles.

This description of qualitative changes in the development of synapses in rat brain has been discussed in terms of current models of brain dysfunction in hypothyroid rat brain which ascribe significance only to quantitative reduction in brain cell growth.

Some characteristics of synaptic membrane acetylcholinesterase from normal rat brain have also been examined in order to evaluate the possible role for lipid in the properties of this enzyme. Non-linear Arrhenius temperature-activity plots, usually associated with lipid dependent enzymes, have been found for acetylcholinesterase. The effects of membrane association, detergents and other lipophilic agents on the activity and kinetic properties of acetylcholinesterase have been evaluated. These data have been interpreted as evidence for a lipoprotein structure for acetylcholinesterase and for

the involvement of lipid in the properties of this enzyme, particularly in the maintenance of a low activation energy state at physiological temperatures.

This lipid has been tentatively identified as cardiolipin (diphosphatidylglycerol) as a phospholipid extracted from partially purified acetylcholinesterase in high salt conditions migrated on two dimensional thin-layer chromatography plates in a similar fashion to a commercial cardiolipin standard.

The low-ionic strength soluble and membrane-bound fractions of synaptic membrane acetylcholinesterase were further examined in order to assess the role of the membrane in enzyme properties. The membrane-enzyme was shown to be much more stable to irreversible thermal inactivation than the soluble. However, this difference was also observed in Lubrol detergent solubilized preparations.

The difference in stability of these fractions has in this case been attributed not to membrane association, but to differences in the oligomeric nature of soluble and membrane-bound acetylcholinesterase.

These properties of acetylcholinesterase have been discussed in terms of a potential model for lipid-protein interactions in general.

GENERAL INTRODUCTION

C.N.S. development in mammals has been shown to be affected adversely by both intrinsic and extrinsic factors. Johnson & Byington (1974) have reported a variety of congenital C.N.S. abnormalities in pigs infected with hog cholera virus. This has been supported by Kurent & Sever (1977) who have reviewed a number of reports of congenital C.N.S. defects in mammals produced by a variety of virus infections. In addition, maternal alcoholism (Jones & Smith, 1973), maternal undernutrition (Dobbing, 1968; Hurley, 1977) diabetes (Takano & Nishimura, 1967) phenylketonuria (Frankenberg, Duncan, Coffelt, Koch, Coldwell & Son, 1968) and immature hypothyroidism (Eayrs & Taylor, 1951) have been said to result in defective brain development in mammals.

The severe nature of some of the defects produced by some of these factors, such as hydrocephalus, spina bifida, cerebral cysts and cerebellar hypoplasia, often result in the young being aborted or born dead, and normally prevent affected animals surviving to a time when brain development would have been fully expressed. However, some conditions, such as immature hypothyroidism and maternal undernutrition produce a more subtle and controllable retardation of brain growth in experimental animals. In these cases affected animals survive to complete brain growth and have provided useful subjects for biochemical investigations throughout development, which have attempted to correlate changes in brain biochemistry with the deficiencies observed in the outward signs of brain function.

It has also been suggested that maternal hyperthermia acts as a teratogenic agent in several species (Edwards & Wanner, 1977) producing predominantly but not exclusively abnormalities of the C.N.S. The effect of elevated maternal body temperature on

brain development in the guinea-pig was particularly interesting. In this species it was reported that elevation of maternal body temperature by a few degrees centigrade on one or two days during the first third of pregnancy resulted in a high incidence of microencephaly in the offspring (Edwards, 1969 (a) & (b)). These microencephalic newborn were shown to survive well into adulthood and to exhibit impaired cognitive abilities as young adults (Johnson, Lyle, Edwards & Penny, 1976). This was a remarkable result of such limited heat exposure in early development, and suggested that the developing nervous system in general was particularly sensitive to heat damage at this stage.

Thus the effect if maternal hyperthermia on brain development in the guinea-pig had similar advantages, as a subject for biochemical analysis, as with hypothyroidism and maternal starvation, in that offspring with brain damage survived to the time of the most rapid phase of brain development and thus expressed fully the effects of any lesions. The effect of hyperthermia also appeared to affect the growth of most of the brain in a general way as heat damage to the C.N.S. was expressed as microencephaly. However, additional properties of the hyperthermia model were particularly interesting. Hypothyroidism and maternal starvation on brain growth are thought to result from stringent metabolic restrictions placed upon brain cells during the rapid phase of brain development, whereas this was unlikely to be the case with hyperthermia as the primary lesion was inflicted at a time remote from this rapid growth phase. This suggests that heat damage to brain cells, early in development, impairs subsequent differentiation. Maternal hyperthermia also has the advantage that general body growth of experimental animals would not be impaired by the general metabolic restrictions of

maternal starvation or hypothyroidism.

As a result of these features it was decided to examine the biochemical state of development of the offspring of heat treated female guinea-pigs. Initially it would be necessary to ascertain the precise conditions of maternal heat exposure which would produce microencephalic animals consistently. This done, it would be possible to determine whether the reduced brain weight resulted from a decrease in brain cell numbers or from a reduction in the extensive elaboration of brain cell dendrites, which is characteristic of the brain growth spurt, or perhaps both of these. It would also be possible to ascertain whether all brain regions were equally affected in this respect.

It was decided to approach this by firstly, measuring the DNA content of microencephalic and control guinea-pig brain and brain regions which would give an index of cell numbers in this tissue, and secondly, by determining the activity of marker enzymes in homogenates of control and microencephalic guinea-pig brain, so as to give an indication of the state of differentiation of brain cells. The choice of marker enzymes was problematical but it was decided to use the plasma membrane enzymes, $Na^+ - K^+$ ATPase, $Ca^{2+} - Mg^{2+}$ ATPase and acetylcholinesterase. This was for two reasons : an increase in the quantity of plasma membrane containing the enzymes is particularly characteristic of brain differentiation and because it would be relatively easy to measure the protein specific activity of these enzymes in membrane fractions isolated from tissue homogenates. The latter was important as the levels of these enzymes increase relative to other membrane proteins during development (Abdel-Latif, Brody & Ramahi, 1967; Abdel-Latif, Smith & Ellington, 1970) and it would be necessary to evaluate any contribution of this effect to enzyme activity in tissue homogenates.

This would ensure that any changes in homogenate activity was proportional to changes in the quantity of nerve cell membrane. Having obtained these values it would be possible to express enzyme activities as a function of cell number and tissue weight for the effect of the heat treatment on the developing brain of the guinea-pig and on the subsequent growth of the brain in response to that initial lesion.

The effects of immature hypothyroidism on the plasma membrane enzyme activity and DNA levels are well characterised, particularly in the case of the rat in which the rapid phase of brain development occurs in the first few weeks of postnatal life. Although hypothyroidism has no effect on cell numbers in the cerebrum and only delays the acquisition of adult cell numbers in the cerebellum (Balazs, Cocks, Eayrs & Kovacs, 1971) the rate of increase in plasma membrane enzyme in brain homogenate was reduced by hypothyroidism (Gell & Timiras, 1967; Valcana & Timiras, 1969). However, the contribution of changes in protein specific activity of these enzymes to the changes in the activities in tissue homogenates have not been examined.

Thus it was decided in the early part of the study to determine the specific activities of the $Na^+ - K^+$ ATPase, $Ca^{2+} - Mg^{2+}$ ATPase and acetylcholinesterase in the synaptic plasma membrane fractions isolated from control and hypothyroid rat brain at various stages during postnatal development. With this information it would be possible to speculate as to whether the primary effect of hypothyroidism on brain development was consistent with a general reduction in nerve cell growth or whether retardation of the qualitative differentiation may also contribute to aberrant brain function.

In the course of characterising the acetylcholinesterase to enable

a rigorous investigation of the effect of heat and hypothyroidism on the activity of this enzyme it was found that the enzyme had some interesting properties. For example, it was found that the rat brain enzyme could exist in two active forms, membrane-bound and membrane-free and these could be characterised separately. The acetylcholinesterase was also found to exhibit non-linear Arrhenius plots. This property has been generally associated with enzymes which are integral membrane proteins such as $Na^+ - K^+$ ATPase and $Ca^{2+} - Mg^{2+}$ ATPase (Charnock, Cook, Almeida & To, 1973; Duncan, 1976), and is not thought to be characteristic of enzymes such as the acetylcholinesterase which have been classed as peripheral membrane proteins by virtue of their weaker association with the membrane (Mitchell & Hanahan, 1966; Singer, 1974).

The precise interpretation of the non-linear temperature plots of integral enzymes such as the $Na^+ - K^+$ ATPase has been the subject of some discussion (Han, 1972). Most workers interpret non-linear Arrhenius plots as representing two straight lines intersecting at a "critical" temperature (Charnock, Almeida & To, 1974). This has been said to result from a thermotropic gel to liquid-crystal phase transition in the hydrophobic fatty-acyl chains in the region of the enzyme (Wyn-Williams, 1976), similar to phase transitions observed for protein-free phospholipid dispersions (Yellin & Levin, 1977). In this case the "critical" temperature would represent the temperature at which the phase change occurred, and the slope of the Arrhenius plot above the transition would represent the enzyme constrained to a low activation energy state by the relatively fluid state of the membrane while the slope below the transition would represent the enzyme constrained to a high activation energy state by the relatively rigid state of the membrane.

However, Arrhenius plots for $Na^+ - K^+$ ATPase presented by Bowler & Duncan (1968) and Tirri & Bowler (1974) clearly show that such plots are curvilinear, with an apparent activation energy of 70 - 80 KJ $mole^{-1}$ at about 37°C which increases progressively with lower temperature to give apparent activation energies of about 150 - 200 KJ $mole^{-1}$ at about 5°C - 10°C. This may be consistent with the more complex thermotropic phase change behaviour observed for natural membranes (Pagano, Cherry & Chapman, 1973).

The Arrhenius plot of rat synaptic membrane acetylcholinesterase activity was clearly not of this form. Although apparently curvilinear, these plots showed an apparent activation energy of only about 13 - 15 KJ $mole^{-1}$ at around 37°C which increased to about 30 - 40 KJ $mole^{-1}$ at about 5°C - 10°C. It was not clear how physical state changes in membrane lipids could produce this effect for the acetylcholinesterase, in the same way as for the $Na^+ - K^+$ ATPase, as both are thought to exhibit completely different forms of membrane association.

To examine this in more detail, it was decided to examine Arrhenius plots for acetylcholinesterase to arrive at a consistent interpretation of such plots, hopefully arriving at quantitative parameters for the form of the transition. Then it would be possible to examine the temperature properties of membrane-dissociated acetylcholinesterase to determine whether non-linear Arrhenius plot of this membrane enzyme was exclusively a membrane phenomenon. If this was the case then rat brain acetylcholinesterase would provide a model for the involvement of the membrane in the non-ideal temperature kinetics of enzymes.

If this was not the case, and solubilized acetylcholinesterase also gave non-linear temperature plots then this may result from the enzyme carrying a 'halo' of lipid molecules in the soluble state or

alternatively non-linear plot, in this case, may result from an entirely protein based phenomenon. Although there would be no method of distinguishing between these alternatives directly, it should be possible to accumulate evidence for or against functional lipid molecules bound to soluble acetylcholinesterase by examining the effect of detergents and other lipophilic agents on enzyme properties.

Although at the outset of this study only circumstantial evidence existed to link membrane phenomena and the properties of the acetylcholinesterase (Farias, Bloj, Moreno, Sineriz & Trucco, 1975) this was encouraging and was inevitably followed by several more during the course of this work. However, these studies were contradictory : Beauregard & Roufogalis (1977) and Reavill, Wooster & Plummer (1978) found non-linear Arrhenius plots for bovine erythrocyte acetylcholinesterase, similar to those found in this study, but Beauregard & Roufogalis (1977) found that the solubilized enzyme also gave similar plots, unlike Reavill et al (1978) who found that solubilized enzyme gave conventional, linear plots. Thus Beauregard & Roufogalis suggested that the bovine enzyme was a lipoprotein whereas Reavill et al (1978) suggested that the temperature properties were determined by membrane lipids. Sihotang (1976) suggested that human erythrocyte acetylcholinesterase was absolutely dependent on lipid association for activity, whereas Beauregard & Roufogalis (1977) and Reavill et al (1978) showed that the activity of acetylcholinesterase was not inactivated by treatments which disturbed lipid bound to this enzyme.

This work clearly did not provide a consistent view point, either because of species differences or because of artefacts of experimental technique. It was thus of considerable interest to examine the rat brain acetylcholinesterase in this context, to at least define

the behaviour of this and hopefully to try to resolve the apparent contradictions of the former studies. To this end it was considered sensible to put much of the developmental effort into this aspect of the project.

GENERAL MATERIALS AND METHODS

CHEMICALS LIST

Sigma (London) Chemical Company

Acetylthiocholine Iodide

Adenosine -5'- Triphosphate (A.T.P.) disodium salt

Sigma grade from equine muscle

Bovine serum albumin, fraction V powder

Chlorpromazine hydrochloride

Deoxycholic acid, sodium salt

Deoxyribonucleic acid, type 1 sodium salt from calf thymus

D.E.A.E. Sephadex A-25

D.E.A.E. Sephadex A-50

Ethyleneglycol-bis(amino-ethyl ether)N N' tetra acetic acid (E.G.T.A.)

Histidine hydrochloride

Imidazole (Grade 1)

Lidocaine

Lubrol WX

Procaine hydrochloride

L α-phosphatidyl choline

Tetracaine hydrochloride

Triton X-100

Tris (hydroxymethyl)-amino methane (tris)

Boehringer Corporation (London)

Deoxyribonucleic acid (DNA)

Supelco

Fatty acid methyl esters (standards for gas-liquid chromatography)

Phospholipid standards (standards for thin-layer chromatography)

I.C.I. dye stuffs division (provided as a gift)

Cirrasol - ALN-WF (Lubrol)

Eastman Kodak

8 anilino-1 naphthalene sulphonic acid (sodium salt) A.N.S.

Pye-Unicam

Poly-ethylene glycol adipate

Applied Science Laboratories

Gaschrom Q

All other reagents were supplied by British Drug Houses (B.D.H.).

ENZYME ASSAYS

Assay of acetylcholinsterase (E.C.3.1.1.7.) activity.

Acetylcholinsterase was assayed essentially by the method of Ellman, Courtney, Andres & Featherstone (1961). The pseudosubstrate acetylthiocholine iodide is hydrolysed, by the enzyme, liberating thiocholine. The free thiol groups are detected by reaction with dithionitrobenzoic acid (D.T.N.B.) which gives a yellow colour, with an absorbance maximum at 410 nm. In some experiments the original assay method was employed in which the reaction is carried out in a thermostated spectrophotometer cuvette. In other experiments the reaction was modified to be carried out in the test tube. In either case the final concentrations of the reagents were as shown below;

i)	Buffers. Sodium phosphate buffer pH 7.5	0.1M
	or tris-hydroxy methylamino methane (Tris) buffer pH 7.5	0.1M
ii)	Dithionitrobenzoic acid DTNB	0.33mM
iii)	Acetylthiocholine iodide	0.75mM

i) Cuvette assay

In this case 3cm^3 of pre-equilibrated buffer was placed in a glass spectrophotometer cuvette. This was then placed in an aluminium cuvette holder through which was circulating water at 37^{o}C in an SP.1800 Pye Unicam spectrophotometer. This was allowed to equilibrate for 10 minutes, after which time 100 ul of DTNB solution and 20 µl of substrate were added. The background rate of hydrolysis of substrate was determined by measuring the rate of increase of absorbance at 410nm on an AR-25 flatbed chart recorder. Then 100 µl of enzyme solution was added and mixed well with a small plastic paddle. The absorbance increase at 410 was measured for several minutes. The enzymic hydrolysis was represented by the difference between this rate and the spontaneous rate of substrate hydrolysis.

ii) Test-tube assay

In this case 3cm^3 of buffer was placed in a glass 10cm^3 test-tube in a water bath maintained at 37°C, and allowed to equilibrate for at least 15 minutes. Then 100 ul of DTNB solution is added followed by 200 ul of enzyme solution. The reaction is initiated with the addition of 200 ul of substrate solution, and allowed to run for between 10 - 30 minutes depending on enzyme concentration. The reaction is terminated by the addition of 3cm^3 of 1.0% sodium dodecyl sulphate solution. The extinction of this solution at 410 nm was measured in a Pye-Unicam SP-1800 spectrophotometer.

The background rate of hydrolysis was measured in a separate tube in which, in the case of isolated membrane preparations, the buffer vehicle was added instead of the enzyme, or, in the case of tissue homogenates the reaction buffer contained 1mM 62C47 acetylcholinesterase inhibitor. This was because no butyrylcholin-esterase activity was detected in membrane preparations but this could not be ruled out for tissue homogenates. With this method it was found that the background rate of hydrolysis of acetyl-thiocholine was about 0.1 absorbance units in 10 minutes and that the reaction was linear up to about 0.8 absorbance units.

The solutions were prepared as follows;

Phosphate Buffer

A solution of 0.1M disodium hydrogen orthophosphate was warmed with continuous stirring to bring the temperature slowly to 37°C. The pH of this was monitored continuously with a Beckman pH-meter, and the pH was brought to pH 7.5 with 0.1M sodium dihydrogen orthophosphate as the temperature of the solution reached 37°C. This was stored at 4°C for no more than one or two weeks, after which time it was discarded. It was discarded sooner in the case

of obvious bacterial contamination.

Tris-HCl Buffer

A solution of 0.1M tris hydroxymethyl amino-methane (Tris base) was warmed to 37°C and simultaneously brought to pH 7.5 with 1.0N hydrochloric acid. The solution was then made up to volume in a volumetric flask.

D.T.N.B.

200 mg of D.T.N.B. and 15 mg of $NaHCO_3$ were dissolved in 100 cm^3 of 0.1M Tris-HCl pH7.0. This was stored at 4°C until needed.

Acetylthiocholine Iodide

Acetylthiocholine iodide was dissolved in distilled water to give 17.5mM and stored at -20°C in 2.0cm^3 aliquots and defrosted as required.

Standard Curve

The relationship between extinction at 410 nm and thiol concentration was determined with a range of concentrations of methionine in the same reaction buffers and D.T.N.B. concentration as used in the assay. This is shown in Figure 1.

Assay of sodium-potassium adenosinetriphosphatase (Na^+ - K^+ATPase) activity (E.C.3.6.1.3.).

The Na^+ - K^+ ATPase activity was determined by the colourimetric estimation of inorganic phosphate released from adenosine triphosphate at 37°C and pH 7.5, in the presence of 130mM NaCl and 20mM KCl. The contaminating Mg^{2+} - ATPase activity was determined in the absence of these ligands.

The reaction media used, contained the following final concentrations;

Solution 1;

Sodium chloride	130mM
Potassium chloride	20mM
Magnesium chloride	3mM
Adenosine triphosphate (Tris-salt)	3mM
Histidine	30mM

Solution 2;

Magnesium chloride	3mM
Adenosine triphosphate ATP (Tris-salt)	3mM
Histidine	30mM

These solutions are prepared as follows;

Ionic reaction-media

Ionic solutions (without ATP) were prepared 2.2x the concentration of the final reaction mixture. These solutions were made routinely in 250cm^3 batches. In this case the solid salts were weighed out on a weighing boat and washed carefully into a glass 250cm^3 beaker. The volume of the solution was brought to about 200cm^3 and the solution warmed to 37^{o}C slowly. As the solution reached 37^{o}C it was brought to pH7.5 with 1N HCl. The solution was allowed to cool and made up to volume in a volumetric flask. The solution was stored at 4^{o}C.

Adenosine triphosphate (Tris-salt)

Sigma grade disodium ATP was weighed out to give 13.2mM in 250cm^3. This was dissolved in about 50cm^3 of deionised water. This was desalted over a bed of activated Dowex ion-exchange resin.

The resin was prepared by suspending 20 gms of resin in 1 litre of 1.0N HCl for 30 minutes with regular stirring. The beads were allowed to settle and the acid poured off. The beads were washed with deionised water until the washings reached pH3-4.

The beads were stored wet at $4^{o}C$.

To desalt one 'batch' of disodium ATP as above, about 5gms of resin were poured over several sheets of Whatman No. 1 paper in a Buchner funnel and washed with several changes of deionised water. The $50cm^3$ of disodium ATP solution was allowed to filter, slowly, through the resin under gravity. The ATP solution was passed through the resin at least six times. The resin was then washed with $150cm^3$ of deionised water and the washings pooled with the original $50cm^3$. This solution was brought to pH7.2 with 1.0M Tris-base, made up to volume, divided into aliquots in boiling tubes, and stored at $-20^{o}C$. This Tris-ATP was also used neat for the Ca^{2+} - Mg^{2+} ATPase assay, but was diluted to 6.6mM for the Na^{+} - K^{+} ATPase assay.

Inorganic phosphate was determined by the method of Atkinson, Gatenby & Lowe (1973), for which the following solutions are prepared;

i) Acid molybdate solution; 1% w/v ammonium molybdate in 0.9M sulphuric acid

(This may be stored at $4^{o}C$)

ii) Cirrasol ALN-WF (Lubrol-W) solution; 1% w/v cirrasol in distilled water

(This was prepared fresh)

A chromogenic solution was made by mixing equal volumes of i) and ii).

PROCEDURE

$1.0cm^3$ of ionic medium and $1.0cm^3$ of 6.6mM Tris ATP were added to a $10cm^3$ glass test-tube, this is equilibrated for at least 15 minutes in a water bath maintained at $37^{o}C$. The reaction is

initiated with the addition of 200ul of enzyme solution and terminated after a suitable time with the addition of $4cm^3$ of chromogenic solution. This mixture was allowed to stand for 5 minutes at room temperature. After this time the extinction of the solution was determined at 390nm in a Pye-Unicam SP.1800 spectrophotometer.

The reaction rate was determined in both solution 1 and 2 for a given enzyme preparation. The difference between these rates was taken to represent the $Na^+ - K^+$ ATPase activity. The relationship between extinction at 390nm and inorganic phosphate concentration was determined using potassium dihydrogen orthophosphate concentrations dissolved in ionic solution 1 without ATP. The colour response was linear up to about 1.0 absorbance unit or 0.6 μ moles cm^{-3} in the assay mixture (Figure 2).

Assay of calcium-magnesium adenosinetriphosphatase ($Ca^{2+} - Mg^{2+}$ ATPase) activity

The $Ca^{2+} - Mg^{2+}$ ATPase activity was determined by the colourimetric estimation of inorganic phosphate released from adenosine triphosphate at 37°C and pH 7.5, in the presence of 1.0mM CaCl, 1.0mM E.G.T.A. and 100mM KCl. The contaminating Mg^{2+} ATPase activity was determined in the absence of 1.0mM CaCl.

The reaction media used, contained the following final concentrations;

Solution 1;

Calcium chloride	1.0mM
E.G.T.A.	1.0mM
Potassium chloride	100mM
Magnesium chloride	3.0mM
Adenosine triphosphate (Tris-salt)	3.0mM
Histidine	30mM

Ouabain (octahydrate) 1.0mM

Solution 2;

As above but without calcium chloride

These solutions were prepared as follows;

Ionic Reaction-media

The ionic solutions (without ATP and ouabain) were prepared at 2.2x the concentration of the final reaction mixture. These solutions were made routinely in 250cm^3 batches. In this case solid salts were weighed out on a weighing boat and washed carefully into a glass 250cm^3 beaker with distilled water. The volume of solution was brought to about 200cm^3 and the appropriate volume of 1M CaCl volumetric solution was added to solution 1. Both solutions 1 and 2 were brought to 37°C slowly and brought to pH 7.5 with 1N HCl as the solutions reached 37°C. The cooled solutions were brought to 250cm^3 in a volumetric flask and stored at 4°C until needed.

Adenosine triphosphate (Tris-salt)

13.2mM Tris-ATP was made as described for the $Na^+ - K^+$ ATPase assay.

Ouabain (octahydrate)

The solution of 4.4mM ouabain octahydrate was made simply by dissolving the solid in distilled water and stored at 4°C.

Phosphate determination

Inorganic phosphate was determined by the method of Atkinson, Gatenby & Lowe (1973), as described for the $Na^+ - K^+$ ATPase assay.

Procedure

1.0cm^3 of ionic medium, 0.5cm^3 of 13.2mM Tris-ATP and 0.5cm^3 of 4.4mM ouabain were added to a 10cm^3 glass test tube, which was equilibrated for at least 15 minutes at 37°C. The reaction was

initiated with the addition of 200 ul of enzyme solution and terminated after a suitable time with the addition of $4cm^3$ of chromogenic solution. This mixture was allowed to stand for 5 minutes at room temperature. After this time the extinction of the solution was determined at 390nm in a Pye-Unicam SP-1800 spectrophotometer.

The reaction rate of a given enzyme preparation was determined in both solution 1 and 2. The difference between these rates was taken to represent Ca^{2+} - Mg^{2+} ATPase activity. The relationship between colour formation and inorganic phosphate concentration was found to be identical with that represented in Figure 2.

The ouabain octahydrate was included to remove any possibility of Na^+ - K^+ ATPase activity. The reaction medium contained 100mM KCl, thus any sodium introduced into the assay tube would then be expressed as Na^+ - K^+ ATPase activity

Protein Assay

The assay procedure was a modified method of McGrath (1972). Samples of protein were hydrolysed to individual amino acids which were measured with ninhydrin. The reagents used for this assay were as follows;

i) Barium hydroxide, octahydrate (finely ground)

ii) 0.5% (w/v) Ninhydrin in 2-methoxyethanol

iii) Acetate Buffer pH 4.7 (with cyanide)

1.32M sodium acetate

1.54M Acetic acid

1×10^{-4}M sodium cyanide

Procedure

Samples of 100mg of barium hydroxide were weighed into 15cm^3 test tubes. 200ul of a given protein solution was pipetted into each of two of such tubes. One of those tubes was heated in a pressure cooker at 120^{o}C for 20 minutes, to hydrolyse the protein. The pressure was released and the sample steamed for a further 20 minutes. After this, 2cm^3 of acetate-cyanide buffer was added to both tubes and the excess barium hydroxide was dissolved. Then 3cm^3 of ninhydrin solution was added to each tube and both tubes were returned to the pressure cooker and heated at 120^{o}C for a further 15 minutes.

The cooled samples were diluted to 25cm^3 with a 60 : 40 (v/v) 2 methoxyethanol : water mixture. The extinction of this solution was determined at 570nm in a Pye-Unicam SP-1800 spectrophotometer. The difference in absorbance between the hydrolysed and unhydrolysed samples was taken to represent the amino-acids released from protein. The system was calibrated with bovine serum albumin (BSA) and the calibration graph (Figure 3) was found to be linear up to 500 µg cm^{-3} BSA.

MEMBRANE ISOLATION PROCEDURES

All animals were stunned, killed by cervical dislocation, and their brains were dissected from the skull and immersed in ice cold buffered iso-osmolar sucrose solution (320mM sucrose, 20mM imidazole, 2mM EDTA pH 7.2 at room temperature). The relevant brain regions were dissected out and homogenised in the above buffered sucrose medium at a tissue concentration of about 10%. A teflon-glass homogeniser was used and care was taken to disrupt the tissue without causing foaming of the solution. The tissue was disrupted with 10 up-and-down strokes of the rotating plunger.

This homogenate was centrifuged at 900 xg in an MSE Mistral 2L centrifuge for 10 minutes at $4^{o}C$. The supernatant was retained and the pellet was washed twice with the same volume of fresh buffered sucrose solution. The supernatants from these washes were retained and pooled with that from the initial homogenates. This supernatant was centrifuged at 20,000 xg for 30 minutes at $4^{o}C$ in an MSE HS18 high speed centrifuge. The supernatant from this spin contains the microsomal and cytosol fractions and the pellet contains intact synaptosomes, mitochondria and myelin membranes.

Microsome Isolation Procedure

The supernatant containing microsomes was diluted with 20mM imidazole 2mM EDTA pH 7.2 at $4^{o}C$ buffer, to give 250mM sucrose. This was centrifuged at 100,000 xg for 1 hour at $4^{o}C$ in either a MSE superspeed 40 preparative ultracentrifuge or a MSE Prepspin 50 preparative ultracentrifuge. The pelleted membranes were rehomogenised in imidazole/EDTA buffer and resedimented by centrifugation at 100,000 xg for 1 hour at $4^{o}C$ as above. The pellet from this spin was suspended by homogenisation in a small volume (3-5 cm^{3}) of imidazole/EDTA buffer and stored at $4^{o}C$, packed in ice.

Synaptic Membrane Isolation Procedure

The material pelleted at 20,000 xg was resuspended by homogenisation in low ionic strength imidazole/EDTA buffer. This lysed the synaptosomes releasing mitochondria and synaptic vesicles. The lysate was centrifuged at 20,000 xg for 30 minutes at $4^{o}C$ in an MSE HS 18 high speed centrifuge. The pellet from this spin was then fractionated to purify synaptic membranes in two ways;

i) Small Scale Isolation Procedure

In the case of the membrane extract from a small number of animals was fractionated on a discontinuous sucrose density gradient. In this case the osmotically lysed pellet was homogenised in $3cm^{3}$ of imidazole/EDTA buffer. This was layered carefully over a gradient consisting of equal volumes of 0.8M sucrose, 0.9M sucrose, 1.0M sucrose and 1.2M sucrose, all dissolved in 20mM imidazole buffer at pH 7.2. The gradient was centrifuged at 100,000 xg for 2 hours at $4^{o}C$ in either an MSE Superspeed 40 ultracentrifuge or an MSE Prepspin 50 ultracentrifuge.

The membranes which floated at the 1.0 - 1.2M sucrose layer was aspirated with a pasteur pipette and diluted with imidazole/EDTA buffer to a sucrose concentration below 0.8M and centrifuged at 100,000 xg for 1 hour at $4^{o}C$. The pellet was rehomogenised in imidazole/EDTA buffer and centrifuged at 100,000 xg for 1 hour at $4^{o}C$. The pellet was homogenised in 3-5 cm^{3} of imidazole/EDTA buffer and stored at $4^{o}C$, packed in ice.

ii) Large Scale Isolation Procedure

In this case the lysed pellet was homogenised in 1.2M sucrose, 20mM imidazole pH 7.2 and centrifuged at 100,000 xg for 2 hours at $4^{o}C$. The supernatant, containing synaptic membranes and myelin, was carefully removed and diluted with imidazole/EDTA buffer to give 1.0M sucrose. This was then centrifuged at

100,000 xg for 1 hour at $4^{o}C$. The synaptic membranes pellet, the myelin membranes remain suspended. The pellet was resuspended in imidazole/EDTA buffer and centrifuged at 100,000 xg for 1 hour at $4^{o}C$. The pellet of synaptic membranes was resuspended in 3-5cm^{3} of imidazole/EDTA buffer and stored, packed in ice, at $4^{o}C$.

GAS CHROMATOGRAPHIC TECHNIQUES

A Pye-Unicam, 104 series gas chromatograph was used throughout this study for the separation of fatty acid methyl esters. The column used was a 1 metre glass column containing 3% Polyethyleneglycol adipate (P.E.G.A.) on Gas Chrom Q solid support, at a temperature of 180°C with a carrier gas (N2) flow rate of 45cm^3 min^{-1}. The chromatograph was fitted with a hydrogen/air flame ionisation detector. The output of the amplifier was displayed on a flatbed chart recorder.

i) Preparation of the Column

The inert solid support was coated with P.E.G.A. in the following manner. 20gms of Gas Chrom Q was added to a 3% (w/v) solution of P.E.G.A. in chloroform. This was stirred for several minutes with a clean glass rod and the mixture was filtered over a glass scinter and the P.E.G.A. coated support material was air dried on the scinter and when dry was placed in an oven at about 100°C for 1 - 2 hours to ensure that all solvent and volatiles were removed. This was stored in a chloroform washed screw-top jar until required.

A length of thick-walled plastic tubing, fitted to metal connector on the glass chromatography column, was connected via a glass wool filled trap, to a vacuum pump. A small quantity of coated support material was introduced into the column under suction and the column was lightly tapped until the powder rested firmly against the glass wool plug. This was repeated until the column was full to within 3 or 4 cm^3 of the top with firmly packed column material. The packed column was then fitted to the chromatograph and flushed with "white-spot" nitrogen at 45cm^3 min^{-1} at 200°C for 24 hours to remove any loosely bound P.E.G.A. After this time the spontaneous column "bleed" was low and a steady baseline

amplifier response was produced at high sensitivity settings.

ii) Calibration of the column

Commercially available fatty acid methyl ester standards (Supelco) were used to obtain calibration curves for this column. This was done after the method of Ackman 1963 (a) and (b) who showed that for a given fatty acid class, the carbon chain length was linearly related to the log of the relative retention time on the column. Although these lines were different for each fatty acid class, they were parallel to each other, as shown in Figure 4. In this Figure retention times were expressed as a function of that for 16 : 0. Lines were drawn for saturated, 1 w9 monounsaturated, 2 w6 diunsaturated and 3 w6 triunsaturated fatty acids with a complete range of standards between C:16 and C:24. The 6 w3 hexaenoic was drawn using commercial 18:6 w3 and 22:6 w3. Other lines were drawn assuming the same relationships between classes as those obtained by Ackman 1963 (a) & (b).

The quantity of any given fatty acid was calculated from the area under the peak as described by Carrol (1961). The areas of all peaks were summed and individual peak areas expressed as a percentage of this value.

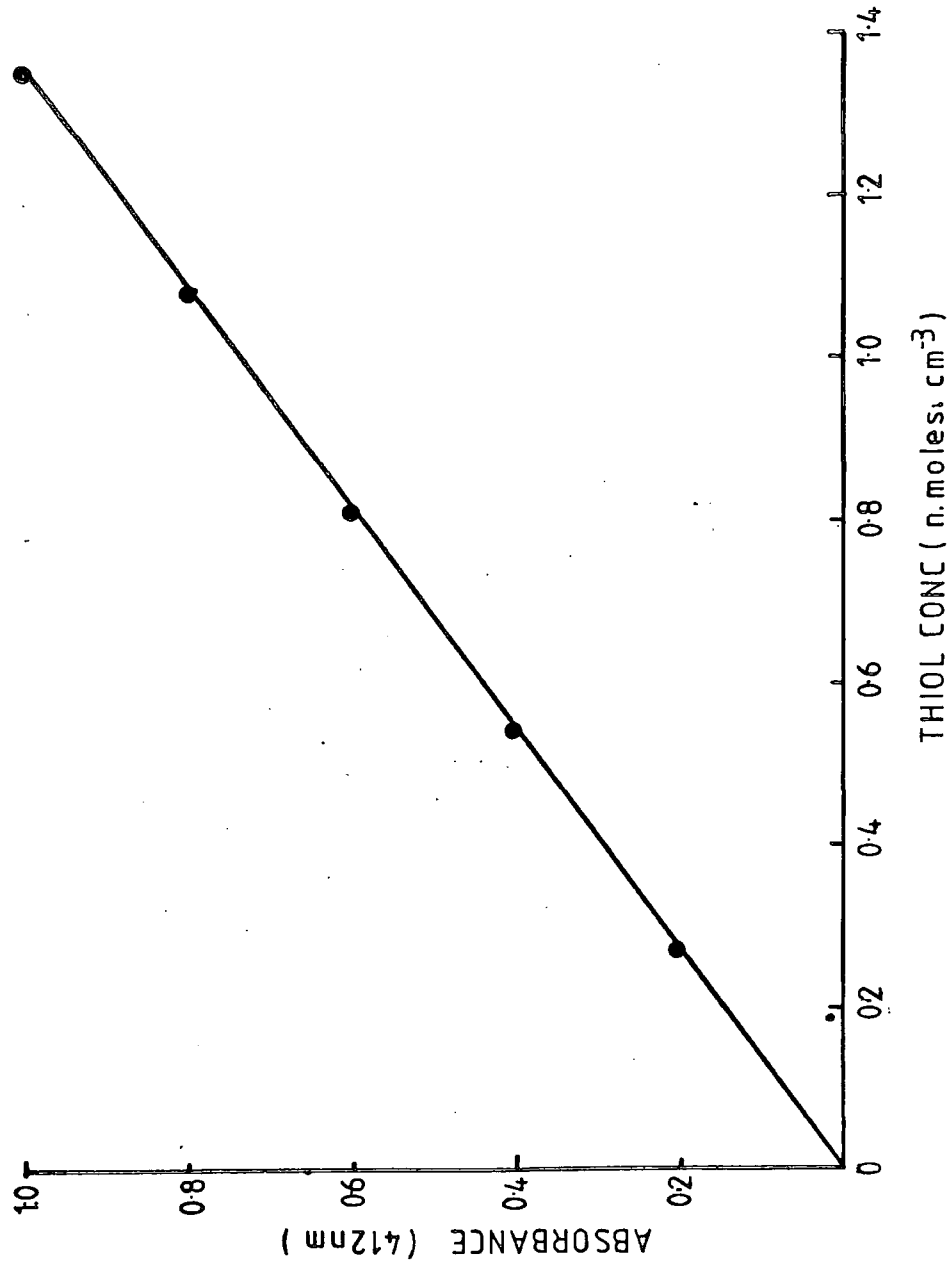

ABSORBANCE (412nm)
1·0
0·8
0·6
0·4
0·2
0
0·2
0·4
0·6
0·8
1·0
1·2
1·4
THIOL CONC (n.moles. cm⁻³)

FIGURE 1.

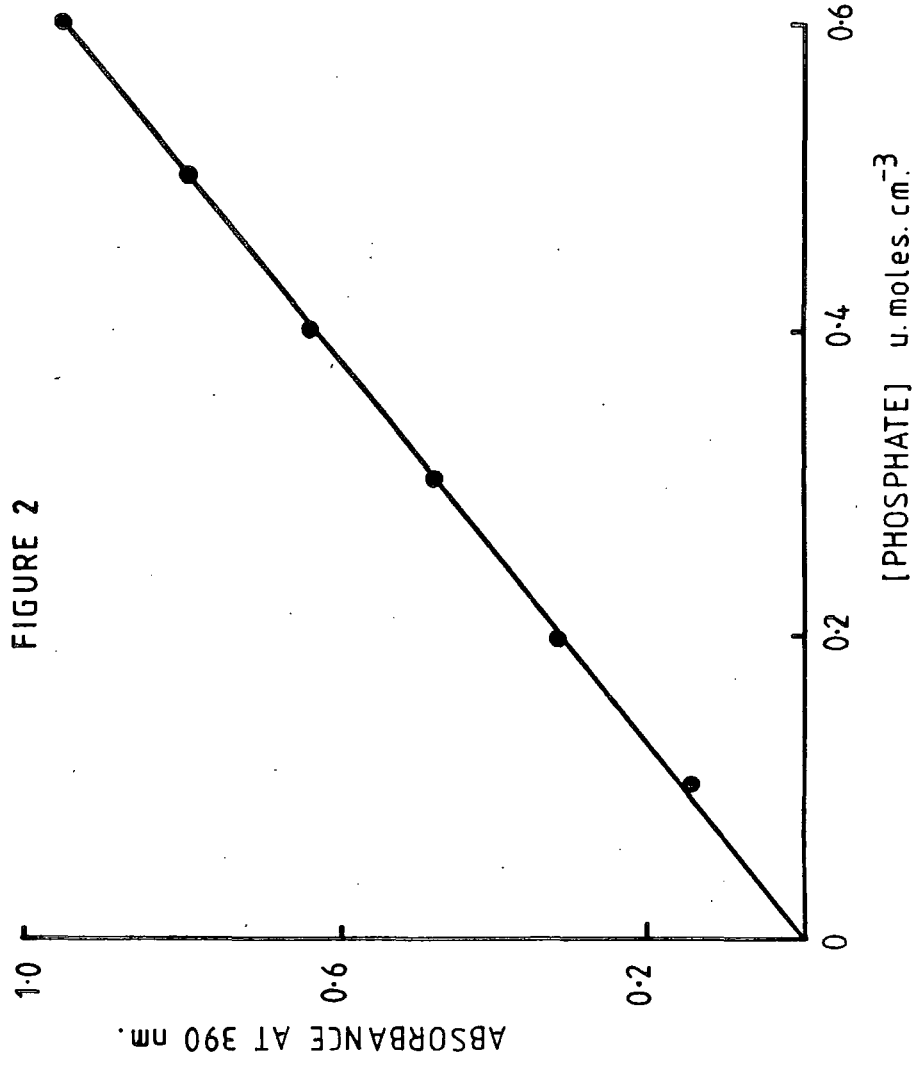


FIGURE 2

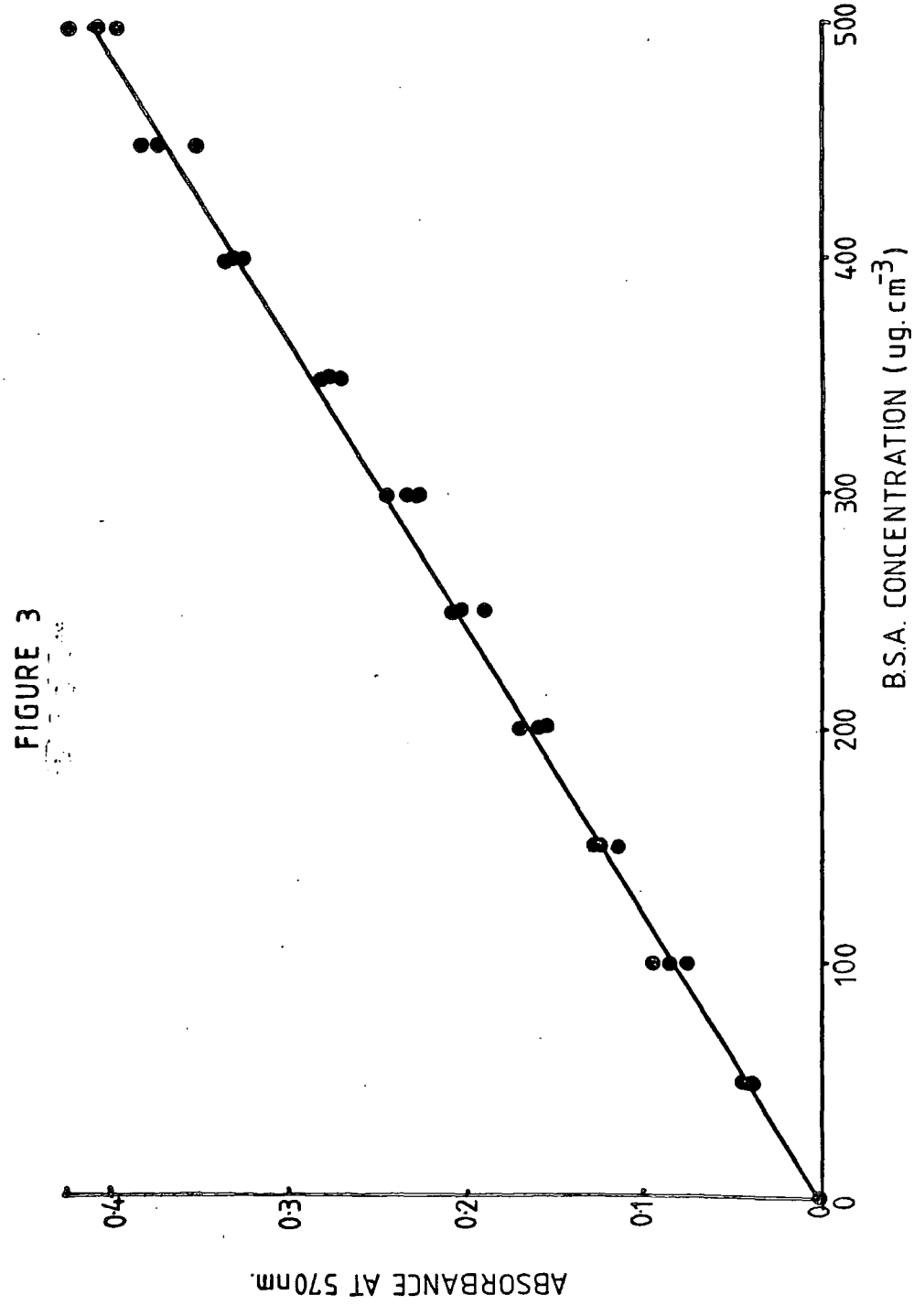

ABSORBANCE AT 570nm.
0·4
0·3
0·2
0·1
0
0
100
200
300
400
500
B.S.A. CONCENTRATION (ug.cm^{-3})

FIGURE 3

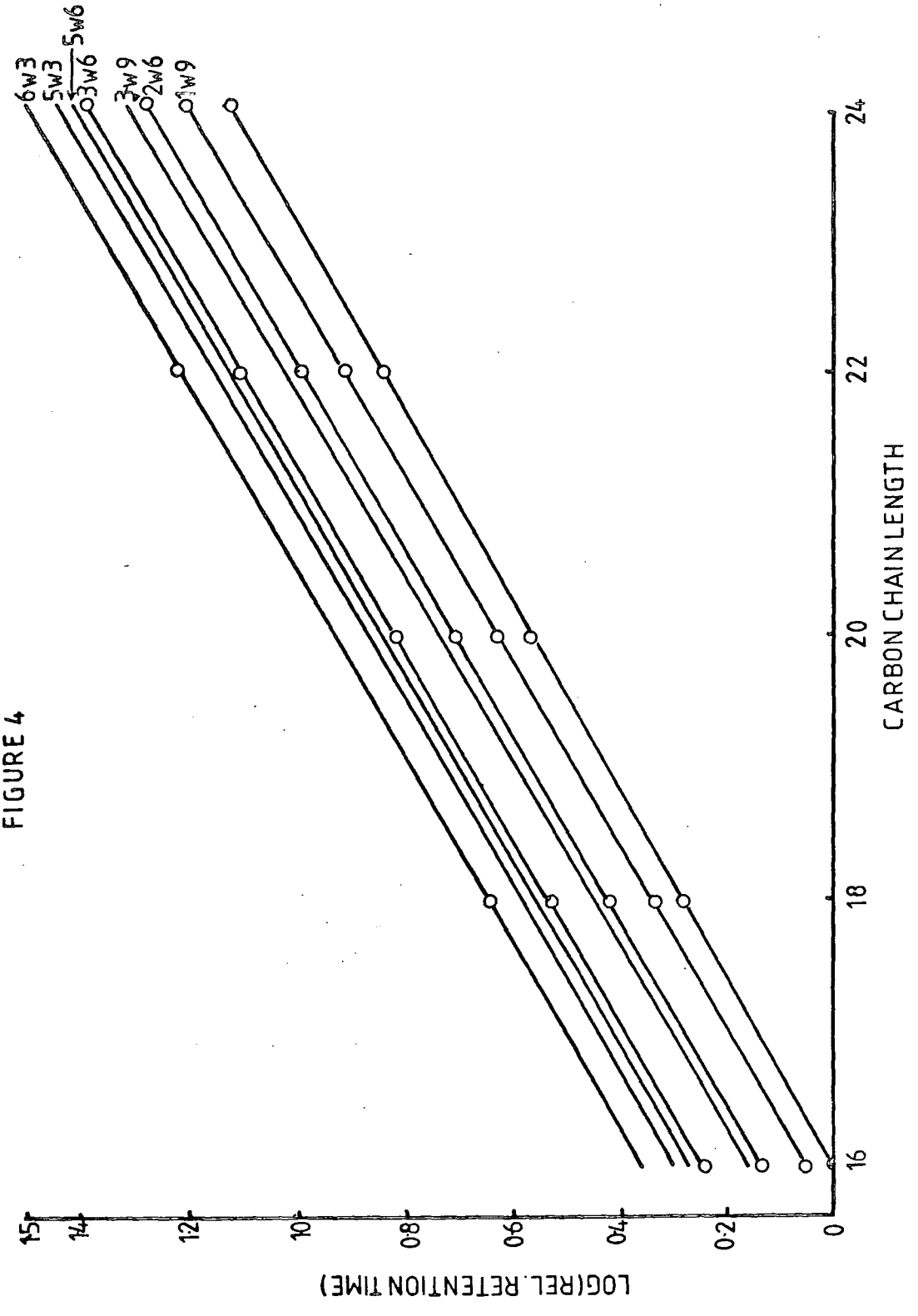

6w3
5w3
5w6
3w6
3w9
2w6
1w9
15
14
12
10
08
06
04
02
0
16
18
20
22
24
LOG(REL. RETENTION TIME)
CARBON CHAIN LENGTH

FIGURE 4

THE EFFECT OF MATERNAL HYPERTHERMIA ON THE DEVELOPMENT OF THE BRAIN OF NEWBORN GUINEA-PIGS

Introduction

A percentage of human foetuses are malformed and a variety of factors are known that induce developmental abnormalities, some genetical and some environmental. Many agents have been tested in animal models for a teratogenic effect, a research field sharpened by the "thalidomide affair". Such work has allowed a controlled examination of the anatomical and biochemical bases for physical and psychological defects in affected offspring to be made. Of a wide variety of possible environmental teratogenic agents that has recently been proposed and studied is that of maternal hyperthermia. However despite a growing literature describing physical defects in experimental animals as a result of maternal hyperthermia at specific periods during gestation, it is not generally recognised that hyperthermia is a teratogenic agent in man. This is not surprising in that the nature of the effect of hyperthermia on the mammalian foetus varies considerably, depending on the particular species involved, the time during gestation at which hyperthermia was applied, and the extent and duration of the elevation of body temperature.

It may well be that the elevation of body temperature commonly experienced during fever in humans is not high enough to produce obvious physical birth defects in large enough numbers to permit direct correlation between temperature and defect. However, Miller, Smith and Shepard (1968) have reported a relationship between maternal hyperthermia and anencephaly in humans. They found that a small but significant incidence of anencephaly apparently correlated with the mother experiencing fever during

the first third of pregnancy. A similar correlation has been shown for babies suffering from meningomyelocele (Change & Smith 1978). The relationship between these birth defects and maternal hyperthermia could never be unequivocal in that, unlike the effects of hypothyroidism and maternal starvation, other factors have been shown to cause anencephaly.

It is interesting that the heat-induced abnormalities discussed above affected the central nervous system. The majority of abnormalities observed in studies on maternal hyperthermia in animals were also defects of the central nervous system. So perhaps this tissue is particularly susceptible to damage by maternal hyperthermia. Also, in the studies quoted above mothers experienced hyperthermia during the first third of pregnancy, which is the same time during pregnancy at which hyperthermia caused most birth-defects in experimental animals (Skreb & Frank, 1963; Edwards, 1967; Edwards, 1968; Umpierre & Dukelow, 1977). Perhaps a particular stage in mammalian brain development is particularly susceptible to elevated maternal body temperature.

That foetal and post-natal hypothyroidism, as well as maternal starvation cause C.N.S. abnormalities in humans is well documented. The effects of these teratogenic agents have also been well characterised in animal models, and have provided crucial information on the periods in development that the C.N.S. is vulnerable to insult. This provides useful information on the mechanism of brain development in mammals by highlighting the consequences of damage to particular anatomical features or the deficient development of particular biochemical parameters of brain growth. It seemed possible

that an animal model of the effect of maternal hyperthermia on brain development could similarly provide general information on the mechanism of the developmental process.

Any species chosen as an animal model for a study of heat-induced brain malformation would ideally require several special properties;

(i) It would be essential that the heat induced defects would not prevent affected animals from being born alive, and also that affected animals were not aborted during gestation.

(ii) It would be useful if the mother would not reject affected animals at birth. Species which give birth to maternally dependent young usually give birth to newborn at a developmental stage prior to the rapid phase of brain development. Hence the level of feeding and amount of maternal care could affect subsequent brain development. This would make suitable controls difficult to obtain.

(iii) The developmental abnormality produced by hyperthermia would need to be specific to the C.N.S. with few other physical defects to complicate measurements.

(iv) The developmental abnormality produced by hyperthermia would need to be consistent in its effect on a brain region or on brain growth in general. The random occurence of a variety of brain growth defects would make it difficult to select a given set of parameters to evaluate brain development.

Studies of the effect of elevated temperature on development have used rats, mice, hamsters, rabbits, guinea-pigs and chicks as experimental animals. When these species are compared for suitability by the criteria described above, it is clear that the guinea-pig represents the most suitable animal model of the effect of maternal hyperthermia on brain

development. In guinea-pigs, maternal heat exposure between the 18th and 25th day of gestation produced a high degree of microencephaly in offspring but with a low incidence of abortion and resorption (Edwards, 1969 (a) and (b)). Also few defects of physical growth were found in microencephalic animals and affected animals survived into adulthood (Edwards, 1969 (a) and (b); Jonson, Lyle, Edwards & Penny, 1976). In the case of the other species mentioned above anencephaly, exencephaly and gross cranial defects resulted from heat exposure, but also resulted in a high incidence of abortion and resorption. Also many animals showing brain abnormalities also showed defects of limb growth (Alsop, 1919; Hsu, 1948; MacFarlane, Pennycuik & Thrift, 1957; Skreb & Frank, 1963; Pennycuik, 1965; Edwards, 1968; Umpierre & Dukelow, 1977).

Another advantage of the guinea-pig is that the young are born after the most rapid phase of brain growth and in this case the effect of heat on the C.N.S. would be fully expressed (Dobbing & Sands, 1970). However, the severity of brain growth defects resulting from heat exposure of rats, hamsters etc., and the necessity for postnatal care in these species would ensure that affected newborn would not survive to undergo the postnatal brain growth spurt (Dobbing, 1968).

Although little evidence exists to show a teratogenic effect of elevated maternal body temperature on human foetal development, the work quoted above has been interpreted as good evidence for such teratogenic effect on laboratory animals (Edwards & Wanner, 1977). The most common effect seems to be that of damage to the developing neuroepithelium giving rise to anencephaly and microencephaly in affected animals. It is clear however, that only the high incidence of microencephaly

in guinea-pigs provides a suitable animal model for the further study of the nature of the reduced brain size.

As stated earlier, guinea-pigs which were born to heat treated females survived into adulthood and were also found to be microencephalic at this stage (Jonson, Lyle, Edwards & Penny, 1976). It was also found that these microencephalic adults performed poorly in psychology tests (Lyle, Jonson, Edwards & Penny, 1973; Jonson, Lyle, Edwards & Penny, 1976). This shows that microencephaly is a reflection of changes in the guinea-pig brain which produces a permanent impairment in brain function.

This work and previous work suggested that heat stress has an interesting effect on the developing central nervous system, but leaves many unanswered questions as to the structure and state of development of the microencephalic brain. It is of interest to enquire as to what makes the brains of heat treated offspring smaller than controls. It may be that they have fewer cells, or that the cell number is normal but cells may be smaller, or microencephaly may result from a combination of these two effects. It was thus the purpose of this study to examine the state of biochemical differentiation of the brains of these microencephalic animals.

This was approached in two ways, firstly by measuring the D.N.A. levels in microencephalic and control guinea-pigs, and by measuring the activities of marker enzymes in these tissues. The increase in levels of marker enzymes has been used commonly as an index of the development of brain tissue (Flexner, 1953; Flexner, 1955; Banik & Davison, 1969; MacDonnell & Greengard, 1974). This has also been used to evaluate defective development of C.N.S. tissue (Sereini, Principi, Perletti & Sereni, 1966;

Adlard & Dobbing, 1971; Ford & Cramer, 1977). However, the choice of marker enzyme is difficult in that the value of an enzyme activity expressed as a function of wet tissue weight may increase with development for more than one reason. Alterations in enzyme levels may be due to a quantitative change in the number of organelles or volume of cytoplasm within a developing cell or as a result of a qualitative change in the enzymology of cytoplasm or an organelle, in which a particular marker enzyme is increased over other proteins. The latter would be associated with an increase in the protein specific enzyme activity in the relevant fraction, in the former case would not. Such increases in specific activity of enzymes have been observed in development (MacDonnell & Greengard,1974).

The choice of marker enzymes used to assess the development of microencephalic guinea-pig brain tissue in this study has been conditioned by two factors. Firstly, the differentiation of mammalian brain is characterized primarily by an increase in the number of membranous neuronal processes (Caley,1971). Secondly, the existing work on brain defects produced by maternal hyperthermia gives no clue as to a defect in any particular cellular process. For these reasons, plasma membrane enzymes were chosen as markers of cellular differentiation, namely the $Na^+ - K^+$ ATPase, $Ca^{2+} - Mg^{2+}$ ATPase and acetylcholinesterase enzymes. These enzymes have been shown to increase in tissue concentration during neuronal differentiation (Samson & Quinn, 1967; Geel & Timiras,1967). Moreover these enzymes also increase in protein specific activity in the synaptic plasma membrane and microsomal fractions from developing mammalian brain (Abdel-Latif, Brody & Ramahi,1967; Abdel-Latif, Smith & Ellington,1970). Using these enzymes as markers, it would be possible to evaluate the development of neuronal processes in newborn microencephalic

guinea-pig brain. Consequently the concentrations of these plasma membrane marker enzymes have been measured in homogenates of cerebrum and cerebellar tissues from control and microencephalic guinea-pigs. Also the protein specific activities of these enzymes have been measured in synaptic membrane and microsomal membrane fractions from these cerebral and cerebellar tissues.

Brain defects which give rise to aberrant learning behaviour in mammals have been shown to be associated with reduced brain cell numbers, as measured by D.N.A. content (Howard, 1966; Balazs, Kovacs, Teichgraber, Cocks & Eayrs, 1968). As it has been observed in guinea-pigs, that hyperthermia on the 21st day of gestation causes an increased pycnotic index in the mitotic cells of the developing neuroepithelium (Edwards, Mulley, Ring & Wanner, 1974), it was possible that microencephaly was, at least partly produced by a reduction in cell numbers.

As a consequence of this, microencephalic guinea-pig brain was analysed for D.N.A. content. It would then be possible to express the brain homogenate enzyme levels from microencephalic animals in terms of D.N.A. content. This would evaluate the relative contributions of reduced cell number and reduced cell growth to the reduction in brain size. The results of these studies are presented below.

MATERIALS AND METHODS

Stock Animal Maintenance

Guinea-pigs (Cava porcellus) used in this study were bred from some six females and two males kindly provided as a gift from the Medical School of the University of Newcastle upon Tyne. The cages and cage racks used were generously provided by a grant from the Smith Kline and French Foundation.

It was found that better synchronised matings of 10 - 12 females was achieved if the male was caged separately from the females, but within their cage for 2 days prior to his release into the main area occupied by the females. The criterion of mating in small mammals is usually the observed formation of a vaginal plug. However, this is unreliable in guinea-pigs as the plug so formed is quickly lost. To compensate for this the females were examined every morning for vaginal opening. When this was observed the female was marked on the back with a felt pen. Marked animals were left with the male for a further 2 days, to increase the chance of successful mating. After this time they were caged and the first day of pregnancy was assumed to be that of the first vaginal opening. The dates of possible mating for each animal were recorded and the dates for heating and projected birth dates calculated assuming a gestation period of 69 days. Animals which were successfully mated gave birth on dates which corresponded well to the calculated dates. However, this was not an efficient process in that only approximately one in every five or six females treated in this way gave birth. However, it is essential to know the day of conception with some accuracy, so that the heating may take place at the correct period in gestation. This meant that many of the animals subjected to the heating procedure described below were not in fact pregnant, however,

those which were pregnant were exposed to a well defined treatment schedule.

Attempts were made to assess pregnancy in guinea-pigs, using a "prognosticon" commercial pregnancy testing kit. However, the intermediate, ambiguous result was always obtained for both mated and unmated animals. A more accurate assessment of successful mating would be very useful as mated guinea-pigs which were not pregnant could be subjected to further mating without wasted effort in fruitless heatings and without waiting for two months before these animals may be mated. Animals giving birth were rested for at least one month before being mated again.

Heating Procedure

Heatings were performed essentially as described by Edwards, 1969 (a) and (b). Animals which were at the eighteenth day of gestation were placed in a water jacketed incubator set at $44.5^{\circ}C$ for one hour. This procedure elevated the body temperature of the females from $38.37 \pm 0.34^{\circ}C$ (n=59) to $43.56 \pm 0.47^{\circ}C$ (n=59). Temperature was measured using a rectal thermistor probe the output of which was displayed on a flat-bed chart recorder. Not more than four animals were heated at any time. Animals treated in this way suffered few ill effects. The heating schedule was repeated once daily until the twenty fifth day of gestation. This has been reported to produce large numbers of microencephalic young without significant effect on visceral growth (Edwards, 1969 (b)).

The body temperature of animals subjected to this procedure was measured before and immediately after the heating. It was difficult to restrain the animals sufficiently to record the body temperature of animals during the heating procedure.

However, this was achieved in two cases, by enclosing the female in a cloth jacket with leg holes, to which the thermistor lead was attached. In these cases the body temperature increased very slowly during the first 45 minutes and then increased rapidly during the last 15 minutes. It is thus essential that the animals were removed after precisely one hours exposure to 44.5°C. In a few cases animals which developed body temperatures close to 44.0°C died although other animals which experienced these temperatures survived without any ill effects. Thus the final body temperature developed by an individual during the heating procedure would depend on the time during this process at which the rapid increase in temperature began.

Neonatal animals were taken for analysis within twelve hours of birth in the case of both control and heat exposed females. The mean litter size, from heat exposed mothers, at 2.63 ± 1.04 ($n = 19$) was not significantly different from that of control animals, which was 3.10 ± 0.88 ($n = 10$). These animals were all found to be in apparent good health and there were no obvious runts. For both brain D.N.A. analyses and enzyme measurements the newborn guinea-pigs were all processed in the following way. The neonates were weighed, alive, in a plastic beaker on a top-pan balance. They were then killed by quickly snapping the spinal cord and decapitated. The brain, including the olfactory bulb was dissected from the skull and separated from the spinal cord by a single cut with a scalpel behind the cerebellum. The brain was then weighed on a chilled watch glass.

Measurement of D.N.A. in control and microencephalic guinea-pig brain

Newborn guinea-pigs which were taken for D.N.A. analysis were initially processed as above. Then the D.N.A. was extracted as described below:-

(i) The brain tissue was homogenized in 10cm^3 of cold 0.5% trichloracetic acid (.T.C.A.) using a teflon-glass tissue homogenizer. This homogenate was tipped into a 15 cm^3 polypropylene centrifuge tube and centrifuged at 3,800xg for 10 minutes at 4^{6}C in an M.S.E. mistral 2L centrifuge. The supernatant contained the deoxyribonucletide pool and was discarded. The pellet contained precipitated protein, carbohydrate,lipid and D.N.A. This pellet was rehomogenised in a further 10cm^3 of 0.5% T.C.A. and centrifuged at 3,800xg for 10 minutes at 4^{o}C. The supernatant was again discarded. In each case the homogenization tube was washed out with 1cm^3 of 0.5% T.C.A. following homogenization and the washings pooled with the initial homogenate prior to centrifugation.

(ii) The pellet from acid precipitation was homogenized in 10cm^3 of cold absolute ethanol. The homogenate was centrifuged at 3,800xg for 10 minutes at 4^{6}C in a mistral 2L centrifuge. This extracts carbohydrate leaving protein, lipid,D.N.A. and R.N.A. in the pellet. The pellet was rehomogenized in a further 10cm^3 of cold absolute ethanol and centrifuged again at 3,800xg for 10 minutes at 4^{o}C. As above, after decanting the homogenate into the centrifuge tube the homogenization tube was washed with 1cm^3 of cold ethanol which was pooled with the homogenate in the centrifuge tube.

(iii) The pellet from ethanol extraction was homogenized in 10cm^3 of ethanol; chloroform; ether (1:1:1) and centrifuged at 3,800xg for 10 minutes at 4^{o}C in a mistral 2L centrifuge.

The supernatant containing lipid was discarded and the pellet rehomogenized in a further 10cm^3 of ethanol: chloroform: ether (1 : 1 : 1). Again after homogenates were decanted into the centrifuge tube, homogenization tubes were washed with 2cm^3 of ethanol: chloroform: ether, which was pooled with the homogenates. This homogenate was centrifuged at 3,800xg for 10 minutes at 4^{o}C.

(iv) The pellet from the above extraction was homogenized in 10cm^3 of 0.3M KOH. The homogenate was placed in a glass test tube, covered with parafilm, and incubated for 16 hours (overnight) at 37^{o}C in a water bath. The following day the homogenate was centrifuged at 3,800xg for 10 minutes at 4^{o}C in a mistral 2L centrifuge. The supernatant, which contained R.N.A. and D.N.A. was retained. The pellet was washed once by rehomogenizing in 10cm^3 of distilled water and centrifuged at 3,800xg for 10 minutes at 4^{o}C. The supernatant from this spin was pooled with that from the previous spin. The pellet was discarded.

(v) The pooled supernatants obtained from the overnight KOH incubation were placed in a 25cm^3 beaker and the pH of the solution was reduced to pH2.0 with careful addition of 0.5N perchloric acid. A white precipitate formed and a characteristic evil smell occurred at this stage. The suspension was then centrifuged at 4,000xg for 1 hour at 4^{o}C in a mistral 2L centrifuge. The supernatant was discarded and the pellet containing the D.N.A. was washed by resuspending the pellet in 10cm^3 of cold 0.5N perchloric acid and was centrifuged at 4,000xg for 1 hour at 4^{o}C.

(vi) The D.N.A. containing pellet which was precipitated at pH2.0 was then homogenized in 4 cm^3 of perchloric acid and heated at 70 - 80^{o}C in a water bath for 30 minutes. This dissolves the D.N.A. out of the pellet. After incubation the suspension was

centrifuged at 3,800xg for 10 minutes at 4°C. The white fluffy material which precipitated at pH2.0 and required 1 hour at 4,000xg to pellet, became a grey granular material in perchloric acid which was easier to sediment. The supernatant from this spin was carefully transferred to a $10cm^3$ volumetric flask using a pasteur pipette. The pellet was rehomogenized in a further $5cm^3$ of 0.5N perchloric acid and was incubated at 70 - 80°C in a water bath for 30 minutes. This suspension was centrifuged at 3,800xg for 10 minutes at 4°C and the supernatant carefully removed with the same pasteur pipette as used previously and transferred to the $10cm^3$ volumetric flask. There was always a slight evaporative loss so the volume was adjusted to precisely $10cm^3$.

This perchloric acid solution contained the deoxyribonucleotides from D.N.A. and was used directly for D.N.A. analysis by the diphenylamine method described below. In all cases D.N.A. was estimated immediately, however, there was no reason to expect the deoxyribose to be unstable in this solution.

D.N.A. Assay Technique

D.N.A. was estimated using the diphenylamine assay of deoxyribose as described by Burton, (1956). Some difficulty was experienced in obtaining a suitable standard solution of D.N.A. Initially highly polymerised calf thymus D.N.A. (Sigma Chemical Corporation) was used as a standard. This fibrous material was weighed out and incubated at 70 - 80°C. Extended incubation failed to dissolve all of the solid material, and standard solutions generated in this way gave variable standard curves in an unpredictable manner.

A more acceptable procedure used a solution of D.N.A. in citrate buffer (Boeringer Chemical Company). This solution precipitated

fibres of D.N.A. when in contact with 0.5N perchloric acid. To avoid handling difficulties the D.N.A. solution was tipped into a 100cm^3 glass beaker and the bottle was washed with a little distilled water. Approximately 50cm^3 of 0.5N perchloric acid was added and the beaker was incubated at 70 - 80°C until the precipitate dissolved. The cooled solution was then made up to 100cm^3 and this solution (100µg/cm^3) was used as a standard. Suitable standard curves were obtained from this solution (figure 1 : 1). An aliquot of this solution was incubated at 90°C in a sealed glass ampoule for several hours and then this solution was subjected to phosphate analysis using the lubrol-acid molybdate solution as described in the general methods chapter. This analysis showed that 100µg/cm^3 ≡ 0.299µ moles D.N.A. - phosphate/cm^3). The results of D.N.A. analysis were expressed as µ moles of D.N.A. - P (phosphate).

D.N.A. Assay Reagent

The assay solution was made up in the following way: 1.5 gms of diphenylamine was dissolved in 100cm^3 of glacial acetic acid. Then 1.5cm^3 of concentrated sulphuric acid was added. 0.1cm^3 of a solution of 16mg/cm^3 acetaldehyde was then added. The acetaldehyde solution was chilled, prior to use, in an ice and water bath, also the pipette used for the acetaldehyde was chilled before use. This solution was the assay solution which was always made freshly and used immediately although it is reportedly stable for many hours at room temperature. It was essential that this solution did not come into contact with water as a white precipitate formed. If the diphenylamine was impure, giving high blanks, it should be recrystallised from petroleum ether.

The assay procedure was as follows: 4cm^3 of assay solution was added to 2cm^3 of D.N.A. in 0.5N perchloric acid. The

mixture was mixed by vortexing and incubated at 37°C overnight. A blue colour develops, the extinction of which was measured at 600 nm using 1cm path length in a Pye Unicam S.P. 1800 spectrophotometer. A reagent blank was used in every estimation.

Enzyme Measurements in Microencephalic and Control Guinea-pigs

Newborn guinea-pigs from control and heat treated mothers taken for enzyme measurement were processed as described previously. The dissected and weighed brain was treated as follows; the cerebral hemispheres and cerebellum were dissected and weighed, the cerebellum was then bisected and the halves were weighed separately. One cerebral hemisphere and half of the cerebellum were taken for D.N.A. analyses. The remaining hemisphere and half cerebellum were each homogenized separately in $10cm^3$ of cold 0.32M sucrose, 20 mM Imidazole buffer, 2mM E.D.T.A. at pH7.2

A $1cm^3$ aliquot of each homogenate was retained for enzyme measurement. These aliquots were then diluted, tenfold in the case of the cerebral hemisphere samples and fivefold in the case of cerebellum sample, with 20mM Imidazole buffer pH7.2. The remainder of each homogenate was processed for the isolation of microsomal and synaptic membrane fraction, as described below. Each brain was processed individually in the case of both control and microencephalic animals.

Membrane Isolation Procedures

General details of the isolation of the membrane fractions mentioned above are contained in the general methods chapter. These were modified as follows so that both microsomes and synaptic membranes may be extracted from a single brain homogenate.

(i) The tissue homogenate remaining from the above procedure was centrifuged at 900xg for 10 minutes at 4°C in a Mistral 2L centrifuge. The supernatant was retained and the pellet was resuspended by homogenization in a further 10cm^3 of 0.32M sucrose in 20mM Imidazole 2mM E.D.T.A. buffer pH7.2 (Imidazole/E.D.T.A. buffer). This was then centrifuged 900xg for 10 minutes at 4°C in a Mistral 2L centrifuge. The supernatants were pooled

(ii) The pooled supernatants were centrifuged at 20,000xg for 30 minutes at 4°C in an H.S.18 centrifuge. The supernatant was retained for microsome isolation and the pellet was resuspended in Imidazole E.D.T.A. buffer without sucrose and homogenized to lyse the synaptosomes. This suspension was then centrifuged at 20,000xg for 30 minutes at 4°C. The pellet was resuspended in 3cm^3 of Imidazole/.E.D.T.A. buffer and layered over a discontinuous sucrose density gradient as described in the general methods section. This gradient was centrifuged at 100,000xg for 2 hours at 4°C in a Prepspin 50 preparative ultracentrifuge. After this time the synaptic membrane fraction was aspirated from the gradient with a pasteur pipette. This suspension was diluted with Imidazole/.E.D.T.A. buffer, and centrifuged at 100,000xg for 1 hour at 4°C, The pellet was resuspended in 5cm^3 of Imidazole buffer, without E.D.T.A. This represents the synaptic membrane fraction. Aliquots of this suspension were diluted and used directly in enzyme activity measurements.

(iii) The supernatant from the 20,000xg centrifugation contained the microsomal fraction. This was diluted with Imidazole/E.D.T.A. buffer to give 0.25M sucrose. This suspension was centrifuged at 100,000xg for 1 hour at 4°C in a Superspeed 40 ultra-centrifuge, or in a Prepspin 50 ultracentrifuge. The pellet was resuspended in 5cm^3 of Imidazole buffer without E.D.T.A.

This represented the microsomic fraction, and aliquots of this suspension were diluted and used directly for enzyme activity measurements.

Enzyme Activity Measurements

The Na^{+} K^{+} ATPase and Ca^{2+} - Mg^{2+} ATPase activities were measured as described in the general methods chapter. The acetylchlorinesterase activity was estimated using either the original method of Ellmanetal,1961 or a modified Ellman method. Both of these are described in the general methods section.

The protein concentrations of these membrane fractions were estimated by a modified ninhydrin method as described in the general methods section.

RESULTS

Brain weights and body weights were obtained for 50 animals born to 19 mothers that had been subjected to hyperthermia during days 18 - 25 of gestation. These data are compared with 31 animals born to 10 mothers which underwent the same manipulation as the heated mothers but were not made hyperthermic at any time in gestation (Table 1 : 1). As can be seen there is no difference in the mean body weights of the two groups of animals. Offspring from heated mothers weighed 91.45 $\pm$ 2.09 gms (n50), whereas offspring from control mothers weighed 94.32 $\pm$ 2.24 gms (n 31).

In contrast however, the mean brain weight of young born to the heat treated mothers was significantly smaller, at 2.30 $\pm$ 0.03 (n5) than the mean brain weight of 2.72 $\pm$ 0.02 (n31) in the control offspring (P = 0.001). It is also of interest to look at the frequency distribution of these data for brain weights as shown in the form of a histogram in figure 1 : 2. As can be seen, the values for the 31 control animals are distributed normally over a range between 2.4 gms and 3.0 gms brain weight. The brain weights for the 50 offspring of heated mothers however, show a more skewed distribution, with a marked trend to lower brain weights. The range in this case was from 1.7 gms to 2.7 gms brain weight.

These data would suggest that microencephalic young have been produced as a result of maternal hyperthermia, however, Edwards, (1969 (b)) has shown that, in the newborn guinea-pigs, brain weight varies directly with body weight in an apparently linear fashion. Consequently it is necessary to show that this relationship holds for both the experimental and control groups of offspring. Figure 1 : 3 shows these individual data for brain weight plotted against body weight.

In the control group a good correlation (r = 0.757) was obtained for a linear relationship between brain and body weight (P = 0.001). The equation of the line, which has been fitted to control data is:

$$Y = 0.00588\ X + 2.16$$

where Y = Brain weight and X = body weight

The importance of obtaining this good correlation was that it enabled the extent of microencephaly in experimental animals to be assessed, without the necessity of obtaining an exact match of body weights between control and experimental animals. Also fitted to the control data in Figure 1 : 3 is a line at 2 standard deviations (2 x S.D.) of brain weight below that calculated for control data. As can be seen from Figure 1 : 3 only in the case of two individuals did the brain weight of offspring from heated mothers fall within 2 x S.D. of the control line. The remainder of the brain weights of experimental animals fell on or below this line.

The data for experimental animals, presented in Figure 1 : 3 also gave significant linear correlation (P = 0.005) between brain weight and birth weight, although in this case the correlation coefficient was lower. The equation of this regression line is:

$$Y = 0.00515\ X + 1.83$$

where Y = brain weight and X = body weight

It is clear that the slopes of the regression lines calculated for control and experimental data are similar (5.88×10^{-3} & 5.15×10^{-3} respectively) but the line for the experimental animals lies significantly below that for controls (Figure 1 : 3). Thus an "expected" brain weight for any newborn guinea-pig can be calculated from the relationship, brain weight = (body weight x 0.00588) + 2.16. When this was done for the offspring of heat-treated mothers, which provided the data presented in Figure 1 : 3, these heat-treated newborn showed brain weight deficits of between 3.4% and 36.1%. The mean deficit was

16.4 ± 1.0% (n50).

A DEFINITION OF MICROENCEPHALY

It is proposed that a newborn guinea-pig whose brain weight falls below two standard deviations below the mean brain weight calculated for its body weight, by the relationship shown above is deemed microencephalic. Thus a newborn guinea-pig showing more than 8% reduction in brain weight would be considered microencephalic.

Thus, in this study, all but two of the guinea-pigs born to mothers exposed to maternal hyperthermia were microencephalic. Having shown a reproducable effect of maternal hyperthermia on guinea-pig brain development, it is pertinent to enquire as to why the brains of these experimental animals were so much smaller than those of control. It may be that they have fewer cells, or that the cell number is normal but that the cells are smaller. Indeed, microencephaly could result from a combination of these two effects.

Many workers have used the quantity of acid-precipitable deoxyribonucleic acid as an index of cell number. This approach has been followed in the present study. The D.N.A. levels in brains from experimental and control newborn guinea-pigs are presented in Table 1 : 2. It is significant that the values for the amount of extractable D.N.A. when expressed as a function of starting weight of brain tissue, were not statistically different between the two groups. The values were 4.79 ± 0.04 μ moles D.N.A. - P/gm (n17) for controls and 4.78 ± 0.06 μ moles D.N.A. - P/gm (n30) for experimental animals. In consequence when the total D.N.A. - P content was expressed per whole brain, for these two groups, then significantly different values were obtained.

The values were 13.04 ± 0.07 μ moles D.N.A. - P/ brain for controls and 11.10 ± 0.12 μ moles D.N.A. - P/ brain for experimental animals (P = 0.001) respectively.

This 14.9% reduction in brain D.N.A. - P in the offpsring of heated mothers is similar to the reduction in brain weight, 15.4% in these animals (Table 1 : 1). This suggests that microencephaly may be simply owing to a reduction in brain cell numbers. However, the degree of microencephaly in these animals varied between 3.4% and 36.1%. In figure 1 : 4, the degree of microencephaly, in terms of percentage brainweight deficit is plotted against brain D.N.A. - P content for individual experimental and control animals. A very good correlation was obtained (r = 0.759) between brain weight deficit and brain D.N.A. levels. The data for control animals were clustered, and at no point does the data for the experimental group overlap that for control animals. Thus even the two experimental animals not considered microencephalic, had lower D.N.A. levels than any of the controls.

This result, showing that the total D.N.A. content of the whole brain was less in the microencephalic animals as compared with control animals, whereas no difference in D.N.A. content was evident when expressed per gm of brain tissue, begs the question of whether different regions of the brain were equally affected. Table 1 : 3 shows data obtained for the cerebral hemispheres and cerebellums of 11 control and 14 microencephalic animals, for D.N.A. content and wet tissue weight. These brain regions were taken, as they form discrete anatomical structures and can be easily separated from the other brain areas. The mean weight of cerebral hemispheres from the microencephalic animals was 0.70 ± 0.02 gms (n 14), 15.7% lower than the control mean

hemisphere weight of 0.83 ± 0.03 gms (n 11). However, the mean D.N.A. level in cerebral hemispheres from microencephalic animals was 1.60 ± 0.06 μ moles D.N.A. - P / hemisphere (n 14), which was 35% less than the mean D.N.A. level in control hemispheres, which was 2.48 ± 0.06 μ moles D.N.A. - P/ hemisphere (n 11). As the percentage deficit in cerebral D.N.A. for microencephalic animals is much greater than the percentage deficit in hemisphere weight for these animals then the mean value of D.N.A. - P per weight of tissue from microencephalic animals (2.30 ± 0.09 μ moles D.N.A. - P / gm wet weight (n 14) is also significantly lower than that for controls (2.98 ± 0.06 μ moles D.N.A. - P/ gm wet weight (n 11)) (table 1 : 3).

In contrast to this, the data for the wet weight and D.N.A. content for cerebellums from microencephalic and control animals, as shown in table 1 : 3, were not significantly different. Now the total D.N.A. content per whole brain for control animals was about 13 u moles, and the D.N.A. content of both hemispheres and the cerebellum was about 10 μ moles, thus the D.N.A. content of the mid-brain region was about 3 μ moles. In the case of the microencephalic animals, the total brain D.N.A. was about 11 μ moles, that from the hemispheres and cerebellum was about 8 μ moles. Thus the D.N.A. content of the mid-brain region was also about 3 μ moles for microencephalic newborn. This shows that the deficit in whole brain D.N.A. levels in microencephalic animals of 15% results from a deficit in cerebral D.N.A. of 35% and normal D.N.A. levels in the remainder of the brain.

These results suggest that, at least in certain brain rggions, microencephalic newborn guinea-pigs have fewer brain cells than controls. It is therefore pertinent to determine whether the microencephalic condition resulted in a change in the development

of brain tissue. This was accomplished by estimating the levels of some membrane-bound enzymes in control and microencephalic newborn guinea-pig brain. Membrane-bound enzymes were chosen as the growth of neurons, particularly during the most rapid phase of brain growth, is characterised by an elaboration of membranous dendritic elements (Caley, 1971). The $Na^+ - K^+$ ATPase, $Ca^{2+} - Mg^{2+}$ ATPase and acetylcholinesterase were chosen for study as these have been shown to increase in activity, in brain tissue homogenates, particularly during the rapid brain-growth phase. (Samson & Quinn, 1966; Geel & Timiras, 1967). Also it is possible to measure the protein-specific activities of these enzymes in microsomal membranes and synaptic membrane fractions isolated from brain tissue. The specific activities of these enzymes have also been shown to increase during development in these membrane fractions (Abdel-Latif, Smith & Ellington, 1969; Bowler & Tirri , 1974). By estimating the specific activity of these enzymes in membrane fractions from control and microencephalic animals it would be possible to assess whether changes in enzyme levels in tissue homogenate could be explained by a qualitative change in the enzyme concentration of a given membrane fraction.

The activities of these enzymes in homogenates of cerebral hemispheres from control and microencephalic guinea-pigs are shown in Table 1 : 4. Taking the data for the $Na^+ - K^+$ ATPase first, it can be seen that the mean activity per mg wet weight of tissue from microencephalic animals is 1.93 ± 0.03 μ moles Pi/mg tissue/hr which is significantly lower than that for control animals, which is 2.12 ± 0.08 μ moles Pi/mg protein/hr. However, this 9.4% reduction is small compared with the reduction in cell number resulting from maternal hyperthermia, and, as can be seen from Table 1 : 4, is significant only at a probability of 0.02.

The $Na^+ - K^+$ATPase level, expressed as μ moles Pi per whole cerebral hemisphere per hour (total activity) is also presented in table 1 : 4. This shows, as would be expected, that the mean total activity in cerebral hemispheres from microencephalic animals was significantly reduced by 19.8% from 1695 ± 39 μ moles Pi/ cerebral hemisphere/hr (n 11) for control animals to 1360 ± 60 μ moles Pi/cerebral hemisphere/hr (n 14) for microencephalic newborn.

The third way in which the activity of the $Na^+ - K^+$ ATPase can be expressed, is as a function of cell number, as measured in the D.N.A.-P level of this tissue (Table 1 : 4). These data show that the level of $Na^+ - K^+$ ATPase per cell has been significantly increased by 15.0% in microencephalic animals from 712 ± 26 μ moles Pi/ μ moles D.N.A.-P/hr for controls to 838 ± 15 μ moles Pi/μ moles D.N.A. - P/hr (n 14) for experimental animals.

The levels of $Ca^{2+} - Mg^{2+}$ ATPase in cerebral tissue homogenates are also presented in table 1 : 4, in the same units as used in the presentation of the $Na^+ - K^+$ ATPase activity. It can be seen that the activity of this enzyme for microencephalic animals is 0.51 ± 0.03 μ moles Pi/mg tissue/hr (n 14) which is not significantly different from the value of 0.46 ± 0.03 μ moles Pi/mg tissue/hr (n 11) for controls. Similarly, the total cerebral hemisphere activity of the $Ca^{2+} - Mg^{2+}$ATPase for microencephalic animals of 340 ± 17 μ moles Pi/cerebral hemisphere/hr (n 14) was not significantly different from the control value of 373 ± 20 μ moles Pi/cerebral hemisphere/hr (n 11). However, the activity of this enzyme expressed as a function of tissue D.N.A. was 222 ± 14 μ moles Pi/μ mole D.N.A.-P/hr (n 14), which was significantly higher than the control value of 154 ± 12 μ moles Pi/μ moles D.N.A. - P/hr (n 11).

Thirdly the levels of acetylcholinesterase in cerebral tissue homogenates from control and microencephalic animals are also presented in table 1 : 4 in the same units as for the previous two enzymes. In this case, the activity per mg tissue for microencephalic animals was 0.75 $\pm$ 0.03 μ moles thiocholine/mg tissue/hr (n 14) which was significantly higher than that for controls, which was 0.57 $\pm$ 0.03 μ moles thiocholine/mg tissue/hr (n 11). However, the total acetylcholinesterase activity was 523 $\pm$ 26 μ moles thiocholine/cerebral hemisphere/hr (n 14) for microencephalic animals and 464 $\pm$ 25 μ moles thiocholine/cerebral hemisphere/hr (n 11) for control animals. These values were not significantly different. The activity per tissue D.N.A. was 324 $\pm$ 12 μ moles thiocholine/μ mole D.N.A.=P/hr (n 14) for microencephalic animals which was as for the previous enzymes, significantly higher than controls value of 192 $\pm$ 10 μ moles thiocholine/ μ moles D.N.A. = P/hr (n 11).

The activities of these three marker enzymes in homogenates of cerebellum from microencephalic and control animals are presented in Table 1 : 5. These activities are presented in the same units as used to express enzyme activities from cerebrum. As can be seen from Table 1 : 5 the activities of each of the three enzymes are not significantly different from controls, whether expressed as a function of tissue weight, D.N.A. level or as total activity per cerebellum. This is in marked contrast to the changes in enzyme activity in cerebral hemispheres from microencephalic animals, but this may not be surprising, as unlike the cerebral hemispheres, no reduction in weight or D.N.A. content of cerebellum from microencephalic animals was observed as compared to controls.

The activities of these enzymes from cerebellum can be compared with those from the control cerebral cortex. The $Na^+ = K^+$ATPase

activity from cerebellum expressed as a function of tissue weight was 2.47 ± 0.12 μ moles Pi/mg tissue/hr (n 11), some 14% higher than the same activity in cerebrum which was 2.12 ± 0.08 μ moles Pi/mg tissue/hr (n 11). The total activity of $Na^+ - K^+$ ATPase from cerebellum was 785 ± 43 μ moles cerebellum/hr (n 11) which is 46% of the cerebral total activity which was 1695 ± 39 μ moles Pi/cerebrum / hr (n 11). The activity per cell D.N.A. in the cerebellum was 156 ± μ moles Pi/μ moles D.N.A. - P/hr (n 11) which was only 22% of the activity per cell D.N.A. for the $Na^+ - K^+$ ATPase from cerebrum which was 712 ± 26 μ moles Pi/μ moles D.N.A. - P/hr (n 11).

The $Ca^{2+} - Mg^{2+}$ ATPase activity from cerebellar tissue showed a similar relationship to the activity of this enzyme from control cerebral hemispheres for the $Na^+ - K^+$ ATPase. The activity of $Ca^{2+} - Mg^{2+}$ ATP ase per tissue weight in cerebellum was 0.53 ± 0.04 μ moles Pi/mg tissue/hr (n 11) which was 15% higher than the cerebral activity of 0.46 ± 0.03 μ moles Pi/mg tissue/ hr (n 11). The total $Ca^{2+} - Mg^{2+}$ ATPase activity from cerebellum was 167 ± 9 μ moles Pi/cerebellum/hr (n 11) which was 44% of the total $Ca^{2+} - Mg^{2+}$ ATPase from control cerebral hemispheres, 373 ± 20 μ moles Pi/cerebrum/hr. The activity per cell D.N.A. in the cerebellum was 33.4 ± 2.5 μ moles Pi/ μ moles D.N.A. - P/hr (n 11) which is again only 22% of the cell specific activity from cerebral tissue which was 154 ± 12 μ moles Pi/μ moles D.N.A. - P/hr (n 11).

As with the previous two enzymes, the acetylcholinesterase activity of the newborn guinea-pig cerebellum was higher than for cerebral hemispheres when expressed per tissue weight and was lower than for cerebral hemispheres when expressed in terms of total activity and D.N.A.-P specific activity, but to a different

extent. The activity of the acetylcholinesterase per cerebellum tissue weight was 0.88 ± 0.03 μ moles thiocholine/mg tissue/hr (n11) which was 54% higher than that for cerebral tissue which was 0.57 ± 0.03 μ moles thiocholine/mg tissue/hr (n11). The mean total activity per cerebellum was 280 ± 15 μ moles thiocholine/cerebellum/hr (n11) which was 60% of activity per cerebrum (464 ± 25 μ moles thiocholine/cerebrum/hr (n11)). The D.N.A. specific activity in cerebellum was 55.5 ± 21 μ moles thiocholine/μ moles D.N.A. - P/hr (n11) which was 28% of the activity from the cerebral hemispheres at 192 ± 10 μ moles thiocholine/μ moles D.N.A. - P/hr (n11).

The data for the three enzymes taken together shows that the concentration of enzymes in cerebellum is higher than in cerebrum, but the total activity is much lower, as is the activity per tissue D.N.A. level.

It was necessary as stated previously, to measure the specific activity of the three marker enzymes in isolated membrane fractions. This was done for microsomes and synaptic membranes from cerebral and cerebellar tissue from control and microencephalic guinea-pigs.

The specific activity data for these three enzymes, determined for cerebral tissue is shown in Table 1 : 6. In no case was any statistically significant difference found between specific activities of those enzymes derived from microencephalic and control animals, whether as microsomes or as synaptic membranes. The specific activity of the $Na^+ - K^+$ ATPase from synaptic membranes (65.9 ± 2.3 μ moles Pi/mg protein/hr) was four times higher than in microsomes (15.7 ± 0.7 μ moles Pi/mg protein/hr). The $Ca^{2+} - Mg^{2+}$ ATPase of synaptic membranes (7.12 ± 0.76 μ moles Pi/mg protein/hr) was 2.3 times higher than in microsomes

(3.07 ± 0.20 μ moles Pi/mg protein/hr). Similarly the specific activity of the acetylcholinesterase from synaptic membranes (15.6 μ moles thiocholine/mg protein/hr) was 1.9 times higher than that of microsomes (8.24 ± 0.22 μ moles thiocholine/mg protein/hr).

The specific data for these three enzymes, determined for cerebellum tissue is shown in Table 1 : 7. As with the data from cerebral membranes, no significant difference was found between specific activities from microencephalic and control cerebellums in the case of either microsomes or synaptic membranes. The specific activity of the $Na^+ \pm K^+$ ATPase from synaptic membranes (56.2 ± 2.2 μ moles Pi/mg protein/hr) was three times that of microsomes (17.7 ± 1.5 μ moles Pi/mg protein/hr). However, unlike membranes from the cerebrum, the specific activity of the $Ca^{2+} - Mg^{2+}$ ATPase from cerebellar synaptic membranes (3.88 ± 0.13μ moles Pi/mg protein/hr) was similar to microsomes (3.20 ± 0.15 μ moles Pi/mg protein/hr). The specific activity of the acetylcholinesterase from cerebellar synaptic membranes (18.13 ± 1.06 μ moles thiocholine/mg protein/hr) was 1.6 times that of microsomes (11.33 ± 0.82 μ moles thiocholine/mg protein/hr).

Thus it can be seen, firstly that there was no change in the specific activity of membrane enzymes in brains from microencephalic, as compared with control animals, and secondly that each brain region and membrane fraction had a characteristic enzyme pattern.

DISCUSSION

The initial observation of this study was that daily maternal hyperthermia in guinea-pigs, applied between days 18 - 25 of gestation produced a 15% reduction in the brainweight of the neonates, with no accompanying effect on body weight (Table 1 : 1). It is clear from Figures 1 : 2 that for most experimental newborn, brainweight was reduced from control value of between 2.6 gms and 2.8 gms to about 2.2 gms to 2.5 gms, but some experimental animals were severely affected, with brainweights from 1.7 gms to 2.1 gms. The true effect was clearer when this data was analysed according to the definition of microencephaly obtained from the birth weight/brain weight relationship in control newborn guinea-pigs. Data presented in Figure 1 : 3 shows that only 2 out of 50 experimental animals were not significantly microencephalic. These results are similar to those obtained by Edwards, (1969) (b), although the mean reduction in brain weight, from that expected for body weight in young born to heat treated mothers (16.4%) in this study was greater than that reported previously. This may be due to an increased innate susceptability to temperature of the inbred strain of guinea-pigs used in this particular study.

These results show a reproducible effect of maternal hyperthermia in guinea-pigs on days 18 and 25 of gestation, in the production of microencephalic young. These animals are otherwise healthy and can serve as subjects for biochemical measurements without the added complications of associated pathological conditions. In this respect the guinea-pig is a good subject, unlike rats and hamsters which are born prior to the rapid phase of brain development (Davison & Dobbing, 1968; Dobbing & Sands, 1970), and require considerable maternal support during this period. Also the heat induced abnormalities in rats and hamsters have been

shown to be less frequent and more severe than those in guinea-pigs (Skreb & Frank, 1963; Edwards, 1968; Umpierre & Dukelow, 1977). Neither rats nor hamsters which were affected by heat exposure would be likely to survive until a stage of brain development equivalent to that of the newborn guinea-pig was reached.

It was possible that microencephaly in guinea-pigs resulted from reduced brain-cell numbers, decrease in cell differentiation or a combination of both of these. Levels of D.N.A. have been used to give estimates of cell number in brain, although because of the high incidence of polyploidy in brain tissue it is not possible to express D.N.A. content directly as cell numbers. Data obtained in this study (Table 1 : 2) for the D.N.A. content of newborn guinea-pig brain (13 μ moles D.N.A.-P/brain) was similar to that obtained by Dobbing & Sands, (1970). The reduction in brain D.N.A. content of 16% in microencephalic animals suggested that the reduction in brain weight resulted, at least in part, from a reduction in cell numbers (Table 1 : 2). This was further supported by the correlation between the degree of microencephaly and whole brain D.N.A. content (Figure 1 : 4). These data are similar to those reported previously by Edwards, Penny & Zevnik, (1971).

The connection between elevated maternal temperature and reduced brain cell number in guinea-pigs has been investigated by Edwards et al (1974) who showed that heat treatment on the 21st day of gestation caused an increase in the pyknotic index of mitotic cells of the developing neuroepithelium. Although their study showed that mitosis increased following heat exposure, it was suggested that repeated heatings around this time during pregnancy may result in a permanent cell deficit in the neuroepithelium. As these

cells later divide to produce the neurons, a reduced neuroepithelium could result in brain cell deficit later in development.

Although whole brain cell numbers were reduced in microencephalic guinea-pigs, it was not clear whether all brain regions were equally affected. Data presented in Table 1 : 3 shows that although the cerebellum contains as many cells as both hemispheres in terms of D.N.A.-P/brain region, there was no reduction in D.N.A. content or weight of this brain region in microencephalic guinea-pigs. In contrast the D.N.A. level of the cerebral hemispheres of the same animals was reduced by 35%, although the wet weight of this region was reduced only by 15%. Hence, as the cerebellum and cerebral hemispheres contain 80% of the total brain D.N.A. then a major cause of microencephaly is a severe reduction in the number of brain cells in the cerebrum. Furthermore, because of this differential reduction of cerebral weight and D.N.A. content, the D.N.A. per cerebral tissue, in microencephalic animals, was lower than for controls. Thus the cerebral cells could be larger than in controls, perhaps this represents a compensation for reduced cell numbers.

These results are in contrast to the work of Edwards et al (1971) who reported that the D.N.A. level of microencephalic cerebrum to be 15% less than controls, a similar reduction to that of tissue weight. However, these authors did not estimate the D.N.A. content of the cerebellum in isolation, and obtained a 7% reduction in the D.N.A. level of cerebellum and brain-stem together. Thus even in their study, not all brain regions were equally affected. It is not possible to resolve these differences as the techniques used for D.N.A. estimation were similar.

In the view of Edwards et al (1974), that hyperthermia affects the dividing cells of the developing foetal nervous system is correct, then it would seem likely that a reduction in cell numbers would primarily affect the cerebrum as these cells are formed early in development (Berry, 1974) whereas the majority of cerebellar neurons are formed by the external granular layer much later, after cerebral neurogenesis is complete. This difference in origin could explain the selective effect of hyperthermia on the cerebrum. Perhaps the neuroepithelium which gives rise to the cerebellum does not undergo significant division between the 18th and 25th days of gestation.

To investigate further the effect of microencephaly on the cellular development of guinea-pig brain, the levels of plasma membrane enzymes were measured in brain tissue homogenates. It was necessary to confirm that the activities of these enzymes conform to values reported in the literature and conform to what would be expected according to the histological findings for the structure of cerebellum and cerebrum. The Na^+/K^+ATPase activity per mg tissue in cerebellum was slightly higher than for the cerebral hemisphere although both were of the same order as that reported for rat brain at equivalent developmental stage (Tables 1 : 4, 1 : 5, Samson & Quinn, 1967). No such data is available for the Ca^{2+} - Mg^{2+} ATPase but the activities per mg tissue for control and cerebrum and cerebellum were similar as with the Na^+ - K^+ ATPase. As the weight of the cerebellum is less than half of a cerebral hemisphere in newborn guinea-pigs, the total activity of both of these enzymes in the cerebellum was less than half that of the cerebrum (Tables 1 : 4 and 1 : 5). However, as the cerebellum contains twice the D.N.A. of a cerebral hemisphere, the activity of these two enzymes, per unit D.N.A. in the cerebellum was less than one quarter of that

of the cerebrum. This suggests that the cells of the cerebellum are more densely packed and have less extensive dendrites than the cells of the cerebral hemispheres.

Histological studies have shown this to be the case, in that the granular layer of the cerebellum consists of very large numbers of small, tightly packed neurons. The Purkinje cells and Golgi cells which have more elaborate arrays of dendrites are fewer in number and therefore represent a small proportion of cerebellar neurons (Ingram, 1976). The neurons of the cerebral cortex are less closely packed and generally possess long branched dendrites (Scholl, 1956).

The $Na^+ - K^+$ ATPase and $Ca^{2+} - Mg^{2+}$ ATPase are markers of plasma membrane in general, but the acetylcholinesterase has been shown to be concentrated primarily on the cytosol surface of synaptic and dendritic membranes of cholinergic neurons (Koelle, 1978). Thus this latter enzyme represents a marker of cholinergic neurons. Unlike the ion activated ATPases, the aceylcholinesterase activity per mg tissue from cerebellum was nearly twice that of the cerebrum, (Tables 1 : 4, 1 : 5), hence the total activity of this enzyme per cerebrum was only slightly higher than that of the cerebellum. This suggests that the cerebellum contains a higher proportion of cholinergic neurons than the cerebral cortex. The activity of this enzyme reported in this study is of the same order as that reported for rat cerebral cortex, at an equivalent stage of development (25 to 30 days postnatal) (Geel & Timiras, 1967).

Although the enzyme activities discussed above are consistent with histological observations of neuronal structure, the cation activated ATPase especially are not exclusively located in

neuronal dendritic and synaptic membrane. However, it has been shown that these enzymes are in particularly high concentration on these membranes. For example the $Na^+ - K^+$ ATPase activity of rat brain microsomes (20 μ moles/mg protein/hr), rabbit brain microsomes (30 μ moles Pi/mg protein/hr), rat brain synaptic membranes (80 - 100 μ moles Pi/mg protein/hr) and mouse brain synaptic membranes (77 μ moles Pi/mg protein/hr), (Bowler & Duncan, 1968; Bowler & Tirri, 1974; Sun & Sun, 1974; Matsumato & Mori, 1976) were much higher than that of rabbit neuronal perikaryal plasma membrane (2.1 μ moles Pi/mg protein/hr) and calf brain oligodendroglial cell plasma membrane (9.8 μ moles Pi/mg protein/hr) (Henn, Hansson & Hamberger, 1972; Podulso, 1975). Thus, provided the specific activities of these enzymes remain similar in microencephalic and control guinea-pig brain membrane fractions, then changes in enzyme activities in tissue homogenates between microencephalic and control animals would predominantly represent changes in neuronal structure.

The specific activity of the $Na^+ - K^+$ ATPase of newborn guinea-pig synaptosomal plasma membranes (56 - 66 μ moles Pi/mg protein/hr) was only slightly lower than that reported for rat brain synaptic membranes (80 - 100 μ moles Pi/mg protein/hr) and mouse brain synaptic membranes (77 μ moles Pi/mg protein/hr) (Bowler & Tirri , 1974; Sun & Sun, 1974). The specific activity of this enzyme of newborn guinea-pig microsomes from cerebrum and cerebellum (16 - 19 μ moles Pi/mg protein/hr) was somewhat less than expected for other mammalian brain preparations (20 - 30 μ moles Pi/mg protein/hr) (Bowler & Duncan, 1968; Matsumato & Mori, 1976). The $Ca^{2+} + Mg^{2+}$ ATPase specific activity from guinea-pig cerebral and cerebellar microsomes (3 μ moles Pi/mg protein/hr) and the cerebral synaptic membranes (7 - 8 μ moles Pi/mg protein/hr)

were similar to those reported previously (Duncan, 1976; Tirri, Lahdekorpi and Bowler, 1976) for rat brain preparations. However, the specific activity of the Ca^{2+} - Mg^{2+} ATPase from cerebellar synaptic membrane (3.5 - 4.0 μ moles Pi/mg protein/hr) was lower than that for cerebral synaptic membranes, and similar to microsomal preparations. There seems to be no reported precedent for this observation.

The specific activities of acetylcholinesterase from cerebral synaptic membranes (16 μ moles thiocholine/mg protein/hr) and cerebellar synaptic membranes (18 μ moles thiocholine/mg protein/hr) were slightly lower than that reported for rat brain (25 μ moles ACH/mg protein/hr) by Goodkin and Howard, (1974). The specific activities of acetylcholinesterase in cerebral microsomes (8 μ moles thiocholine/mg protein/hr) and cerebellar microsomes (11 μ moles thiocholine/mg protein/hr) were similar to that observed for rat brain microsomes (8 μ moles ACH/mg protein/hr) by Abdel and Latif et al (1970). Thus, allowing for species variation in enzyme specific activity and variations in the precise methods of membrane extraction the values presented in tables 1 : 6 and 1 : 7 compare favourably with those previously published values, quoted above.

Data presented in tables 1 : 6 and 1 : 7 show that the specific activities of the three enzymes from microencephalic and control animals were not significantly different, whether from synaptic membrane or microsomes. Consequently any change in the activities of these enzymes in tissue homogenates, between microencephalic and control guinea-pig brain, would represent changes in cell structure, predominantly of neuronal structure.

The activities of these three enzymes in homogenates of cerebellum tissue from microencephalic and control guinea-pigs were not

significantly different (Table 1 : 5)。 This is consistent with the finding that although hyperthermia resulted in a reduction in whole brain weight and D。N。A。 levels, no such effect was found in the case of the cerebellum (Tables 1 : 1 and 1 : 2)。

The $Na^+ - K^+$ ATPase activity per mg of cerebral tissue from microencephalic animals was slightly lower than that for controls, and the total activity of $Na^+ - K^+$ ATPase was also lower in microencephalic newborn (Table 1 : 2)。 This is consistent with the reduction in cell numbers in microencephalic cerebrum (Table 1 : 2)。 However, the activity of this enzyme per cell was higher in microencephalic cerebrum than in controls, thus the reduction in the amount of membrane bearing $Na^+ - K^+$ ATPase was not in proportion to the reduction in cell number。 A similar result was found for the $Ca^{2+} - Mg^{2+}$ ATPase as, although the activity per mg tissue and total activity in microencephalic animals was not significantly different from controls, the activity per u mole D。N。A。 was higher than for controls。 The low intrinsic activity of $Ca^{2+} - Mg^{2+}$ ATPase, and consequent larger variation in values may have masked a reduction in total activity of this enzyme。 Taken together the results for these enzymes suggests that, firstly the cerebral hemisphere from a microencephalic animal contains less cell membrane than for control cerebrum and secondly that cells from the microencephalic cerebrum have more extensive processes than those of controls。

The case of the acetylcholinesterase is somewhat different in that the activity per mg cerebral tissue for microencephalic animals was higher than controls, such that even the total activity was higher than controls (Table 1 : 4)。 Consequently the activity per u mole D。N。A。 for microencephalic animals was almost twice that of controls。 This confirms the view that the

cells of the microencephalic cerebral cortex have more membranous material than controls and suggests that there may be a disproportionate increase in dendrites and synapses from cholinergic neurons.

It appears therefore that the cells of the microencephalic forebrain develop more processes than cells of control tissue. It is not possible to differentiate between neuronal and glial contributions to this suggested cell hypertrophy, however, the acetylcholinesterase measurements confirm that some neuronal hypertrophy occurs as the membrane markers used appear to be predominantly associated with dendrites and synaptic structure. It is not possible to say whether other neuronal cell types may be affected, although this seems likely as the time during gestation at which heat treatment was applied was before any neuronal specificities would be apparent.

It is interesting to speculate as to the reason for the increased neuronal processes in microencephalic guinea-pigs. It seems possible that a decrease in neuronal numbers as a result of maternal hyperthermia may also decrease the probability of neuronal interaction. It may be that as a response to the lack of neuronal connections, the neuron may increase the size and perhaps degree of branching of the dendritic tree. This may compensate in some way for the cell deficit. Although this is not completely successful as the total amount of tissue remains reduced, and brain function is impaired (Jonson, Lyle, Edwards & Penny, 1976).

If the increase in dendritic growth occurs as a compensatory response to the deficit in cell numbers then the enzyme activity per cell ought to increase with the degree of microencephaly. The values for microencephaly for animals used in this part of the

study varied between 15.2% and 28%. The data concerned was processed for linear regression analysis, but no significant correlation was obtained. The data obtained in this study does not support the view that the increase in Na^{+} - K^{+} ATPase, Ca^{2+} - Mg^{2+} ATPase and acetylcholinesterase activity per u mole D.N.A. in microencephalic forebrain is directly related to the severity of retardation of brain growth. Perhaps the sample size is too small in this case. However, it was not possible to increase the number of animals in the experimental group as the ratio of females giving birth to females exposed to heat treatment was low, as described previously.

TABLE 1 : 1

Brain weights and body weights of newborn guinea-pigs born to control and heat treated mothers.

	Body weight (gms)	Brain weight (gms)	n
Control Animals	94.3 ± 2.2	2.72 ± 0.02	31
Experimental Animals	91.3 ± 2.1	2.3 ± 0.03	50
P	n.s.	0.001	

TABLE 1 : 2

DNA content of brains from newborn guinea-pigs

	DNA-P (μ moles/brain)	DNA-P (μ moles/gm. brain tissue)	
Control Animals	13.04 ± 0.07	4.79 ± 0.04	n 17
Experimental Animals	11.10 ± 0.12	4.78 ± 0.06	n 30
	P = 0.001	n.s.	

TABLE 1 : 3

DNA content of cerebral and cerebellar brain tissue from control and microencephalic guinea-pigs

	CEREBRAL HEMISPHERE			CEREBELLUM			n
	DNA-P (μ moles/ cerebrum)	DNA-P (μ moles/gm. wet weight)	Weight of Cerebrum	DNA-P (μ moles/ cerebellum)	DNA-P (μ moles/gm. wet weight)	Weight of Cerebellum	
Control Animals	2.48±0.06	2.98±0.06	0.83±0.03	5.00±0.19	15.86±0.40	0.32±0.02	11
Microencephalic Animals	1.60±0.06	2.30±0.09	0.70±0.02	5.10±0.09	15.62±0.54	0.34±0.02	14
P	0.001	0.001	0.01	n.s.	n.s.	n.s.	

Values expressed as ± one standard error

TABLE 1 : 4

Activities of marker enzymes in homogenates of cerebral tissue for control and microencephalic newborn guinea-pig brain

Enzymes	Control Animals	Microencephalic Animals	P
$Na^+ - K^+$ATPase			
µ moles Pi/mg. wet weight/hr	2.12 ± 0.08	1.93 ± 0.03	0.05
µ moles Pi/ cerebrum/hr	1695 ± 30	1360 ± 60	0.001
µ moles Pi/ µ moles DNA-P/hr	712 ± 26	838 ± 15	0.001
$Ca^{2+} - Mg^{2+}$ATPase			
µ moles Pi/mg. wet weight/hr	0.46 ± 0.03	0.51 ± 0.03	n.s.
µ moles Pi/ cerebrum/hr	373 ± 20	340 ± 17	n.s.
µ moles Pi/ µ moles DNA-P/hr	154 ± 12	222 ± 14	0.01
Acetycholinesterase			
µ moles thiocholine/ mg. wet weight/hr	0.57 ± 0.03	0.75 ± 0.03	0.001
µ moles thiocholine/ cerebrum/hr	464 ± 25	523 ± 26	0.001
µ moles thiocholine/ µ moles DNA-P/hr	192 ± 10	324 ± 12	0.001
n	11	14	

TABLE 1 : 5

Activities of marker enzymes in homogenates of cerebellar tissue from control and microencephalic guinea-pig brain

Enzyme	Control Animals	Microencephalic Animals	P
$Na^+ - K^+$ ATPase μ moles Pi/mg. wet weight/hr	2.47 ± 0.12	2.23 ± 0.13	n.s.
μ moles/Pi/ cerebellum/hr	785 ± 43	741 ± 42	n.s.
μ moles Pi/ μ moles D.N.A-P/hr	156 ± 8	143 ± 8	n.s.
$Ca^{2+} - Mg^{2+}$ ATPase μ moles Pi/ mg.wet weight/hr	0.53 ± 0.04	0.56 ± 0.03	n.s.
μ moles Pi/ cerebellum/hr	167 ± 9	177 ± 8	n.s.
μ moles Pi/ μ moles D.N.A. - P/hr	33.4 ± 2.5	35.9 ± 1.9	n.s.
Acetylcholinesterase μ moles thiocholine/ mg.wet weight/hr	0.88 ± 0.03	0.96 ± 0.05	n.s.
μ moles thiocholine/ cerebellum/hr	280 ± 15	316 ± 12	n.s.
μ moles thiocholine/ μ moles D.N.A.-P/hr	55.5 ± 2.1	61.5 ± 2.9	n.s.
n	11	14	

TABLE 1 : 6

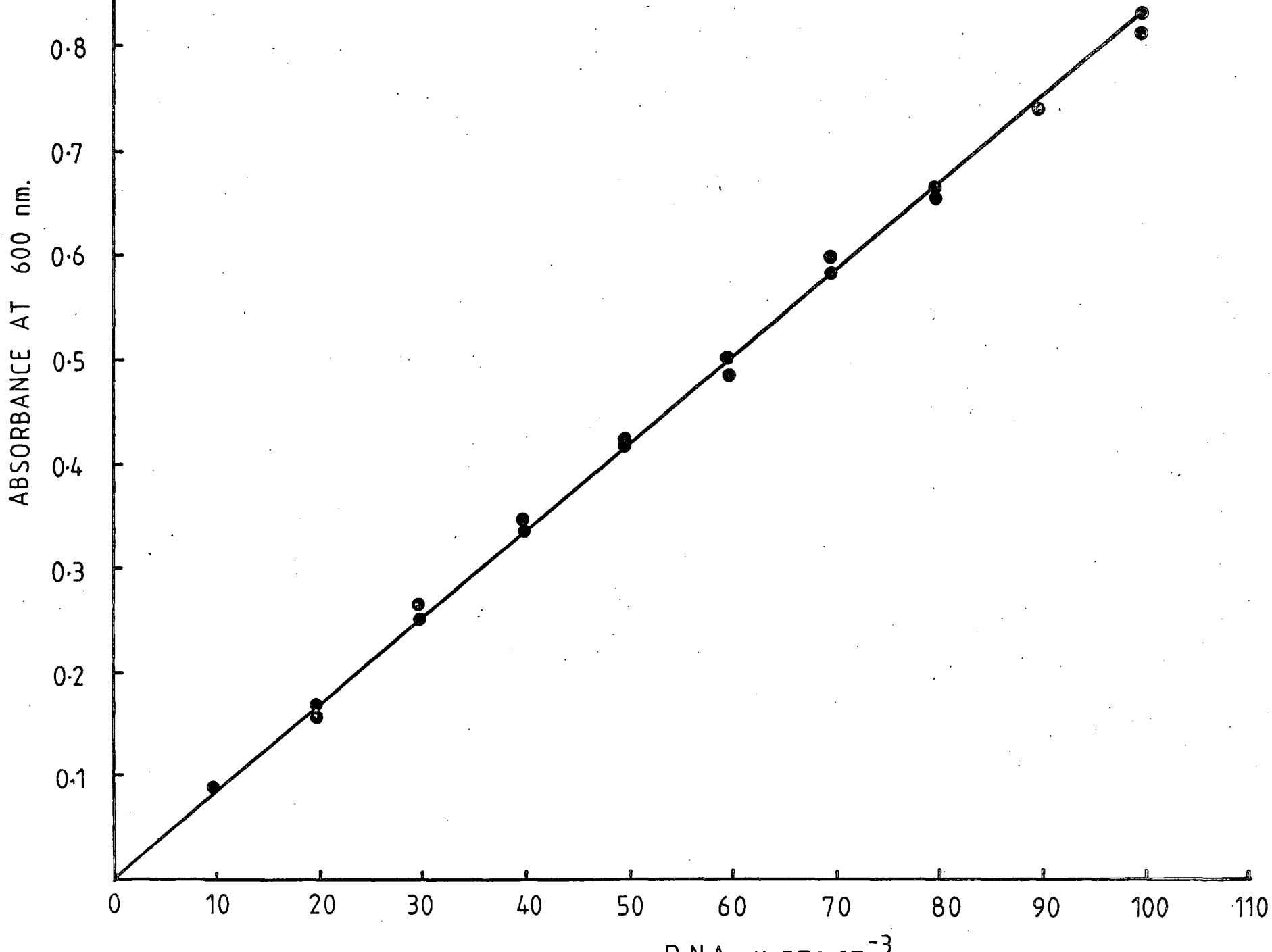
Specific activities of marker enzymes in isolated membrane fractions from cerebral tissue for control and microencephalic guinea-pig brain

	Control Animals	Microencephalic Animals	P
$Na^+ - K^+$ ATPase µ moles Pi/mg protein/hr			
Synaptic Membranes	65.9 ± 2.3	59.7 ± 2.1	n.s.
Microsomes	15.7 ± 0.7	17.7 ± 1.5	n.s.
$Ca^{2+} - Mg^{2+}$ ATPase µ moles Pi/mg protein/hr			
Synaptic Membranes	7.12 ± 0.76	8.46 ± 1.1	n.s.
Microsomes	3.07 ± 0.20	2.92 ± 0.17	n.s.
Acetylcholinesterase µ moles thiocholine/ mg protein/hr			
Synaptic Membranes	15.6 ± 1.4	13.6 ± 0.5	n.s.
Microsomes	8.24 ± 0.22	8.45 ± 0.47	n.s.
n	6	14	

TABLE 1 : 7

Specific activities of marker enzymes in isolated membrane fractions from cerebellar tissue for control and microencephalic guinea-pig brain

	Control Animals	Microencephalic Animals	P
Na^+-K^+ATPase μ moles Pi/mg. protein/hr			
Synaptic Membranes	56.2 ± 2.2	55.0 ± 3.4	n.s.
Microsomes	18.7 ± 1.5	20.4 ± 1.2	n.s.
Ca^{2+}-Mg^{2+}ATPase μ moles Pi/mg. protein/hr			
Synaptic Membranes	3.88 ± 0.13	3.60 ± 0.18	n.s.
Microsomes	3.20 ± 0.15	2.75 ± 0.20	n.s.
Acetylcholinesterase μ moles thiocholine/ mg.protein/hr			
Synaptic Membranes	18.1 ± 1.1	16.9 ± 0.7	n.s.
Microsomes	11.3 ± 0.8	11.7 ± 0.7	n.s.
n	6	14	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0·7
0·6
0·5
0·4
0·3
0·2
0·1
0
10
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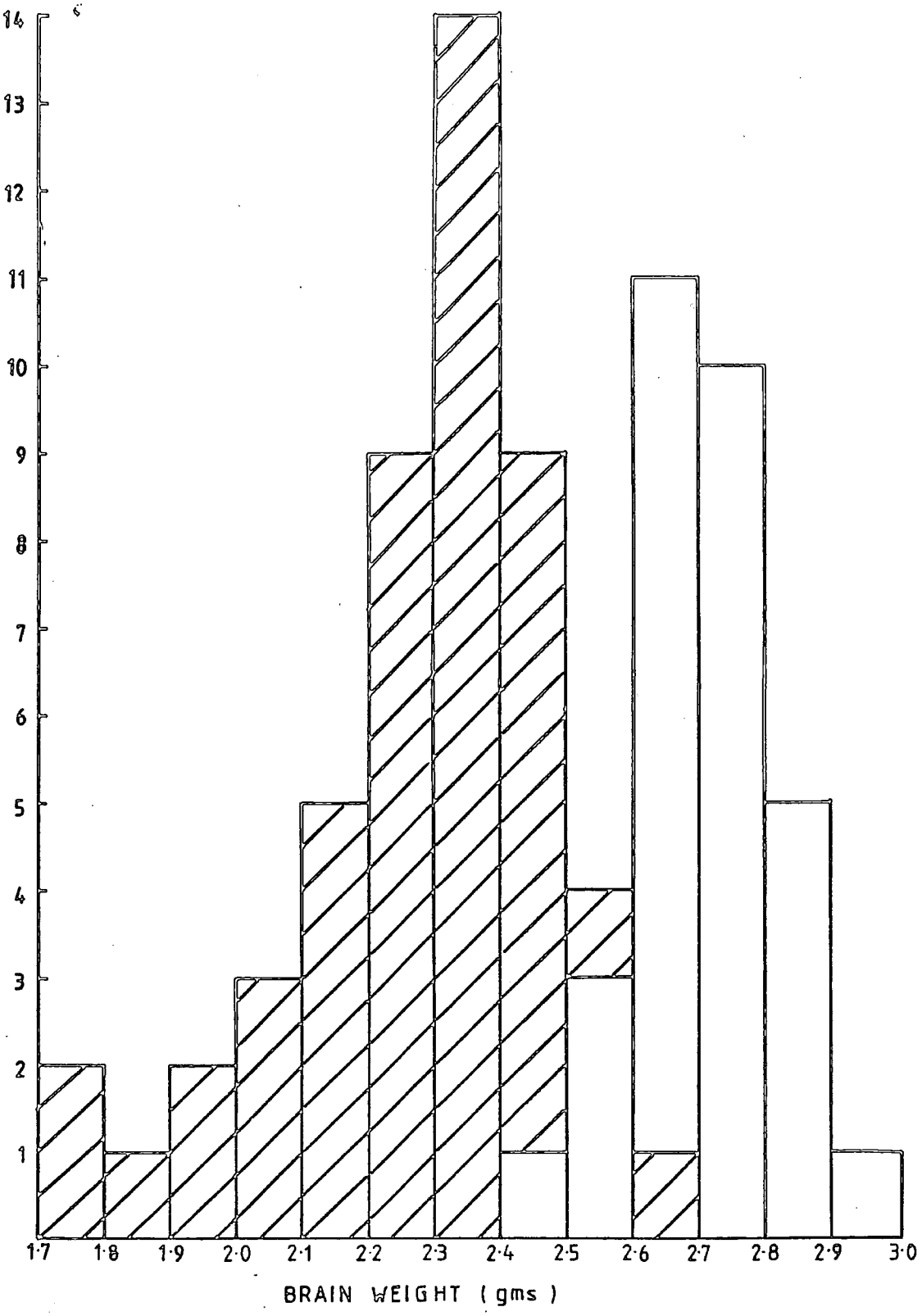
30
40
50
60
70
80
90
100
110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1
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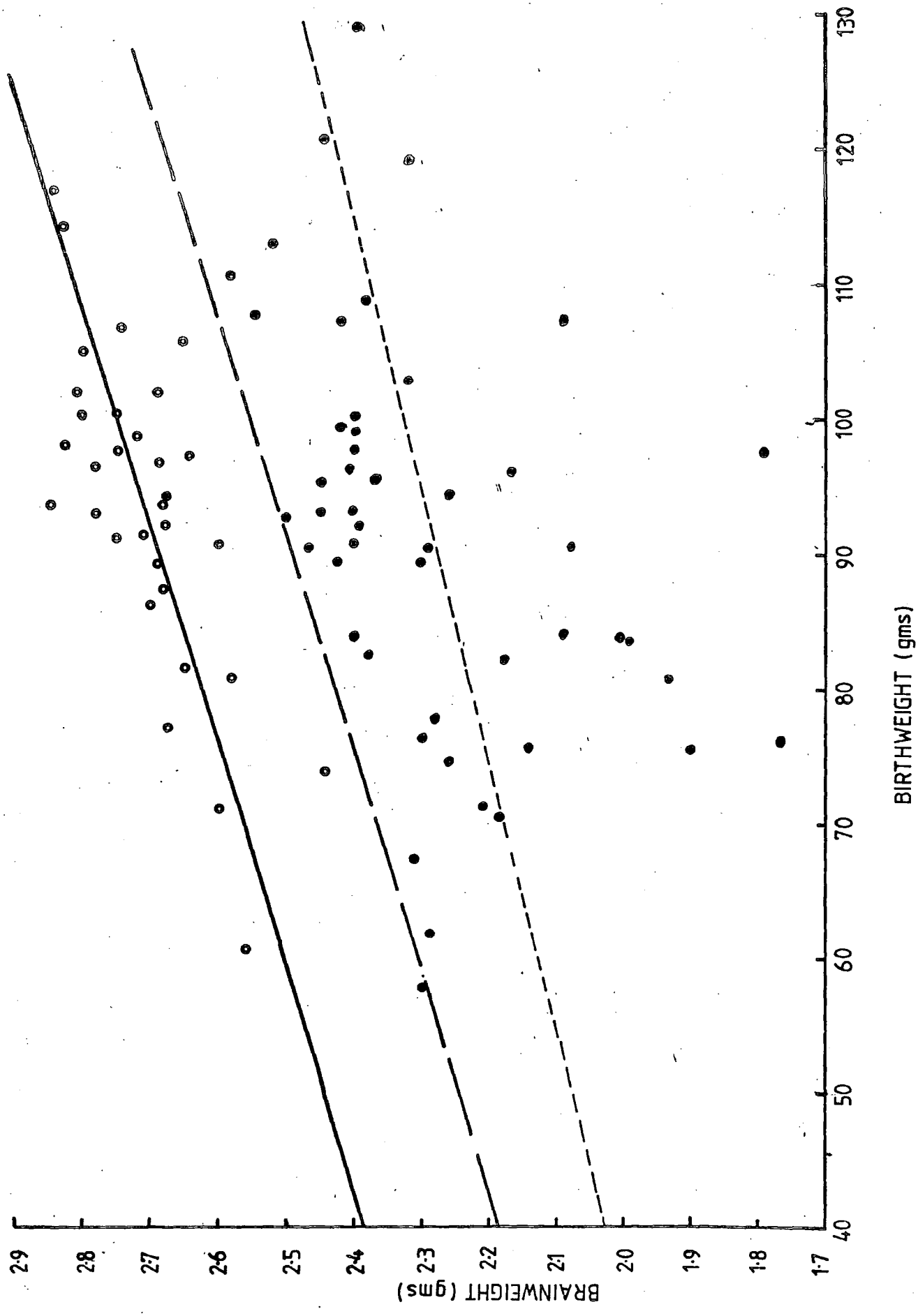
2·4
2·5
2·6
2·7
2·8
2·9
3·0
BRAIN WEIGHT (gms)


BRAINWEIGHT (gms)
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2·8
2·7
2·6
2·5
2·4
2·3
2·2
2·1
2·0
1·9
1·8
1·7
BIRTHWEIGHT (gms)
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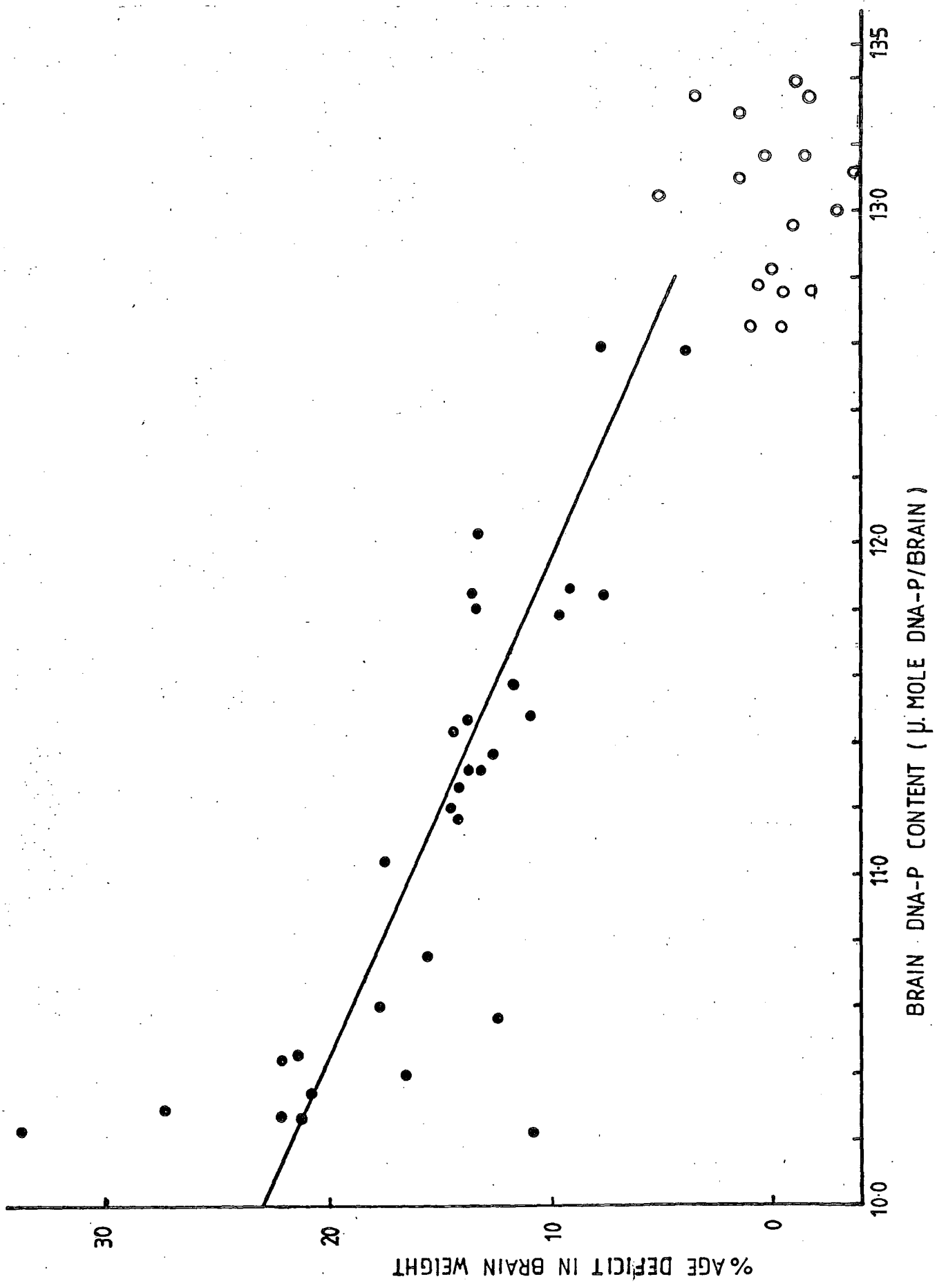
50
60
70
80
90
100
110
120
130


% AGE DEFICIT IN BRAIN WEIGHT
30
20
10
0
10·0
11·0
12·0
13·0
13·5
BRAIN DNA-P CONTENT (μ. MOLE DNA-P/BRAIN)

THE DEVELOPMENT OF SYNAPTIC MEMBRANE ENZYME ACTIVITIES IN HYPOTHYROID RAT BRAIN

Introduction

It is well recognised that thyroid hormones have both metabolic and developmental morphological effects on target tissues. In particular the development and proper function of C.N.S. in mammals has been shown to have a requirement for thyroid hormones, for their deficiency during the prenatal and perinatal period causes mental retardation in man (Fagge, 1871; Topper, 1951) and in mild cases thyroid hormone replacement therapy restored normal mental capacity, although in severe cases, subnormality remained despite thyroid hormone therapy (Lewis, 1937). In other laboratory animals, hypothyroidism has been shown to reduce dramatically the growth of body and brain (Eayrs and Taylor, 1951; Balays, Kovacs, Teichgraber, Cocks and Eayrs, 1968).

In the case of the rat, these effects were permanent unless replacement therapy was begun before the tenth postnatal day (Horn, 1954; Eayrs, 1961 Also many characteristic signs of rat development, such as eye opening, onset of fur growth, growth of dentition, development of reflexes and learning abilities were temporarily delayed by hypothyroidism or were sensitive to hormone replacement thereapy late in life (Eayrs & Taylor, 1951; Eayrs & Lishman, 1955; Eayrs, 1966).

From this and other work it has been found that the rat is a particularly suitable model for the study of the relationship between thyroid hormones and brain development. The pup is born before the phase of maximum brain growth, which occurs within the first few postnatal weeks (Davson & Dobbing, 1968). This combined with the observation that the foetal thyroid gland only begins to function close to the time of birth allows the selective administration of surgical or chemical thyroidectomy. The subsequent evaluation of brain growth and maturation is then not complicated by the hormonal state in utero.

Several studies indicate that thyroidectomy, or hypothyroidism occurring before the tenth postnatal day in the rat, causes permanent effects on brain development. Rabinowitz and Rosvold, (1951), using the Hebbs-Williams closed field test (said to measure cerebral function in experimental animals) showed that in hypothyroid rats the cognitive abilities were permanently reduced. Eayrs, (1961) also showed, in rats thyroidectomised at birth, that replacement therapy was only successful if initiated before the tenth postnatal day.

It is now generally agreed that hypothyroidism has numerous and similar effects on all the neurons of the cerebral cortex and cerebellum of the rat brain. In the cerebral cortex, where neuronal types have been difficult to define, it has been shown that these cells were smaller, more closely packed, had less elaborate dendritic trees, and made fewer synapses (Eayrs & Taylor, 1951; Horn, 1954; Eayrs & Horn, 1955; Eayrs & Goodhead, 1959; Eayrs, 1960; Eayrs, 1966; Cragg, 1970; Shapiro, Vukovitch & Globus, 1973: Bass, Pelton & Young, 1977; Ruiz-Marchos, Sanchez-Toscano, Escobas del Rey, Monreale De Escobar, 1979). In the cerebellum where cell-types have been well defined, hypothyroidism also produced smaller neurons, reduced synaptogenesis and reduced dendritic aborization (Legrand, 1967 (a), (b) and (c); Tusque, Lefranc & George, 1967; Mugnaini, 1969; Crepel, 1972; Nicholson & Altman, 1972 (a), (b) and (c); Rebiere & Legrand, 1972 (a) and (b); Close & Legrand, 1973; Crepel, 1973; Hajos, Patel & Balazs, 1973; Rebiere & Legrand, 1973; Rebiere & Dainat, 1976; Lauder, 1977; Lauder, 1978; Rabie, Faure, Clavel & Legrand, 1979).

One difference between hypothyroidism in cerebral cortex and cerebellar cortex is associated with neurogenesis. In the rat cerebral cortex, neurogenesis is complete by birth (Berry, 1974) and consequently hypothyroidism has been shown to have no effect on cell

numbers in this brain region (Balazs, Kovacs, Teichgraber, Cocks & Eayrs, 1968; Balazs, Cocks, Eayrs & Kovacs, 1971). However, the immediate postnatal period is a time of considerable cerebellar neurogenesis. Cells are formed in a region called the external granular layer (E.G.L.). In the cerebellum, hypothyroidism delays the acquisition of adult cell number (Balazs et al, 1968; Balazs et al, 1971), this is eventually attained only by the E.G.L. persisting beyond the normal time in development (Legrand, 1967; Hamburgh, 1968; Nicholson & Altman, 1972 (a); Lewis, Patel, Johnson and Balazs, 1973).

This dramatic effect of hypothyroidism on brain morphology has stimulated much reasearch into the mode of action of thyroid hormones at the brain cell level. Thyroxine is known to cross the blood-brain barrier (Ford, 1968) and be taken up into nerve cells (Oklund & Timiras, 1977) and consequently it may act directly upon intracellular processes (Hamburgh & Bunge, 1966; Hamburgh, 1969). Several authors have suggested the thyroxine acts at the level of m R.N.A. synthesis (Balazs & Cocks, 1967; Geel & Timiras, 1967; Pascquini, Kaplun, Garcia-Argiz & Gomez, 1967; Balazs et al 1968; Geel, 1975) whilst other workers suggest that its developmental effects are mediated through an effect on protein synthesis (Geel, Valcana & Timiras, 1967; Dainat, Rebiere & Legrand, 1970; Szijan, Kalverman & Gomez, 1971). That these processes have an interdependance makes these separate proposals difficult to interpret.

Many workers have attempted to show that the level of a particular enzyme was affected by hypothyroidism. The technique adopted was to measure the activity of enzymes in homogenates of forebrain or cerebellum tissue from hypothyroid and control rats. The level of almost every enzyme measured was reduced by immature hypothyroidism, These include cytosol enzymes (Schwark, Singhal & Ling, 1971; Schwark, Singhal & Ling, 1972 (a), (b) and (c); Weimer & Neims

1977), mitochondrial enzymes (Hamburgh & Flexner, 1957; Garcia-Argiz, Pasquini, Kaplan & Gomez, 1967), ion pumping enzymes (Garcia-Argiz et al, 1967; Valcana & Timiras, 1969), and the enzymes of neurotransmitter metabolism (Geel & Timiras, 1967; Balazs et al, 1968; Rastogi & Singhal, 1974; Pesetsky & Burkart, 1977).

The significance of these studies was not clear as the levels of these enzymes in tissue homogenates were expressed either as a function of cell number (tissue D.N.A.) or as a function of wet tissue weight. However, it was clear from histological studies that the cells of both cerebrum and cerebellum of hypothyroid rats were smaller than controls (Eayrs & Horn, 1955).

A reduction in enzyme levels per cell may simply reflect this fact. Similarly it is not clear what fraction of the wet weight of tissue was occupied by neurons in hypothyroid rat brain. It remains a possibility that reduced tissue specific enzyme levels do not reflect a significiant decrease in the concentration of these enzymes within their respective subcellular fractions. In fact there is some evidence that hypothyroidism does not produce such a reduction in enzyme concentration (Szijan, Chepelinsky & Piras, 1970; Verity, Brown, Huntsman & Smith, 1976).

It was not clear how a decrease in the cell concentration of a glycolytic or mitochondrial enzyme would effect neuronal metabolism, or how an affect on metabolism would delay the acquisition of normal behaviour, and permanently reduce cognitive abilities in hypothyroid rats. It was easier to believe that reduced levels of the enzymes associated with neurotransmitter metabolism may produce nervous dysfunction, but even in this case the reduction in tissue and cell specific levels of these enzymes may simply reflect the decreased number of synapses found in hypothyroid rat brain, not a qualitative change in existing synapses.

In this study, it was proposed to assess the effect of hypothyroidism on the qualitative development of synapses in rat brain-tissue. It is possible to isolate a vesicle fraction from homogenates of nervous tissue rich in "synaptosomes", which are thought to be the synaptic structures seen in electron micrographs which have been "pinched off" during homogenisation (review Jones, 1975). Following hypotonic lysis, the plasma membrane surrounding these synaptosomes can be further isolated from mitochondria and myelin by floatation (Rodriguez de Lores Arnais et al, 1967). This membrane has been shown to be a rich source of ion-activated A.T.P.ases and acetylcholinesterase (Whittaker, 1965; Bowler & Tirri, 1974; Gookin & Howard, 1974).

The $Na^+ - K^+$ ATPase has been shown to increase in specific activity during the immediate postnatal period in a manner which correlated with the onset of electrical activity in brain (Abdel-Latif, Brody & Ramahi, 1967). It has been suggested that the rise in $Na^+ - K^+$ ATPase enzyme over other synaptic membrane proteins is essential for the normal function of synapses. This work shows that in normal rats, synapses develop qualitatively as well as in quantity during the rapid phase of brain growth.

It was proposed to measure the development of $Na^+ - K^+$ ATPase, $Ca^{2+} - Mg^{2+}$ ATPase and acetylcholinesterase in synaptic membrane preparations from hypothyroid rat brain. This preparation was particularly suitable for the analysis of the effect of hypothyroidism on synapses, as the membranes were only from synaptic structures and the enzymes in question have been associated with synaptic function. A change in the levels of these enzymes could be the connecting link between the effect of thyroid hormones on neuronal

metabolism, and the impaired mental performance observed in the whole animal. Thus this data would determine whether brain dysfunction could be explained entirely in terms of reduced synaptogenesis or whether an immature form of neuron persists in full term hypothyroid rats.

MATERIALS AND METHODS

Production of experimental hypothyroidism

Rats used in this study were born to stock females. Pregnant rats were caged individually and the date on which they gave birth was noted. Litters were only used when at least two females gave birth on the same day. In this case, one litter was designated as a control litter and the second as an experimental litter.

Hypothyroidism was produced in experimental neonates by daily injections of propyl-thio-uracil (P.T.U.) described by Nicholson & Altman, (1972 (a)). These rats were injected subcutaneously starting on the second day of life, and the quantity of P.T.U. injected varied with the age of the animal in the following manner.

Animals between 2 and 10 days of age were injected daily with 50 ul of 0.2% P.T.U., in 1% carboxymethylcellulose, 0.9% saline. From 11 to 20 days this was increased to 100 ul of the same 0.2% P.T.U. solution. After 21 days of age experimental animals were injected with 100 ul of 0.4% P.T.U. in 1% carboxymethylcellulose, 0.9% saline. Control neonates received the carboxymethylcellulose vehicle in appropriate volumes.

Synaptic plasma membrane isolation

Hypothyroid and control rat pups from age-matched litters were processed for membrane isolation simultaneously. At least two control and two hypothyroid rats were taken at each time and processed in an exactly similar fashion. Thus enzyme preparations from age matched control and hypothyroid rats were subjected to the same manipulation and were assayed in the same media.

Rats were weighed on a top-pan balance and then killed either by decapitation (prior to eye-opening) or by stunning, snapping the spinal cord and decapitation (in older animals). The brain was

dissected from the cranium intact and weighed. The whole brain was then placed in ice-cold 20mM imidazole 2mM E.D.T.A. pH 7.2 buffer (imidazole E.D.T.A. buffer).

The cerebral hemispheres and cerebellum were dissected from each brain on an iced glass petri-dish. The cerebral hemispheres from hypothyroid rats were pooled, as were the cerebellums from hypothyroid animals, the cerebrums from controls and the cerebellums from controls, giving four tissue samples.

In initial experiments the synaptic membrane fraction was isolated from rat brain tissue using the discontinuous sucrose density-gradient method described in detail in the general methods chapter, which is essentially that of Rodruiguez de Lores-Arnais et al,(1967). However, the specific activities of synaptic membrane enzymes isolated in this way were very variable. This was clearly owing to different degrees of mitochondrial contamination of the synaptic membrane fraction. These organelles were visible on sucrose density-gradients at the 1.0M/1.2M sucrose interface as a yellowish band of membranous material, either within or immediately below the usual band of synaptic membranes, and was also seen as a brown spot in pellets of synaptic membranes produced when membranes were washed free of sucrose.

It was clear that, when larger amounts of tissue were processed, the dense band of synaptic membranes sedimenting at the 1.0M/1.2M sucrose interface acted as a barrier to mitochondria. A modification of the isolation technique was devised to avoid producing a dense band of synaptic membranes. This modification has been described in detail in the general methods chapter and is identical to the method of Rodruiguez de Lores-Arnais et al, (1967) except that the osmotically lysed synaptosome and myelin membranes for the 20,000 xg pellet were not resuspended in buffer and layered on the sucrose density gradient, instead this pellet was resuspended in 1.2M sucrose. This was

centrifuged at 100,000 xg for 2 hours. The mitochondria pellet while the synaptic membranes remain suspended. The supernatant was diluted with imidazole buffer to give 1.0M sucrose. This was centrifuged at 100,000 xg for 1 hour, in this case the synaptic membranes sediment leaving the myelin membranes suspended. The pellet was washed once by homogenizing in imidazole/EDTA buffer and centrifuged at 100,000 xg for 1 hour at $4^{o}C$. The final synaptic membrane pellets were resuspended in 5 cm^{3} of imidazole/EDTA buffer and stored overnight at $4^{o}C$ packed in ice. This gave membrane suspensions at protein concentrations of between 1 and 3 mg. cm^{-3}.

The stored membrane fractions were diluted to about 250 ug. protein cm^{-3} and used directly in enzyme analyses. Membranes intended for Ca^{2+} Mg^{2+} ATPase assay were diluted with 20 mM imidazole buffer pH 7.2 without E.D.T.A. This was to ensure that the free calcium level in the reaction medium was controlled only by the E.G.T.A. levels of the reaction medium.

The Na^{+} - K^{+} ATPase and Ca^{2+} - Mg^{2+} ATPase activities of synaptic membranes were assayed as described in the general methods chapter. The acetylcholinesterase activity was assayed by the method of Ellman, (1961) modified to avoid running the assay in a spectrophotometer cell, as described in general methods chapter.

Protein was assayed by the modified ninhydrin procedure described in the general methods section.

In order to estimate the protein and phospholipid specific activity of rat brain Na^{+} - K^{+} ATPase from different membrane sources, microsomes and synaptic membranes were isolated from the same homogenates of normal adult rat brain in the following manner. Twelve normal female rats weighing about 250 grammes were chosen at random from

stock. They were stunned from a blow to the head and killed by decapitation. The brains were quickly dissected from the skull and placed in ice-cold imidazole/E.D.T.A. buffer. When all the animals had been killed and the brains removed, the cerebral hemispheres were dissected from the brains and scraped free of white matter. Four hemispheres were homogenized in 20 cm^3 of 0.32 M sucrose, 20 mM imidazole, 2 mM E.D.T.A. pH 7.2 using a teflon - glass - tissue homogenizer. The homogenates were processed according to the modification of the synaptic membrane isolation method which avoided the use of sucrose density gradients, except that the supernatant remaining from when the synaptosomes, mitochondria and myelin were first sedimented at 20,000 xg was retained. This supernatant was centrifuged at 100,000 xg for 1 hour in an M.S.E. prepspin 50 preparative ultracentrifuge. The supernatant was discarded and the pellet was rehomogenized in imidazole/E.D.T.A. buffer and resedimented at 100,000 xg for 1 hour in a prepspin 50 preparative ultracentrifuge. The supernatant was discarded and the pellet was taken up in 3 cm^3 of imidazole E.D.T.A. buffer by homogenization. This suspension was used for enzyme assay of microsomal Na^+ - K^+ ATPase by diluting to about 250 $\mu g/cm^3$ protein with imidazole E.D.T.A. buffer, and was used neat (1 - 3 mg/cm^3) for phospholipid analysis.

The pellet produced by centrifugation at 20,000 xg was subjected to hypo-osmotic lysis and further processed for synaptic membrane isolation. In this way six preparations of adult rat brain synaptic membranes and microsomes were obtained and were assayed for Na^+ - K^+ ATPase activity and protein content as described previously and were assayed for phospholipid phospohorous content as described below.

Phospholipid assay

Phospholipid phosphorous can be estimated in intact phospholipids essentially as described by Raheja, Charanjut Kaur, Ajit Singh & Bhatia, (1973).

A sample of membranes in buffer (200 µl containing about 200 ug protein) was dried onto a clean test tube in an oven, at 100°C 0.5 cm^3 of chloroform was added and the dried membranes were resuspended by vortexing. The chloroform was evaporated in a boiling water bath. This procedure dispersed membrane phospholipid. The dried sample was suspended in 0.4 cm^3 of chloroform to which was added 200 ul of chromogenic solution, prepared as described below. This was mixed by vortexing and incubated in a boiling water bath for 2 to 3 minutes. The mixture was then cooled, 3 cm^3 of chloroform were added and phases separated by centrifugation at 500 xg for 2 to 3 minutes. The chloroform layer was recovered and the extinction of this phase at 716 nm was measured in a Pye Unicam SP-1800 spectrophotometer.

A standard curve was produced by dissolving a commercial preparation of egg lecithin (Sigma Chemical Company) in chloroform. Linear colour response was found in range of 0.10 ug of lipid phosphorous per tube. The curve produced in this way is presented in Figure 3 : 1.

The chromogenic solution for this assay was made as follows:-

Sol I 16 g of ammonium molybdate was dissolved in 120 cm^3 of distilled water. The addition of a few drops of conc. HCl may be necessary to dissolve the ammonium molybdate completely.

Sol II 10 cm^3 of redistilled mercury were added to a mixture of 40 cm^3 of conc. HCl and 80 cm^3 of solution I. The mixture was stirred for 30 to 45 minutes and filtered through double layers of Whatman No. 1 filter paper. The reddish brown filtrate was solution II.

Sol III Add 200 cm^3 of conc. H_2SO_4 to 40 cm^3 of Sol I and the mixture to Sol II, slowly, with careful stirring. The dark green solution formed is Solution III.

The Chromogenic Solution

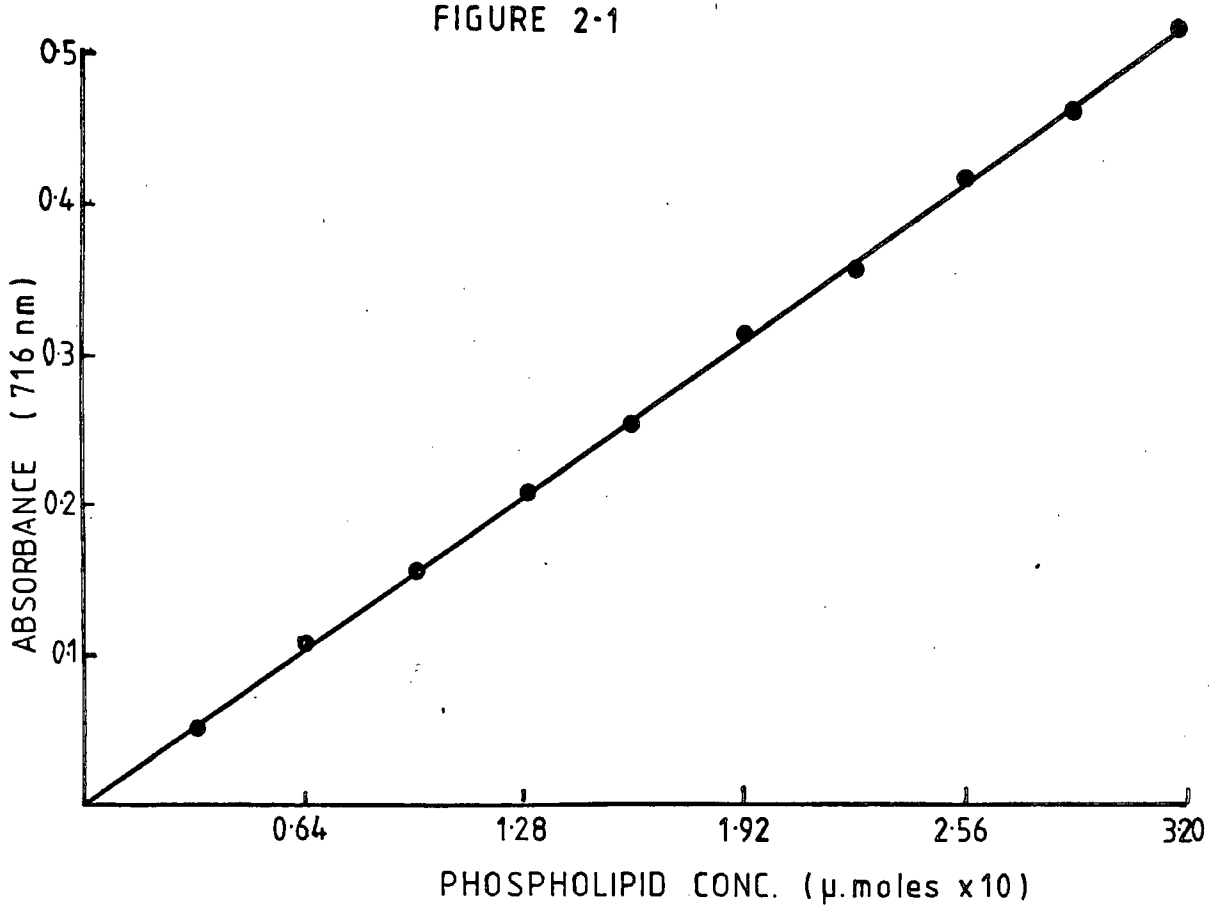
The chromogenic solution was prepared by mixing 450 cm^3 of methanol, 5cm^3 of chloroform, 20cm^3 of distilled water and 25 cm^3 of Solution III. The dark green solution produced is the chromogenic solution which has an expected shelf life of at least six months.

Sodium dodecyl sulphate activation of $Na^+ - K^+$ ATPase activity

The "latent" active sites of $Na^+ - K^+$ ATPase in synaptic membrane preparation were exposed by incubating aliquots of membrane suspension in low concentrations of sodium dodecyl sulphate (S.D.S.). Aliquots of each preparation were incubated with a range of S.D.S. concentrations between 150 $\mu g/cm^3$ and 15 $\mu g/cm^3$, as the precise S.D.S. concentration for maximum activation of enzyme activity depends on the precise concentration of the membrane sample and on the length of incubation.

The reaction media for these experiments consisted of 500 ul of 50 mM imidazole buffer pH 7.2 containing the appropriate S.D.S. concentrations, to which was added 250 µl of 13.2 mM tris - A.T.P. This was allowed to equilibrate at 20°C for at least 15 minutes in a glass test-tube. Then 250 µl of membrane suspension (approximately 1 mg protein/cm^3) was added to each tube and the samples were incubated at 20°C for 40 minutes. The incubation was stopped by placing the test-tubes in an ice-water mixture. Aliquots (200 µl) of these samples were used directly in place of crude synaptic membranes suspension in the routine assay of $Na^+ - K^+$ ATPase activity as described above.

FIGURE 2-1


0·5
0·4
0·3
0·2
0·1
ABSORBANCE (716 nm)
0·64
1·28
1·92
2·56
3·20
PHOSPHOLIPID CONC. (μ.moles x10)

RESULTS

In this study, rats made hypothyroid during the early postnatal period showed a marked reduction in the rate of body and brain growth. Rats from sham injected control litters increased their body weights by 7.5 to 8.0 fold, from 18 to 20 gms to about 150 gms between the 7th and the 44th postnatal day (Table 2 : 1, Figure 2 : 2). In the case of hypothyroid rats, body weight increased much more slowly after the end of the second postnatal week. P.T.U. treated animals only managed to double their body weight to about 40 gms by the 44th postnatal day (Table 2 : 1, Figure 2 : 2). Thus by this stage, hypothyroid rats are only $\frac{1}{3}$ as heavy as control animals.

The lack of thyroid hormone has a similarly dramatic effect on the development of brain weight. The control rats showed a sharp rise in brain weight between the second and fourth postnatal weeks (Figure 2 : 3). The brain weight of control animals rose by 40% in the period studied, from about 1.1 gms to about 1.8 gms (Table 2 : 1). This sharp rise in brain weight was absent in hypothyroid animals (Table 2: 1, figure 2: 3). The brain weights of P.T.U. treated animals were similar to controls for the first two postnatal weeks, after which time, the brain growth occurred very slowly. By the 44th postnatal day the brain weights of hypothyroid rats were only 70% of the brain weights of control rats (Table 2: 1).

The relative development of brain weight and body weight in control and hypothyroid animals are given in Figure 2 : 4. This shows that in control animals the ratio of brain weight to body weight falls throughout the developmental period. This reflects the larger change in body weight during development.

The brain weight/body weight ratio for hypothyroid animals also falls in development but to a lesser extent than for control animals (Figure 2 : 4). This shows, as would be expected from data presented in Table 2 : 1, that hypothyroidism had a larger effect on the development of body weight.

As it was clear that this experimental regime brought about a dramatic reduction in brain growth, then animals so treated were used for enzyme measurements. The synaptic membrane fraction was isolated from homogenates of cerebral and cerebellar brain regions taken from hypothyroid and control rats, at several stages of development. The membrane fractions were used as a source of the $Na^+ - K^+$ ATPase, $Ca^{2+} - Mg^{2+}$ ATPase and acetylcholinesterase enzymes, and were used directly in enzyme specific activity measurements. The $Na^+ - K^+$ ATPase of cerebral synaptic membranes rose from about 40 μ moles Pi/mg protein/hr on day seven to more than 80 μ moles Pi/mg protein/hr by day 44 in control animals (Table 2 : 2). This latter value is similar to adult values obtained for uninjected euthyroid animals later in this study (Table 2 : 5). The $Na^+ - K^+$ ATPase levels in cerebral synaptic membrane preparations from these animals increased steadily throughout the postnatal period (Figure 2: 5).

The $Na^+ - K^+$ ATPase specific activity from the cerebral synaptic membranes from hypothyroid rat pups was slightly lower than that from control animals even at 11 days of age, but was about 20% lower than controls at subsequent points in the postnatal period (Table 2 : 2, figure 2 : 5). Thus by 44 days the $Na^+ - K^+$ ATPase activity of cerebral synaptic membranes from these animals had risen to only about 65 μ moles Pi/mg protein/hr (Table 2 : 2).

The specific activity of $Na^+ - K^+$ ATPase of synaptic membranes derived from the cerebellum of control animals increased from about 40 μ moles Pi/mg protein/hr on day 11 to between 90 to 100 μ moles Pi/mg protein/hr by day 36 (Figure 2 : 6). This was slightly higher than the values obtained for the later stages in the development of this enzyme in cerebral synaptic membranes.

The values for the specific activity of $Na^+ - K^+$ ATPase in synaptic membranes from hypothyroid rat cerebellum also increased more slowly when compared with controls after the 11th postnatal day (Figure 2 : 6). In fact, these values were similar to those for the cerebral synaptic membranes of hypothyroid animals, where values of about 65 μ moles Pi/mg protein/hr were obtained by 44 days of age (Table 2 : 3). Thus the percentage deficit in $Na^+ - K^+$ ATPase specific activity was larger in the cerebellum than in the cerebrum of hypothyroid animals, at about 30% (Table 2 : 3).

The specific activity of the $Ca^{2+} - Mg^{2+}$ ATPase of synaptic membranes from both the cerebrum and cerebellum of control rats increased from about 7 μ moles Pi/mg protein/hr on day 11 to about 11 to 12 μ moles Pi/mg protein/hr on the 44th day of life (Table 2 : 2, Table 2 : 3). The specific activity values of the $Ca^{2+} - Mg^{2+}$ ATPase in membranes from these tissues from hypothyroid animals increased more slowly than controls, such that by 44 days, the $Ca^{2+} - Mg^{2+}$ ATPase was about 20% lower than controls (Figure 2 : 7, Figure 2 : 8).

The specific activity of the acetylcholinesterase from cerebral synaptic membranes from control animals increased from about 8 μ moles thiocholine/mg protein/hr on the 11th postnatal day to about 25 μ moles thiocholine/mg protein/hr on day 44 (Table 2 : 2).

This is similar to adult values for acetylcholinesterase specific activity observed in this study (Chapter 3).

The specific activity of acetylcholinesterase from the cerebral tissue of hypothyroid animals increased from about 7 μ moles thiocholine/mg protein/hr to about 13 μ moles thiocholine/mg protein/hr over the period studied (Table 2 : 2, Figure 2 : 9). Thus at about 44 days of age the specific activity of the acetylcholinesterase of cerebral synaptic membranes is lower than controls by about 50%.

In synaptic membrane preparations extracted from control cerebellar tissue the acetylcholinesterase increased from about 8 μ moles thiocholine/mg protein/hr to only 15 μ moles thiocholine/mg protein/hr between days 11 and 44 of postnatal life (Table 2 : 3). As with the cerebral membranes, the specific activity of acetylcholinesterase from cerebellar synaptic membranes from hypothyroid animals, increased more slowly than controls. In this case the acetylcholinesterase increased from about 6 μ moles thiocholine/mg protein/hr on day 11 to about 10 μ moles thiocholine/mg protein/hr on day 44 (Table 2 : 3, Figure 2 : 10). By day 44 the specific activity of acetylcholinesterase of hypothyroid rat cerebellum synaptic membranes was about 30% lower than controls.

The relative time-course of the increase in marker enzyme activities for control cerebral and cerebellar synaptic membrane preparations is presented in Figures 2 : 11 and 2 : 13. This was done by taking the increase in the activity of each enzyme between 11 and 44 days as 100%, in each case. The activity of each enzyme at each age between 11 and 44 days was expressed as a fraction of this 100% value. From Figures 2 : 11 and 2 : 13 it can be seen that each enzyme activity in preparations from control animals increased with a characteristic time course, and that these are similar in the case

of control cerebral and cerebellar membrane preparations.

The $Na^+ - K^+$ ATPase activity increased almost linearly throughout the period studied, whereas the $Ca^{2+} - Mg^{2+}$ ATPase showed a low relative rate of increase between 11 and 23 days followed by a sharp increase, to reach adult values by 30 days of age (Figures 2 : 11 and 2 : 13). The acetylcholinesterase activity, as with the $Ca^{2+} - Mg^{2+}$ ATPase, showed a low relative rate of increase between 11 and 23 days, also the subsequent relative rate of increase in activity was somewhat lower than for the $Ca^{2+} - Mg^{2+}$ ATPase, reaching adult levels by only 44 days of age.

The time course of the increase in marker enzyme activity for cerebral and cerebellar synaptic membrane preparations from hypothyroid rats are presented in Figures 2 : 12 and 2 : 14. In the case of cerebral membranes, the relative rate of increase in the $Na^+ - K^+$ ATPase with age was similar to that for control preparations, whereas in the case of cerebellar membranes, the relative rate of increase in $Na^+ - K^+$ ATPase was lower than for control preparations between the 11th and 30th postnatal days (Figure 2 : 14). The relative rate of increase in $Ca^{2+} - Mg^{2+}$ ATPase in cerebellar membrane preparations from hypothyroid rats was similar to that for control preparations (Figure 2 : 14), however, the lag in the increase in $Ca^{2+} - Mg^{2+}$ ATPase activity in the case of cerebral membranes from hypothyroid rats was more pronounced than for control preparations, and the rapid increase in activity was relatively greater than for controls (Figure 2 : 12). Finally, the time course of the increase in acetylcholinesterase activity from hypothyroid rat cerebral synaptic membranes was similar to that for cerebellar synaptic membranes (Figures 2 : 12 and 2 : 14). However, in both cases this is different from the time course of the increase in acetylcholinesterase in control preparations, in that the relative rate of increase in acetycholinesterase activity in

hypothyroid rat brain membranes was greater than for control preparations after the 23rd postnatal day such that the final threshold values were reached by 30 days of age.

Further aliquots of synaptic membrane preparations from hypothyroid and control rat brain have been incubated in low concentrations of sodium dodecyl sulphate (S.D.S.) in order to expose latent $Na^+ - K^+$ ATPase sites. Indeed Figures 2 : 15 - 2 : 18 show that incubating these membrane preparations with between 15 and 150 μg cm^{-3} S.D.S. produced an apparent stimulation of $Na^+ - K^+$ ATPase activity. In each case a maximum stimulation was followed by a fall in activity at higher S.D.S. concentrations.

The maximum percentage stimulation of $Na^+ - K^+$ ATPase activity by S.D.S. was about 40 - 50% for all preparations from both control and hypothyroid cerebral cortex (Table 2 : 4). The maximum stimulation of this enzyme was only about 20 - 40% for preparations from control and hypothyroid cerebellum (Table 2 : 4). However, as the apparent stimulation of $Na^+ - K^+$ ATPase activity was similar for membranes from control and hypothyroid animals then the reduced extent of normal development of $Na^+ - K^+$ ATPase activity observed in S.D.S. treated preparations from hypothyroid rats, as compared with control preparations (Figures 2 : 19, 2 : 20) was similar to that observed in crude synaptic membrane preparations (Figures 2 : 5 and 2 : 6).

The $Na^+ - K^+$ ATPase activities from six preparations of synaptic membranes and microsomal membranes from euthyroid normal adult rats are represented in Table 2 : 5. In this case the activity is expressed as a function of membrane protein, as for previous preparations, and as a function of membrane - lipid phosphorus. The microsomal enzyme activity is 38% of that for synaptic membranes, when expressed as a function of protein concentration and is 26% of that

of synaptic membranes when expressed as a function of membrane phospholipid.

DISCUSSION

The induction of immature hypothyroidism in rats by daily injections of the goitrogen propyl-thiouracil, clearly reduced the rate of body and brain growth (Figures 2 : 2 and 2 : 3). This was similar to that reported previously, where other methods were used to induce hypothyroidism (Eayrs & Taylor, 1951; Balazs et al, 1968). The reduction in body weight was differentially greater than that of brain weight, as the brain weight/body weight ratio for hypothyroid animals was consistantly higher than that of controls following the 10th postnatal day (Figure 2 : 4). However, it has been suggested that the lower rate of growth of body weight is caused by a reduction in growth hormone production in hypothyroidism whereas the lack of growth hormone alone had no effect on brain development in rats (Diamond, 1968; Rebiere & Legrand, 1970). This suggests that the direct effect of hypothyroidism is primarily in reducing the rate of brain development. The data presented in Figures 2 : 2 and 2 : 3 shows that the hypothyroid condition produced in the rats used in this study compares favourably with that reported previously.

The synaptic structures isolated as synaptosomes from nervous tissue have been shown to undergo a process of "maturation", characterised by an increase in the specific activities of marker enzymes present in the synaptosome fraction (Abdel-Latif et al, 1967, 1970). The development of synaptic structures from hypothyroid and control rat brain have been evaluated, in this study, by estimating the specific activities of marker enzymes in preparations of synaptic membranes from the brain tissue of rats at different ages. It was found that sufficient material could be extracted from the brains of rats of 10 - 11 days of age and over.

The specific activity of the $Na^+ - K^+$ ATPase of synaptic membranes from cerebellar and cerebral tissue from control animals increased about two-fold between the 11th and 44th postnatal days (Tables 2 : 2 and 2 : 3) when adult values of 80 - 90 μ moles Pi/mg protein/hr were reached. An increase in the $Na^+ - K^+$ ATPase activity with development has been reported for rat brain synaptosome preparations (Abdel-Latif et al, 1967), however, in this case, activity only reached a maximum value of 13 μ moles Pi/mg protein/hr by the 20th day of life, whereas the activity of this enzyme in cerebral and cerebellar synaptic membranes only reached adult values by the 44th postnatal day (Figures 2 : 5 and 2 : 6). This discrepancy could result from a developmental increase in proteins, which, although present in synaptosomes are not present in synaptic membrane preparations. Such a change would tend to mask true increases in the specific activity of synaptic membrane $Na^+ - K^+$ ATPase.

It can be seen from Tables 2 : 2 and 2 : 3 that the specific activity of the synaptic membrane $Na^+ - K^+$ ATPase from cerebral cortex was slightly lower than that of cerebellum. The techniques used to extract the membranes and assay enzyme activity were identical, thus it is unlikely that this difference was an artifact of preparation. In consequence the higher activity could result from either an increase in the number of $Na^+ - K^+$ ATPase units in cerebellar synaptic membranes and/or a lower level of other proteins in cerebellar synaptic membranes.

In both cerebral and cerebellar tissue from control rats the specific activity of the synaptic membrane $Ca^{2+} - Mg^{2+}$ ATPase increased between the 10th and 44th day of gestation (Tables 2 : 2 and 2 : 3). The activities developed by 44 days of age

(11 - 12 μ moles Pi/mg protein/hr) were similar to those reported for adult rat synaptic membranes by Duncan (1976) and Tirri et al. (1976). This confirms that the enzyme composition of rat synaptic membranes changes with development, and suggests that synaptic membranes are enriched in Ca^{2+} - Mg^{2+} ATPase as well as Na^{+} - K^{+} ATPase enzyme molecules.

As in the case of the Na^{+} - K^{+} ATPase data, the Ca^{2+} - Mg^{2+} ATPase activity from cerebellar synaptic membranes was slightly higher than for cerebral synaptic membranes. As with the Na^{+} - K^{+} ATPase, this could be owing to a larger number of Ca^{2+} - Mg^{2+} ATPase units in cerebellar synaptic membranes and/or to a lower level of other membrane proteins.

The specific activity of the cerebral synaptic membrane acetylcholinesterase increased from about 8 μ moles thiocholine/mg protein/hr on day 11 to about 25 μ moles thiocholine/mg protein/hr on day 44 (Table 2 : 2). The latter value is similar to that reported for adult rat cerebral cortex synaptic membranes by Goodkin & Howard (1974). The specific activity of the cerebellar synaptic membrane acetylcholinesterase also increased during this period, but from about 8 μ moles thiocholine/mg protein/hr to about 15 μ moles thiocholine/mg protein/hr (Table 2 : 3). This suggests that as with the cation activated ATPases, the number of acetylcholinesterase molecules in synaptic membranes increases faster than other membrane proteins, during brain development.

The observation that the specific activity of acetylcholinesterase from control rat cerebellar synaptic membranes was lower than that from cerebral membranes was different from the case for newborn guinea-pig brain (Tables 1 : 6 and 1 : 7). The

activity of acetylcholinesterase from guinea-pig cerebellar synaptic membranes was slightly higher than for control membranes. It is difficult to resolve these differences as these data, and data for enzyme levels in homogenates of guinea-pig brain suggested a higher proportion of cholinergic neurons in cerebellum than in cerebrum, whereas results from rat brain suggest a lower proportion of these neurons in rat cerebellum.

It is clear from Figures 2 : 11 and 2 : 13 that in preparations from control animals, each enzyme exhibited a characteristic time-course of development. The increase in $Na^+ - K^+$ ATPase activity for both cerebral and cerebellar membranes was curvilinear throughout the period studied, unlike that shown by Abdel-Latif et al (1967) for whole synaptosomes. This difference may be explained, as previously, in terms of the heterogeneous composition of whole synaptosomes. The time-course of development of synaptic membrane $Ca^{2+} - Mg^{2+}$ ATPase from cerebral cortex was similar to that for cerebellum, for control animals. This activity showed a lag in development between 11 and 23 days, followed by a sharp increase to adult activity by 30 days of age.

The time-course of the increase in synaptic membrane acetylcholinesterase activity for cerebral cortex was similar to that for cerebellum (Figures 2 : 11 and 2 : 13). In both cases there was a lag in the increase in activity between 11 and 23 days followed by a sharp increase between 23 and 44 days of age. However, the relative rate of increase of acetylcholinesterase after the 23rd day was less than that for $Ca^{2+} - Mg^{2+}$ ATPase and this suggests that the development of cholinergic neurons, as defined by the increase in acetycholinesterase specific activity, is initiated at a

later time than the development of nerve cells in general. However, the possibility remains that the ion activated enzymes increase in activity prior to any increase in the levels of the enzymes of neurotransmitter metabolism.

The data shown in Figures 2 : 11 and 2 : 13 is different from the time course of the increase in acetylcholinesterase activity in whole synaptosomes (Abdel-Latif et al, 1970). This study showed that by 20 days of age, the activity of this enzyme in rat brain synaptosomes had reached adult values. However, the specific activity of the enzyme at the adult stage for synaptosomes (3 μ moles thiocholine/mg protein/hr) was only a small fraction of that obtained for synaptic membranes from adult rat brain (25 μ moles thiocholine/mg protein/hr). Changes with development of the large excess of protein present in synaptosomes could account, not only for the reduced specific activity but also could alter the time-course of the apparent increase in acetylcholinesterase with brain development.

The foregoing data have established the normal condition for the development of synaptic membrane enzymes, and have validated their use as subcellular markers. These data can be compared with similar data for hypothyroid rat brain. In this case the specific activity of cerebral and cerebellar synaptic membrane $Na^+ - K^+$ ATPase increased in development to a lesser extent than for control preparations such that the activity for hypothyroid rat brain was lower than controls after the 11th day (Tables 2 : 2 and 2 : 3, Figures 2 : 5 and 2 : 6). This effect was similar to that observed for $Na^+ - K^+$ ATPase activity in deoxycholate extractable membranes from 22 days old hypothyroid rat brain by Valcana &

Timiras (1969). However, this is in contrast to the work of Verity, Brown, Cheung, Huntsman & Smith (1976), who reported that the specific activity of the $Na^+ - K^+$ ATPase for whole rat brain synaptosomes from hypothyroid rats was not significantly different from that of control rats, when assayed at 14 and 30 days of age. Once again, this could be explained in terms of hetergeneous composition of synaptosomes. As well as synaptic membrane, these structures have been shown to contain mitochondria, synaptic vesicles, cytosol proteins including glycolytic enzymes, also microtubules and microfilaments (Jones 1975). This excess of protein not associated with synaptic membrane results in the low specific activity for $Na^+ - K^+$ ATPase (11 - 12 μ moles Pi/mg protein/hr) and for acetylcholinesterase (3 μ moles acetycholine/mg protein/hr) reported for synaptosomes, (Abdel-Latif et al, 1967; 1970; Verity et al, 1976) compared with that reported in this study for synaptic membranes (80 - 90 μ moles Pi/mg protein/hr and 25 μ moles thiocholine/mg protein/hr respectively) (Tables 2 : 2 and 2 : 3). Thus if changes in synaptosomal proteins could explain the differences between the time-course of development of enzyme activities in this study and that reported for synaptosomes, then this could also mask variations in enzyme activity due to hypothyroidism.

The time-course of the development of the $Na^+ - K^+$ ATPase of hypothyroid rat cerebral synaptic membranes was similar to that of control preparations (Figures 2 : 11 and 2 : 12) whereas the time-course of the development of this activity in cerebellar membranes from hypothyroid rat brains showed a lower relative rate of increase between 11 and 30 days of age than that observed for control preparations (Figures 2 : 13 and 2 : 14). Thus not only did the absolute value for

$Na^+ - K^+$ ATPase activity in the case of synaptic membranes from hypothyroid rat brain increase to a lesser extent than that for controls after 11 days of age, but in the case of cerebellar membranes from hypothyroid rats the increase in $Na^+ - K^+$ ATPase occurred at a later time in development.

The increase in the specific activity of the $Ca^{2+} - Mg^{2+}$ ATPase for synaptic membranes from hypothyroid rat brain was, as in the case of the $Na^+ - K^+$ ATPase, lower than that for control rat brain (Tables 2 : 2 and 2 : 3, Figures 2 : 7 and 2 : 8). This activity in cerebellar synaptic membranes was lower than for control brain by 11 days of age, whereas the activity for cerebral membranes from hypothyroid rat brain was lower than controls after day 11.

The time-course of the development of $Ca^{2+} - Mg^{2+}$ ATPase activity of synaptic membrane preparations from hypothyroid rat cerebellum and cerebrum were similar (Figures 2 : 12 and 2 : 14). Also the pattern of the increase of this activity in hypothyroid cerebellar membranes was similar to controls. However, it can be seen from Figure 2 : 12 that hypothyroidism slightly delays the onset of the rapid phase of the relative increase in $Ca^{2+} - Mg^{2+}$ ATPase activity in cerebral synaptic membranes.

The specific activity of synaptic membrane acetylcholinesterase for hypothyroid rat brain, as with the ion activated ATPases, increased more slowly during postnatal development than for control preparations (Tables 2 : 2, 2 : 3, Figures 2 : 9 and 2 : 10). This was the case for synaptic membranes from both cerebral and cerebellar tissues. In both cases a slight reduction in acetylcholinesterase activity was already

apparent by 11 days of age. Figures 2 : 12 and 2 : 14 show that in hypothyroid rats the relative rate of increase in acetylcholinesterase specific activity was greater than that for controls between 23 and 30 days of age. This suggests that not only did the level of acetylcholinesterase in nerve endings from hypothyroid rats increase at a lower absolute rate but that the increase which did occur was confined to a shorter period of time in development and reached a threshold by 30 days of age instead of 44 days, in the case of controls.

The interpretation of the reduced rate of increase of these three enzymes with brain development in hypothyroid rats depends on the mechanism by which enzyme activity increases in normal rats. It has generally been assumed that the increase in marker enzyme specific activity is due to an increase in the level of marker enzyme protein over other membrane proteins, however, an alternative view is possible. This increase in activity could result from a reduction in other membrane proteins.

It has been shown that synaptic vesicles fuse with presynaptic membranes during the release of neurotransmitter and that synaptic plasma membrane is retrieved and recycled to the synaptic vesicle pool (Fried & Blaustein, 1978). Thus the composition of the synaptic membrane could alter during prolonged cycles of neurotransmitter release. In this case the increase in enzyme specific activity would simply be due to the replacement of synaptic membrane with membranes containing these enzymes and not other proteins. Thus the decrease in the specific activity of these enzymes in hypothyroid rat brain would not reflect decreased synthesis of enzyme proteins but a reduced rate of neurotransmitter release.

It would be possible to test this possibility by expressing the activity of a membrane marker enzyme as a function of membrane phospholipid rather than membrane protein, for synaptic and microsomal membranes. Microsomes do not undergo the membrane recycling process as do synaptic membranes, and the $Na^+ - K^+$ ATPase activity of microsomes has been shown to be lower than that of synaptic membranes (Bowler & Duncan, 1968; Tirri & Bowler, 1974). The microsomes of grey matter isolated under conditions which maintain synaptosomal, mitochondrial and nuclear integrity would originate from dendrites. Thus if the above scheme is true then the phospholipid specific activity of the $Na^+ - K^+$ ATPase from microsomes and synaptic membranes would be similar. However, data presented in Table 2 : 5 shows that the protein specific activity of $Na^+ - K^+$ ATPase from microsomes (29.6 μ moles Pi/mg protein/hr) was lower than from synaptic membranes. (78.8 μ moles Pi/ μg phospholipid/hr) was also lower than for synaptic membranes (42.0 μ moles Pi/μg phospholipid/hr).

The activity per μmole of phospholipid for microsomes

Thus the increase in synaptic membrane enzymes does reflect an increase in the density of enzyme molecules on a given "patch" of membrane. This is confirmed by the observation that the percentage increase in enzyme activity in synaptic membranes from control animals between the 10th day and 44th day of life, is 2.1 fold for the $Na^+ - K^+$ ATPase, 1.4 fold for the $Ca^{2+} - Mg^{2+}$ ATPase and 2.0 - 3.0 fold for the acetylcholinesterase. If the normal developmental increase in enzyme activity was owing to a decrease in general membrane protein then the percentage increase between the 10th and 44th day would have been the same in each case.

Yet another possibility remains to account for the lower rate

of increase of enzyme activity in hypothyroid rat brain. Data presented in Figures 2 : 15 and 2 : 17 show that when $Na^+ - K^+$ ATPase is assayed in conventional media, about 40 - 50% of control cerebral synaptic membrane $Na^+ - K^+$ ATPase and 30 - 40% of control cerebellar synaptic membrane $Na^+ - K^+$ ATPase were "latent", hidden from substrate within membrane vesicles. This apparent activation of $Na^+ - K^+$ ATPase of these membranes, from both cerebral and cerebellar tissue, by S.D.S. treatment was similar at each stage of development (Tables 2 : 2, 2 : 3 and 2 : 4). Thus it was possible that the decreased specific activity of $Na^+ - K^+ -$ ATPase in hypothyroid rat brain membranes resulted from a difference in the number of occluded, latent active sites from that of control tissue.

Data presented in Figures 2 : 16 and 2 : 18 and in Table 2 : 4, shows that the percentage activation of $Na^+ - K^+$ ATPase, from hypothyroid rat brain synaptic membranes by S.D.S. treatment was similar to that for control membranes for both brain regions. Thus if the time course of development of $Na^+ - K^+$ ATPase from hypothyroid and control preparations is plotted using the maximum values obtained by S.D.S. treatment, it can be seen that the pattern of development of enzyme activity obtained (Figures 2 : 19 and 2 : 20) was similar to that for crude membrane preparations (Figures 2 : 5 and 2 : 6). Thus a change in the physical state of membranes in hypothyroid rat brain cannot account for the depressed activities of membrane enzymes in these animals.

From conclusions drawn in the previous section it is likely that the increase in synaptic membrane enzyme activity during brain development in normal rats results from an increase in

the density of enzyme molecules, those used in this study and perhaps others relevant to synaptic function, on synaptic membranes. This could result from a change in the enzymology of existing synapses or from the replacement of immature rat brain synapses, having low enzyme activity, with a mature form of synapse, having higher levels of these enzymes. In either case it can be assumed that the higher levels of these enzymes would be essential for normal adult brain function. If this was true then one would also expect that the markedly reduced levels of these membrane enzymes in hypothyroid rat brain would suggest that synaptic function was impaired by hypothyroidism.

Although this study has focused on enzymes which are indicative of synapse development in rat brain, it is not suggested that these represent the exclusive expression of the effect of thyroxine on rat brain development. Although it has been shown that a block to synaptic transmission could reduce the development of postsynaptic neurons (Black & G een, 1974), it is more likely that thyroxine broadly controls protein synthesis in developing rat brain and the marker enzymes measured in this study, along with general neuronal growth, are reduced by hypothyroidism. Nevertheless this does not detract from the potential effect of reduced synaptic membrane enzyme levels on the function of synapses, and the possibility remains that this effect of hypothyroidism represents a significant contribution to impaired brain function.

In conclusion, it is clear from these data presented here that it is not possible to discuss the consequences of impaired brain growth in hypothyroidism simply in terms of quantitative changes in neuronal circuitry, as these data have shown

significant inhibition of the qualitative development of neurons in hypothyroid rat brain.

Data for body and brain weights for control and hypothyroid rats.

Age (days)	Body weight (gms)				Brain weight (gms)			
	Control Animals	n	Hypothyroid Animals	n	Control Animals	n	Hypothyroid Animals	n
7	17.9 ± 0.2	3	19.4 ± 1.0	3	1.22 ± 0.01	3	1.12 ± 0.03	3
11	20.5 ± 1.8	9	16.2 ± 1.0	9	1.06 ± 0.03	9	1.02 ± 0.04	9
13	20.6 ± 0.4	4	15.1 ± 0.5	4	1.15 ± 0.05	4	1.05 ± 0.01	4
16	37.0 36.4		17.2 20.6		1.29 1.07		0.87 0.97	
23	61.3 ± 3.8	4	32.3 ± 2.8	4	1.42 ± 0.03	4	1.03 ± 0.03	4
25	58.5 ± 1.2	4	23.1 ± 0.7	6	1.51 ± 0.03	4	1.07 ± 0.03	6
30	72.1 ± 2.6	4	24.6 ± 0.5	4	1.59 ± 0.04	4	1.14 ± 0.01	4
36	92.8 ± 1.2	4	23.8 ± 1.0	5	1.59 ± 0.02	4	0.98 ± 0.04	5
38	117.4 113.3		27.2 ± 1.8	3	1.70 1.56		1.11 ± 0.02	3
44	151.3 ± 4.8	4	44.1 ± 3.6	7	1.76 ± 0.06	4	1.26 ± 0.04	7

Values expressed as ± 1 x S.E.

TABLE 2 : 2

Development of rat cerebral synaptic membrane enzyme activities in hypothyroid and control rats.

	$Na^+ + K^+$ ATPase *		$Ca^{2+} + Mg^{2+}$ ATPase *		Acetylcholinesterase +	
Age (days)	Control	Hypo-thyroid	Control	Hypo-thyroid	Control	Hypothyroid
11	43.8	30.3	9.9	7.0	9.6	7.1
	38.8	35.7	10.2	7.3	8.3	7.4
	44.9	37.4	8.5	7.4	7.6	6.6
23	64.4	50.6	10.7	7.4	10.1	9.1
	65.3	47.3	10.1	6.7	10.6	9.0
25	65.0	46.1	10.1	8.4	11.2	9.5
	67.4	45.9	11.5	8.5	12.9	10.0
30	67.7	51.0	11.3	9.1	21.8	12.7
	65.8	55.0	11.7	9.3	20.4	13.3
36	73.0	59.6	11.4	9.7	20.2	14.3
	76.7	57.0	10.9	9.1	21.6	14.9
44	82.2	63.5	11.3	9.0	24.4	13.2
	82.6	66.8	11.5	9.2	25.1	13.5

* Activities expressed as μ moles Pi, mg. protein $^{-1}$, hr $^{-1}$.

\+ Activities expressed as μ moles thiocholine, mg. protein^{-1}, hr^{-1}.

TABLE 2 : 3

Development of rat cerebellar synaptic membrane enzyme activities in hypothyroid and control rats.

Age (days)	Na^+ - K^+ ATPase *		Ca^{2+} - Mg^{2+} ATPase *		Acetylcholinesterase +	
	Control	Hypo-thyroid	Control	Hypo-thyroid	Control	Hypo-thyroid
11	41.8	37.3	7.8	5.8	8.6	7.2
	46.7	40.5	7.3	8.4	8.1	6.5
	45.8	41.7	8.8	7.0	7.8	5.7
23	75.4	48.1	8.3	7.3	8.4	6.2
	71.3	47.6	8.9	7.5	8.6	6.3
25	77.2	52.1	9.5	8.3	9.7	7.0
	71.5	47.7	9.3	8.6	10.8	7.6
30	84.3	50.9	11.8	9.8	12.8	10.0
	87.8	51.0	11.9	9.9	11.4	9.6
36	101.2	61.4	12.2	10.1	12.3	11.6
	91.6	65.2	11.5	9.3	12.5	10.4
44	95.7	63.8	11.7	9.5	15.0	9.9
	99.5	65.7	12.1	9.9	15.4	10.9

* Activities expressed as μ moles Pi, mg. protein^{-1}, hr^{-1}.

\+ Activities expressed as μ moles thiocholine, mg. protein^{-1}, hr^{-1}.

TABLE 2 : 4

Maximum activity of S.D.S. activated rat brain

$Na^+ - K^+$ ATPase

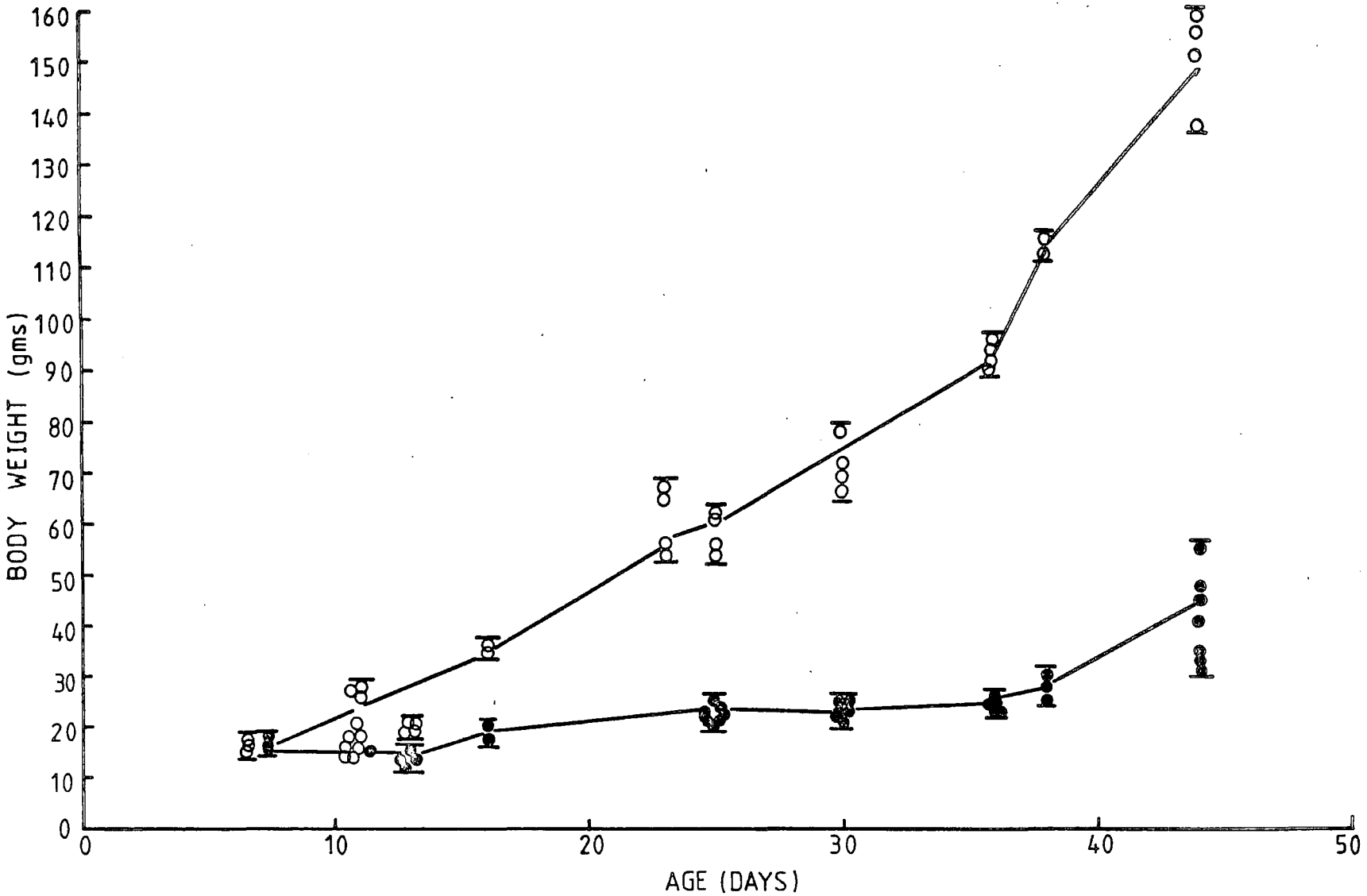
	CEREBRAL CORTEX		CEREBELLUM	
Age (Days)	Control Rats	Hypothyroid Rats	Control Rats	Hypothyroid Rats
11	75	60	63	52
	81	58	69	58
	85	56	74	59
23	119	89	95	57
	109	81	99	63
30	143	93	108	75
	136	90	111	80
36	152	109	140	84
	147	112	147	90
44	183	118	162	110
	179	121	175	112

Values for enzyme activity are expressed as μ moles Pi/mg. protein/hr.

TABLE 2 : 5

$Na^+ - K^+$ ATPase activities of adult rat brain microsomal and synaptic membrane preparations.

	$Na^+ - K^+$ ATPase activity		n
	μ moles Pi/mg. protein/hr	μ moles Pi/μg. phospholipid/hr	
Microsomal Membranes	29.6 ± 0.8	11.3 ± 0.4	6
Synaptic Membranes	78.8 ± 1.9	42.0 ± 1.7	6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10
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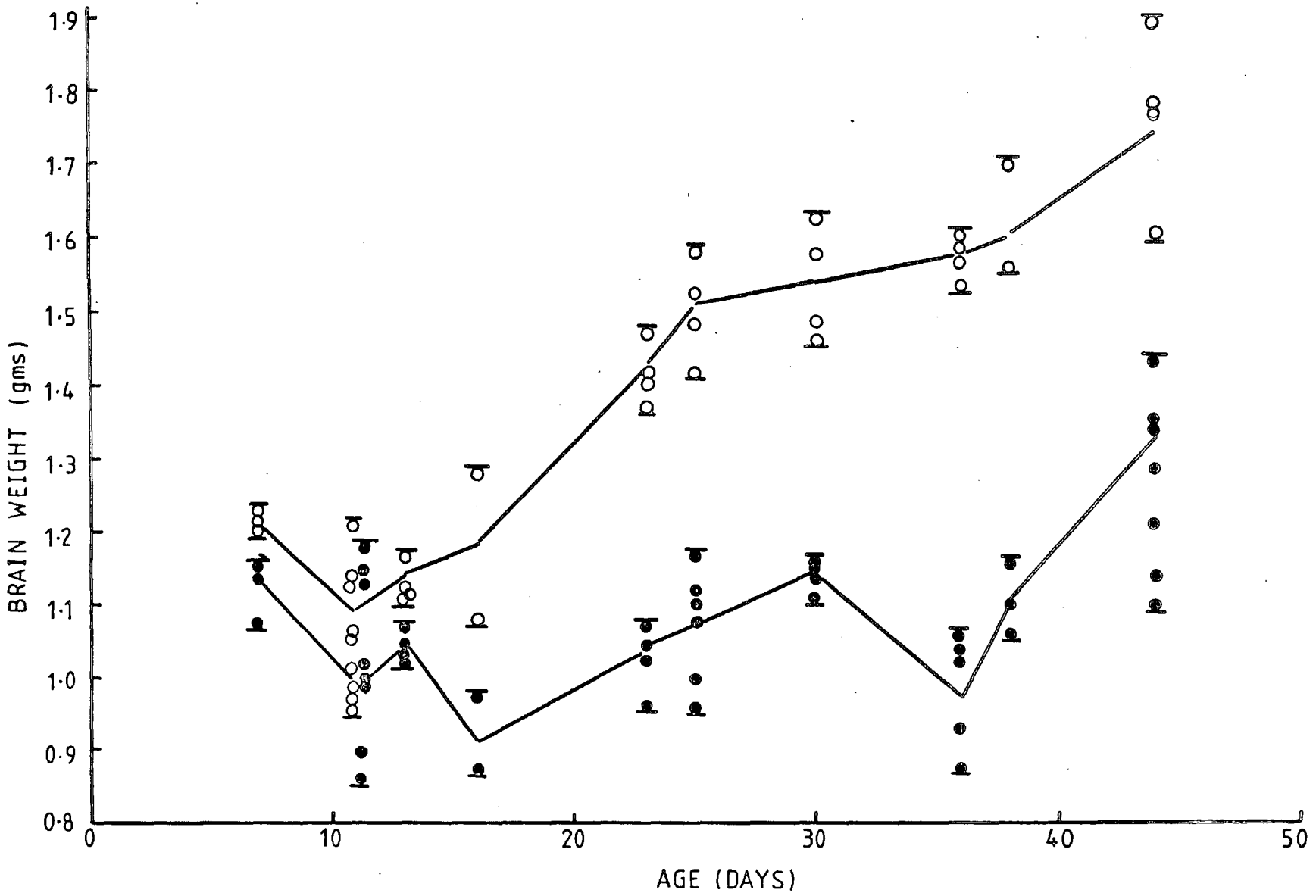
30
40
50
AGE (DAYS)


BRAIN WEIGHT (gms)
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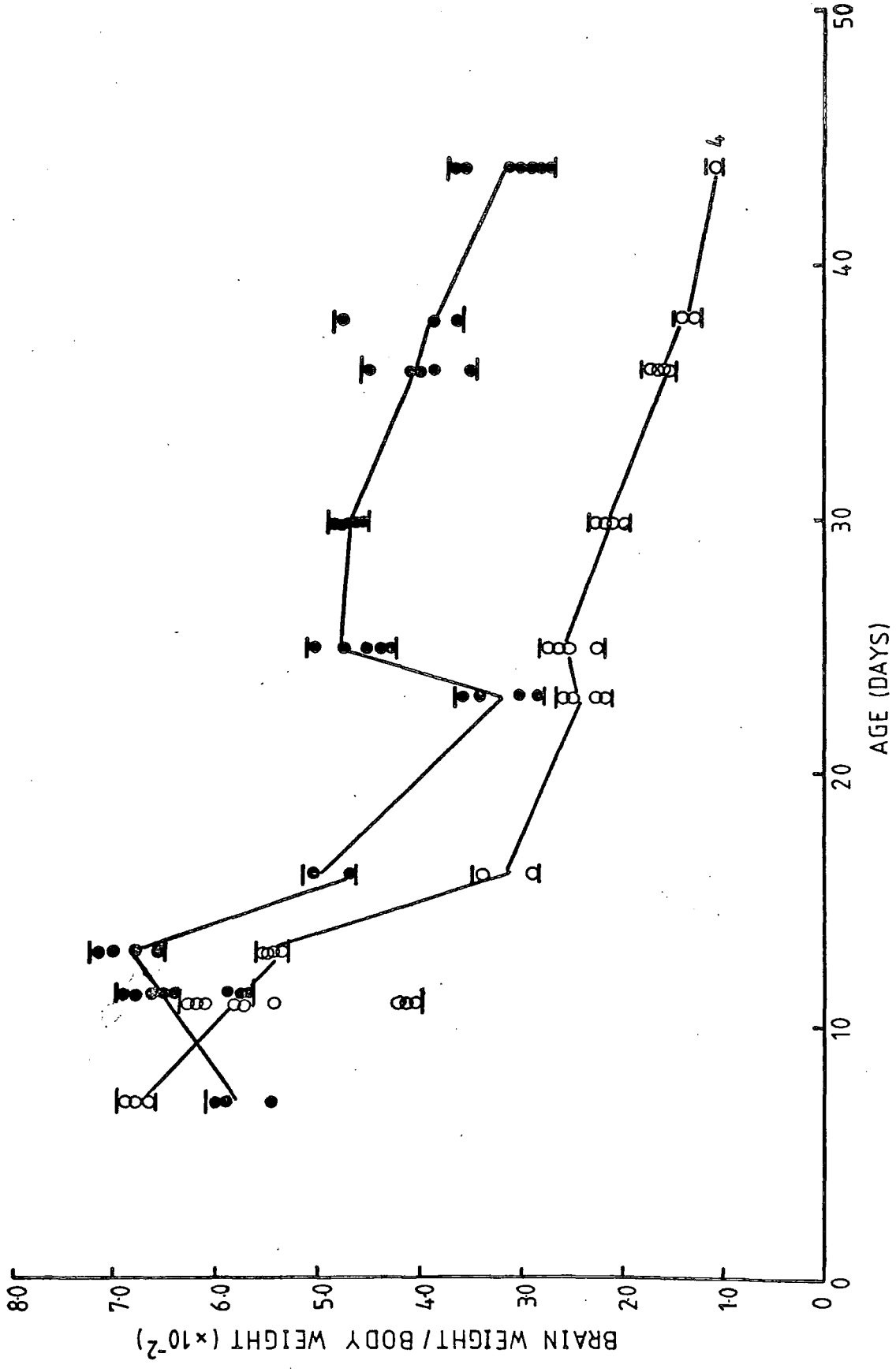
0·8
0
10
20
30
40
50
AGE (DAYS)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6·0
5·0
4·0
3·0
2·0
1·0
0
10
20
30
40
50
AGE (DAYS)

SPECIFIC ACTIVITY
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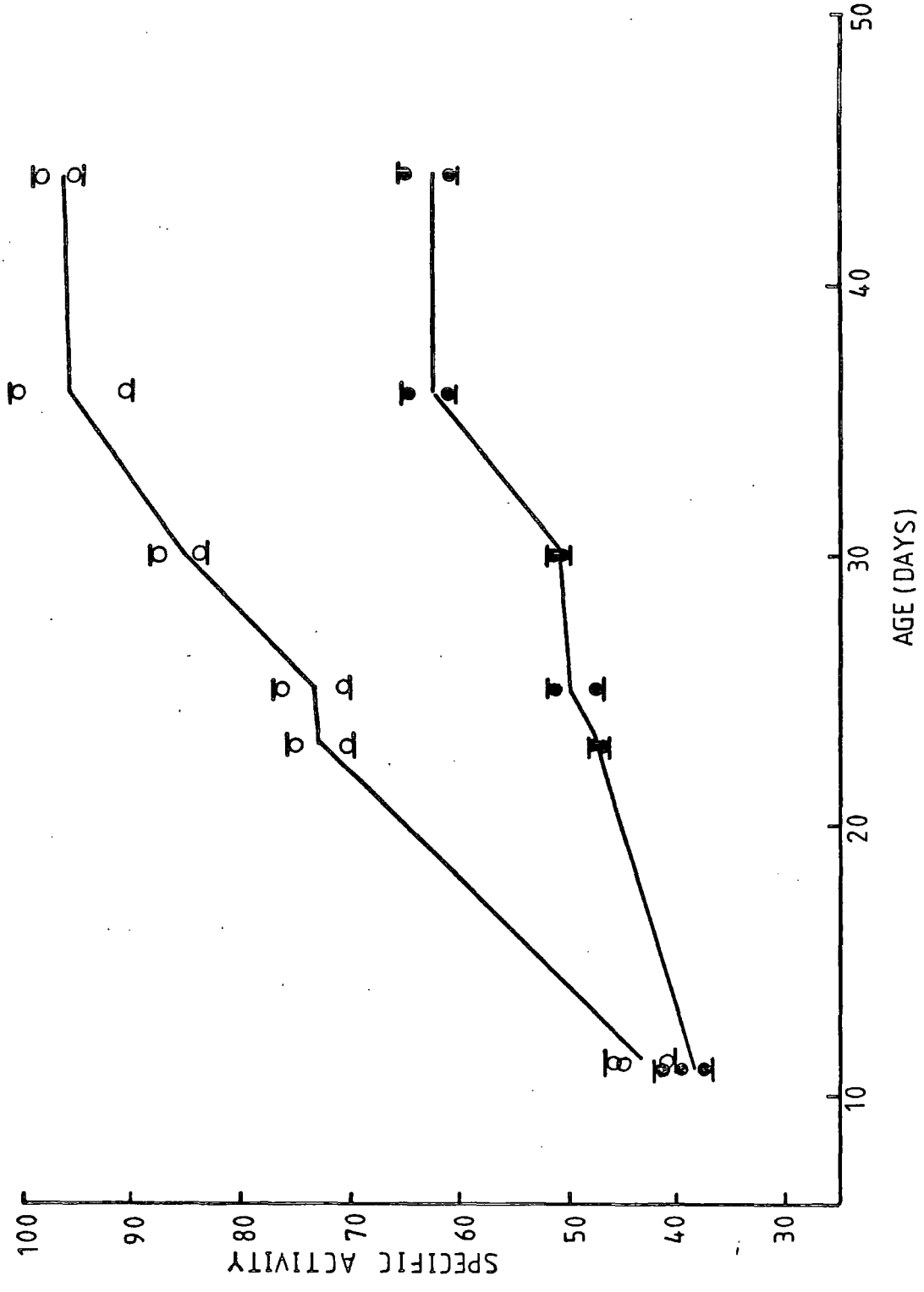
80
70
60
50
40
30
20
10
20
30
40
50
AGE (DAYS)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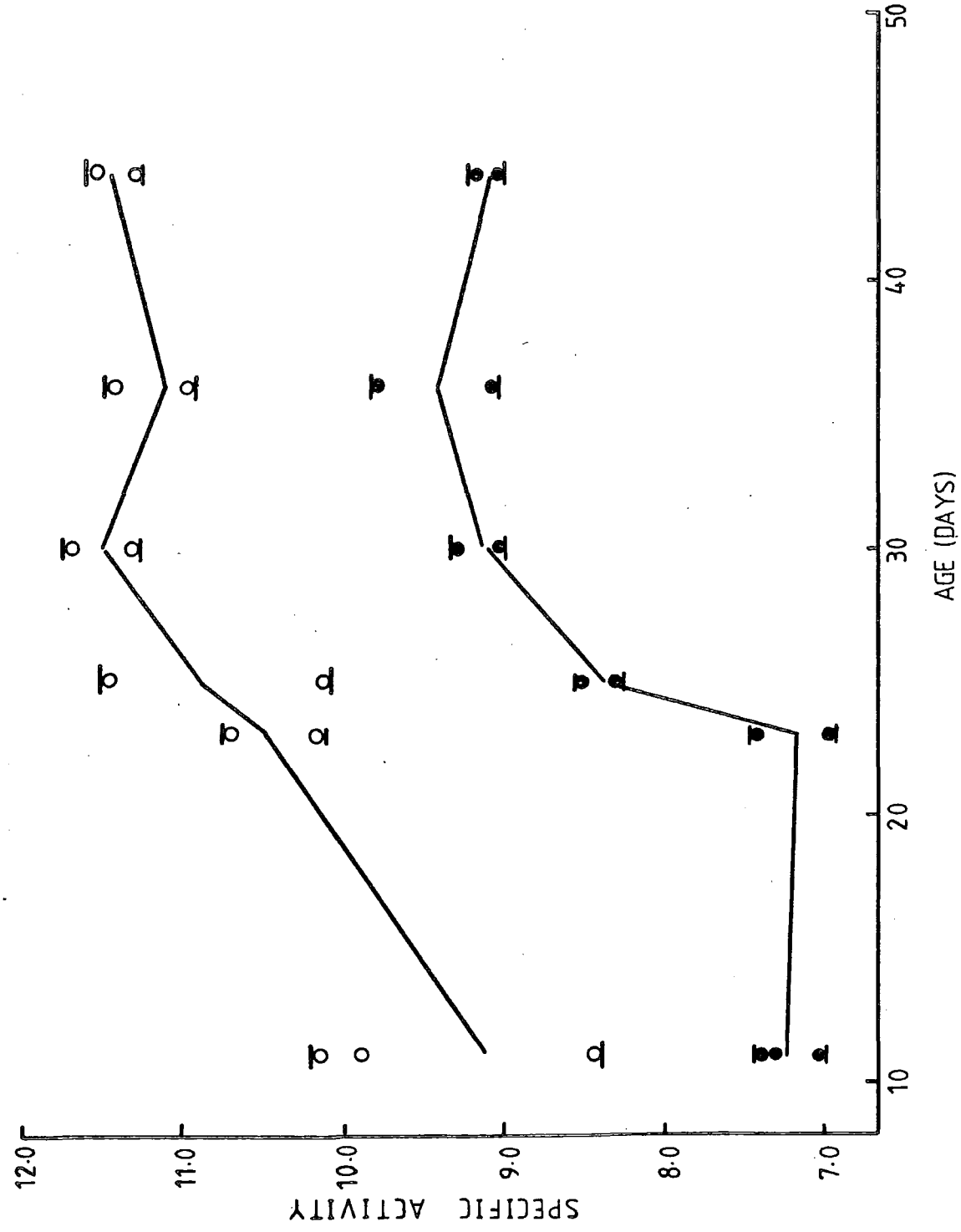
30
40
50
AGE (DAYS)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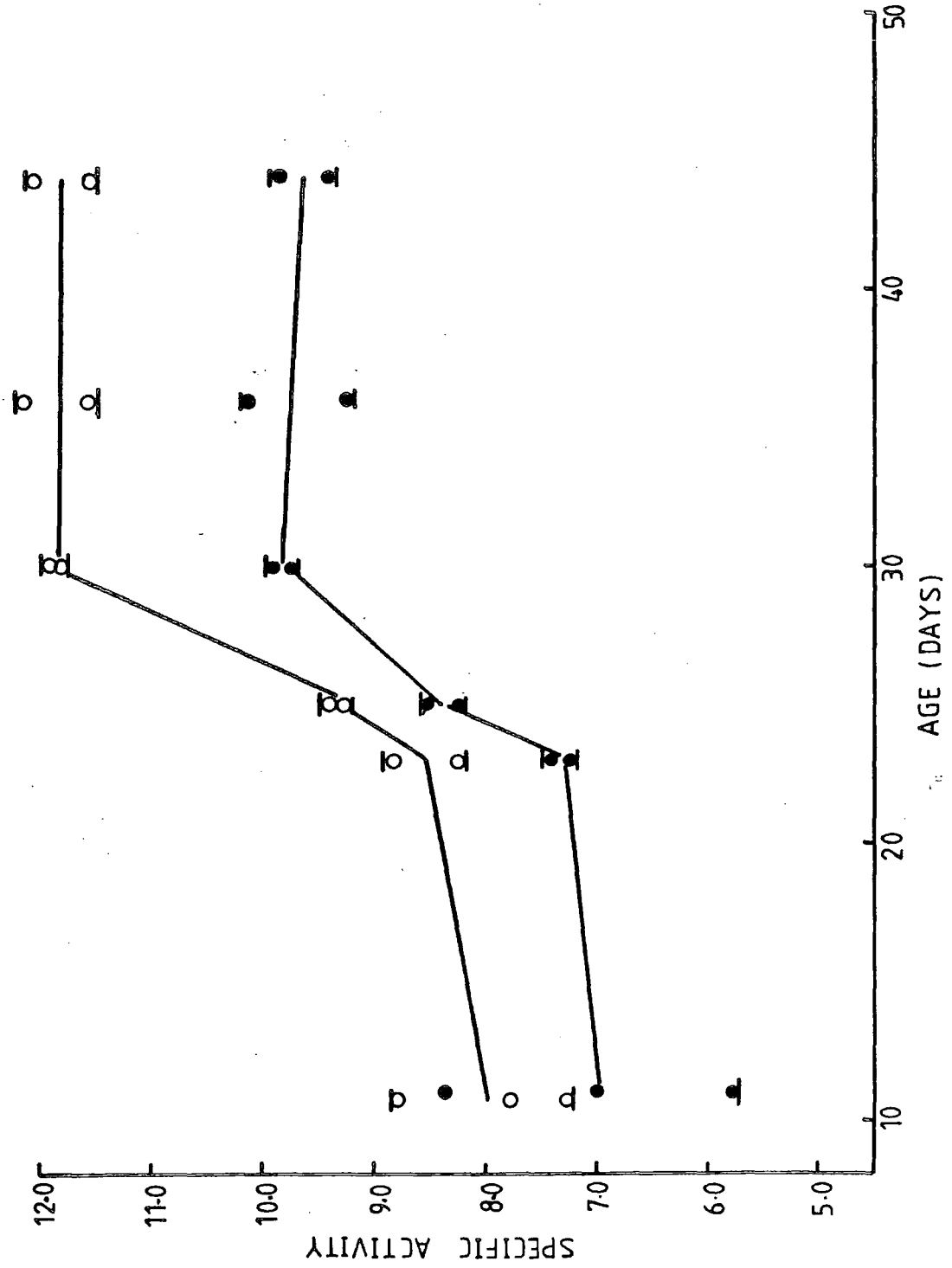
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10
20
30
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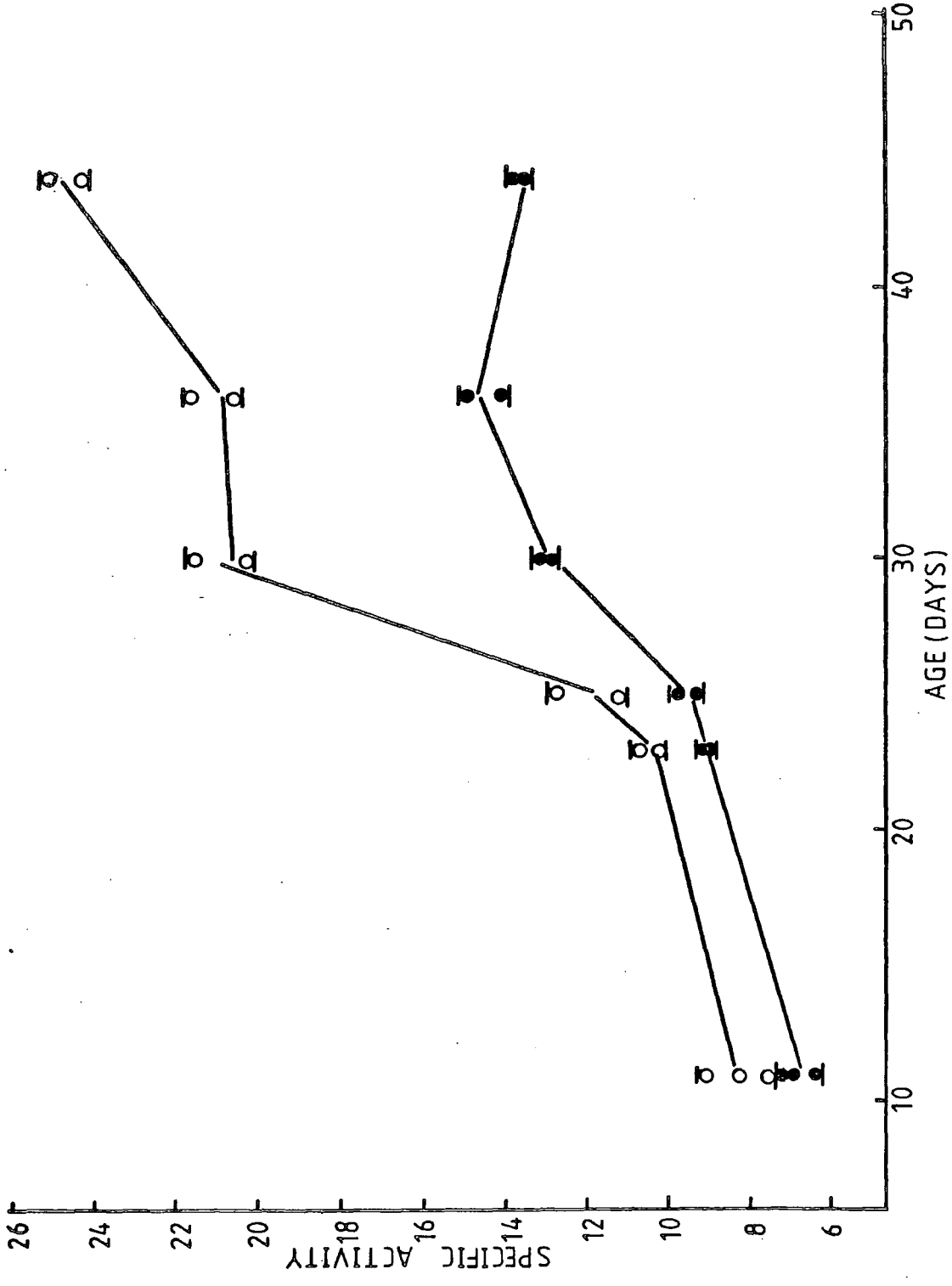
AGE (DAYS)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AGE (DAYS)
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8
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40
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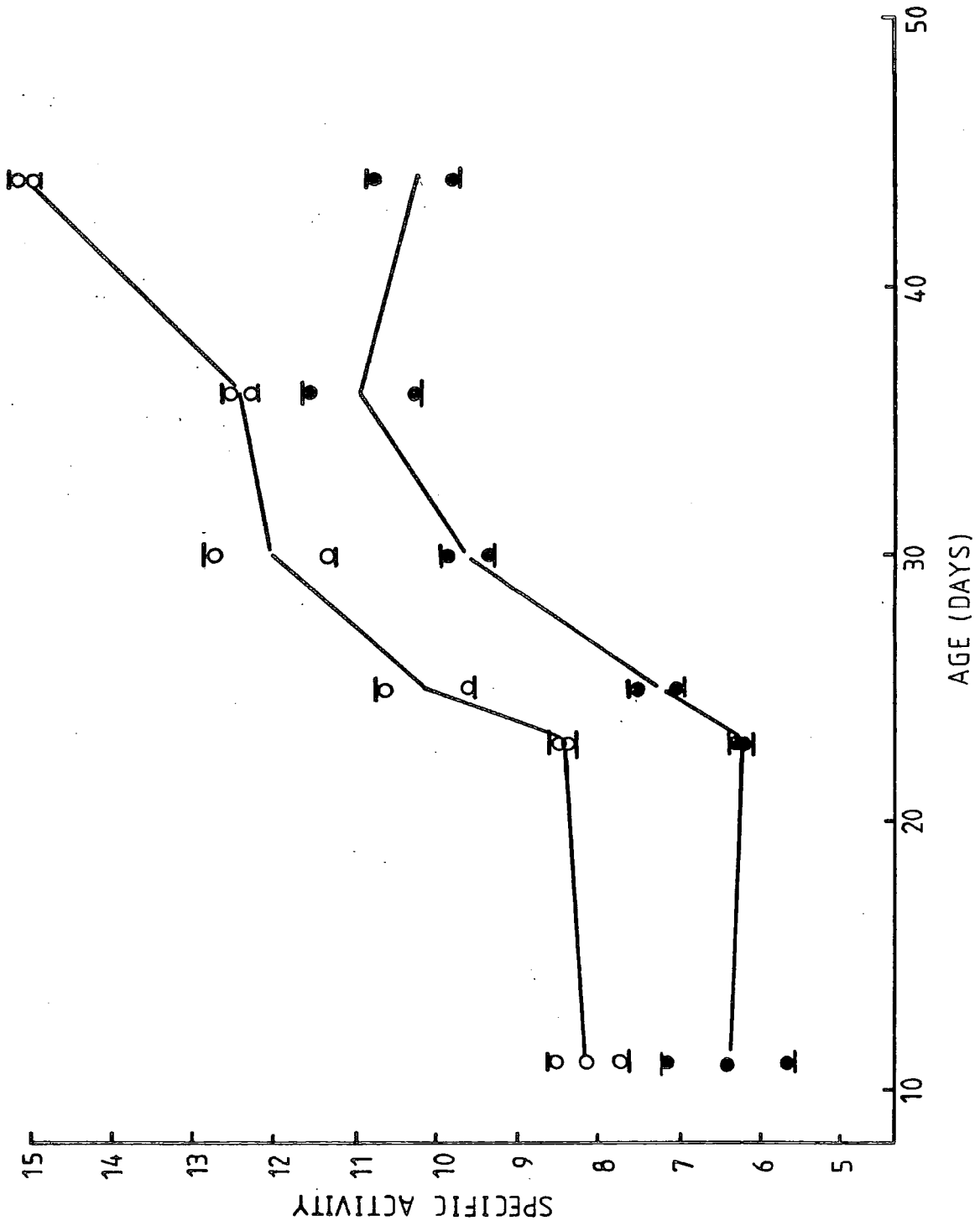
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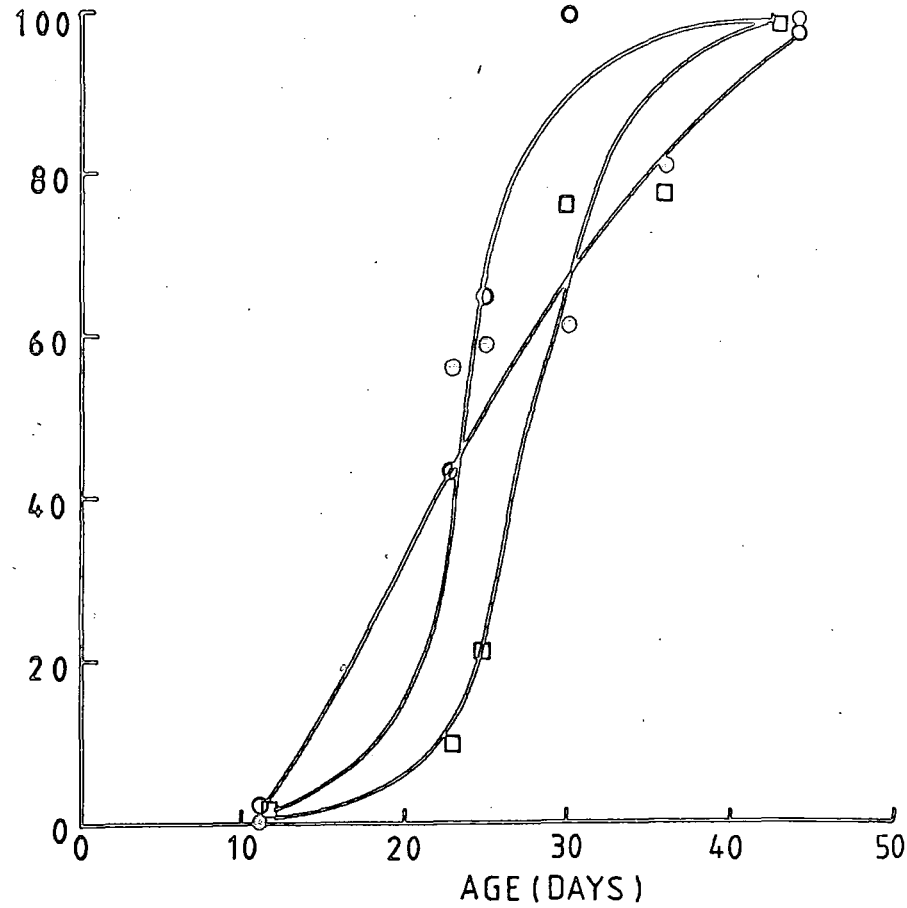
30
40
50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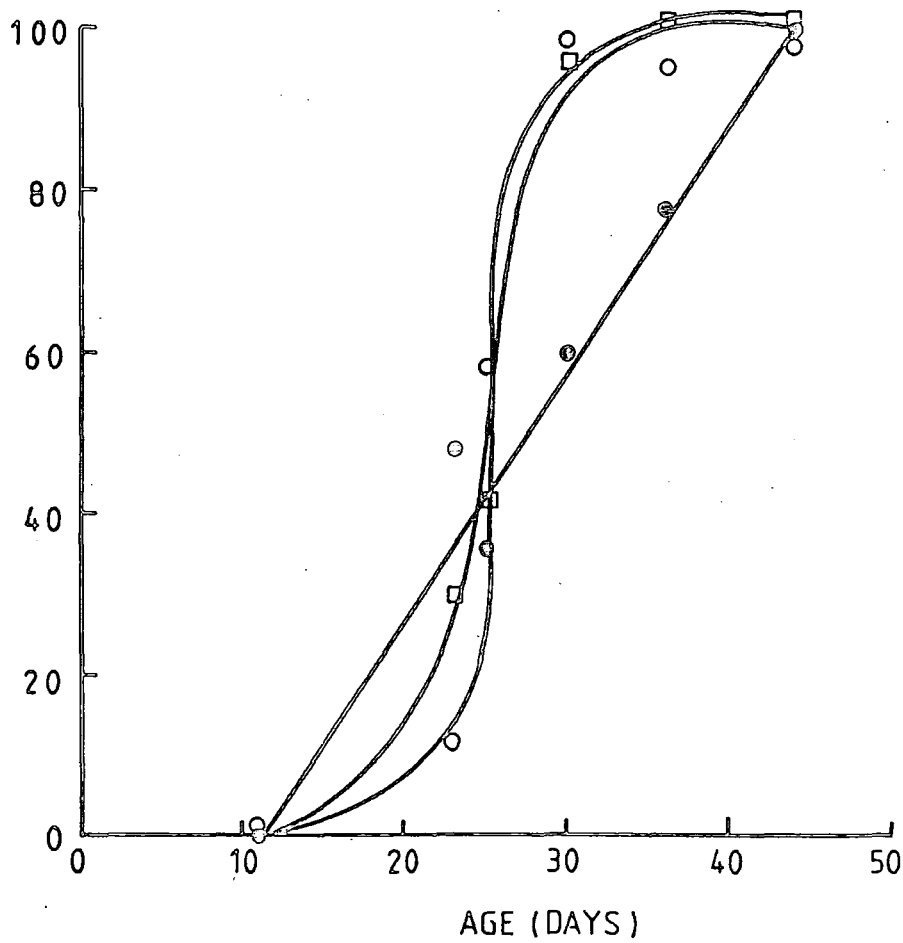
AGE (DAYS)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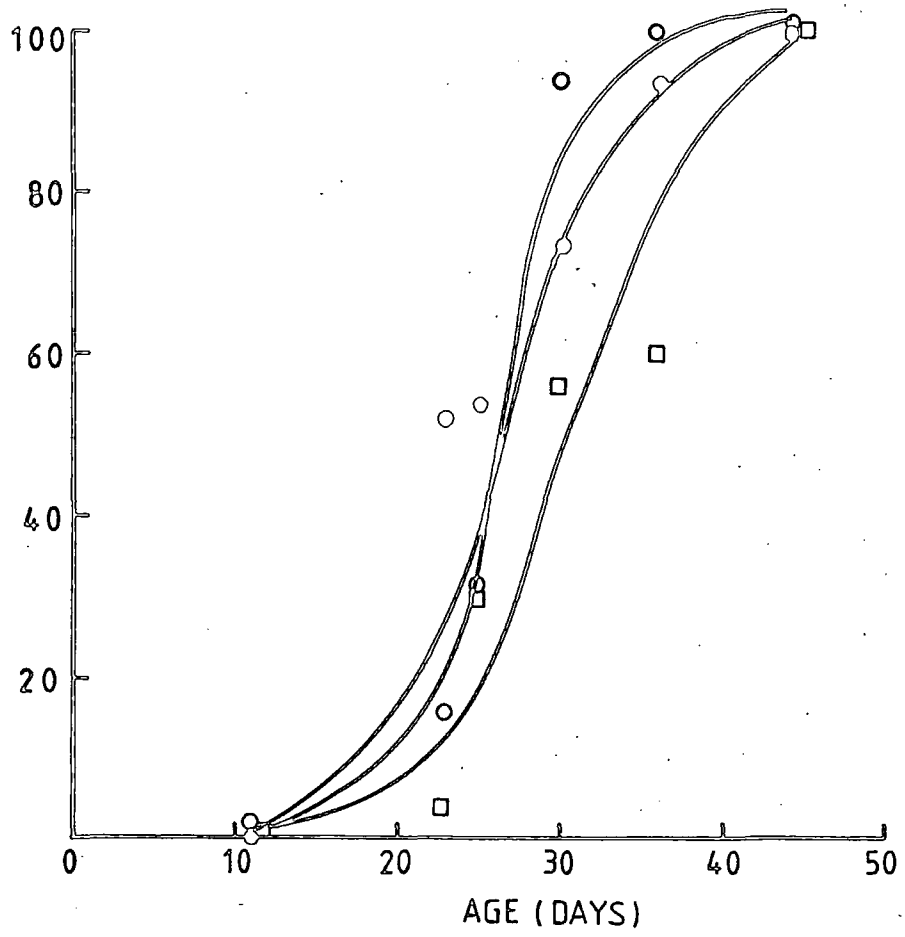
AGE (DAYS)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40
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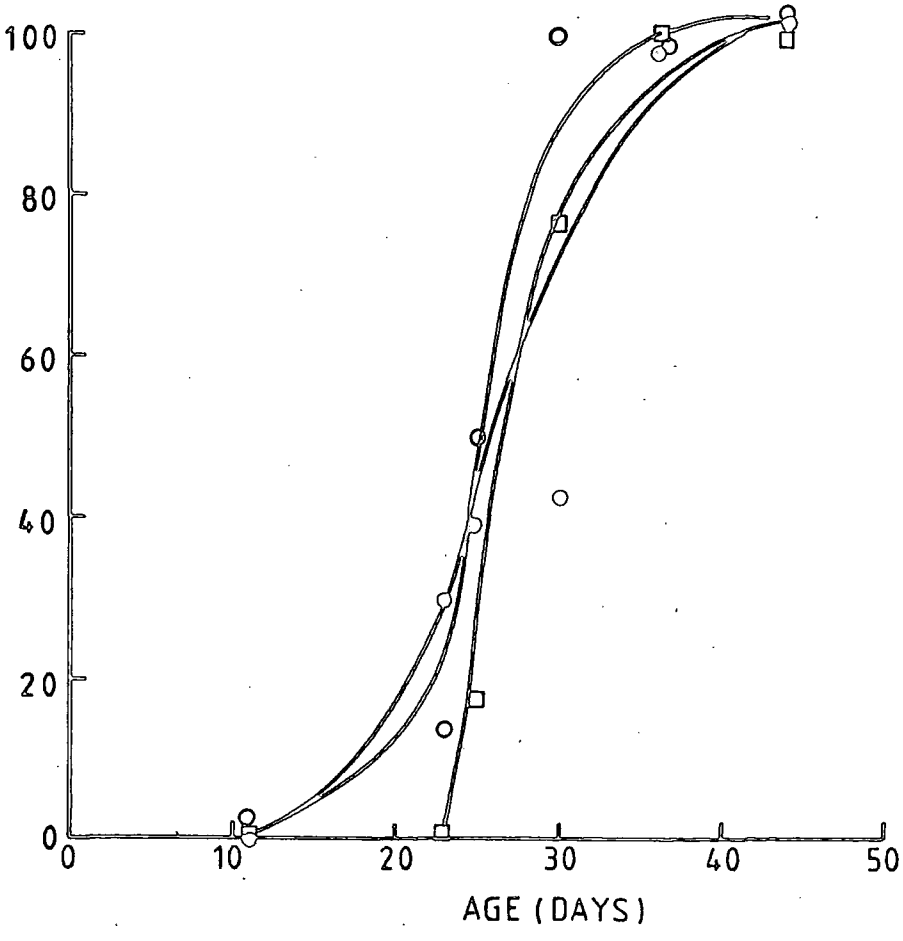
AGE (DAYS)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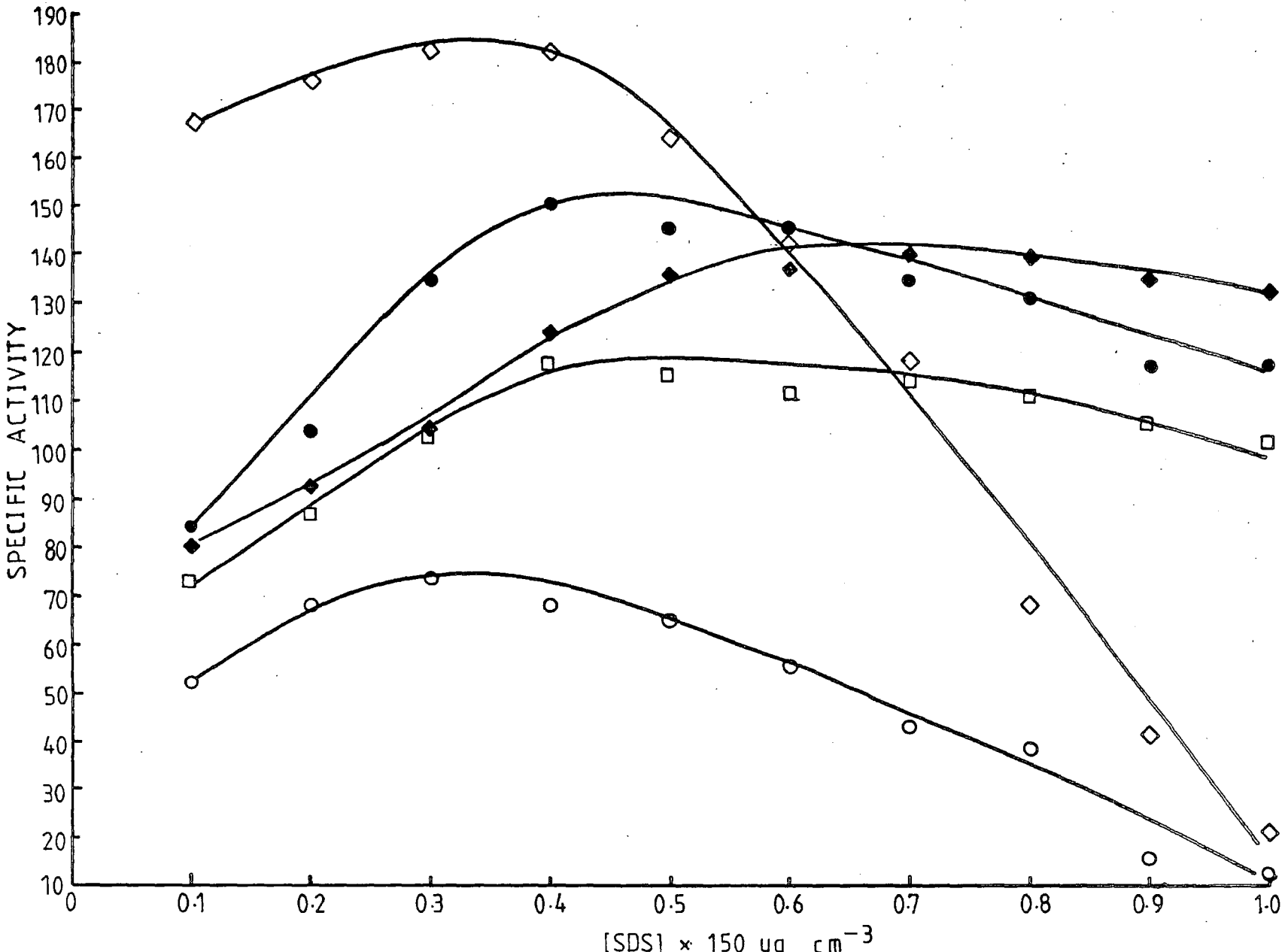
40
50
AGE (DAYS)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0·9
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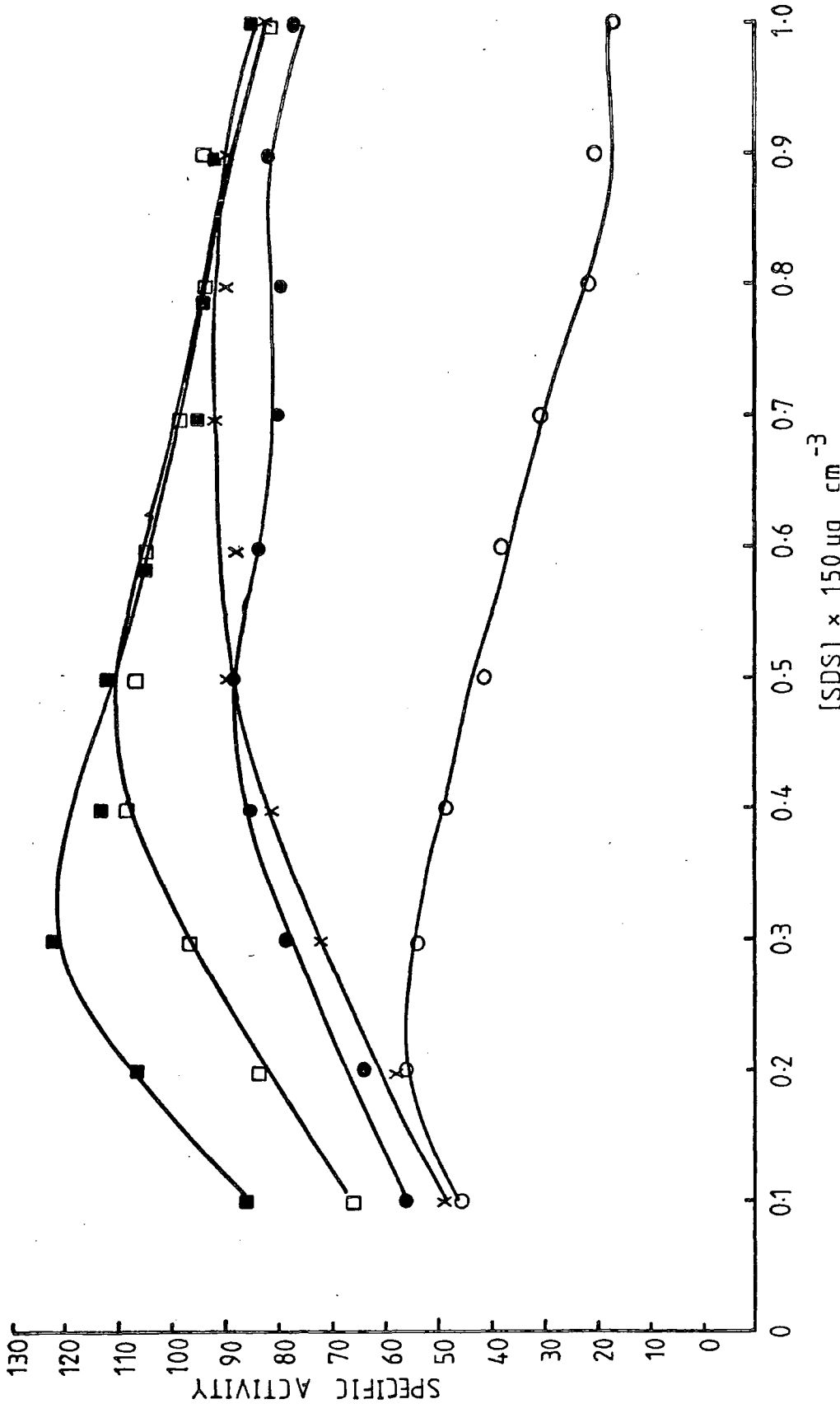
[SDS] × 150 ug cm^{-3}


SPECIFIC ACTIVITY
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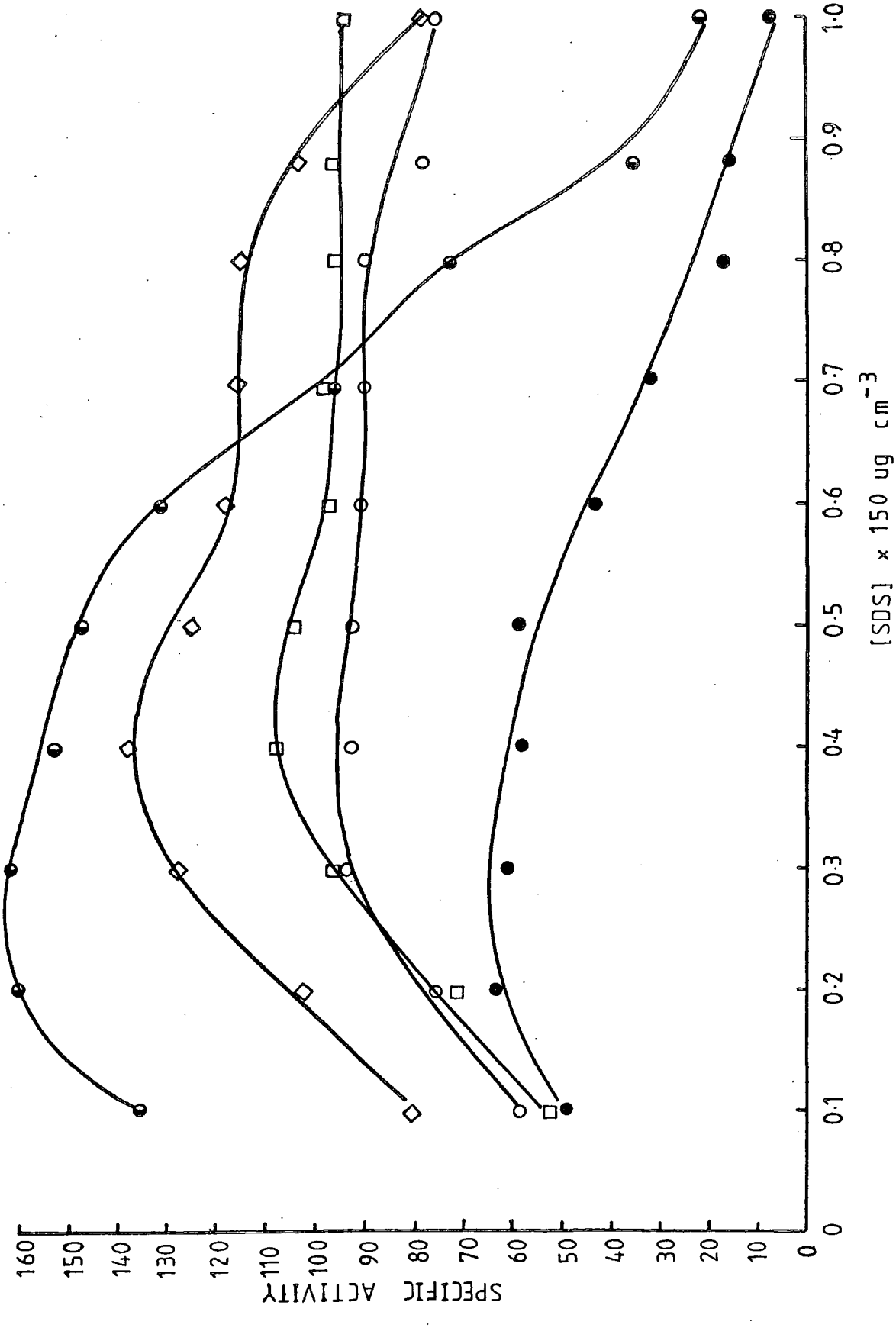
[SDS] x 150 μg cm^{-3}


SPECIFIC ACTIVITY
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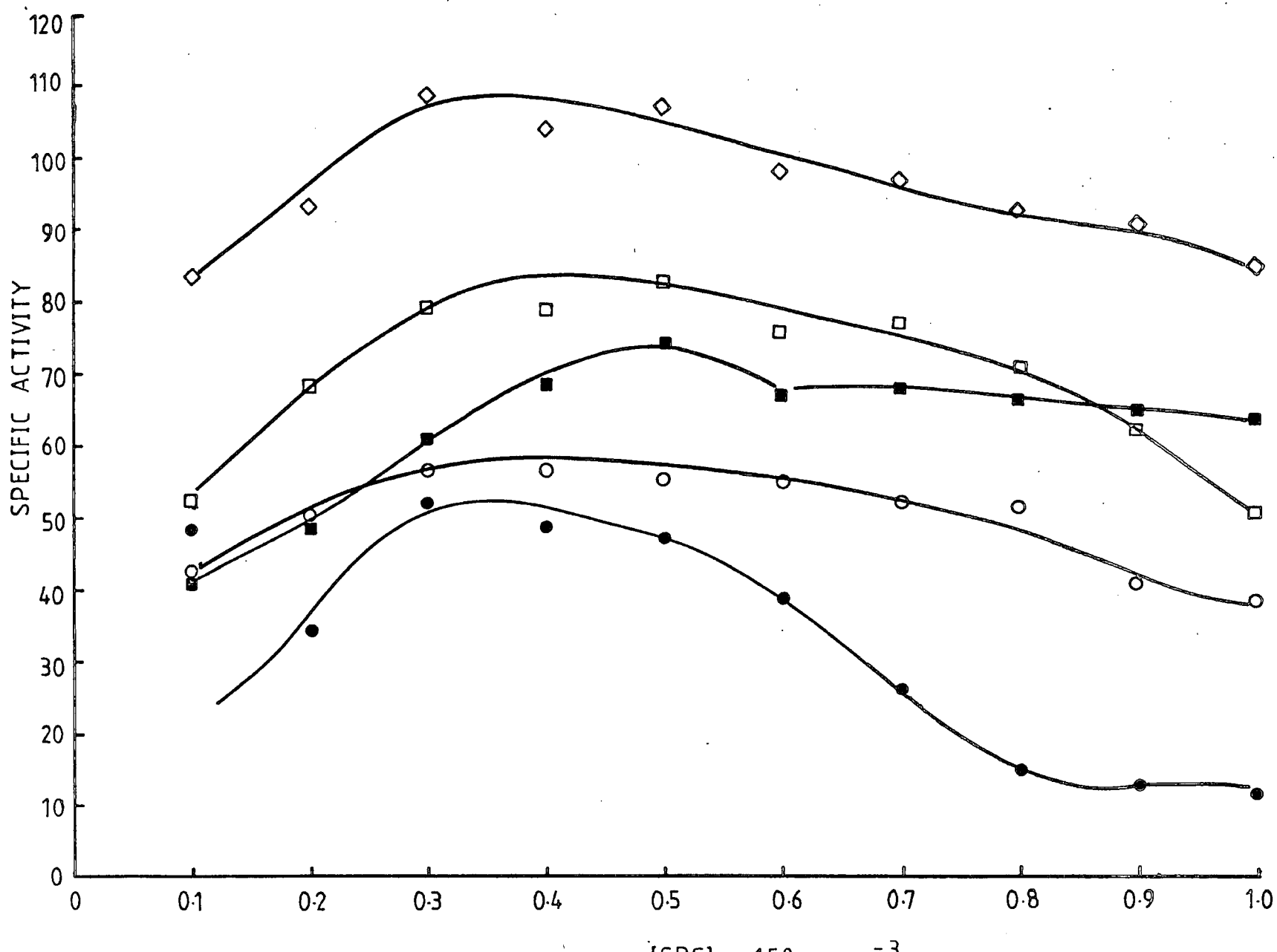
[SDS] x 150 ug cm^{-3}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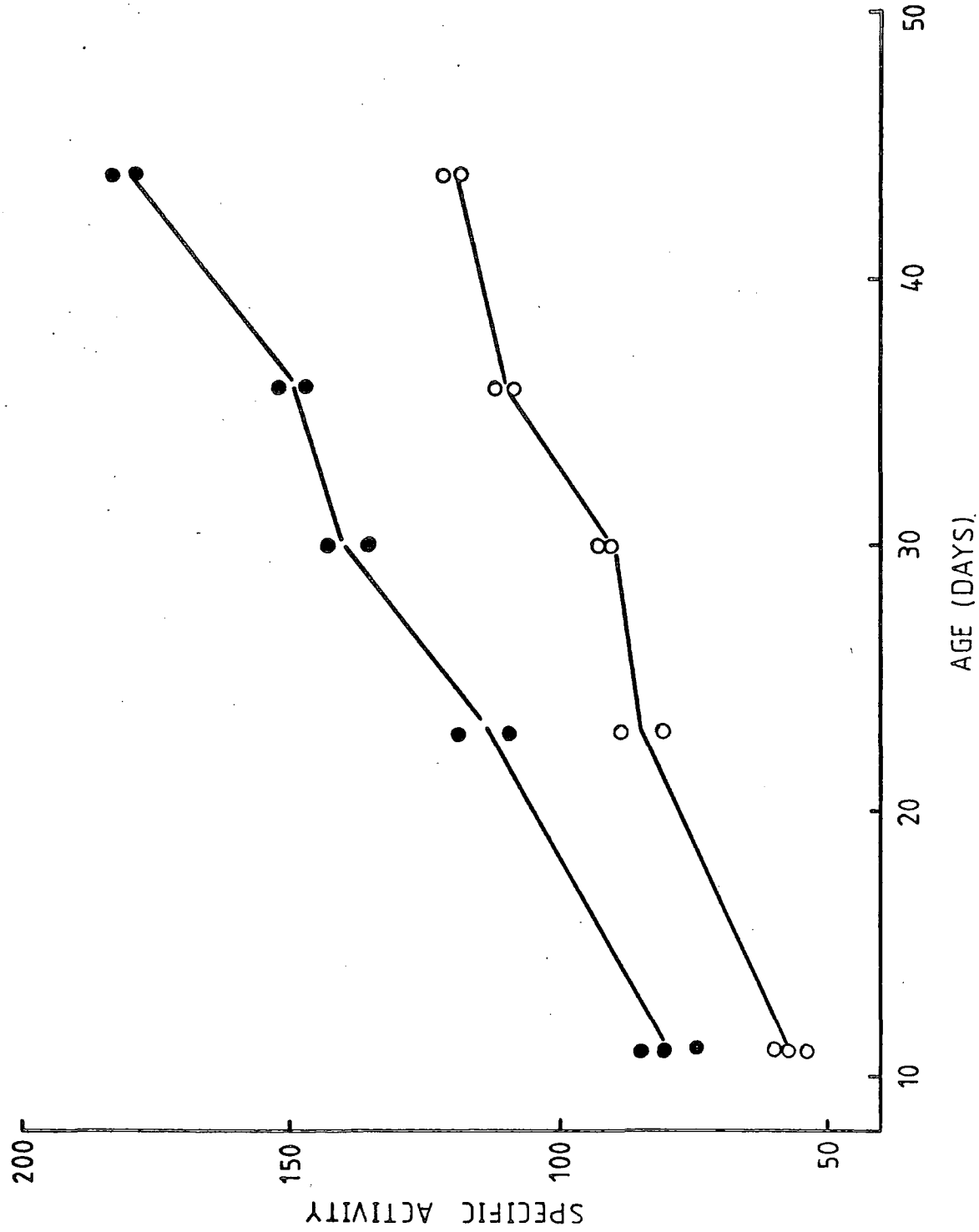
SPECIFIC ACTIVITY
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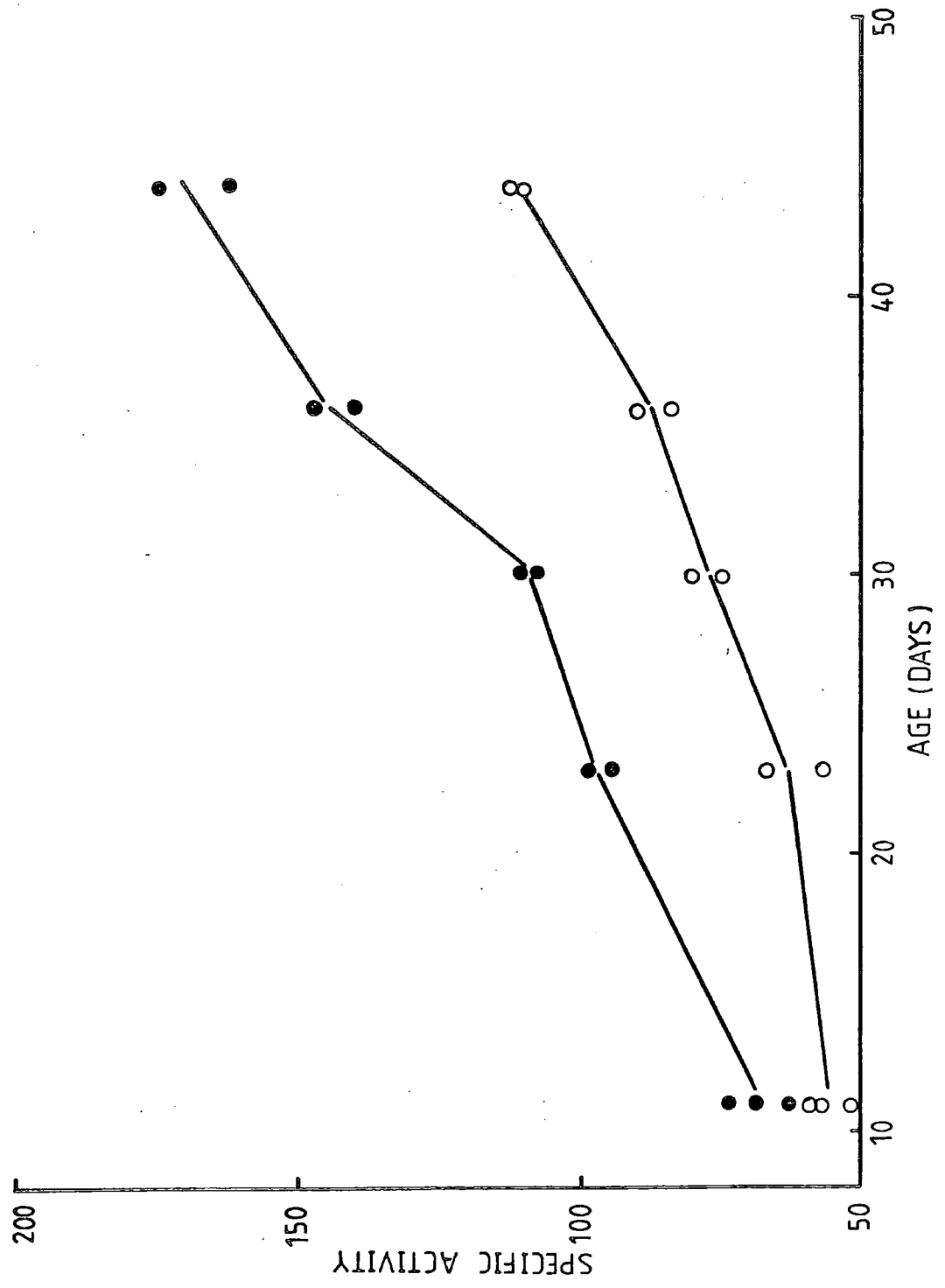
AGE (DAYS)


SPECIFIC ACTIVITY
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AGE (DAYS)

An analysis of the non-linear Arrhenius kinetics of the rat synaptic membrane acetylcholinesterase.

An analysis of the temperature dependence of the initial rate of an enzyme activity can provide information as to the enthalpy factor involved in the formation of the rate-limiting step of an enzyme mechanism. Thus the value for the activation energy of this rate-limiting step forms an important part of the characterization of a particular enzyme reaction mechanism.

Provided that a given enzyme activity is measured at saturating concentration of substrate, at optimal pH and in a temperature range at which the enzyme would be stable to irreversible thermal denaturation, then the temperature dependence of the enzyme activity may be analysed using the rate equation of Arrhenius (1889). This relationship, originally developed for gas phase collision theory by Arrhenius and Van't Hoff, has been applied to reaction mechanism modelled by transition state theory, and to many enzyme catalyced reactions.

In this case the rate of reaction is related to temperature by the relationship below:

$$\text{Enzyme Rate} = A.e^{\frac{-\mu}{RT}}$$

T = absolute temperature

A = proportionality constant

R = the gas constant

μ = apparent activation energy

In the case of an enzyme which obeys this relationship in an ideal fashion, the log of the rate would be linearly related to $\frac{1}{T}$ with a slope of $\frac{-\mu}{R}$. This constitutes a graphical method for representing the relationship between enzyme rate and temperature. Also when data are presented in this graphical form deviations

from ideal Arrhenius kinetics are more obvious than would be the case for other methods of graphical representation.

The value 'μ' calculated by this method does not necessarily correspond to the simple activation energy of the transition state of a bimolecular reaction. The reaction sequence of an enzyme will often involve several steps, comprising a series of transition states each with a characteristic enthalpy of activation. The μ value calculated for this reaction scheme will be the energy difference between the initial state, and that of the transition state of the rate limiting step in the reaction mechanism. This value is usually termed the "apparent activation energy" (Ea).

Most enzymes exhibit orthodox Arrhenius temperature kinetics. However, a number of enzymes have been shown to exhibit deviations from the ideal behaviour predicted by Arrhenius kinetics, in that the activity at low temperatures was lower than predicted (Charnock, Cook & Opit, 1971; Charnock, Doty & Russel, 1971; Hoare, 1971; Charnock, Cook, Almeid & To, 1973; Lacko, Wittke & Geck, 1973; Hsung, Huang, Hoy & Haug, 1974; Charnock, Almeida & To, 1975). As these enzymes do not display simple temperature dependence of activity then they cannot be modelled directly in terms of the Arrhenius rate equation.

A variety of kinetic models have been proposed to account for this non-linearity of the classical semi-logarithmic plot. However, no single model has been able to account for all cases of non-linear Arrhenius kinetics and it appears that several reasons may exist for this behaviour. A superficial examination of the nature of reported non-linear temperature plots may cast light on the reasons for this disharmony and suggest a course of action for the investigator wishing to

solve this type of problem.

The enzymes which have been shown to display non-linear Arrhenius plots fall into two main classes. First, non-linear plots have been described for a group of soluble enzymes. These plots show a small curvature across the normal temperature range of $0^{o}C$ to $40^{o}C$ with sharper curvature between $0^{o}C$ and $20^{o}C$. Examples of enzymes which have been shown to exhibit this behaviour are trypsin, sodium -α- glycerophosphatase and invertase (Kavanau, 1951). The second class of enzyme exhibiting this form of plot are membrane-bound enzymes and membrane transport processes. These processes display markedly non-linear temperature plots across the range from $0^{o}C$ to $40^{o}C$. Much attention has been directed towards the possible association between cold-sensitivity of enzymes and their association with biological membranes.

Some of the membrane processes which have been shown to exhibit this latter type of non-linear Arrhenius plot are; mitochondrial transport (Kemp, Groot & Rietsma, 1969; Lyons & Raison, 1970 (a) and (b); Raison, Lyons & Thompson, 1971; Raison, 1972; Lenaz, Sechi, Porenti-Castelli, Landi & Bertoli, 1972; Smith, 1973; McMurchie, Raison & Cairncross, 1973), cation transport (Weith, 1970), glucose transport (Sen & Widdas, 1962; Dawson & Widdas, 1964; Bolis, Luly, Pethica & Wilbrant, 1970; Lacko, Wittke & Geck, 1973) and amino-acid transport (Hoare, 1972). Also the Na^{+} - K^{+} ATPase (Greuner & Avi-Dor, 1966; Bowler & Duncan, 1968; Charnock, Cook & Opit, 1971; Charnock, Doty & Russell, 1971; Charnock, Cook, Almeida & To, 1973; Hsung, Huang, Hoy & Haug, 1974; Charnock, Almeida & To, 1975) and the Ca^{2+} - Mg^{2+} ATPase (Charnock & Frankel, 1971; Duncan, 1976; Tirri, Lahdekorpi & Bowler, 1976) are two of the membrane enzymes which have been shown to exhibit non-linear temperature kinetics of this second type.

Previously published work has described the non-linear Arrhenius plots of the former soluble group of enzymes in different terms from that of the latter membrane bound group. As the temperature plots of the soluble enzymes becomes markedly non-linear below 0°C, this effect has been attributed to the effect of the structure of water on proteins, (Kavanau, 1951) whereas the non-linearity observed for the membrane enzymes, which is marked between 0°C and 40°C has been said to be due to the effect of the membrane environment on these proteins. The ATPase enzymes, electron transport and transport proteins operate across the membrane bilayer. In consequence it is thought that such proteins have a large hydrophobic region which is in contact with the hydrophobic fatty acyl chains of the membrane phospholipids. This interaction has been said to render intrinsic membrane enzymes sensitive to the physical state of the membrane lipids, which is temperature dependent (Charnock, Cook & Opit, 1971; Kumamoto, Raison & Lyons, 1971; Charnock, Cook, Almeida & To, 1973; Barnett & Parlazzotto, 1974; Wyn-Williams, 1976; Boldyrev, Ruuge, Smirnova & Tabak, 1977). This does not aid in the interpretation of non-linear Arrhenius plots, however, as the normal application of the Arrhenius equation becomes invalid.

Several workers have attempted to fit the data from temperature measurements of intrinsic membrane-bound enzymes to theoretical models designed to account for non-linear Arrhenius plots. However, this has not resulted in a comprehensive model which can account for all forms of non-linear temperature plots. Hence it is essential to examine the fit of temperature data from an individual enzyme to each of the available models and to decide from this, which may be rejected. This does not necessarily provide for an unambiguous analysis, and eventually relies on

a subjective decision. However, a working model is useful as it allows comparison of temperature data from different enzymes, the analysis of membrane-bound enzymes in different lipid micro-environments, and analysis of the effect of external agents on a membrane-bound enzyme, when changes in the Arrhenius plot are not intuitively obvious.

Some of the models proposed to account for non-linear Arrhenius kinetics are summarised below;

(i) Crozier (1924, 1926) suggested that non-linear Arrhenius kinetics was due to a temperature-induced shift between consecutive reactions. These reactions would have distinct activation energies and the shift between these would occur at a specific "critical temperature". Belehradek (1957) showed that a shift between two simultaneously occurring processes would describe a catenary curve, without a sharp transition at a critical temperature.

(ii) Kavanau(1951) suggested that non-linear Arrhenius kinetics resulted from a reversible thermal inactivation of the enzyme. In this case the activity at a given temperature would depend on the equilibrium between the active form of the enzyme predominating at high temperatures, and the inactive form, predominating at low temperature.

(iii) Several workers have interpreted non-linear Arrhenius kinetics of membrane bound enzymes as consisting of two straight lines, intersecting at a transition temperature. Although rejecting the consecutive reaction mechanism of Crozier (1924), it is suggested that this behaviour is due to a phase change in the system at a place remote from the active site of the enzyme (Kumamoto, Raison & Lyons, 1971; Wyn-Williams, 1976). This phase change would produce a sharp change in the activation energy at a system-specific

transition temperature.

A considerable number of studies have sought to associate non-linear Arrhenius kinetics with thermotropic phase changes in the hydrophobic portion of the membrane bilayer. Sharp thermotropic gel-liquid crystal phase transitions have been observed in pure phospholipid dispersions and in artificial pure phospholipid vesicles (Hubbell & McConnell, 1969; Ladbroke & Chapman, 1969; Veatch & Stryer, 1977; Fahey & Webb, 1978). Similar thermally induced changes have been observed in the physical state of complete biological membranes (Reinart & Stein, 1970; Chapman & Urbina, 1971; Raison, Lyons, Melhorn & Keith, 1971; Eletr & Inesi, 1972; Inesi, Millman & Eletr, 1973; Borochov, 1977).

As the majority of enzymes reportedly exhibit non-linear Arrhenius kinetics are intrinsic membrane proteins, it has been suggested that the phase change in the membrane is responsible for the change in the temperature properties of these enzymes (Kumamoto, Raison & Lyons, 1971; Charnock, Cook & Opit, 1971; Wyn-Williams, 1976; Boldyrev, Ruuge, Smirnova & Tabak, 1977). Support for this suggestion has come from the observation that the fatty acyl chain composition of the membrane phospholipids was responsible for the temperature at which the phase change occurs, in that a larger proportion of unsaturated fatty acids has been shown to cause the fluid state of membrane lipids to persist at lower temperatures, thus lowering the transition temperature.

Further support for this idea has come from studies on fatty acid auxotrophs. For example the temperature properties of mitochondrial enzymes from Saccharomyces cerevisiae were dependent on the nature of the fatty acid supplement of the

growth medium (Janki, Aithal, McMurray & Tustanoff, 1974). Similar results have been obtained for the membrane ATPase of Mycoplasma mycoides membranes (Rottem, Cirillo, De Kruyff, Shinitsky & Razin, 1973).

Similarly it has been shown that the Arrhenius profiles of plasma membrane enzymes for poikilotherms show less sharply defined non-linear kinetics (Smith, 1967) or linear kinetics (Lyons & Raison, 1970; McMurchie, Raison & Cairncross, 1973). This has been correlated with the observation of a greater proportion of unsaturated fatty acyl chains in membrane phospholipids from such species (Cossins, 1977; Cossins & Prosser, 1978; Cossins, Christianson & Prosser, 1978; Lewis, 1978). This is said to produce lower activation energies for biological rate processes at lower temperatures. Indeed it has been suggested that thermal acclimation in poikilotherms is mediated, at least in part, by changes in the fatty acyl-chain composition of certain membrane phospholipids, which would tend to compensate for the increase in activation energy of membrane enzymes with decreasing temperature (Cossins, 1977; Cossins & Prosser, 1978; Cossins, Christianson & Prosser, 1978).

Studies on purified membrane enzymes have shown that many membrane enzymes have an absolute requirement for phospholipid for activity, and that when the purified constituent proteins of these enzymes were reconstituted with phospholipid dispersions and artificial membranes, these enzymes adopted temperature properties characteristic of the reconstituting lipids (Seelig & Hasselbach, 1971; Tanaka & Tenuya, 1973; Kimelberg & Papahadjopoulos, 1974).

Thus a considerable body of evidence has sought to link the temperature properties of membrane enzymes reactions to the

physical properties of the membrane microenvironment. This has led many workers to interpret non-linear Arrhenius plots as two straight lines, the slopes of which represent the activation energy of the reaction above and below the phase change. The interesection of these lines is termed the "critical temperature" at which the membrane phase change occurs. This has been observed to occur at about 20°C for mammalian systems (Massey, Curti & Ganther, 1966; Kumamoto, Raison & Lyons, 1971; Raison, Lyons & Thomson, 1971; Wyn-Williams, 1976). This forms the most common treatment of non-linear Arrhenius plots.

Some work on the temperature properties of mitochondrial processes has been interpreted as showing two non-interesecting straight lines (Kumamoto, Raison & Lyons, 1971). This suggests that the enzyme converts entirely from a state specific to higher temperatures to a state specific to lower temperatures over an infinitely small temperature range. However, this is not a general observation for membrane bound enzymes.

(iv) A fourth possible mechanism proposed to account for non-linear Arrhenius kinetics is that of the effect of temperature on the substrate affinity constant (Silvius, Read & McElhaney, 1978). It is proposed that the substrate binding constant exhibits considerable temperature sensitivity, and that this could modify the rate of reaction at lower temperatures such that the apparent activation energy would increase, producing a catenary curve for Arrhenius plots.

The stimulus for this analysis was the observation that the acetylcholinesterase enzyme of rat synaptic membrane exhibits non-linear Arrhenius kinetics, which was an unexpected result,

having at that time, no precedent in previous work. It was of considerable interest to examine the possible role of membrane lipids in this temperature effect and to evaluate the effect of membrane attachment on these temperature measurements.

Clearly the views presented above do not represent a consistent interpretation of the temperature kinetics of enzyme catalysed reactions. A comprehensive review of such kinetic models by Han (1972) also comes to this conclusion. It was necessary therefore, to assess the observed temperature kinetics of acetylcholinesterase in the light of each of these possible interpretations in order to obtain a working model for non-linear Arrhenius kinetics, and in this way to attempt to produce enzyme specific parameters from temperature measurements. This would facilitate comparison of the temperature properties of different membrane bound enzymes or the possible effects of membrane perturbing agents on the temperature properties of membrane-bound enzymes.

It was considered inadvisable to consider the acetylcholinesterase in isolation, as little was known of the temperature properties of this enzyme. Consequently the acetylcholinesterase was compared at each stage with $Na^+ - K^+$ ATPase. This membrane enzyme has been found in high concentrations in the same synaptic membrane preparation as would be used for studies on the acetylcholinesterase (Albers, Rodriguez de Lorez Arnaiz & De Robertis, 1965).

Non-linear Arrhenius kinetics have been observed for the $Na^+ - K^+$ ATPase activity of several mammalian tissue sources (Greuner & Avi-Dor, 1966; Bowler & Duncan, 1968; Charnock, Cook & Casey, 1971), and has been shown to have many properties

in common with other enzymes displaying cold-sensitive temperature properties. Also this enzyme has been shown to exhibit absolute requirement of membrane lipid for activity (Askari & Frantantoni, 1964; Emmelot & Bos, 1968), presumably because the enzyme activity is a product of an oligomeric system of at least two proteins (Kyte, 1971; Hokin, 1974) and the membrane lipid is required for correct orientation of these proteins within the membrane. This then is similar to many membrane bound enzymes in these respects.

The results presented in the following section were obtained when the observed temperature profiles of the acetylcholinesterase and $Na^+ - K^+$ ATPase activities from synaptic membranes were considered in terms of the several kinetic models proposed to account for non-linear Arrhenius behaviour of enzyme catalysed reactions.

MATERIALS AND METHODS

Synaptic membrane preparations

Animals used in this study were adult albino rats and guinea-pigs taken from animal house stock. In a typical rat experiment, about 25 rats were killed and brains were removed and immersed in 0.32M sucrose, 20mM imidazole - 2 mM EDTA, pH 7.2. After all animals were killed, the cerebral hemispheres were dissected and scraped free of white matter. The grey matter from three hemispheres was homogenized in $10cm^3$ of imidazole/EDTA buffered 0.32M sucrose. When all tissue had been homogenized, all homogenates were processed together for the bulk isolation of synaptic membranes as described in the general methods section. The final synaptic membrane pellets were routinely resuspended in $5cm^3$ of 20mM imidazole - 2mM EDTA pH 7.2. This gave membrane protein concentrations of about 1-3 mgs. cm^{-3}.

For guinea-pig synaptic membrane preparations at least two guinea-pigs were killed and their brains dissected out and placed in ice-cold imidazole - EDTA buffered 0.32M sucrose. As above the cerebral hemispheres were dissected away from the brain, scraped free of white matter. The grey matter from one hemisphere was homogenized in $10cm^3$ of imidazole - EDTA buffered 0.32M sucrose. When all guinea-pig tissue had been processed, the homogenates were treated, as for the rat, for the bulk extraction of synaptic membranes. The final membrane pellets were pooled and resuspended by homogenization in $3cm^3$ of 20mM imidazole - 2mM EDTA buffer at pH 7.2, to give protein concentration of about 1 mg cm^{-3}.

Aliquots of both of these membrane preparations were routinely

prepared for use in enzyme assay by dilution with 20mM imidazole - 2mM EDTA pH 7.2 buffer to protein concentrations of about 250 ug cm^{-3} and used directly for assay.

Enzyme assays

Acetylcholinesterase activity was assayed according to the modified method of Ellman et al, (1961). $Na^+ - K^+$ ATPase activity was assayed using the colourimetric estimation of inorganic phosphate (Atkinson et al, 1973). Both assays are described in detail in the general methods section.

Measurement of the temperature kinetics of Enzyme Reactions

Arrhenius profile measurements were made on a temperature gradient. This gradient was constructed on an aluminium Forbes bar, drilled with regularly placed holes. One end of the bar protruded into a water bath maintained $\pm$ 0.1°C at about 43°C, the other end was placed in an ice and water mixture. The bar was allowed to reach equilibrium for 3 to 4 hours. The temperature gradient produced in the bar was between about 42°C to 40°C at the hot end to 3°C to 4°C at the cold end. Tubes containing buffered reaction media were placed in holes in the equilibrated bar and allowed to equilibrate for at least 1 hour, before initiating assays. Media for both enzymes were buffered at four temperatures (35°C, 25°C, 16°C and 7°C), to cover the temperature range. This avoids variation of pH with temperature in media. Assays were then performed as described above. In the case of $Na^+ - K^+$ ATPase, at temperatures between 40°C and 20°C normal enzyme concentrations of about 250 mg cm^{-3} were used, at temperatures lower than 20°C double enzyme concentrations were used. In the case of acetylcholinesterase the same enzyme concentration was used at all temperatures.

pH Dependence of enzyme activity

Acetylcholinesterase; the pH dependence of acetylcholinesterase was measured using the assay method of Ellman et al (1961), as described in general methods chapter, using a temperature controlled spectrophotometer cuvette holder in a Pye-Unicam SP 1800 spectrophotometer. The cuvette holder was maintained at 37°C. The colour development in the cuvette was displayed on an AR - 25 flat bed chart recorder, and the rate of product formation with time was measured, by hand, from this trace.

For the pH profile, the standard reaction medium buffer at pH 7.5 was replaced with 0.1M phosphate buffers between pH 6.4 and pH 8.0 at 37°C. The background rate of hydrolysis of acetylthiocholine, in the absence of enzyme, was carefully determined for each pH, as this rate is faster at higher pH.

$Na^+ - K^+$ ATPase; the $Na^+ - K^+$ ATPase activity pH dependence was measured by replacing the conventional histidine buffer with 50mM B.E.S. between pH 6.4 and pH 7.6 and 50mM Bicine between pH 7.6 and pH 8.2. In all cases the pH of the buffers was determined at the reaction temperature (37°C).

Substrate kinetics of the acetylcholinesterase

Substrate activation kinetics were measured for the acetylcholinesterase in the conventional media. A substrate solution (5×10^{-4}M) was used to prepare 20 dilutions down to 7.2×10^{-6}M acetylthiocholine. Assays were performed according to the modified Ellman procedure. Data were used only if less than 10% of the substrate was consumed. The resulting data were analysed according to the Michaelis-Menten equation by a computer assisted least-squares error minimisation procedure.

The scheme above was performed at 37°C and 5°C, to obtain the

Michaelis constant at these temperatures. For assays at 5°C the conventional buffer was replaced with 0.1M tris - HCl buffer at pH 7.5 at 5°C.

RESULTS

The rat brain synaptic membrane preparation was found in this study to be a rich source of $Na^+ - K^+$ ATPase and acetylcholinesterase enzymes. The specific activities of these enzymes obtained for membrane preparations quoted in this study are similar to those reported in the literature (Table 3 : 1).

Typical examples of Arrhenius plots obtained for these two enzymes are presented in Figure 3 : 1 for the acetylcholinesterase and Figure 3 : 2 for the $Na^+ - K^+$ ATPase. It is clear that these profiles do not represent the linear plots expected from the Arrhenius rate equation, however, it is not obvious that any individual theory or analytical method could account for the anomalous behaviour of these Arrhenius plots. Indeed a smooth curve may fit the data well although two intersecting straight lines could be fitted arbitrarily, as is customary. However, it is suggested that it would be better to examine critically the possible causes of deviation from a single straight line before fitting a line or lines to these data. In that way subjective explanation of this anomalous enzyme behaviour may be avoided.

(i) It was necessary initially to establish that both enzymes were stable to irreversible thermal denaturation under the conditions used in the enzyme assays. This was tested by measuring the time-dependent rate of product formation, at temperatures at the high end of the temperature range (37^oC). Such measurements are presented in Figures 3 : 3 and 3 : 4. These data show that the rate of product formation, at 37^oC, was linear for considerably longer than the maximum assay duration, at

protein concentrations commonly used for Arrhenius plot measurements.

These data argue for a considerable degree of thermal stability for both $Na^+ - K^+$ ATPase and acetylcholinesterase.

(ii) A second factor considered to be of importance was the pH of the assay medium. In a recent comprehensive and critical treatise on many aspects of enzyme kinetics (Cornish-Bowden, 1976), no kinetic treatment of the temperature dependence of enzyme catalysed reactions was presented. This was said to be on the grounds that the dissociation constants of the various charged amino-acid side groups, essential for the tertiary structure and catalytic activity of enzymes, are temperature dependent. If this effect were sufficiently large then the decay in catalytic activity with decreasing temperature would depend, not on the enthalpy of activation of a single rate-limiting step but on complex changes in the protein as a whole. This effect can be examined with reference to the pH profiles of these enzymes. These profiles are presented in Figure 3 : 5 for the acetylcholinesterase and Figure 3 : 6 for the $Na^+ - K^+$ ATPase. Both of these enzymes exhibit a broad pH maximum, indicating that catalytic activity of these enzymes is not sensitive to considerable changes in charge equilibria of ionizable amino-acid side groups. Thus only small changes in enzyme activity occurred between pH 7.0 and pH 8.0 for both $Na^+ - K^+$ ATPase and acetylcholinesterase, and in consequence, this effect cannot account for non-linear Arrhenius plots for either of these enzymes.

(iii) Silvius Read & McElhaney, (1978) suggested that the temperature dependence of substrate binding to an enzyme could result in a change in Arrhenius u with temperature. A kinetic model for this possibility had already been suggested by Han, (1972). This model shows that the observed Arrhenius u (u obs) would be given by the relationship shown below;

$$u(obs) = u - \Delta H \left(\frac{1}{1 + \frac{[S]}{Km}} \right) \qquad 1$$

where u = activation energy of rate limiting step

ΔH = enthalpy of substrate binding

Km = Michaelis-Menten constant

$[S]$ = Substrate concentration

This equation predicts a catenary curve for the Arrhenius plot of an enzyme, provided that the variation in Km with temperature is large. At one extreme where [S] was large with respect to Km then u(obs) = u, and the Arrhenius plot will tend to a straight line of slope u, also if Km becomes large compared with S then u obs = u - ΔH then the Arrhenius plot will tend to a second straight line of slope u - ΔH.

In practical terms it would be necessary to obtain values for the magnitude of the term $\Delta H \left(\frac{1}{1 + \frac{[S]}{Km}} \right)$ at extremes of the temperature range used in Arrhenius plots. This can be done by measuring the Km at these temperatures. In practice, then the heat of substrate binding (ΔH) can be calculated from the Van't Hoff isotherm. Assuming ΔH does not vary with temperature, the term $\Delta H \left(\frac{1}{1 + \frac{[S]}{Km}} \right)$ may be calculated for these temperatures, and the possible contribution of this term to the observed non-linearity of Arrhenius plots assessed.

It has been observed that in the case of the Na^+ - K^+ ATPase

the Km for ATP at 43°C was 2×10^{-4}M, whereas at 1°C it was 5×10^{-6}M (Neufeld & Levy, 1970). Also these authors report that ATP is the most temperature sensitive of the activating ligands. Thus the enthalpy of ATP binding calculated for these values using the Van't Hoff isotherm was 63 KJ $mole^{-1}$. As this was the most temperature sensitive ligand this would represent the largest ΔH term for all the activating components of the $Na^+ - K^+$ ATPase. Thus allowing for experimental variation an upper limit of 80 KJ $mole^{-1}$ was assumed for the ΔH of ATP binding. This value was then used to calculate the Km values at 1°C for the ion activators of $Na^+ - K^+$ ATPase (Mg^{2+}, Na^+ & K^+), as shown in Table 3 : 2. Literature values were used for the Km at 37°C for each of these ions. This shows that the maximum change in the observed Arrhenius u which could be accounted for in terms of substrate binding was about 17 KJ $mole^{-1}$. From Table 3 : 3 it can be seen that the observed u at temperatures around 37°C was about 70KJ $mole^{-1}$ and about 170 at around 4°C, giving a change of u (obs) of approximately 100 KJ $mole^{-1}$. Clearly this is too large to be due to variation in substrate affinity with temperature.

With the acetylcholinesterase the Kms for acetylthiocholine at 37°C and 5°C were determined and found to be 1.0×10^{-4}M and 5.0×10^{-5}M respectively. The heat of substrate binding was found in this case, to be 15.7 KJ $mole^{-1}$. The $\Delta H \left(\frac{1}{1 + \frac{[S]}{Km}}\right)$ terms were calculated and are shown in Table 3 : 2. In this case the maximum change in u (obs) which could result from changes in enzyme substrate dissociation constant would be 1.2 KJ $mole^{-1}$. From Table 3 : 3 it can be seen that the value for u (obs) above 20°C is about 19 KJ $mole^{-1}$ and about 37 KJ $mole^{-1}$ at temperatures below 20°C. Thus the change in u (obs), across this temperature

range was 18 KJ mole^{-1}. As with the $Na^+ - K^+$ ATPase this is too large to result from the actual change in substrate affinity with assay temperature.

(iv) The temperature plot data for $Na^+ - K^+$ ATPase and acetylcholinesterase, examples of which are shown in Figure3 3 : 1 and 3 : 2, were processed according to the phase change effect. This was done by attempting to fit two straight lines to the plots, which yield activation energy values for the high and low temperature ranges, and a 'critical' temperature at which the enzyme shifts from one activation energy state to the other. Data was fitted using a computer assisted least squares minimisation scheme. Graphical representations of Arrhenius plots, modelled in this way, are presented in Figures 3 : 7 and 3 : 8. The values for activation energies and critical temperatures for several preparations of each of these enzymes are presented in Table 3 : 3.

The conclusions from these data, processed in this way are that the $Na^+ - K^+$ ATPase undergoes a thermal transition between an activation energy of about 70 KJ mole^{-1} at higher temperatures to an activation energy of about 170 KJ mole^{-1} at lower temperatures, with a transition temperature at about 20^{o}C. The acetylcholinesterase undergoes a similar transition but between energies of about 15 KJ mole^{-1} at higher temperatures and about 40 KJ mole^{-1} at lower temperatures. The transition temperature also occurred at about 20^{o}C in this case.

The higher values for the activation energies for the $Na^+ - K^+$ ATPase as compared with the acetylcholinesterase indicates that the former is much more temperature sensitive than the acetylcholinesterase. At low temperatures the $Na^+ - K^+$ ATPase activity approaches zero, whereas the

acetylcholinesterase activity is still relatively high at temperatures close to 0°C. At 4°C the activity of the $Na^+ - K^+$ ATPase decreased to only 0.4% of the value at 37°C. In contrast, the activity of acetylcholinesterase at 4°C is still 20% of the value at 37°C. Thus a superficial examination of these two Arrhenius plots could be taken to suggest that the transition model is satisfied.

The detailed kinetic consequences of the phase transition model has been discussed by Kumamoto et al (1971) and Wyn-Williams (1976). Their conclusions may help in critical interpretation of the above treatment of non-linear Arrhenius plots. The molar free energy or chemical potential of the system undergoing a phase change is given by the relationship below;

$$U = Uo - RT \ln K \qquad 2$$

where: U = molar free energy of the system

Uo = standard molar free energy of the system

T = absolute temperature

R = gas constant

K = system specific constant

As the phase change is considered to represent a thermotropic conversion between pure systems, each with a chemical potential defined by equation 2. The phases will be in equilibrium at the transition temperature where the chemical potentials of both systems will be separated into "macroscopic domains", as described by Kumamoto et al (1971). Changes in the physical state of the system will occur only at the critical temperature.

In this model, Arrhenius plots would exhibit constant slope between 40°C and 20°C, where an abrupt change would occur to a different slope between 20°C and 0°C.

This view has been tested for both of these enzymes by calculating the Arrhenius 'u' of small sections of individual Arrhenius plots at a number of temperatures across the range normally used in temperature studies. This was done by drawing a short line, by eye through the three or four points around the chosen temperature points at $5^{o}C$ intervals between $40^{o}C$ and $5^{o}C$. The slope of this line was calculated to give the Arrhenius u values in this temperature region. The graphical representations of these calculations for Na^{+} - K^{+} ATPase are presented in Figure 3 : 9 and for acetylcholinesterase in Figure 3 : 10. The dotted lines on these figures represent the predicted ideal behaviour for the phase change effect.

From Figure 3 : 9 it can be seen that the Na^{+} - K^{+} ATPase exhibits relatively constant activation energy of about 70 KJ $mole^{-1}$ between $40^{o}C$ and $20^{o}C$, however, below $20^{o}C$ the activation energy increases progressively with extremely high values of about 230 KJ $mole^{-1}$ at temperatures around $5^{o}C$. The trend in this case is of increasing activation energy with decreasing temperature. Thus this enzyme does not exhibit temperature kinetics consistent with a simple phase change effect.

From Figure 3 : 10 it can be seen that the acetylcholinesterase exhibits a different relationship between u and temperature than that for the Na^{+} - K^{+} ATPase. In this case, the activation energy remains constant at about 35 KJ $mole^{-1}$ at low temperatures, and decreases with increasing temperature with very low values of 5 - 8 KJ $mole^{-1}$ at about $40^{o}C$. This suggests that as with the Na^{+} - K^{+} ATPase the temperature kinetics of acetylcholinesterase cannot be explained in terms of a phase change effect. As a result, further possible models have been examined which may

account for the temperature dependence of Arrhenius u as described in Figures 3 : 9 and 3 : 10.

(v) It has been suggested that non-linear Arrhenius kinetics could result from a thermodynamic equilibrium between different active states of an enzyme (Kistiakowsky & Lumry, 1949; Han, 1972). In this case, it is proposed that an enzyme can exist in a state A, which predominates at high temperatures and in a state B, which predominates at low temperatures, then the equilibrium between these species would be defined by an equilibrium constant Ke; which is given by the relationship in equation 3.

$$Ke = \frac{[B]}{[A]} \qquad 3$$

If these states (A & B) have different activation energies then the Arrhenius plot for such an enzyme would be non-linear. The temperature plot of an enzyme with this property would take the form of linear segments at the extremes of the plot, jointed by a smooth curve. The slopes of the extremities would approximate to the activation energy of A at high temperatures and to that of B at low temperatures. The breadth of the transition between these values would depend on the magnitude of the ΔH component in the equilibrium constant Ke (Han, 1972). Thus the relationship between the activation energy and temperature would be of the form described in Figure 3 : 11. However, it can be seen from Figures 3 : 9 and 3 : 10 that neither the $Na^+ - K^+$ ATPase nor the acetylcholinesterase conformed very closely with this model.

(vi) It has been suggested that non-linear Arrhenius kinetics can be produced by a reversible thermal inactivation of a portion of the enzyme molecules (Johnson, Eyring & Williams, 1942; Kavanau, 1950). As in the previous section, it is assumed that the enzyme can exist in two states A and B in

equilibrium as defined by the equilibrium constant Ke, as in equation 3. However, unlike the previous section either A or B can be considered to be an inactive stable species. The kinetic models resulting from both of these schemes are examined below;

In either case the total enzyme concentration is given by the sum of enzyme in state A and that in state B, as shown in equation 4.

$$[Et] = [A] + [B] \qquad 4$$

In the case of an inactive species at low temperatures, the reaction rate would depend on the concentration of state A, as in equation 5. By substituting

$$v = Ka\,[A] \qquad Ka = \text{rate constant for active species A.} \qquad 5$$

the term for B in equation 3 into equation 4 we obtain a term for A as equation 6

$$[A] = \frac{[Et]}{1 + Ke} \qquad 6$$

This term for A is substituted into equation 5, as in equation 7. If this enzyme

$$v = \frac{Ka\,[Et]}{1 + Ke} \qquad 7$$

activity is expressed as an enzyme concentration specific term V, where $V = \frac{v}{Et}$, then equation 7 is expressed as in equation 8. This related the specific activity of enzyme

$$V = \frac{Ka}{1 + Ke} \qquad 8$$

to the rate constant of the active species and the equilibrium constant for the enzyme states. These terms may be expressed in terms of their temperature dependence in the following manner. Ka as a rate constant can be expressed in terms of the Arrhenius equation, whereas Ke as a thermodynamic parameter may be expressed in its component enthalpy and entropy terms. In this way equation 8 becomes as written in equation 9.

$$= \frac{Ae^{\frac{-\mu a}{RT}}}{1 + \left(e^{\frac{\Delta S}{R}} e^{\frac{-\Delta H}{RT}} \right)}$$

where ua is the activation energy of the active species A 9

The Arrhenius plot of this equation will be given by taking logs and differentiating with respect to $\frac{1}{T}{}^{o}K$. The slope of this plot will thus be given by equation 10. In this case the μ,

$$\text{slope} \left(\frac{\mu \text{ (obs)}}{R} \right) = \frac{-\mu a}{R} + \frac{\Delta H}{R} \left(\frac{Ke}{1 + Ke} \right) \qquad 10$$

observed at any temperature will depend on the magnitude of Ke at that temperature. At low temperatures the B form of the enzyme predominates and from equation 3 it can be seen that Ke would be large. In this case, from equation 8, it can be seen that the specific activity would be small and from equation 10 the activation energy at low temperatures would tend to $\mu a = \Delta H$. At high temperatures Ke would tend to zero, the specific activity would depend on Ka. The activation energy in this case would approach ua.

This relationship would account for the behaviour of the $Na^{+} - K^{+}$ ATPase as described in Figure 3 : 9. At high temperatures the $Na^{+} - K^{+}$ ATPase shows a limiting activation energy of about 60 - 70 KJ $mole^{-1}$, with increasing activation energy at lower temperatures, also the activity of the $Na^{+} - K^{+}$ ATPase becomes small at low temperatures. This agrees with the predictions of this model. However, no limiting value of activation energy at low temperatures ($\mu a = \Delta H$) was observed. This may be because at temperatures below $4^{o}C$ the enzyme activity becomes too small to measure with accuracy.

The model described above cannot account for the observed temperature dependence for μ for the acetylcholinesterase (Figure 3 : 10). A better fit may be obtained by considering the possibility of a reversible inactivation at high temperatures.

In this case, the equilibrium constant Ke would be related to the concentrations of A and B as in equation 3, but the rate of enzyme activity, v, would be given by equation 11.

$$v = Kb\,[B] \qquad \text{where Kb is the rate constant for the active species B} \qquad 11$$

A term for B can be obtained by substituting for A in equation 3 with equation 4 (equation 12). This is substituted into equation 11, to give equation 13.

$$[B] = \frac{[Et]}{\left(1 + \frac{1}{Ke}\right)} \qquad 12$$

$$v = \frac{Kb\,[Et]}{\left(1 + \frac{1}{Ke}\right)} \qquad 13$$

The specific activity of the enzyme (V) can be expressed as $V = \frac{v}{Et}$. Therefore equation 13 becomes as in equation 14.

$$V = \frac{Kb}{\left(1 + \frac{1}{Ke}\right)} \qquad 14$$

The temperature dependence of this relationship can be expressed by expressing the rate constant Kb in terms of the Arrhenius equation, and the equilibrium constant Ke, in terms of its component enthalpy and entropy terms. The slope of the Arrhenius plot of this relationship can be obtained by taking logs and differentiating with respect to $\frac{1}{T}{}^{\circ}K$, resulting in the relationship shown in equation 15. In this case, at high temperatures

$$\text{slope}\left(\frac{\mu\ obs}{R}\right) = \frac{\mu b}{R} + \frac{\Delta H}{R}\left(\frac{1}{1 + Ke}\right) \qquad 15$$

Ke would be small, then the observed activation energy would tend to $\mu b + \Delta H$ and the activity (V) would tend to zero. At low temperatures Ke would be large and the slope of the temperature plot would tend to μb, the activation energy of the low temperature species.

The observations for the acetylcholinesterase presented in Figure 3 : 10 agree with this interpretation. The value for Arrhenius 'μ' was constant at low temperatures and decreased progressively with increasing temperature. However, the activation energy at high temperatures did not tend to a particular value. This may be because the range of temperatures at which reversible thermal denaturation occurs, overlaps with the range at which irreversible inactivation occurs. Thus this latter model can account for the observed temperature properties of the acetylcholinesterase better than the previous models.

A computer assisted least squares error minimisation scheme was available which fitted enzyme temperature data to either of the reversible inactivation models. The data obtained for the $Na^+ - K^+$ ATPase was processed according to reversible inactivation at low temperatures, data for acetylcholinesterase was processed according to reversible inactivation at high temperatures.

The values for the activation energy of the active species, and the enthalpy and entropy parameters of the equilibrium constant, for the transition, are presented in Table 3 : 4. A typical curve generated by such an analysis of $Na^+ - K^+$ ATPase data, is presented in Figure 3 : 12. This computer generated curve gives a good fit to the available data, and gives reproducible values for enthalpy, entropy and activation energy parameters.

Similarly, the data for the acetylcholinesterase gave good fit to the computer generated curve (Figure 3 : 13) and consistent values for the activation energy of the active species and for the enthalpy and entropy of the transition (Table 3 : 4).

These latter models account best for the observed temperature properties of the $Na^+ - K^+$ ATPase and acetylcholinesterase.

The model describing non-linear Arrhenius kinetics for the acetylcholinesterase in terms of a reversible thermal inactivation at high temperatures was suitable for the analysis of rat brain enzyme data. However, it was of interest to examine the acetylcholinesterase activity of another mammalian species. This would indicate whether non-linear Arrhenius kinetics, was a more general property of mammalian brain acetylcholinesterase, and whether the data generated for acetylcholinesterase from this source also gave good fit to the kinetic model used to process the rat brain enzyme data. This was particularly important as when this work was initiated no precedent for the finding of non-linear Arrhenius kinetics existed in the literature.

From Figure 3 : 14 it can be seen that the Arrhenius profile of the guinea-pig enzyme was obviously non-linear, and that the computer generated curve gives good fit to the data. Also the values for the activation energy of the low temperature species, and the enthalpy and entropy of the transition were consistent for several preparations (Table 3 : 5). These values were similar to those obtained for the rat brain enzyme (Tables 3 : 3 & 3 : 4).

The significance of these results for the further examination of the non-linear Arrhenius plots of the rat brain enzyme are discussed below.

DISCUSSION

The $Na^+ - K^+$ ATPase specific activities from the synaptic membrane preparations used in this study were similar to that quoted for synaptic membranes by other workers (Morgan, Wolfe, Mandel & Gombos, 1971; Levitan, Mosynski & Ramirez, 1972; Bowler & Tirri, 1974). As can be see from Figure 3 : 2 the Arrhenius plot of the $Na^+ - K^+$ ATPase describes a smooth curve with no obvious sharp inflexion. Similar curves have been attributed to rat brain $Na^+ - K^+$ ATPase (Bowler & Duncan, 1968; Bowler & Tirri, 1974) and rat brain $Ca^{2+} - Mg^{2+}$ ATPase (Tirri, Lahdekorpi & Bowler, 1976).

The plot in Figure 3 : 7 can be compared with other reported observations of the temperature properties of the $Na^+ - K^+$ ATPase as the majority of authors have considered their plots to be represented by two intersecting straight lines. The values for the activation energies for $Na^+ - K^+$ ATPase temperature plots, processed according to the phase change effect were about 70 KJ $mole^{-1}$ for the high temperature range and about 140 KJ $mole^{-1}$ for the low temperature range with a critical temperature of about 20°C. These data agree with the values reported for the $Na^+ - K^+$ ATPase by other workers (Charnock, Cook & Casey, 1971; Charnock, Cook, Almeida & Toh, 1973; Bowler & Tirri, 1974). Thus the Arrhenius profile presented in Figure 3 : 7 did not differ markedly from these reports. Several other enzymes and biological transport processes have been shown to exhibit high temperature and low temperature activation energies, similar to those quoted here for the $Na^+ - K^+$ ATPase. For example Azobacter nitrogenase (Ceuterick, Peetes, Heremans, De Smedt & Olbrechts, 1978) leucine transport and glucose transport in erythrocytes (Hoare, 1971; Lacko, Wittke & Geck, 1973) and the $Ca^+ - Mg^{2+}$ ATPase (Tirri, Lahde korpi &

Bowler, 1976).

The fact that few published Arrhenius plots for the $Na^+ - K^+$ ATPase obviously describe the curved system quoted here is likely to be a reflection of the small number of temperature points used to establish those plots. It is clear that judicious selection of a small number of temperature points can result in an Arrhenius plot with an apparently sharp transition. This does not totally invalidate measurements of the activation energies in the region of 37°C and 10°C but attributed inappropriate significance to the intersection of the lines describing these activation energies. It was considered necessary therefore, to process the existing data for the $Na^+ - K^+$ ATPase temperature plots in terms of the various theoretical models proposed in the literature and discussed fully in the results section, in order to arrive at a less subjective and therefore more rigorous method of treating this type of data.

The acetylcholinesterase preparation from rat cortical synaptic membranes used in this study had a similar specific activity to that quoted for Ficoll-gradient extracted rat synaptic membranes by Goodkin & Howard, 1974 (Table 3 : 1). The Arrhenius plot of acetylcholinesterase shown in Figure 3 : 1 was also obviously non-linear and this observation was the stimulus for this work in that this enzyme was thought to be an extrinsic membrane protein characteristic of neuronal plasma membrane, as well as muscle membranes and erythrocytes (Bellhorn, Blumenfeld & Gallop, 1970; Goodkin & Howard, 1974; McIntosh & Plummer, 1976; Rieger & Vigny, 1976; Adamson, 1977; Ott & Brodbeck, 1978), but was not thought to be associated

with membrane lipids. However, as non-linear Arrhenius kinetics have often, but not exclusively been reported as a feature of membrane lipid dependency for enzymes, it was of interest to investigate a possible role of lipid in the properties of the acetylcholinesterase.

Fitting two intersecting straight lines to this Arrhenius profile for the acetylcholinesterase gives values of about 18 KJ $mole^{-1}$ for the high temperature activation energy and about 35 KJ $mole^{-1}$ for the low temperature activation energy. These lines intersect at about 20°C. At the time of these initial observations, no anomalous Arrhenius kinetics had been reported for this enzyme, however subsequently two separate reports of non-linear Arrhenius plots for acetylcholinesterase have been made and the values obtained for activation energies over the same temperature ranges are similar to those obtained in this study (Table 3 : 2). These preparations were from bovine erythrocytes and pig brain (Beauregard & Roufogalis, 1977; Reavell, Wooster & Plummer, 1978).

Both of these reports treated the non-linear Arrhenius plots obtained as if they derived from two intersecting straight lines, although examination shows a smooth curve would fit these data better than the suggested biphasic plot. Thus the acetylcholinesterase was treated in a similar fashion to the $Na^+ - K^+$ ATPase, as if it displayed biphasic Arrhenius kinetics, this may be owing to the small number of temperature points used, so producing an incompletely defined plot. It is clear, however, that a more systematic approach to the data is necessary. It was therefore decided to eliminate or verify the various models of non-linear Arrhenius kinetics proposed in the literature by testing the observed temperature properties of the $Na^+ - K^+$ ATPase and acetylcholinesterase on

each model in turn.

It was possible that the non-linearity in temperature plots for both enzymes could have occurred by an irreversible thermal inactivation. The question in this case was whether the $Na^+ - K^+$ ATPase or acetylcholinesterase underwent significant loss of activity at any of the temperatures in temperature plots. Thermal inactivation at low temperatures would have been revealed as the enzyme preparation was routinely stored on ice for several days without loss in activity. Thus it was thought that the only significant inactivation would occur at the highest assay temperatures used.

In this case the extent of high temperature inactivation would presumably depend on the duration of exposure to a given temperature and the activation energy of the decay process. This effect can be seen to be negligible as the rate of product formation with time was linear for at least 30 minutes at the protein concentrations used routinely (100 - 200 µg/ml) (Figures 3 : 3, 3 : 4). Consequently, it can be concluded that no significant thermal inactivation occurred during the incubation times used in the Arrhenius plots of both of these enzymes, and that irreversible thermal inactivation cannot account for the non-linear Arrhenius plots shown in Figures 3 : 1 and 3 : 2.

It has also been suggested that the properties of all enzymes vary with temperature because the dissociation constants of the various amino-acid side groups responsible for tertiary protein structure and enzyme activity vary with temperature (Cornish-Bowden, 1976). Thus the measurement of enzyme reactions at various temperatures would be pointless as the kinetics would be too complex to model. However, it

can be seen from Figures 3 : 5 and 3 : 6 that neither acetylcholinesterase nor $Na^+ - K^+$ ATPase show changes in activity with considerable changes in pH. In other words the shifts in the charge equilibriae of charged amino acid side groups did not affect the activity of these two enzymes. Thus this effect cannot account for non-linear temperature plots in these cases.

It has been noted that large changes in the substrate binding affinity with temperature would produce non-linear temperature kinetics for an enzyme (Silvius et al, 1978; Han, 1972). The constribution of this effect in producing the temperature plots obtained in this study was estimated as described by Han, (1972) by evaluating the heat of substrate binding and thus the term $\Delta H \left(\frac{1}{1 + \frac{[S]}{Km}}\right)$ (equation 1). The maximum value for this term, in the case of $Na^+ - K^+$ ATPase was 17 KJ $mole^{-1}$ (Table 3 : 2). This is clearly much less than the 100 KJ $mole^{-1}$ required to produce the observed effect for this enzyme, (Table 3 : 3). The value of this term $\Delta H \left(\frac{1}{1 + \frac{[S]}{Km}}\right)$ for the acetylcholinesterase was only 1.2 KJ $mole^{-1}$ (Table 3 : 2) between 37°C and 5°C, which is also much less than the approximately 20 KJ $mole^{-1}$ difference in activation energies between these temperatures (Table 3 : 3). Thus this effect cannot account for the observed data and may be ignored.

The existing temperature measurements on both the $Na^+ - K^+$ ATPase and acetylcholinesterase activity were also processed according to the phase change model and the computer generated lines to fit these data, are presented in Figures 3 : 7 and 3 : 8. These both superficially give good fit to these data, and are similar to those previously published (Gruener & Avi-Dor, 1966; Swanson, 1966; Bowler & Duncan, 1968; Neufeld & Levij, 1970; Charnock, Cook,

Almeida & To, 1973). Also the activation energy values at the high and low temperature ranges of 70 - 80 KJ $mole^{-1}$ and 160 - 180 KJ $mole^{-1}$ respectively are similar to those reported in the literature for rat brain Na^+ - K^+ ATPase and for bovine erythrocyte and pig-brain acetylcholinesterase (Beauregard & Roufogalis, 1977; Reuvill, Wooster & Plummer, 1978) of 15 - 21 KJ $mole^{-1}$ and 30 - 40 KJ $mole^{-1}$ respectively. Also the critical temperatures at which the shift between activation energy states for data obtained in this study was, at about 20°C, similar to that quoted in these reports.

However, the phase change effect would predict a constant 'μ' value across the high temperature range which would shift over a very short temperature range to a higher value for 'μ' at low temperatures. It is clear from Figures 3 : 9 and 3 : 10 that the change in Arrhenius 'μ' with temperature for both Na^+ - K^+ ATPase and acetylcholinesterase did not fit that expected either for the phase change effect or for the model based on conversion between active states (Figure 3 : 11). The 'μ' value between 40°C and 20°C was relatively constant, in the case of the Na^+ - K^+ ATPase, and in this way conformed to the phase change model, but at temperatures lower than 20°C the 'μ' value increased progressively, reaching 200 - 250 KJ $mole^{-1}$ at about 5°C, which is much higher than the value of about 180KJ $mole^{-1}$ calculated for this temperature range.

Although the variation of 'μ' with temperature for the acetylcholinesterase does not agree with that predicted for the phase change effect or the conversion between active forms of the enzyme (Figures 3 : 10 & 3 : 11), it is also different from that observed for the Na^+ - K^+ ATPase. In this case the 'μ' value is relatively constant between 5°C and 20°C at the value predicted for this range (32 - 38 KJ $mole^{-1}$) by the

phase change effect, but over 20°C, the 'μ' value decreases progessively until at around 40°C the 'μ' value is about 5 - 9 KJ mole^{-1}. This is much less than the 15 KJ mole^{-1} predicted for this temperature range by the phase change effect.

In consequence although the temperature kinetics of Na^+ - K^+ ATPase and acetylcholinesterase may result from a thermotropic change in the physical state of the biomembrane, the temperature properties of these enzymes cannot be modelled in terms of a simple phase change. Also it is clear that any model which successfully accounts for the change in Arrhenius 'μ' with temperature for the Na^+ - K^+ ATPase (Figure 3 : 9) would differ significantly from that which would explain the change in 'μ' with temperature for the acetylcholinesterase (Figure 3 : 10).

The reversible thermal inactivation model of Han, (1972) explained the behaviour of the "Arrhenius μ temperature" plot for the Na^+ - K^+ ATPase better than any of the other models suggested in the literature. The computer generated curve when Na^+ - K^+ ATPase temperature data was processed according to this model gives a consistently good fit to the data points (Figure 3 : 12), also the activation energies of the high temperature form of the enzyme and the enthalpy and entropy value for the transition between active and inactive states were consistent for several preparations (Table 3 : 4). The activity of the Na^+ - K^+ ATPase tends to zero at temperatures close to 4°C, which argues for an inactivation process. Similarly the model predicts that the Arrhenius 'u' at temperatures below 20°C would not represent the activation energy of the rate limiting step of the enzyme activity. This is confirmed by the actual value of 'μ' in this temperature range which, at about 150 - 200 KJ mole^{-1}, is

much larger than expected for a biological rate process.

Although the dependence of the temperature properties of $Na^+ - K^+$ ATPase on a simple membrane phase change has been rejected, the possibility remains that non-linear plots for this enzyme are the expression of a membrane effect. The value for the transition enthalpy (200 KJ $mole^{-1}$) was larger than that for reversible thermal transitions in proteins (Maier, Tappel & Volman, 1955) but less than calorimetrically determined enthalpies (30 - 40 KJ $mole^{-1}$) involved in phase transitions in pure lipid systems (Hinz & Sturtevant, 1972; Chapman, Peel, Kingston & Lilley, 1977). However, thermal transitions in cholestrol rich protein containing natural biomembranes are more complex than in pure phospholipid systems (Pagan, Cherry & Chapman, 1973; De-Kruyff, Demel, Slotboom, Van Deenen & Rosenthal, 1973; Feinstein, Fernandez & Sha'afi, 1973). In biomembranes, broader and more complex phase transitions have been observed. Also the enthalpies of transition calculated from indirect spectral data have been higher than those derived from direct measurements. This has been taken to indicate that phospholipids behave as co-operative units of between 10 - 70 molecules (Trauble, 1971; Hinz & Sturtevant, 1972; Yellin & Levin, 1977). In these terms the lipid phase transition enthalpy can easily account for the transition enthalpy of $Na^+ - K^+$ ATPase activity. Thus the non-linear temperature kinetics of $Na^+ - K^+$ ATPase is consistent with a physical change in the membrane.

In the case of the acetylcholinesterase, the reversible thermal inactivation model with an inactive form at high temperatures accounted most satisfactorily for the relationship between

Arrhenius 'μ' and temperature for this enzyme (Figure 3 : 10). The Arrhenius curves generated by the computer assisted least-squares error minimization scheme show good fit to the data points (Figure 3 : 13), and the activation energies of the low temperature active form transition enthalpies and entropies calculated for several preparations were consistent (Table 3 : 4). The ΔH parameter (85 KJ $mole^{-1}$) and ΔS parameter (266 J^{o} K^{-1} $mole^{-1}$) for this enzyme were smaller than those obtained for the transition in Na^{+} - K^{+} ATPase activity (ΔH = 196 KJ $mole^{-1}$, ΔS = 683 J^{o} K^{-1} $mole^{-1}$), this shows that the transition in acetylcholinesterase activity was broader than in temperature plots of Na^{+} - K^{+} ATPase activity.

Interpretation of the reversible inactivation model for the acetylcholinesterase temperature properties is more difficult than that for the Na^{+} - K^{+} ATPase. The value for the transition enthalpy (85 KJ $mole^{-1}$) is consistent with both the enthalpy of reversible conformational changes in proteins and the transition enthalpy of a lipid phase change assuming some co-operativity. It would not be possible to distinguish between these possibilities without further work. However, the main concern is to assess the significance of a high temperature reversible thermal inactivation. It is relatively easy to conceive of a reversible inactivation of Na^{+} - K^{+} ATPase as unphysiological membrane states exist at low temperatures and this enzyme exhibits the activation energy of the active species at 37°C. However, it is more difficult to imagine why, in the case of acetylcholinesterase, a portion of the total number of enzyme molecules should be inactive at 37°C. It is not clear why such an equilibrium should exist. If the 'μ' value at the temperature around 37°C (13 KJ $mole^{-1}$) represents a true activation energy then the Arrhenius plot

would represent a transition between active species, yet this transition would be incompletely defined in existing plots as the high temperature asymptote would overlap with the temperatures at which irreversible thermal denaturation occurred. However, if this is so, then the activation energy of the high temperature species would be very small and would approximate to the model already discussed. A reduced activation energy in the region of 37°C would facilitate the functioning of the acetylcholinesterase, by reducing the rate constant represented by the Arrhenius equation.

It was of interest to test whether the unusual temperature properties of acetylcholinesterase were specific to the rat brain enzyme. Non-linear temperature plots somewhat similar to those obtained in this study were reported for pig-brain acetylcholinesterase and bovine erythrocyte acetylcholinesterase (Beauregard & Roufogalis, 1977; Reavill, Wooster & Plummer, 1978) although these data were reported in insufficient detail to enable testing in terms of this model. Consequently the temperature properties of acetylcholinesterase from another mammalian source, namely guinea-pig brain synaptic membranes, were examined. It can be seen from Figure 3 : 14 that the temperature kinetics of acetylcholinesterase from this source were obviously non-linear and gave a_A good fit to the model, as shown by the computer generated line. Also the activation energy, ΔH and ΔS parameters calculated for this enzyme from guinea-pig were similar to that for the rat brain enzyme (Tables 3 : 5 and 3 : 3). These tables show that values for apparent activation energies and critical temperatures, obtained by processing this data according to the phase change effect

were similar to those obtained for the rat brain enzyme (Tables 3 : 5 and 3 : 3). This suggests that non-linear temperature plots could be a property of mammalian acetylcholinesterase in general, and that the theoretical treatment used in this chapter could be applied to these enzymes in general.

It is clear from this chapter that more than one kinetic model can account for the non-linear Arrhenius plots of membrane-bound enzymes. When examining experimental observations of this type, it is essential to make as few assumptions as possible and to analyse the data critically. Although no conclusions can be drawn from this chapter as to the possible association between rat synaptic membrane acetylcholinesterase and membrane lipid, a systematic approach to the observed temperature properties of this enzyme has been devised, which can assist in the assessment of the effects of membrane perturbing agents on the acetylcholinesterase.

Table 3 : 1

Specific activities of rat brain synaptic membrane enzymes.

Enzyme		Specific * Activity	n	Source
Acetylcholinesterase	i)	25.3 ± 0.4	6	ref.1
	ii)	26.4		ref.2
Na^+-K^+ ATPase	i)	94.3	5	ref.1
	ii)	120.0	—	ref.3
	iii)	78.9-103.1	—	ref.4
	iv)	≃ 100		ref.5

* activity = μ moles product/ mg.protein/hr

References

1 present study

2 Goodkin & Howard,(1974).

3 Levitan, Musynski & Ramirez, (1972).

4 Morgan, Wolfe, Mandel & Gombos, (1971).

5 Bowler & Tirri, (1974).

TABLE 3 : 2

Changes in Arrhenius 'μ' attributable to temperature sensitivity of substrate and activator binding

Substrate or Ligand	Assay Concentration (M)	Km (M)		$\Delta H \left(\frac{1}{1 + \frac{[S]}{Km}} \right)$		Max chang μ
		37°C	1°C	37°C	1°C	
Δ Mg^{2+}	$3x10^{-3}$	$8x10^{-4}$	$8x10^{-6}$	16.8	0.2	16.6
X ATP	$3x10^{-3}$	$2x10^{-4}$	$2x10^{-6}$	5.0	0.05	4.95
□ Na^{+}	$1.3x10^{-1}$	$6x10^{-3}$	$6x10^{-5}$	3.53	0.04	3.49
+ K^{+}	$2.0x10^{-2}$	$9x10^{-4}$	$9.2x10^{-6}$	3.45	0.04	3.41
		37°C	5°C	37°C	5°C	
Acetylthiocholine	$5x10^{-4}$	$1x10^{-4}$	$5x10^{-5}$	2.67	1.46	1.22

Δ Robinson J.D. (1974) Biochim. Biophys. Acta. 341: 232-247

+ Robinson J.D. (1974) A.N.Y. Acad. Sci. 242: 185.

□ Bakkeren & Bonting (1968) Biochim. Biophys. Acta. 150: 460-466

X Neufeld & Levey (1970)

TABLE 3 : 3

Parameters calculated for Arrhenius plots processed according to the phase change effect.

Enzyme	n	App.Activation Energy of High Temp.Range ($KJ\ mole^{-1}$).	App.Activation Energy of Low Temp.Range ($KJ\ mole^{-1}$).	Critical Temperature (^{o}C)
$Na^{+} - K^{+}$ ATPase	5	72.2 ± 1.1	172.9 ± 7.6	18.8 ± 1.0
Acetylcholinesterase	4	18.8 ± 3.9	36.5 ± 4.4	21.4 ± 2.0

Values expressed as ± 1 x S.E.

TABLE 3 : 4

Parameters from Arrhenius plots processed according to the reversible thermal inactivation model.

Enzyme	n	Activation Energy of the active species of the enzyme KJ $mole^{-1}$	Enthalpy Parameter (ΔH) KJ $mole^{-1}$	Entropy Parameter (ΔS) $J^{o}K\ mole^{-1}$
$Na^{+} - K^{+}$ ATPase	5	67.3 ± 1.5	-196 ± 14	-683 ± 50
Acetylcholinesterase	4	38.8 ± 9.0	-84.6 ± 1.7	-266 ± 6

Values expressed as $\pm$ 1 x S.E.

TABLE 3 : 5

Parameters calculated for the Arrhenius plots of guinea-pig synaptic membrane acetylcholinesterase

i) Data processed according to the phase transition model.

App. Activation Energy at High Temperature Range (KJ $mole^{-1}$)	App. Activation Energy at Low Temperature Range (KJ $mole^{-1}$)	Critical Temperature (^{o}C)	n
20.4 ± 2.4	40.6 ± 2.9	19.2 ± 1.7	4

ii) Data processed according to the reversible thermal inactivation model

Activation Energy of Low Temperature Active Form (KJ $mole^{-1}$)	Enthalpy Parameter ΔH (KJ $mole^{-1}$)	Entropy Parameter ΔS ($J^{o}K^{-1}mole^{-1}$	
38.6 ± 2.0	88.7 ± 3.7	281 ± 13	

Values expressed as $\pm$ 1 x S.E.

FIGURE 3 : 4

Time dependent rate of product formation for rat synaptic membrane acetylcholinesterase.

two dilutions of a single preparation *

- o - = 200* $\mu g.cm^{-3}$

... - □ - = 100 $\mu g.cm^{-3}$

Values expressed as μ moles of thiocholine produced in a single assay tube.

U MOLES OF PRODUCT
0·2
0·4
0·6
0·8
1·0
1·2
1·4
0
10
20
30
40
50
60
65

FIGURE 3 : 3

Time dependent rate of product formation for rat synaptic membrane $Na^+ - K^+$ ATPase

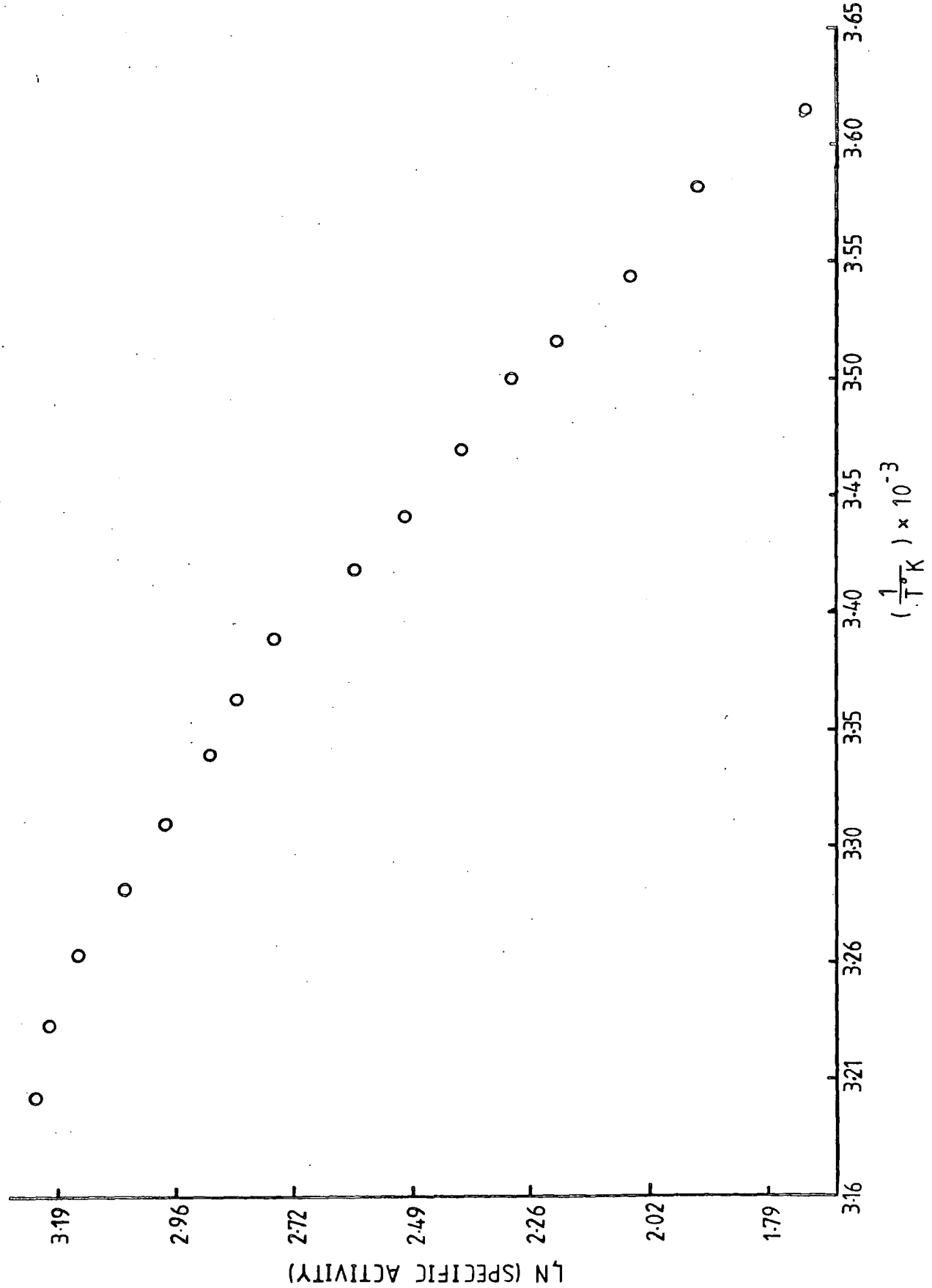
protein concentrations of preparation

- □ - 200 $\mu g . cm^{-3}$

- ○ - 100 $\mu g . cm^{-3}$

- ● - 50 $\mu g . cm^{-3}$

Values represent μ moles of inorganic phosphate in a single assay tube.


LN (SPECIFIC ACTIVITY)
3·19
2·96
2·72
2·49
2·26
2·02
1·79
3·16
3·21
3·26
3·30
3·35
3·40
3·45
3·50
3·55
3·60
3·65
$(\frac{1}{T°K}) \times 10^{-3}$

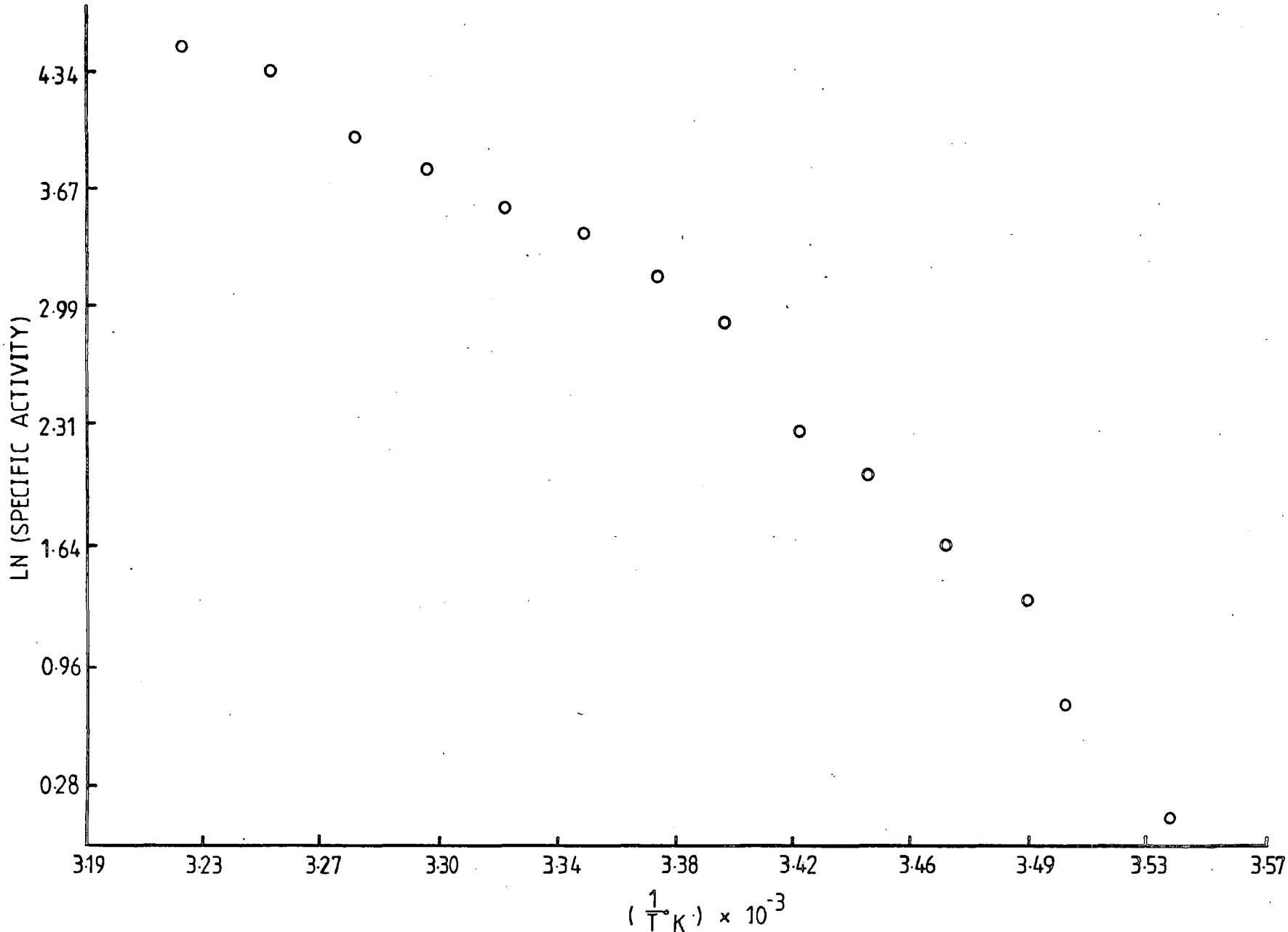

LN (SPECIFIC ACTIVITY)
4·34
3·67
2·99
2·31
1·64
0·96
0·28
3·19
3·23
3·27
3·30
3·34
3·38
3·42
3·46
3·49
3·53
3·57
$(\frac{1}{T}°K) \times 10^{-3}$

FIGURE 3 : 2

Typical Arrhenius plot of rat synaptic membrane $Na^+ - K^+$ ATPase

- o - = $Na^+ - K^+$ ATPase activity

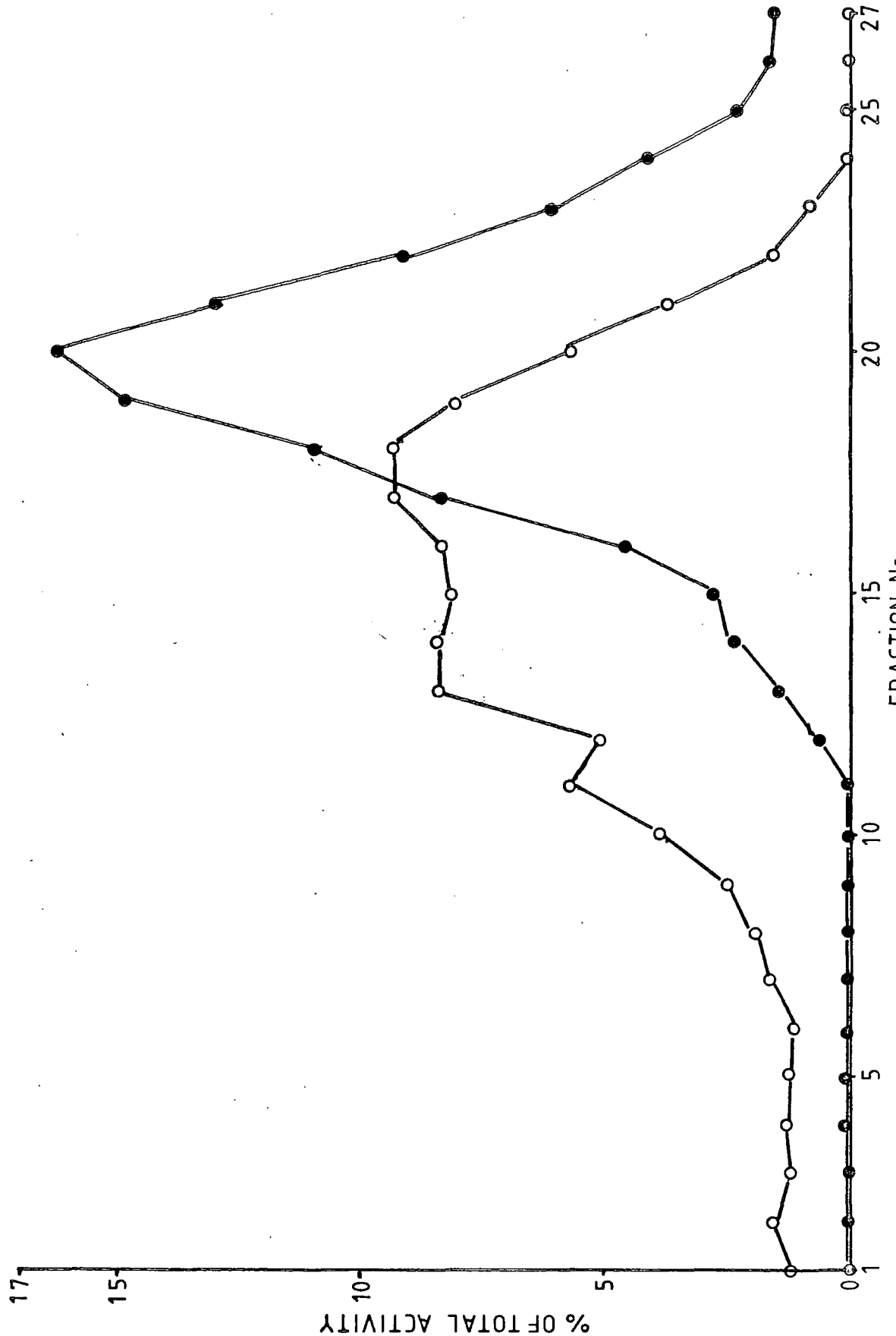
enzyme activity units = μ moles Pi/mg. protein/hr.

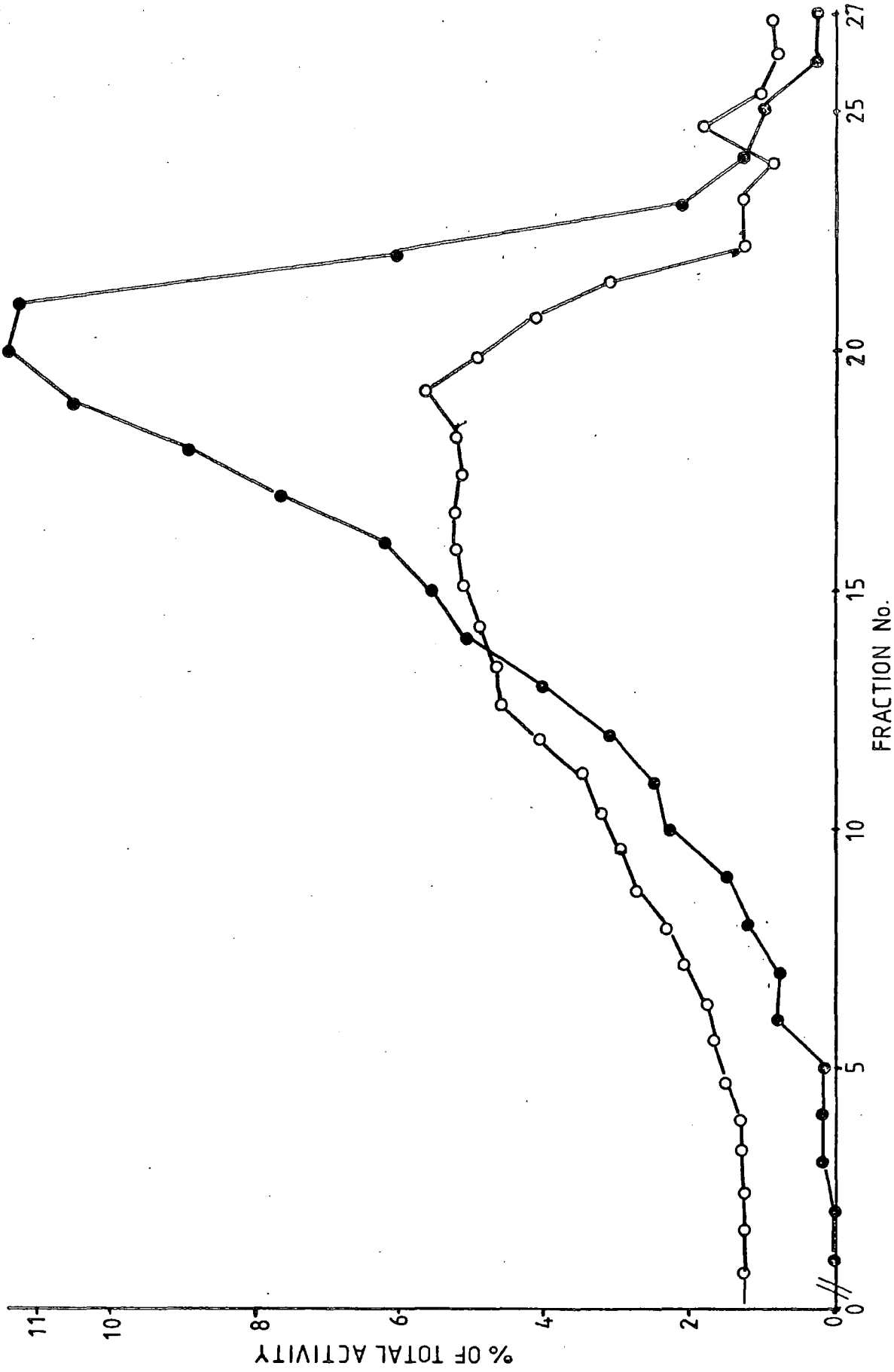
FIGURE 3 : 1

Typical Arrhenius plot of rat synaptic membrane acetylcholinesterase

- O - = Acetylcholinesterase activity

enzyme activity units = μ moles thiocholine/mg protein/hr.


% OF TOTAL ACTIVITY
17
15
10
5
0
1
5
10
15
20
25
27


% OF TOTAL ACTIVITY
0
2
4
6
8
10
11
0
5
10
15
20
25
27
FRACTION No.

GENERAL DISCUSSION

A large literature exists showing that abnormally high temperatures during development can have teratogenic effects. Anderson & Horsfall, 1963 showed that thermal stress during the larval development in the mosquito can influence the subsequent appearance of male anatomical structures. Tsukuda (1960) has shown that slight elevation of environmental temperature produced vertebral column abnormalities in guppies. Similar results have been found for the stickleback by Lindsey (1962). However, the best characterised model of abnormalities produced by high temperatures is that of Drosophila. Mild heat shock at specific stages of embryological development has been shown to induce phenocopies in adults.

The term 'phenocopy' was suggested by Goldschmidt (1935) to describe morphological abnormalities, produced by environmental stresses, which closely resembled morphological changes produced by mutation. Moreover, heat shock applied at specific stages of embryological development induced specific phenocopies (Gloor, 1947; Hadorn, 1955; Milkman, 1963 & 1966; Mitchell & Lipps, 1978).

In phenocopy production heat treatment results in a general reduction of RNA and protein synthesis, it has been demonstracted that new RNA and protein synthesis is induced at specific loci (Ish-Horowicz, Holden & Gehring, 1977). In this case different loci would be heat-inducable at different stages of embryological development producing different phenocopies (Tissiere, Mitchell & Tracy, 1974). It has also been suggested by Tissiere et al (1974) that at specific stages in development the expression of specific loci are repressed by temperature sensitive factors, and that phenocopy production originates from a lack of recovery of the repressors in time to control further development correctly.

Developmental abnormalities resulting from slight elevations of environmental temperatures have also been observed in several species of mammals. In early studies Hsu (1948) reported microphthalmia in rat pups from mothers subjected to hyperthermia at 10 - 11 days of gestation, and Kreshover & Clough (1953) found defects of dentition in rat pups which experienced 40 - 41°C after the 9th day of gestation.

In more systematic studies, Skreb & Frank (1963) and Skreb (1965) immersed the exposed uteri of pregnant rats in saline at 40 - 41°C at times between the 8th and 16th days of gestation. A range of abnormalities were obtained depending on the precise time during foetal development when heat was applied. Anophthalmia and microphthalmia followed heat treatment on day 8 of gestation, whereas anencephaly resulted from treatment on day 10. Heat treatment on day 11 produced cellular abnormality in the prosencephalon and also abnormal tail growth. Abnormal growth of retina and brain resulted from heat treatment on day 12 but were accompanied by some limb growth abnormality. By day 13 of gestation heat treatment resulted in cleft palate, and a wider range of well defined limb abnormalities.

Edwards (1968) was able to obtain a similar range of abnormalities in rats simply by placing pregnant females in an incubator at temperatures of up to 43°C for 1 hr. on one day between the 9th and 14th days of gestation. This study also showed that brain abnormalities resulted from heat treatment at about day 10, whereas limb bud abnormalities and cleft palate predominated in rats heated at day 13.

Edwards (1969 (a) & (b)) found a wide range of abnormalities in newborn guinea-pigs after the mothers had experienced hyperthermia of two or three degrees. In these studies, heat

treatment before 11 days of gestation resulted in a large incidence of resorption. Heat treatment daily between days 11 and 25 produced young with a high incidence of microencephaly and also some with both microencephaly and abnormal bone growth in the hind limbs. In addition, some hydrocephalus was noticed following hyperthermia at this period. The growth of the forelimbs, thorax, abdomen and the neck were sensitive to heat between the 26th and 31st days of gestation, whereas the growth of the hindlimbs and lumbar region were more sensitive during days 30 - 46 of gestation.

Pennycuik (1965) observed some abnormalities in young mice following maternal hyperthermia (42°C). Microphthalmia was observed in mice heated on day 8 of gestation, whereas abnormalities resulting from hyperthermia later in development, days 12 - 15 were localised to the limbs and vertebrae. Embryonic and foetal resorptions resulting from maternal hyperthermia have also been reported in rabbits by Brinsmade & Rubsaamen (1957) and in pigs by Omtveldt, Nelson, Edwards, Stephens & Turman (1971).

In principle, these effects of hyperthermia on mammalian development were similar to those on Drosophila development, in that particular organs and anatomical structures were sensitive to temperature at specific stages of development. In mammals, hyperthermia produces abortion and resorption, at the earliest stages in development after which the CNS becomes sensitive and later in development limb and skeletal abnormalities predominate. However, developmental abnormalities in mammals were generally gross defects as opposed to the phenocopy abnormalities of Drosophila. Furthermore, significant details are known of the mechanism of heat induced phenocopy production, whereas little is known of the mechanism by which heat causes abnormality in mammalian

development nor what may be the consequences to the function of a given tissue with a non-lethal abnormality.

It is clear from the studies detailed in Chapter 1 on the effect of maternal hyperthermia on brain development in guinea-pigs, that mild heat treatment on eight successive days, between the 18th and 25th days of gestation, produced, to a somewhat variable extent, a significant reduction in the brainweight of the offspring. This is in agreement with the general conclusions of Edwards & Wanner (1977), that maternal hyperthermia, if applied at a critical stage in development, can be a teratogenic agent in mammals.

It is interesting to speculate on how a mild heat treatment at such an early stage of development can produce developmental abnormality. It is an essential property of developing organisms that for a given organ, the many constituent parts of that organ each undergo a sequential series of changes, such that each constituent attains a state capable of interacting to form the entire, functional organ. If for some reason any of the stages in development of any of the constituents is delayed or interrupted, then the interaction of the constituents will be deficient and the form and function of the organ would be disturbed.

This developmental pattern applies equally to the mammalian brain. Although the precise details of brain development in the guinea-pig are not as well understood as for the rat, the processes are thought to be essentially similar, in that the rat serves as a general model for mammalian brain development (Berry,1974). In this case brain development consists of three stages, cell proliferative phase, a cell migration phase and a stage of cell differentiation.

Through the early part of the proliferative stage, when the cells of the developing brain form a pseudoepithelium (days 7 - 13 of gestation in the rat, and days 17 - 24 in the guinea-pig), all cells are of a uniform type (Sauer 1935 (a) & (b)). These cells undergo cell division, with a division time of about 12 hours (Shimada & Langman, 1970). The migratory phase begins when one of the cells produced by cell division migrates to the pial surface, forming a defined layer which does not participate in further division. One of the daughter cells of the next set of divisions also migrates past this layer to the pial surface to form a second layer of undividing cells. This process continues throughout the migratory phase until, in the case of the cerebral cortex, seven layers of neurons have been formed in this 'inside out' fashion. The inner layers being formed first (Berry & Rogers, 1965; Berry, 1974).

Once all of the cells have been produced by division and have migrated to their appropriate position, the neurons differentiate by putting out branching dendrites and making synapses. The dendrites grow at swellings at their tips called growth cones (Morest, 1969 (a) & (b)). The number of dendrites associated with a single cell reaches adult numbers quite quickly after the end of migration (12 days post partum in the rat) (Eayrs & Goodhead, 1959), whereas the extension of dendrites continues up to 30 days of age. Synapse formation begins at about the 7th or 8th day of life, in the rat, and is very rapid between the 10th and 35th days of gestation (Aghajanian & Bloom, 1967).

Van der Loos (1965) and Berry & Eayrs (1966) have shown that the architecture of the dendritic tree is not influenced greatly by the cellular environment and are presumed to be under genetic

control, whereas the formation of synapses may be influenced by environmental factors (Hubel & Wiesel, 1963; Wiesel & Hubel, 1963).

In this case it may be possible to interpret the results concerning the effect of maternal hyperthermia on guinea-pig brain development presented in Chapter 1 in the context of the nature and time-course of brain development discussed above. In fact, Chapter 1 shows that maternal hyperthermia applied daily between the 18th and 25th days of gestation resulted in a reduction in brainweight in the newborn guinea-pigs of up to 36%, with a mean reduction of 15%. This degree of microencephaly was similar to that found by Edwards (1969 (b)). Apparently, this resulted at least in part from a reduction in cell numbers, as the DNA content of microencephalic guinea-pig brain was reduced by a mean of 15% Surprisingly, not all brain regions were equally affected; the weight and DNA content of the cerebral hemispheres of microencephalic animals were considerably reduced but these were unaffected in the case of the cerebellum.

Thus hyperthermia was applied at a time in gestation, during the proliferative phase, when the cells form a pseudostratified epithelium, and before any of the products of cell division are committed to form a particular cortical layer. It is reasonable to assume thst the reduction in cell numbers occurs at this stage. Edwards et al (1974) have proposed that the lesion induced by maternal hyperthermia in guinea-pigs results from the death of the dividing cells in the neuroepithelium. It may be that this effect was localised to the cerebrum as the majority of cerebellar cells are produced much later in development.

A further finding was that the activities of plasma membrane marker-enzymes in homogenates of cerebral cortex, expressed as a function of DNA content, were higher in cortical tissues from

microencephalic animals than for control animals (Chapter 1). No such effect was found for cerebellum tissue. This strongly suggests that the subsequent form of cortical neurons can be affected by reduction in cell numbers in this brain region. This change in enzyme activity was not found to be produced by changes in the specific activity of these enzymes (Chapter 1) in the cell membrane.

The change in dendritic architecture is consistent with the view that extra dendritic membrane is developed in microencephalic brain. The membrane for dendritic growth comes from the growth cone (Morest 1969 (a) & (b)). Del Cerro & Snider,(1968) suggested that the membrane vesicles within the growth cone fuse with the tip of the cone to extend the dendrite, whereas Morest (1969 (b)) suggested that membranous filopodia on the growth cone contract, and the membrane forms the main shaft of the dendrite. It has been suggested by Morest (1968, 1969 (a)) that the dendrite grow until the filopodia connect, by some unknown mechanism, with a suitable synaptic site and a synapse forms. If the cell number in the cerebrum are reduced, it is conceivable that the appropriate sites on axons or dendrites for a growing dendrite are lower than for control brain. In this case it would be reasonable to assume that the dendrite would continue to grow to locate a suitable site. This may account for the increase in the enzyme activity per cell number from microencephalic brain (Chapter 1).

This in turn suggests that although the bulk of dendritic growth may be under genetic control, the fine structure of the dendritic array may be sensitive to the microenvironment of brain tissue.

It is also pertinent to ask whether hyperthermia exerts a direct effect on brain cells or via some indirect, hormonal or metabolic

effect. However, it is not possible to differentiate between these possibilities with certainty. Some indication has been given from the work of Cockroft & New (1975) have shown that the developing CNS of explanted and cultured rat foetuses was particularly sensitive to temperatures of 41°C - 42°C at a time in gestation (9 - 10 days) equivalent to the time during development when guinea-pig brain was sensitive to temperature (18 - 25 days). This does not prove a direct effect but at least argues against an effect acting exclusively through maternal metabolism. This also argues to some extent against an endocrine mediated effect as the foetal endocrines would be very immature at this stage.

This study clearly represents a preliminary examination of the effects of hyperthermia on guinea-pig brain development. The major areas of further study suggested by this would be a histological study of the cells of microencephalic guinea-pig cerebra, in the newborn and adult animals. It may also be possible to determine whether complex physiological processes which rely on CNS integration such as the hormonal processes controlled through the hypothalamic-hypophysial axis, and thermoregulation, are affected in microencephalic animals.

The effects of the hypothyroid condition on the rat have constituted one of the most closely investigated models of abnormal brain development. This is because the developmental changes in the hypothyroid rat are analogous to those observed in human cretins (Myant, 1971). The characteristic effects of hypothyroidism on the rat are a severe reduction in the rate of growth of both body and brain weights after the second postnatal week (Balazs et al 1968), although the brain is generally thought to be more sensitive to thyroid hormones than general body growth, in that thyroid hormone therapy, initiated at any time up until

the fourth postnatal week, can eventually restore normal body weight, whereas such therapy must be initiated before the 10th postnatal day to restore completely normal brain development (Eayrs, 1968).

This reduction in brain weight is known not to be an expression of reduced cell numbers, as the DNA content of the cerebrum remains normal in the hypothyroid rat (Balazs et al, 1968; Balazs, et al 1971). This is not particularly surprising as the effects of hypothyroidism induced both before and after birth were similar, and cell division in the rat cerebrum is largely completed by birth (Berry, 1974). In the rat cerebellum where most neurogenesis is postnatal, a transient reduction in cell numbers has been observed, but the adult cell complement is attained by 35 days of age (Legrand, 1967; Hamburgh, 1968; Nicholson & Altman, 1972 (a), Lewis et al, 1973).

It is clear that reduced brain weight in hypothyroidism results from a general hypotrophy of brain cells. Almost every aspect of the biochemical and histological development of both neurones and glia have been shown to be adversely affected by hypothyroidism. Dendritic development is severely impaired in both the cerebrum (Eayrs & Taylor, 1951) and cerebellum, (Legrand, 1967; Rebiere & Legrand, 1972) and synaptogenesis is impaired in both brain regions (Cragg, 1970; Nicholson & Altman, 1972 (b)). Consequently the glial space is increased and abnormal astrocytes are observed (Legrand, 1967; Clos & Legrand, 1973; Pesetsky, 1973). Hypothyroidism also affects myelination as Hamburgh et al (1977) have reported a decreased number of myelin figures in hypothyroid rat cerebrum, and several authors have reported lower yields of myelin isolated from hypothyroid rat brain (Balazs, Brooksbank, Davison, Eayrs & Wilson 1969; Matthieu, Reier, Shawchak, 1975; Rosman & Malone, 1975). However, Balazs et al (1969) reported

no abnormalities in the maturation of the myelin from hypothyroid rat brains.

As many enzymes, associated with developing neuronal structures, show a marked increase in tissue concentration during the immediate postnatal period in the normal rat, the activities of many of these enzymes have been measured in homogenates of hypothyroid rat brain. Most enzyme activities, expressed either as a function of cell number (DNA content) or tissue weight, were reduced in hypothyroid brain. This included glycolytic enzymes (Schwark, Singhal & Ling, 1971; Schwark, Singhal & Ling, 1972 (a) & (b)), mitochondrial enzymes (Hamburgh & Flexner, 1957; Garcia-Argiz et al, 1967), enzymes of neurotransmitter metabolism (Geel & Timiras, 1967; Balazs et al, 1968; Rastogi et al, 1974) and $Na^+ - K^+$ ATPase (Garcia-Argiz et al, 1967; Valcana & Timiras, 1969).

This work shows that no single enzyme, or organelle or cell type constitutes the site of thyroid hormone action. The reductions in enzyme levels are simply consistent with the histological findings of reduced cell growth in hypothyroid rat brain.

Despite the severity of cellular hypotrophy the outward signs of impaired brain function in hypothyroidism are surprisingly slight. Eayrs & Lishmann (1955) and Eayrs & Levine (1963) have shown that the appearance of several markers of behavioural development was only delayed in hypothyroid rats, whereas quite subtle tests are necessary to detect the permanently impaired cognitive abilities in these animals (Eayrs, 1961). Indeed it is not clear how the reduced cell growth relates to brain function. Eayrs (1966) suggested that the decrease in neuronal growth argued for a decrease in cell interaction and therefore synaptogenesis,

of some 70%, whereas Cragg (1970) observed only a 20% decrease in synapses. Thus if the link between cellular hypertrophy and brain malfunction in hypothyroidism is at the synaptic level, then the observations presented in Chapter 2 shich show aberrant qualitative development of synapses are of particular interest.

The increase in the specific activity of synaptic plasma membrane marker enzymes during normal postnatal development in the rat have been associated with the development of adult synaptic function during synaptogenesis (Abdel-Latif et al, 1967). Two models could account for this change as either immature synapses of low enzyme activity are replaced by different synapses, characteristic of adult brain, which exhibit higher enzyme activity or alternatively, individual synapses may change so as to manifest a higher enzyme activity during development.

Specific activity in this case was enzyme activity expressed as a function of other membrane proteins, thus an increase in this value may result from a loss of membrane proteins other than the marker enzyme during development, as well as a selective increase in the quantitity of marker enzyme molecules during development. However, the former interpretation is considered less likely as the protein/phospholipid ratio of adult rat brain microsomal and synaptic membranes were similar (Chapter 2) indicating no differential loss of membrane proteins during synaptogenesis and it is more likely that the higher specific activities of marker enzymes reflects an increase in enzyme molecules in the membrane.

In this case, the reduced rate of increase in the specific activity of synaptic membrane enzymes in hypothyroid rat brain presented in Chapter 2, would represent a qualitative change in the synapses as a result of thyroid hormone deficiency. In addition, in hypothyroid rat brain the level of plasma membrane marker enzymes

in tissue homogenates is not proportional to the quantity of membrane material in homogenates and the decreased activities in hypothyroid rat brains do not solely reflect a decrease in cell size or degree of branching. Although it is quite possible that the reduction in neuronal size may cause defective brain function in hypothyroidism, it is also possible that this may be produced by a reduced capacity of individual neurons to participate in neuronal pathways.

Although the precise mechanism of action of thyroid hormones in mammalian tissues is not understood, it is clear that the action of thyroid hormones in amphibian metamorphosis involved the expression of a novel set of genes. It would seem reasonable to assume that thyroid hormones exert a similar mode of action in mammalian tissues. However, it is clear that there is no absence of particular gene products in hypothyroid rat brain. Therefore, it is possible that thyroid hormones either switch on previously inactive copies of particular genes, or that in mammalian tissues thyroxine stimulates protein synthesis at the translational level. In either case the conclusion of these studies presented in Chapter 2 are that one of the most important effects of hypothyroidism for mammalian brain function would be an inhibition of the qualitative development of synapses.

It is possible to compare the effect of transient hyperthermia on the brain development of the guinea-pig, with the effect of prolonged hypothyroidism on rat brain development. Both newborn, heat treated guinea-pigs and the hypothyroid rat at an equivalent stage of development (45 - 50 days post partum) have lower brain weights than would be expected for their respective controls. However, in hypothyroid rats the weight of the cerebellum is reduced (Chapter 2) whereas in microencephalic guinea-pigs no reduction in cerebellar weight was observed (Chapter 1). Furthermore, results presented in Chapter 1 have shown that the reduction in cerebral

weight in microencephalic guinea-pigs and hypothyroid rats do not have the same cause, as the former results primarily from a considerable reduction in cell numbers, whereas in the latter case no such deficiency has been found (Balazs et al, 1968). It is therefore interesting that both phenomena produce offspring with impaired cognitive abolities (Rabinowitz & Rosvold, 1951; Jonson et al, 1976).

In addition, both hypothyroidism and hyperthermia produced different effects on the enzymology of brain tissue. In the hypothyroid rat brain the activities of plasma membrane marker enzymes, expressed as a function of cell numbers were lower than for controls (Garcia-Argiz et al 1967; Geel & Timiras, 1967; Valcana & Timiras, 1969), also the specific activities of these enzymes in isolated plasma membrane fractions were lower for hypothyroid rat brain than for controls. In the microencephalic guinea-pig however, there was no change in specific activities of these enzymes in similar fractions, and the activities as a function of cell number was higher than for controls. This suggests that the different form of lesion have different subsequent effects on cell structure.

Indeed, histological studies on hypothyroid rat brain have clearly shown that the dendritic arrays of cortical and cerebellar neurons in hypothyroid rat brain are less elaborate and extensive than for controls (Eayrs & Taylor, 1955) which is consistent with the interpretation of the enzyme data. No such histological observations exist for microencephalic guinea-pigs, but the enzyme data would suggest that in this case surviving neurons exhibit increased dendritic growth.

From this point the discussion will concern the studies on rat synaptic membrane acetylcholinesterase. The 'fluid-mozaic' model of membrane structure proposed by Singer & Nicholson (1972) and reviewed in detail by Singer (1974) was able to explain membrane observations such as the sensitivity of membrane lipids in intact membranes to phospholipases, data from fluorescent probe studies, and the ability of proteins to flow through the membrane in phenomena such as antigen 'capping' in lymphocytes. This view of membrane structure has been largely confirmed by electron microscopic studies of 'freeze-fractured' membranes.

The physical state of the membrane lipids have been shown to be sensitive to temperature, becoming more rigid at lower temperatures, in a manner analagons to the thermotropic gel-liquid crystal phase changes found for artificial phospholipid membrane (Papahadjopoulos & Watkins, 1967). This viscosity of the membrane lipid has also been suggested to influence the properties of membrane enzymes, in producing non-linear Arrhenius plots (Wynn-Williams, 1970). In this case the apparent activation energy increases at lower temperatures. The fluid-mozaic model would argue that the more rigid lipid at lower temperatures constrains the membrane proteins in contact with it to a high apparent activation energy state. It has been suggested in Chapter 3 that this view cannot explain the observations from Arrhenius plots for the $Na^+ - K^+$ ATPase enzyme of rat synaptic membranes. The non-linearity of the temperature plots are likely to be due to changes in the membrane lipid, but not a pure phase transition as suggested by Wynn-Williams (1970). However, the apparent activation energy of this enzyme in the physiological temperature range was very high (approximately 80 KJ $mole^{-1}$) compared to soluble enzymes, and the even higher values found at lower temperatures (150 - 200 KJ $mole^{-1}$) were unlikely to represent a true rate constant, and that such values

are better explained by a reversible thermal inactivation process (Chapter 3). However, this work confirmed the general conclusion that non-linear Arrhenius plots were characteristic of membrane-bound enzymes.

A more surprising observation was that non-linear Arrhenius plots are obtained for rat synaptic membrane acetylcholinesterase. Within the Singer-Nicholson model, membrane proteins are classified firstly as integral, or intrinsic, in that they have significant proportions of their structure in contact with the hydrophobic portion of the membrane lipid and secondly, as peripheral or extrinsic, in that the enzyme is associated with the membrane lipid polar head groups or other membrane proteins. The acetylcholinesterase is a peripheral protein by this classification. It was difficult to see how non-linear Arrhenius plots could be due to lipid changes in the case of acetylcholinesterase. This was further complicated by the observation, in Chapter 4, that the low-ionic strength solubilized fraction of acetylcholinesterase also exhibited non-linear Arrhenius plots, which also suggested that this property had nothing to do with membrane lipid.

This view was contradicted by observations, also presented in Chapter 4, that various detergents and lipophilic agents interact with both soluble and membrane-bound acetylcholinesterase and tend to abolish the non-linearity of the temperature plots. In addition high ionic strength treatment extracted a single phospholipid species from Lubrol solubilized acetylcholinesterase (Chapter 5). These observations have been reconciled by suggesting that the rat brain acetylcholinesterase is a lipoprotein, and that this lipid interacts with the enzyme to form a low activation energy state at physiological temperatures, in a manner consistent with the model described in Chapter 3.

Lipoprotein structure has also been suggested to explain the properties of bovine erythrocyte acetylcholinesterase by Beauregard & Roufogalis (1977). Furthermore, several other studies have attempted to link membrane association or lipid effects with the properties of acetylcholinesterases from various species. From this it is clear that some properties of this enzyme are species-specific. For example, the human erythrocyte enzyme cannot be dissociated in low-ionic strength media (Mitchell & Hanahan, 1966), whereas 50% of the rat brain enzyme can be dissociated in this way (Chapter 4) and more than 80% of bovine erythrocyte enzyme can be dissociated by this treatment (Beauregard & Roufogalis, 1977). In addition, the substrate kinetics of the soluble and membrane-bound rat brain acetylcholinesterases were found to be similar (Chapter 6), whereas Reavill et al (1978) showed that this property was membrane dependent in the case of the pig brain enzyme.

Other differences between the properties of the rat brain acetylcholinesterase presented in Chapters 3 - 6, and those revealed in other studies cannot be attributed to species differences. In some cases solubilisation of acetylcholinesterase with detergents produced enzymes with linear Arrhenius plots (Reavill et al, 1978), whereas in other cases Arrhenius plots for solubilised enzyme were non-linear (Beauregard & Roufogalis, 1977). These differences may be reconclied in terms of results presented in Chapter 4 which suggest that the thermotropic transition in acetylcholinesterase activity was sensitive to the nature of the solubilising detergent. Thus it may be possible to interpret each of the studies which suggest lipid involvement in the properties of acetylcholinesterase may each be interpreted in terms of a lipoprotein structure for mammalian acetylcholinesterase. (Sihotang, 1976; Beauregard & Roufogalis, 1977; Reavill et al, 1978).

It is possible that similar properties to those described in this study may have been observed if the techniques used had been better characterised.

The work presented in Chapter 3 - 6 seeks to describe a series of properties characteristic of the rat brain acetylcholinesterase which extend the range of possible protein lipid interactions in biological membranes. It is suggested that sufficient lipid is bound to acetylcholinesterase to produce phase change behaviour and suggests that the physical properties of a relatively small number of phospholipids in the vicinity of a membrane enzyme may influence enzyme properties rather than the bulk of the membrane lipid.

In a recent review, Massoulie (1980) has postulated that all acetylcholinesterases, not only from mammalian sources, have a common structure. In this model the enzyme is said to consist of three components, firstly an easily solubilised enzyme dimer, non-covalently bonded to a second enzyme dimer which is covalently attached to the third component, a collagen-like tail which is embedded deeply into the bilayer. This model has been derived largely from studies on the well characterised acetylcholinesterase from the electric organ of Torpedo and Electrophorus, and also draws upon molecular-size data of aggregates obtained from mammalian tissue sources.

The data obtained in this study do not directly contradic this view. In fact the surcrose density gradient analyses presented in Chapter 6 show that the low and high molecular weight aggregates exist in rat brain acetylcholinesterase extracts. But this study has suggested that other factors such as phospholipid association to be important in consideration of the properties and structure of rat brain acetylcholinesterase, and probably

also for acetylcholinesterases from other mammalian tissues. No such lipid relationships have been suggested for the eel enzyme (Rosenberry, 1976). In addition, the membrane affinity of the rat enzyme and that from other species are so different as to argue against a common structure for all acetylcholinesterases.

Perhaps the most interesting prospect revealed by this study is the possibility of using acetylcholinesterase as a model for the characterisation of lipid-protein interactions in general. It is clear that such interactions in biological membranes, are of physiological significance. There is evidence that membrane proteins require optimum membrane lipid fluidity for function (Sinensky, 1971; Cossins, 1977), and also that specific lipids are required for the activity of membrane-bound enzymes (Wheeler & Whittam, 1970). However, it has been difficult to study the effect of lipids on membrane proteins, as although a number of membrane-bound enzymes have been purified and shown to require phospholipids for activity (Garland & Cori, 1972; Hinkle, Kim & Racker, 1972; Kimelberg & Papahadjopoulos, 1972; Lu, Kuntzman, Wost & Jacobson, 1972; Neilson & Fleischer, 1973, Rogers & Strittmatter, 1973, Hokin 1974; Gorski & Kasper, 1978). However, purification has inevitably entailed disturbing the enzyme lipid relationship, and in these cases activity could only be restored by reconstitution with phospholipid dispersions and the complexes so generated are not very stable.

The acetylcholinesterase, on the other hand could be used to avoid these problems as the enzyme has been shown in the present study to be stable in the low ionic-strength solubilised and Lubrol detergent solubilised forms as well as in the membrane-bound form. This system offers the opportunity to examine the

effects of factors such as phospholipid head group composition , fatty acid composition and cholestrol content on the binding of proteins to liposomes and on enzyme function with the minimum of manipulation. Such data would certainly be of significance for the analysis of membrane-protein function in general.

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FIGURE 6 : 22

Sucrose density-gradient analysis of Lubrol W - X treated low ionic-strength soluble and membrane-bound rat synaptic membrane acetylcholinesterase.

The membrane-bound fraction, after Lubrol W - X treatment, was centrifuged at 100,000 xg for 1 hour. The supernatant was layered onto the gradient.

- ● - = soluble enzyme

- O - = membrane-bound enzyme

Enzyme activity in each fraction is expressed as a percentage of the total activity added to each gradient.

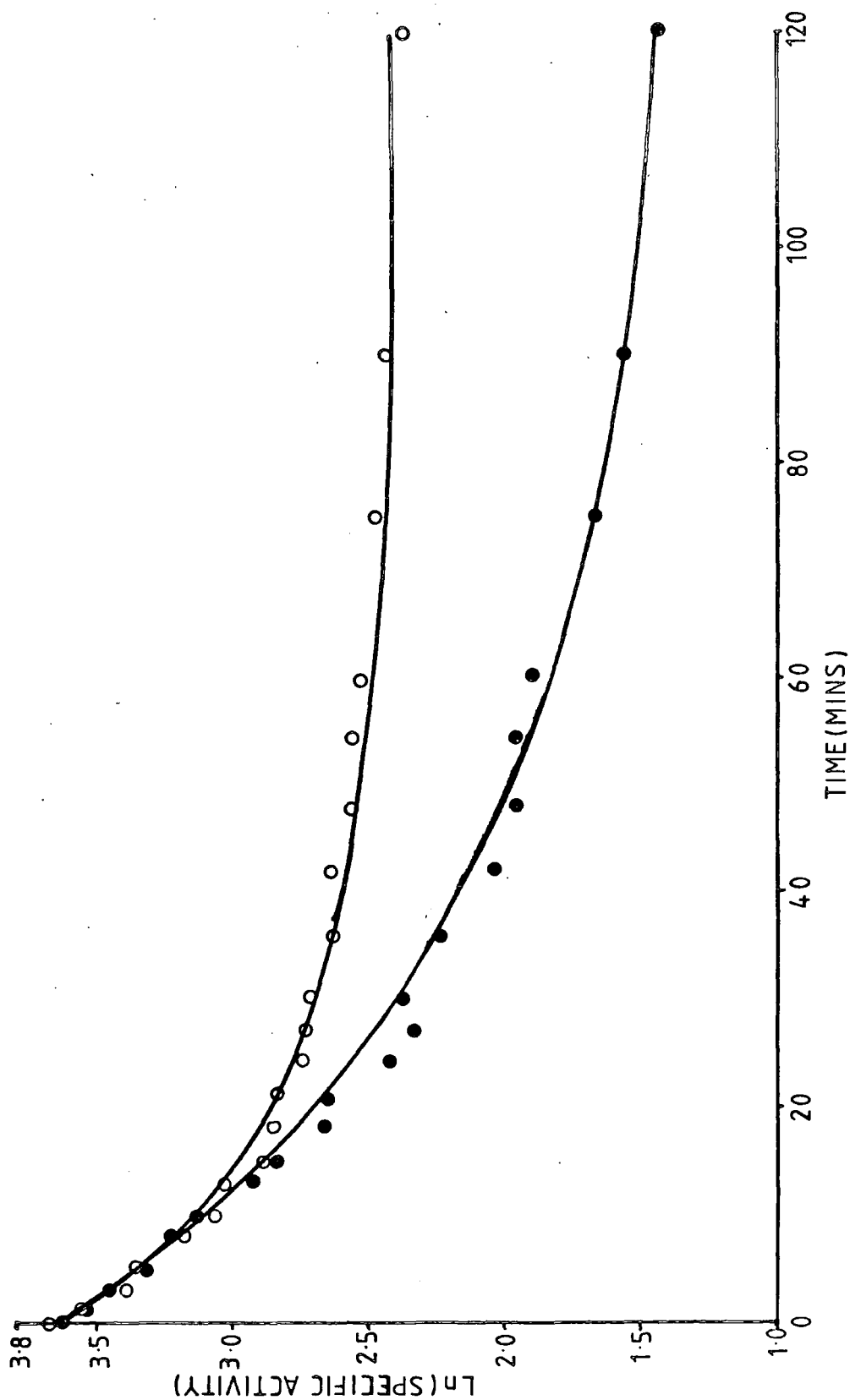

Ln (SPECIFIC ACTIVITY)
3·8
3·5
3·0
2·5
2·0
1·5
1·0
0
20
40
60
80
100
120
TIME (MINS)

FIGURE 6 : 21

The effect of Lubrol W - X treatment on the isothermal inactivation of the low ionic-strength soluble rat synaptic membrane acetylcholinesterase.

- ● - = control soluble preparation at 47°C

- O - = Lubrol W - X treated soluble preparation at 47°C

Specific activity expressed in units of μ moles thiocholine/ mg protein/hr.

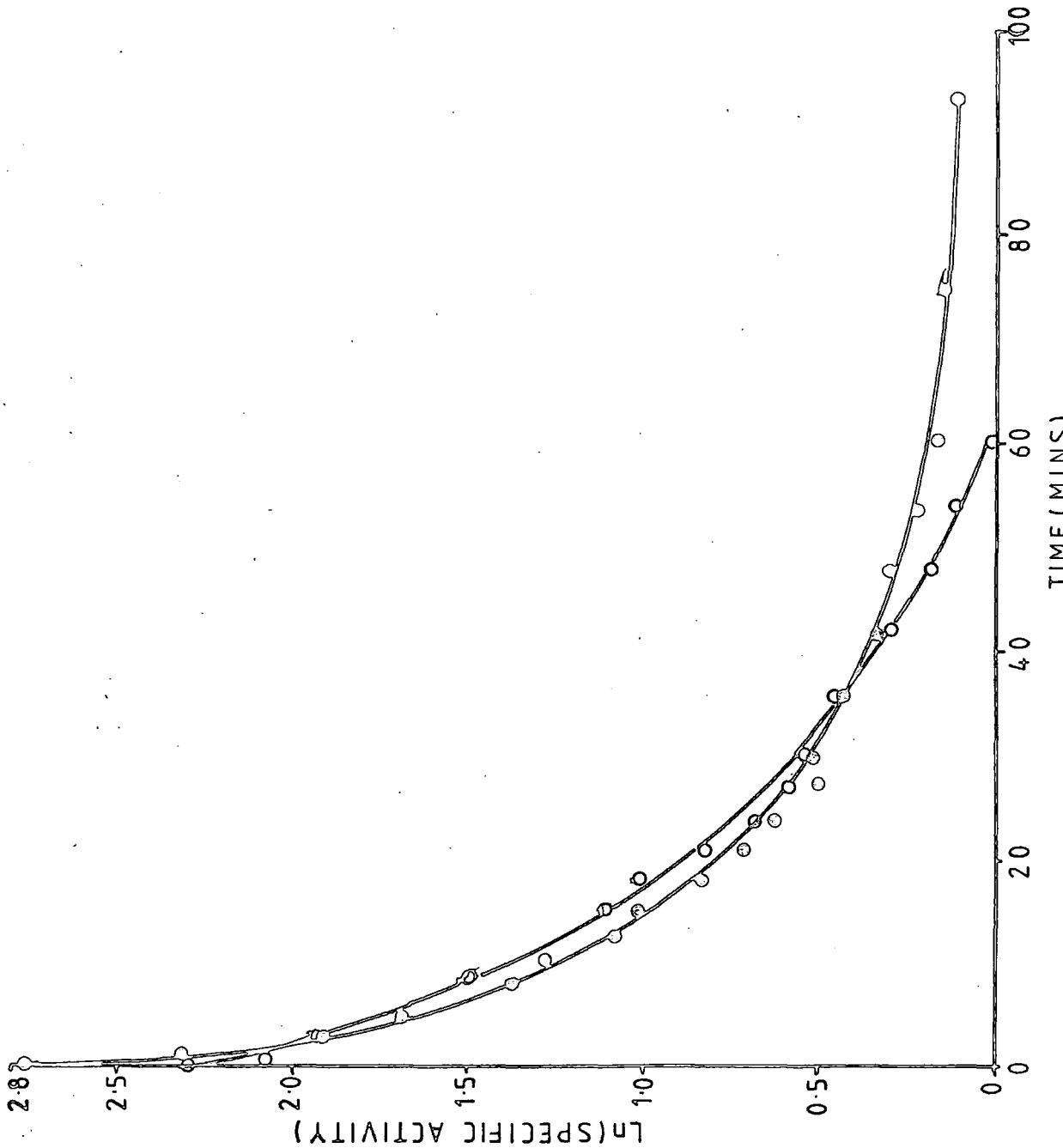

Ln(SPECIFIC ACTIVITY)
2·8
2·5
2·0
1·5
1·0
0·5
0
0
20
40
60
80
100
TIME (MINS)

FIGURE 6 : 20

The effect of Lubrol W - X treatment on the isothermal inactivation of membrane-bound rat synaptic membrane acetylcholinesterase.

- O - = control membrane-bound preparation at 57°C

- ⊙ - = Lubrol W - X treated membrane-bound preparation at 57°C

Specific activity expressed in units of μ moles thiocholine/ mg protein/hr.

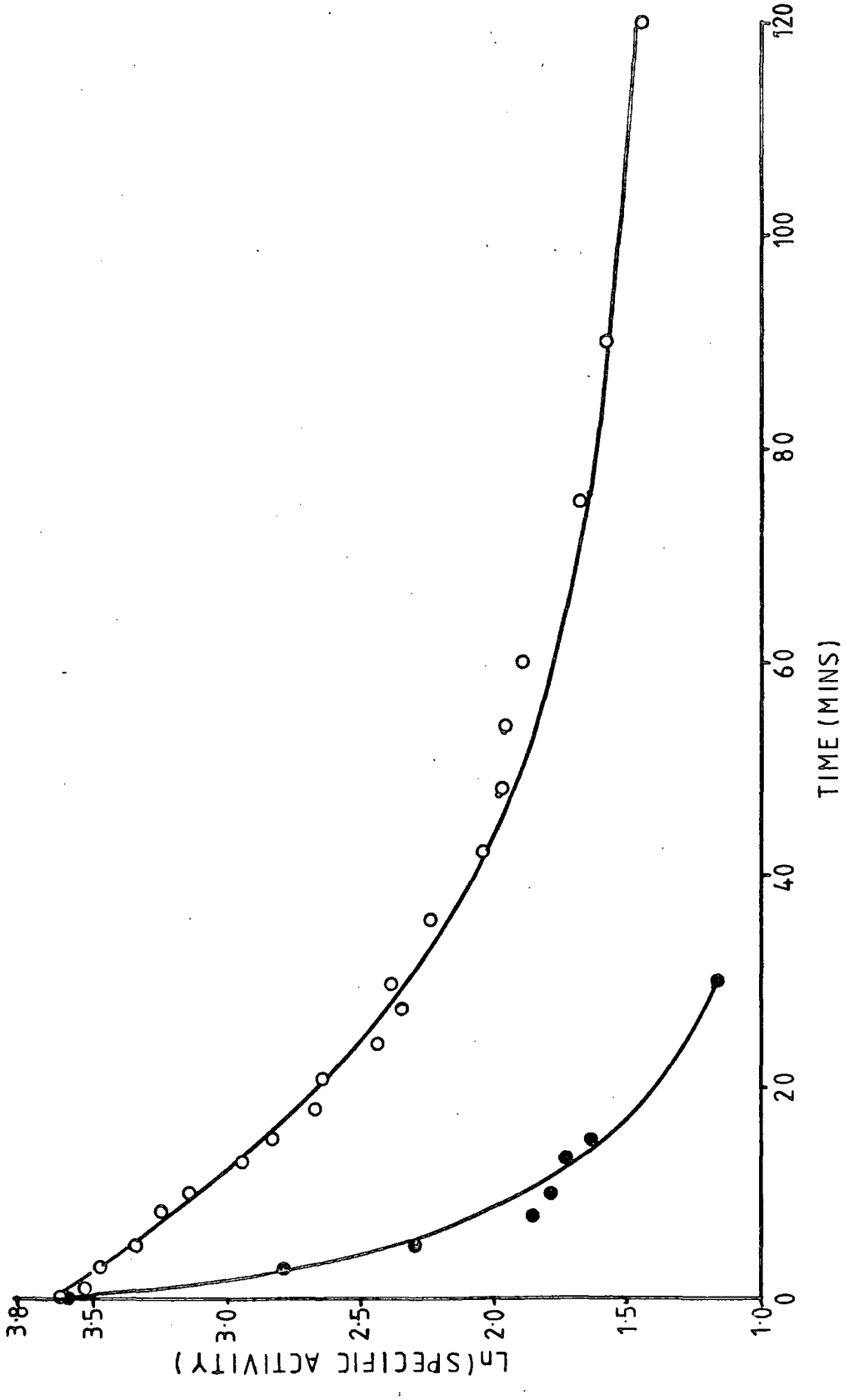

Ln(SPECIFIC ACTIVITY)
3·8
3·5
3·0
2·5
2·0
1·5
1·0
0
20
40
60
80
100
120
TIME (MINS)

FIGURE 6 : 19

The effect of Triton x - 100 treatment on the isothermal inactivation of the low ionic strength soluble rat synaptic membrane acetylcholinesterase.

- O - = control soluble preparation at 47°C

- ● - = Triton x - 100 treated soluble preparation at 47°C

Specific activity expressed in units of μ moles thiocholine/ mg protein/hr.

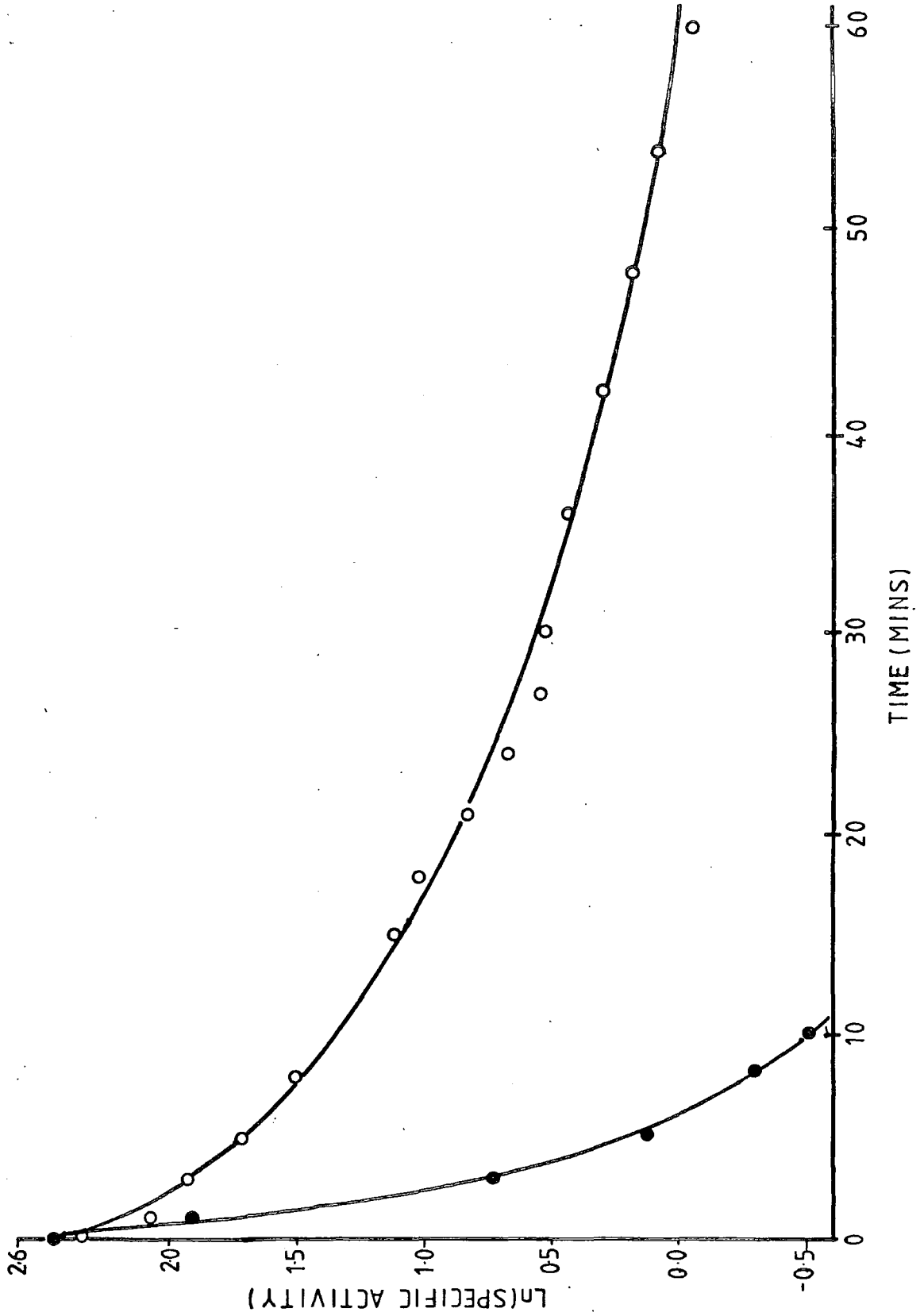

Ln(SPECIFIC ACTIVITY)
2·6
2·0
1·5
1·0
0·5
0·0
-0·5
0
10
20
30
40
50
60
TIME (MINS)

FIGURE 6 : 18

The effect of Triton x - 100 treatment on the isothermal inactivation of membrane-bound rat synaptic membrane acetylcholinesterase.

- O - = control membrane-bound preparation at 57°C

- ⊛ - = Triton x - 100 treated membrane-bound preparation at 57°C

specific activity expressed in units of μ moles thiocholine/ mg protein/hr.

Ln(SPECIFIC ACTIVITY)
1.2
2.0
3.0
3.8
30
60
90
TIME/MINS

FIGURE 6 : 17

The effect of sodium deoxycholate treatment on the isothermal inactivation of low ionic strength soluble rat synaptic membrane acetylcholinesterase.

- ○ - = control soluble preparation at 47°C

- ◉ - = deoxycholate-treated soluble preparation at 40°C

- □ - = deoxycholate-treated soluble preparation at 47°C

Specific activity expressed in units of μ moles thiocholine/ mg protein/hr.

Ln(SPECIFIC ACTIVITY)
0
1·0
2·0
2·6
0
0·2
0·4
0·6

FIGURE 6 : 16

The effect of sodium deoxycholate treatment on the isothermal inactivation of membrane-bound rat synaptic membrane acetylcholinesterase.

- o - = control membrane preparation at 57°C

- ● - = deoxycholate-treated membrane preparation at 52°C

- □ - = deoxycholate-treated membrane preparation at 57°C

Specific activity is expressed in units of μ moles thiocholine/mg protein/hr.

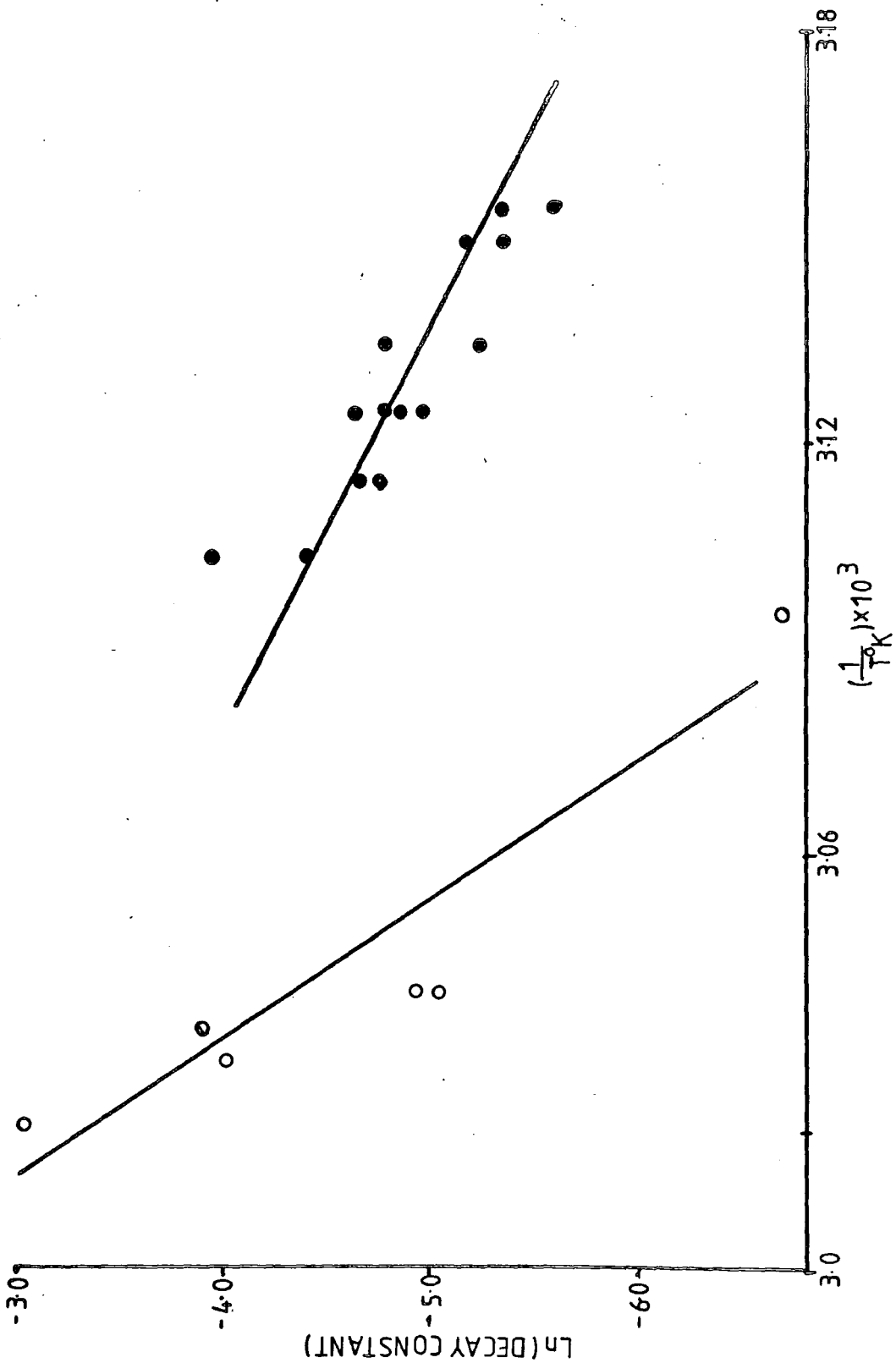

Ln (DECAY CONSTANT)
-3·0
-4·0
-5·0
-6·0
3·0
3·06
3·12
3·18
$(\frac{1}{T^{\circ}K})\times10^{3}$

FIGURE 6 : 15

Temperature dependence of the decay constants of the stable-state of low ionic-strength soluble and membrane-bound forms of rat synaptic membrane acetylcholinesterase.

i) -O- = membrane-bound acetylcholinesterase

Ea of decay process = 410 KJ $mole^{-1}$

ii) -◉- = soluble acetylcholinesterase

Ea of decay process = 135 KJ $mole^{-1}$

Decay constants are expressed in units of min^{-1}.

Lines were fitted by linear regression analysis.

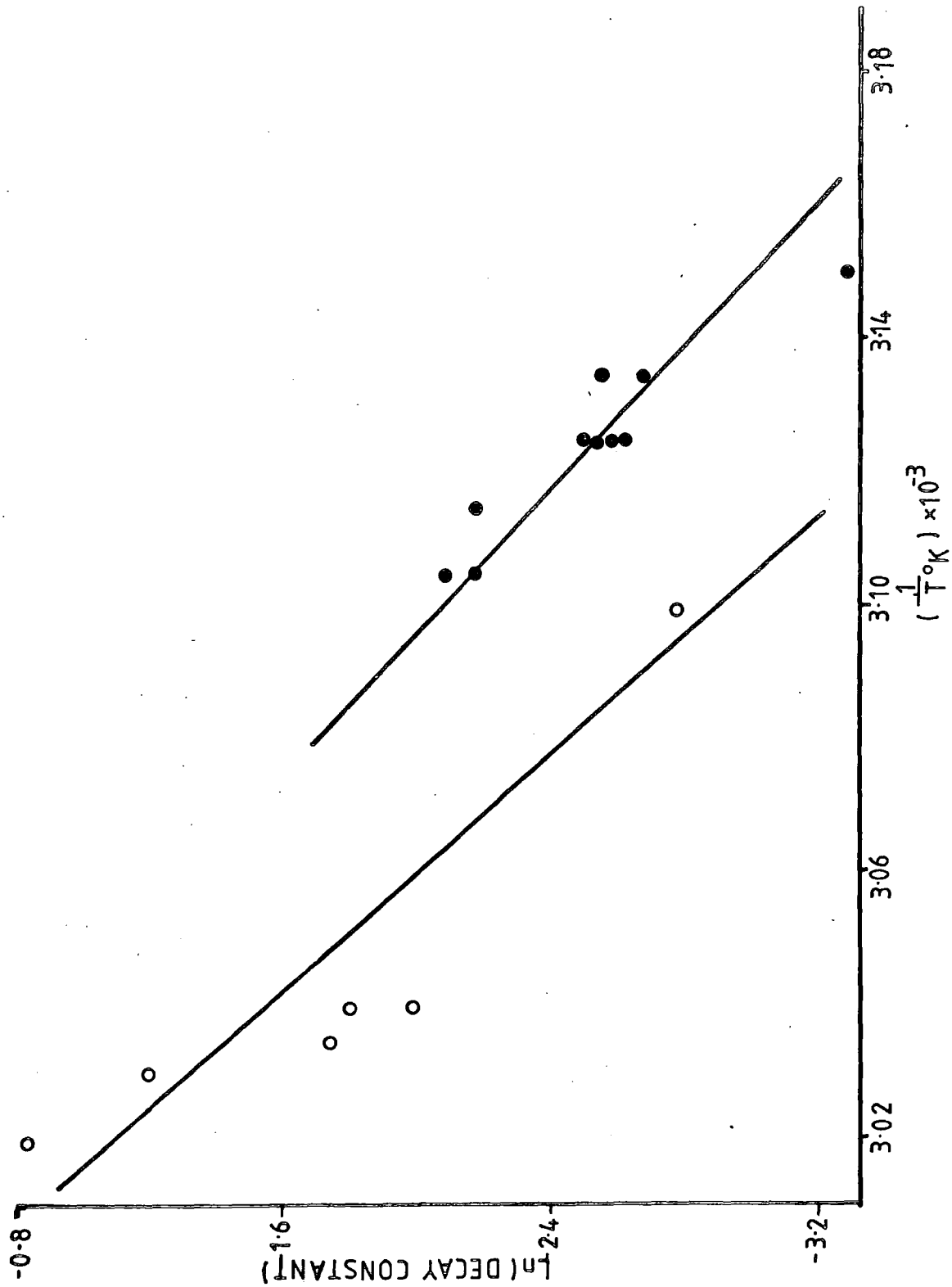

-0·8
-1·6
-2·4
-3·2
ln(DECAY CONSTANT)
3·02
3·06
3·10
3·14
3·18
(1/T°K) ×10⁻³

FIGURE 6 : 14

The temperature dependence of the decay constants of the labile state of low ionic-strength soluble and membrane-bound rat synaptic membrane acetylcholinesterase.

- O - decay constants for membrane-bound acetylcholinesterase

- ● - decay constants for soluble acetylcholinesterase

Decay constants are expressed in units of min^{-1}.

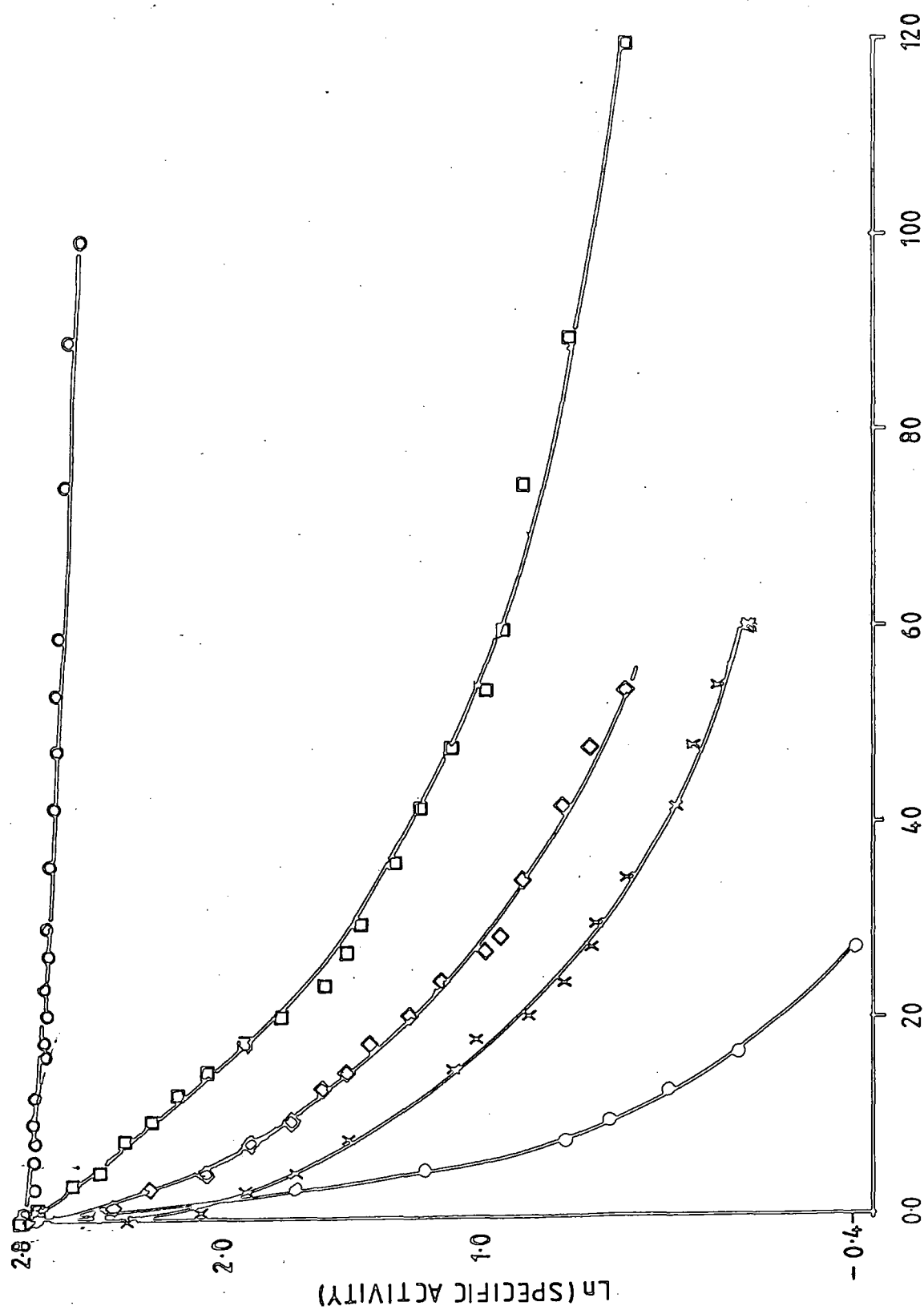

Ln (SPECIFIC ACTIVITY)
2·8
2·0
1·0
- 0·4
0·0
20
40
60
80
100
120

FIGURE 6 : 13

Typical isothermal inactivation profiles for membrane-bound rat synaptic membrane acetylcholinesterase.

Inactivation was carried out at the following temperatures;

- O - = 50°C

- □ - = 56°C

- ◇ - = 56.5°C

- X - = 57°C

- ⊜ - = 58°C

Specific activity expressed as μ moles thiocholine/mg protein/hr.

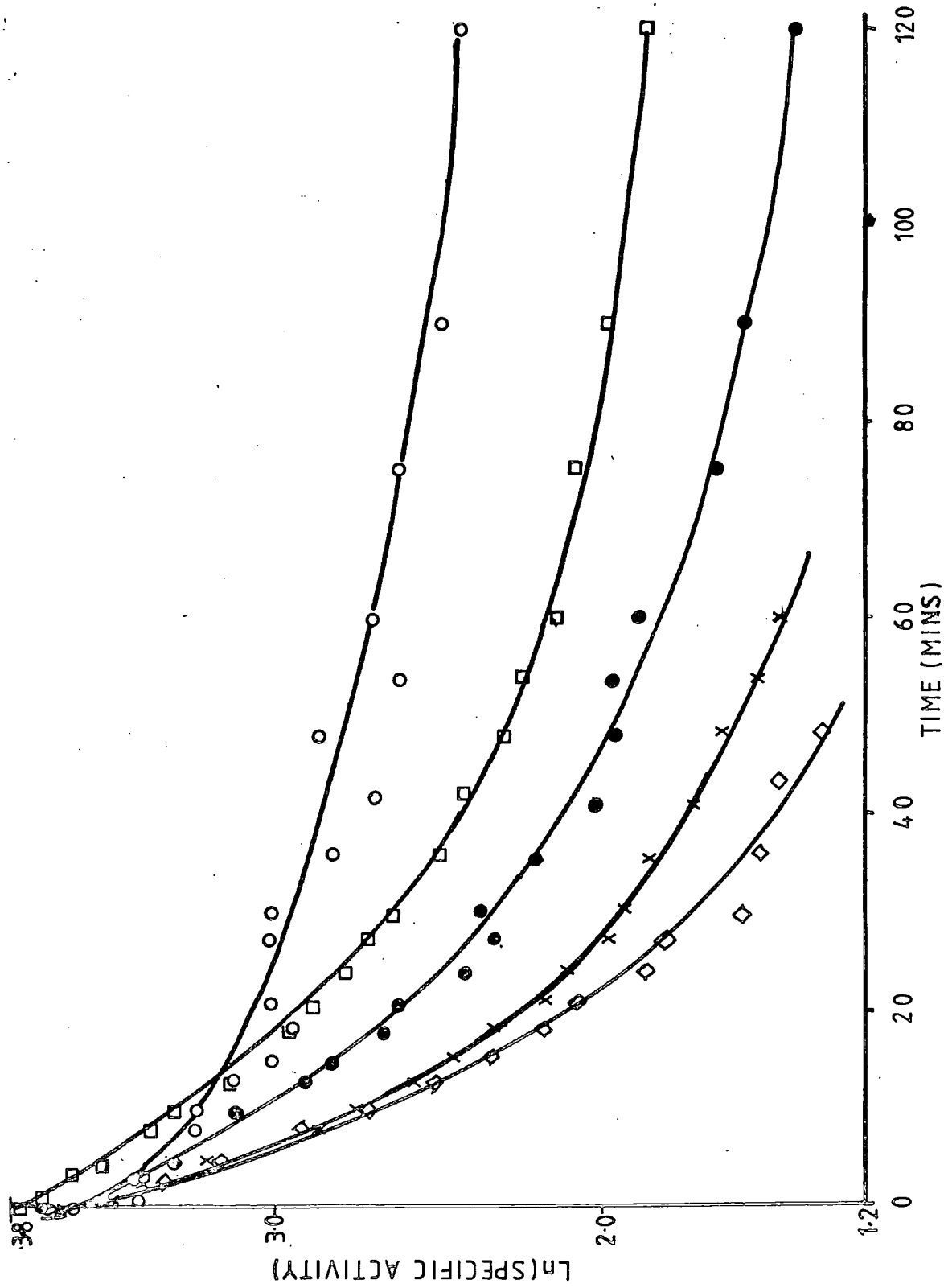

Ln(SPECIFIC ACTIVITY)
3·8
3·0
2·0
1·2
0
20
40
60
80
100
120
TIME (MINS)

FIGURE 6 : 12

Typical isothermal inactivation profiles for low ionic-strength soluble rat synaptic membrane acetylcholinesterase.

Inactivation was carried out at the following temperatures;

- O - = 44°C

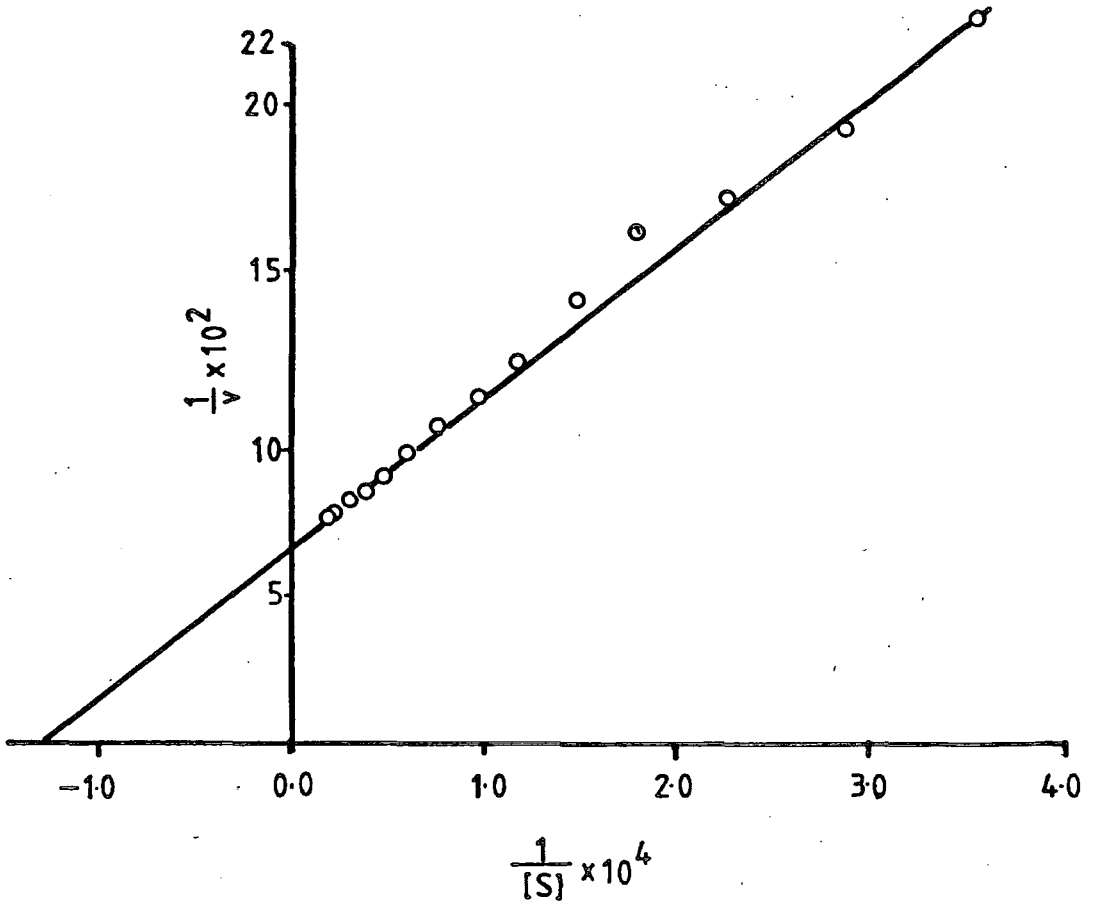
- □ - = 45°C

- ● - = 47°C

- X - = 48°C

- ◇ - = 49°C

Specific activity expressed as μ moles thiocholine/mg protein/hr.


22
20
15
10
5
$\frac{1}{v} \times 10^2$
−1·0
0·0
1·0
2·0
3·0
4·0
$\frac{1}{[S]} \times 10^4$

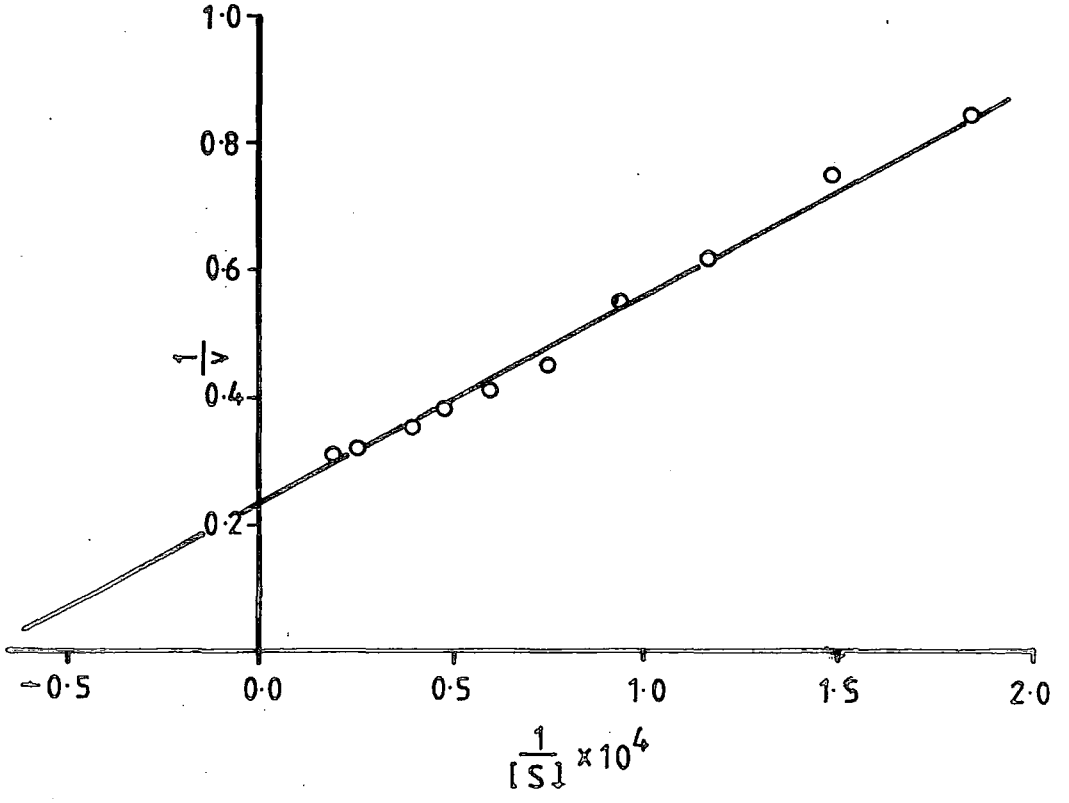

1·0
0·8
0·6
0·4
0·2
$\frac{1}{v}$
−0·5
0·0
0·5
1·0
1·5
2·0
$\frac{1}{[S]} \times 10^4$

FIGURE 6 : 10

Substrate inactivation of membrane-bound rat synaptic membrane acetylcholinesterase. A typical Lineweaver - Burk plot is presented. Km was calculated and the line fitted by a computer assisted fit to the Micahelis - Menten relationship.

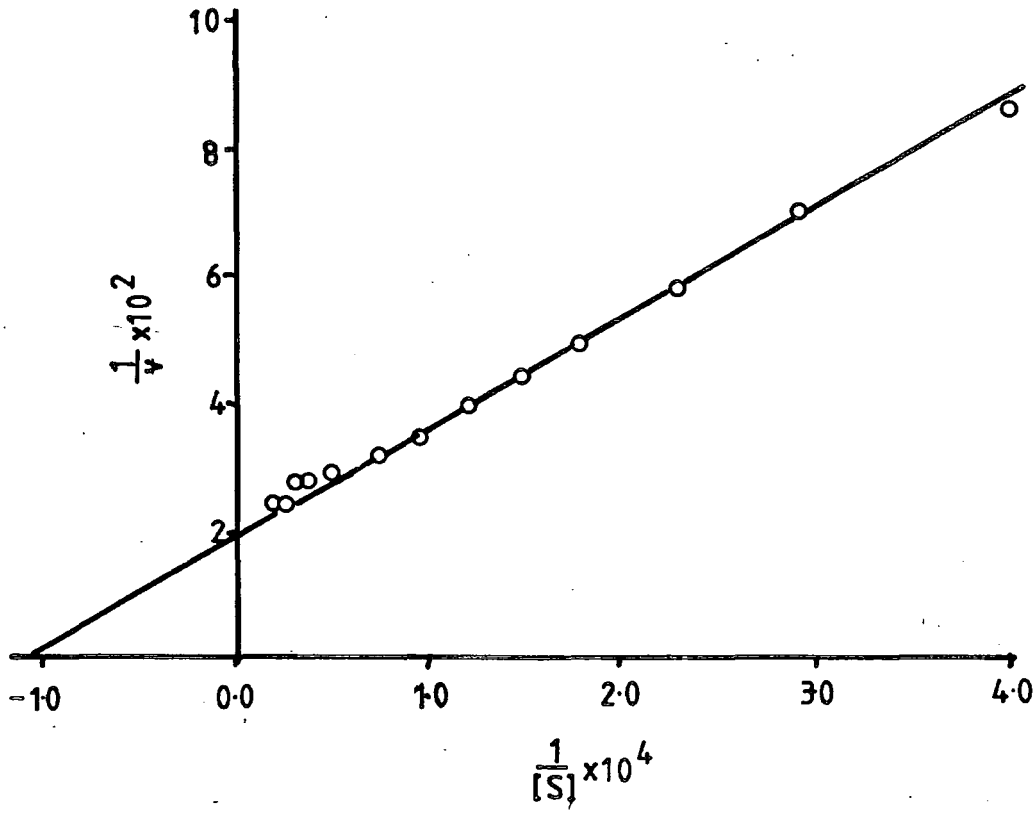
$$Km = 7.35 \times 10^{-5} M$$

FIGURE 6 : 11

Substrate activation of partially heat inactivated membrane-bound rat synaptic membrane acetylcholinesterase. A typical Lineweaver - Burk plot is presented.

Enzyme was incubated at $55^{\circ}C$ for 60 minutes prior to assay. Km was calculated, and line fitted by a computer assisted fit to the Michaelis - Menten relationship.

$$Km = 1.38 \times 10^{-4} M$$


10
8
6
4
2
$\frac{1}{v} \times 10^2$
-1·0
0·0
1·0
2·0
3·0
4·0
$\frac{1}{[S]} \times 10^4$

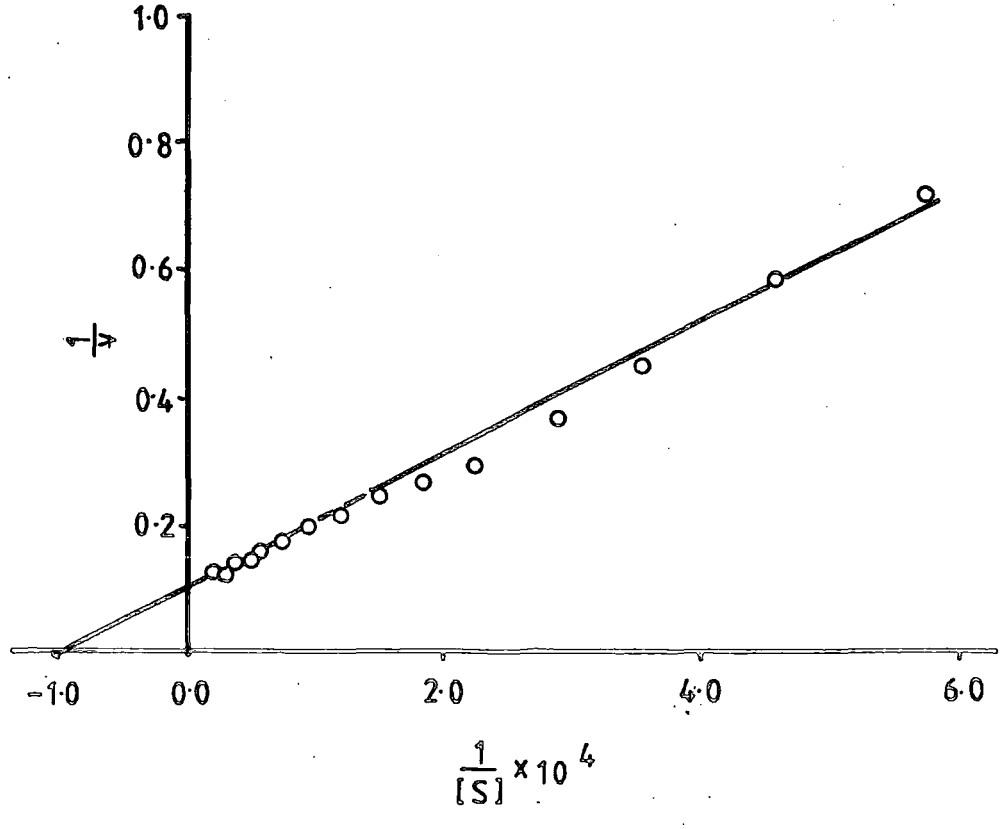

1·0
0·8
0·6
0·4
0·2
$\frac{1}{v}$
-1·0
0·0
2·0
4·0
6·0
$\frac{1}{[S]} \times 10^4$

FIGURE 6 : 8

Substrate activation of low ionic-strength soluble rat synaptic membrane acetylcholinesterase. A typical Lineweaver - Burk plot is presented. Km was calculated, and the line fitted by a computer assisted fit to the Michaelis - Menten relationship.

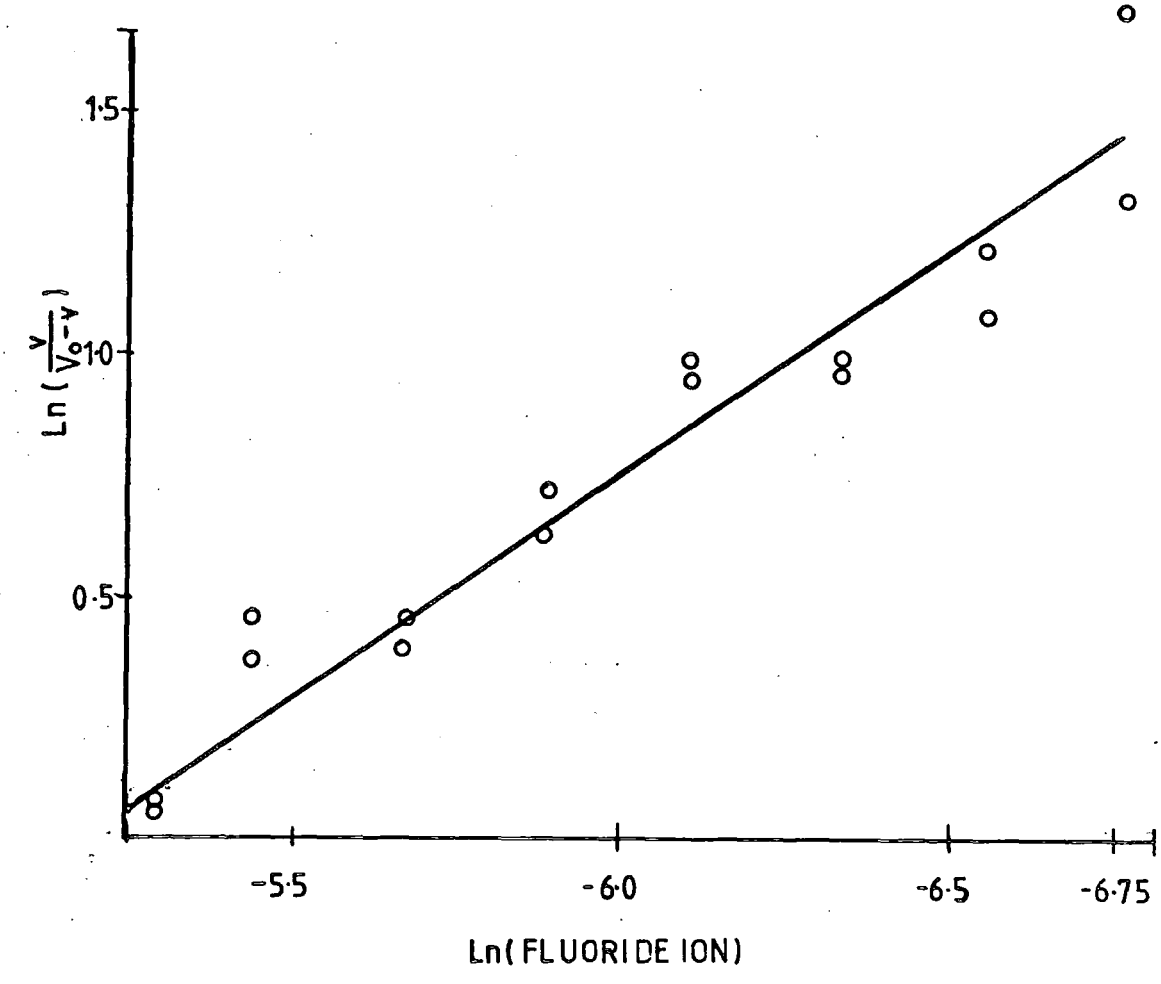
$$Km = 8.77 \times 10^{-5} M$$

FIGURE 6 : 9

Substrate activation of partially heat inactivated, low ionic-strength soluble rat synaptic membrane acetylcholinesterase. A typical Lineweaver - Burk plot is presented.

Enzyme was incubated at $47^{\circ}C$ for 60 minutes prior to assay. Km was calculated, and line fitted by a computer assisted fit to the Michaelis - Menten relationship.

$$Km = 9.80 \times 10^{-5} M$$


1·5
1·0
0·5
Ln (V/Vo-V)
-5·5
-6·0
-6·5
-6·75
Ln(FLUORIDE ION)

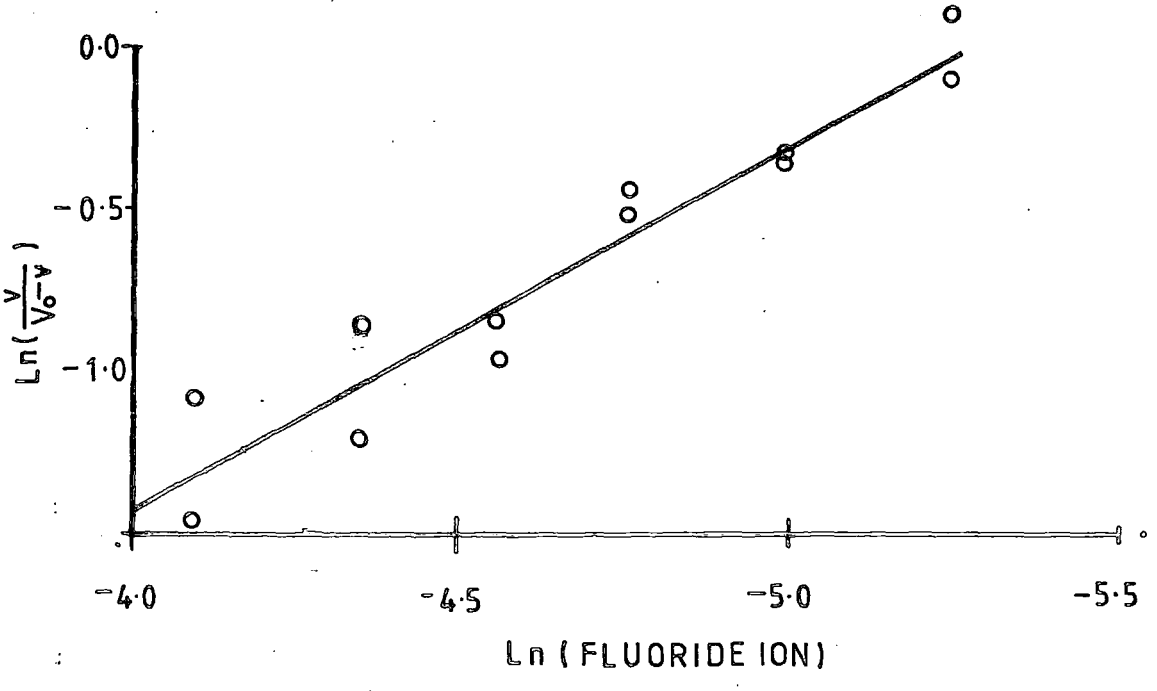

0·0
-0·5
-1·0
Ln (V/Vo-V)
-4·0
-4·5
-5·0
-5·5
Ln (FLUORIDE ION)

FIGURE 6 : 6

Hill plot of fluoride - ion inhibition of membrane-bound rat synaptic membrane acetylcholinesterase.

Data for two separate preparations are presented. The line was fitted by linear regression analysis.

Hill co-efficient = 0.92

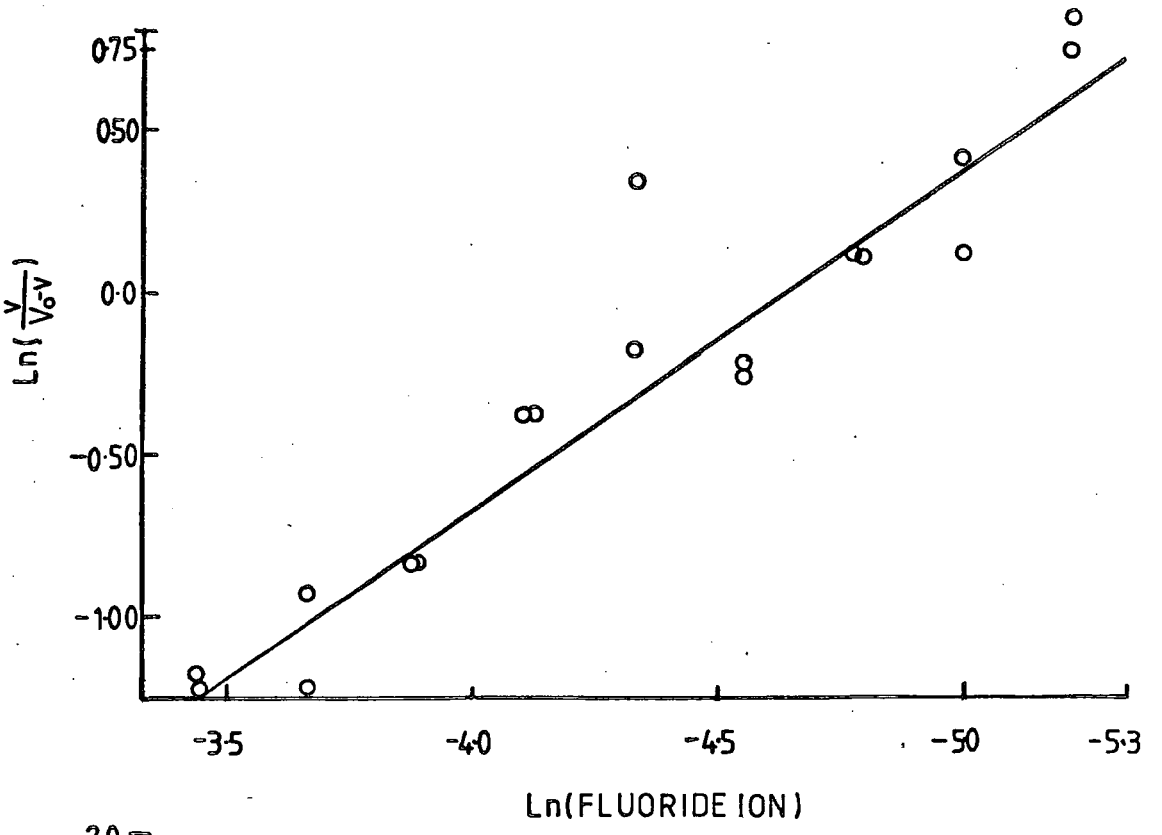
FIGURE 6 : 7

Hill plot of fluoride - ion inhibition of partially heat-inactivated membrane-bound rat synaptic membrane acetylcholinesteras

Enzyme was incubated at 55°C for 60 minutes prior to assay in the presence of various concentrations of fluoride - ion.

Data for two separate preparations are presented. The line was fitted by linear regression analysis.

Hill co-efficient = 1.10


0·75
0·50
0·0
-0·50
-1·00
Ln(V/Vo-V)
-3·5
-4·0
-4·5
-5·0
-5·3
Ln(FLUORIDE ION)

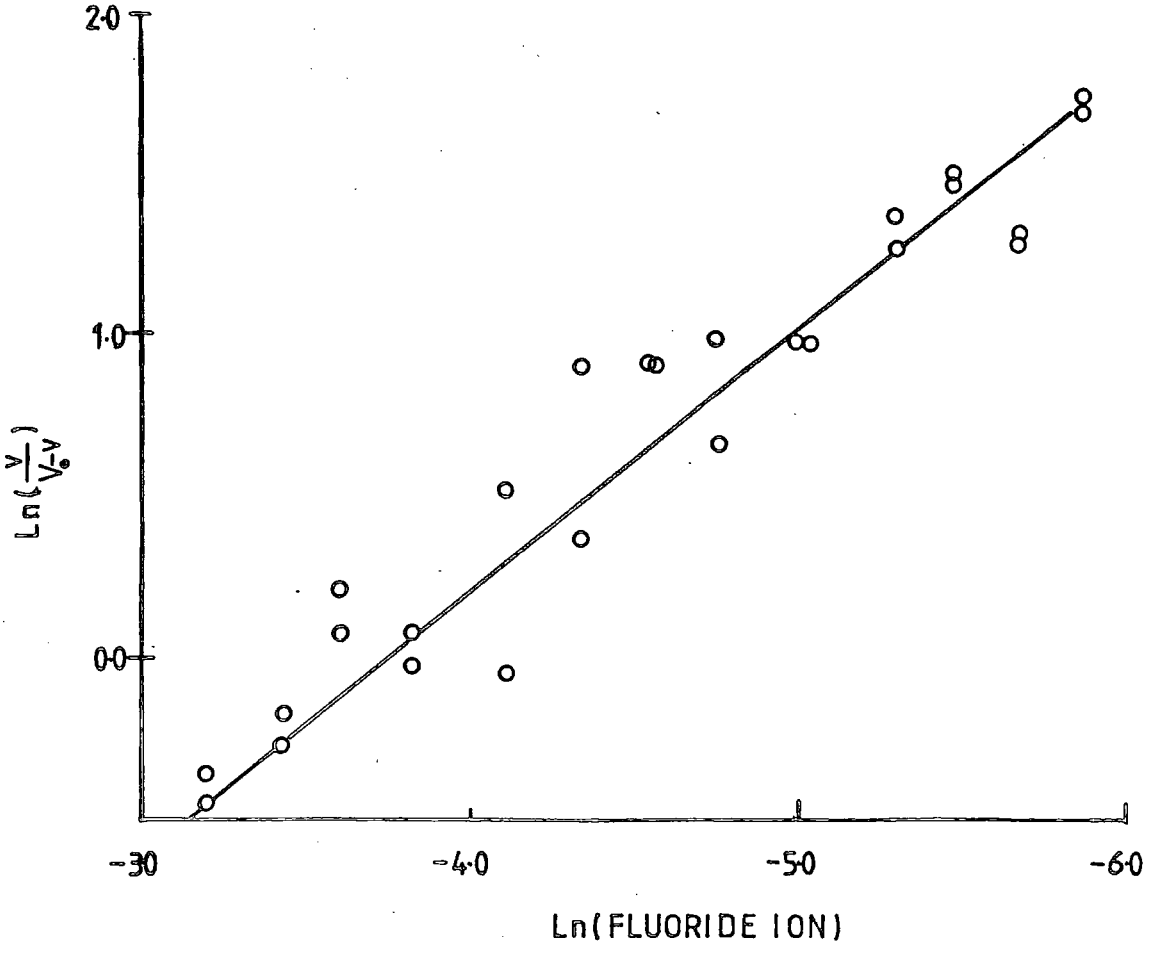

2·0
1·0
0·0
Ln(V/Vo-V)
-3·0
-4·0
-5·0
-6·0
Ln(FLUORIDE ION)

FIGURE 6 : 4

Hill plot of fluoride - ion inhibition of low ionic-strength soluble rat synaptic membrane acetylcholinesterase.

Data for two separate preparations are presented. The line was fitted by linear regression analysis.

Hill co-efficient = 1.06

FIGURE 6 : 5

Hill plot of fluoride - ion inhibition of partially heat-inactivated, low ionic-strength soluble rat synaptic membrane acetylcholinesterase.

Enzyme was incubated at $47^{o}C$ for 60 minutes prior to assay in the presence of various concentrations of fluoride - ion.

Data for two separate preparations are presented. The line was fitted by linear regression analysis.

Hill co-efficient = 0.80

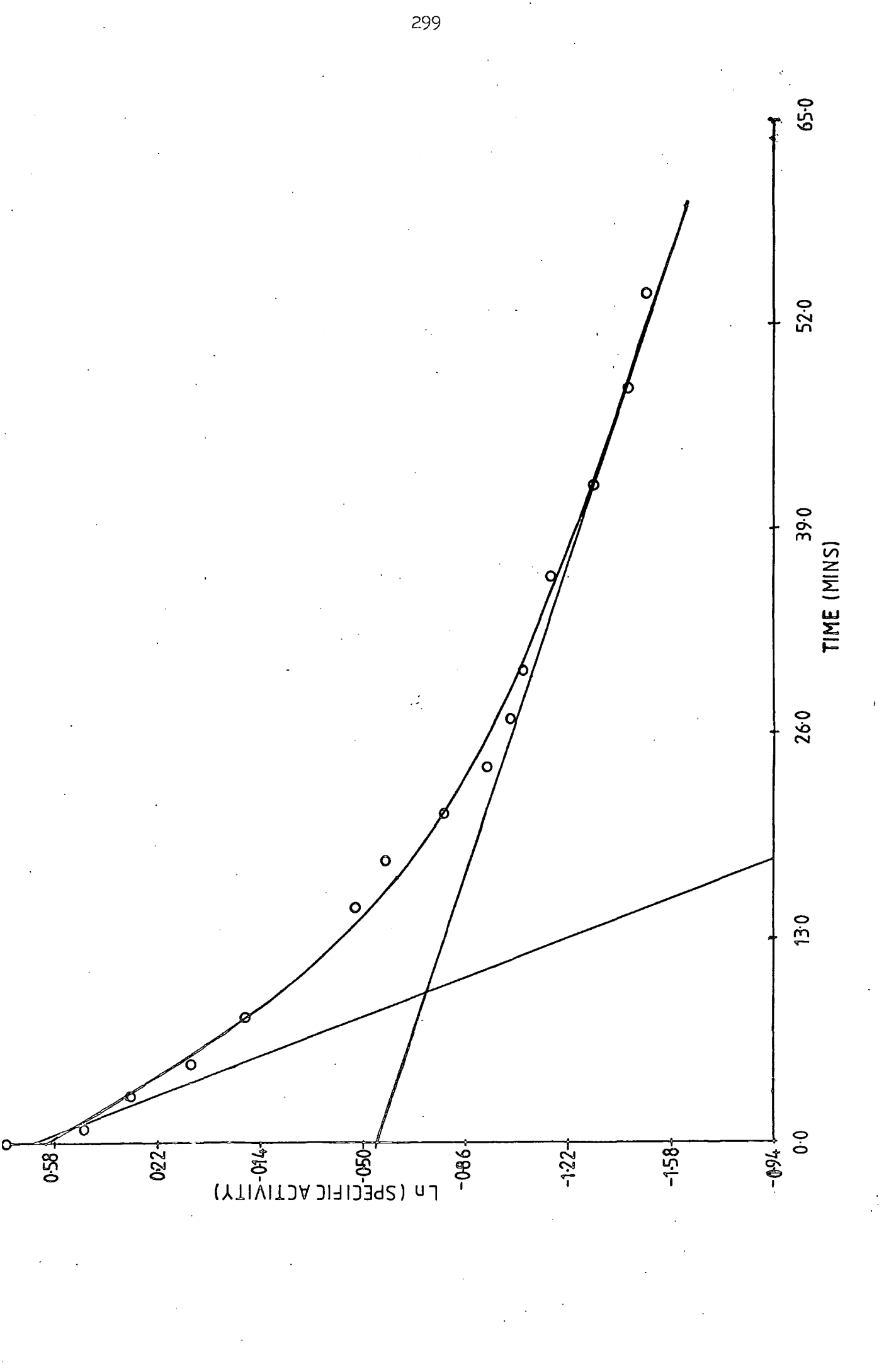

0·58
0·22
-0·14
-0·50
-0·86
-1·22
-1·58
-1·94
Ln (SPECIFIC ACTIVITY)
0·0
13·0
26·0
39·0
52·0
65·0
TIME (MINS)

FIGURE 6 : 3

A typical isothermal irreversible inactivation profile of membrane-bound rat synaptic membrane acetylcholinesterase at 57°C.

Data was analysed according to a computer assisted sequential inactivation model.

Parameters calculated for the profile were;

'labile-state' decay constant = $0.144\ min^{-1}$

'stable-state' decay constant = $0.182 \times 10^{-2}\ min^{-1}$

Specific activity expressed as μ moles thiocholine/mg protein/hr.

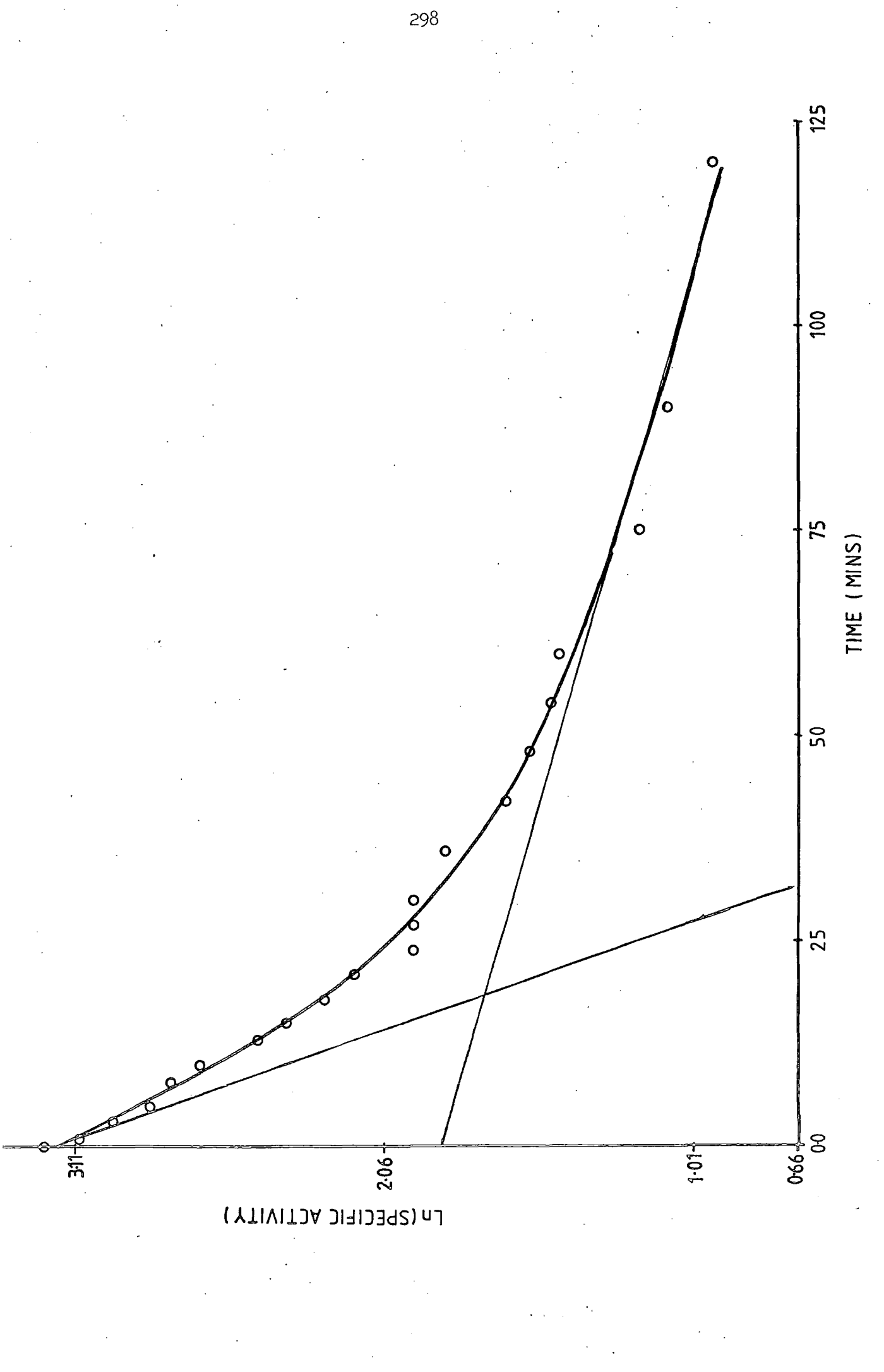

3·11
2·06
1·01
0·66
0·0
25
50
75
100
125
TIME (MINS)
Ln (SPECIFIC ACTIVITY)

FIGURE 6 : 2

A typical isothermal irreversible inactivation profile for low ionic-strength soluble rat synaptic membrane acetylcholinesterase at $47^{o}C$.

Data was analysed according to a computer assisted sequential inactivation model.

Parameters calculated for this profile were;

'labile-state' decay constant = 0.789×10^{-1} min^{-1}

'stable-state' decay constant = 0.802×10^{-2} min^{-1}

Specific activity expressed as μ moles thiocholine/mg protein/hr.

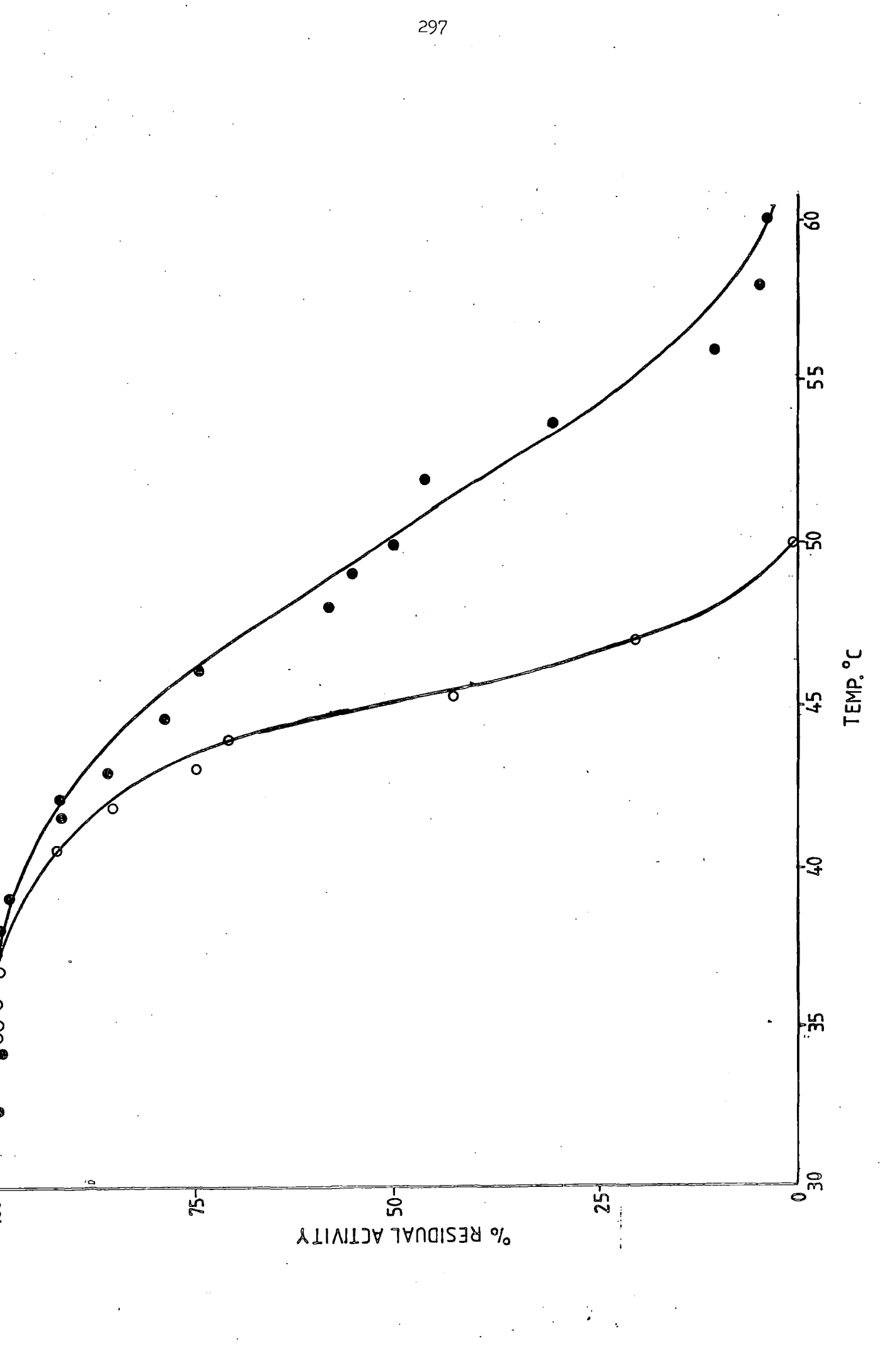

% RESIDUAL ACTIVITY
0
25
50
75
30
35
40
45
50
55
60
TEMP. °C

FIGURE 6 : 1

Typical irreversible thermal inactivation profiles of the low ionic-strength and membrane-bound acetylcholinesterase.

Samples of enzyme were incubated for 10 minutes at the temperatures shown.

Activity is expressed as a percentage of the uninactivated enzyme.

- O - = soluble acetylcholinesterase

- ● - = membrane-bound acetylcholinesterase

in Figure 6 : 22 were similar to those for rat superior cervical ganglion acetylcholinesterase by Vigny, Gisigier & Massoulie, (1978). Consequently it is likely that a structural difference between enzyme fractions accounts for the difference in thermal stability and not membrane association. It is not possible to say whether the oligomeric nature produces the increased affinity for the membrane.

This enables the effect of Triton x - 100 and sodium deoxycholate to be examined further. If the stability of the acetylcholinesterase is increased by the association of monomeric units then it is possible that these detergents could increase the thermal sensitivity of acetylcholinesterase by promoting dissociation of oligomeric units. However, Figure 6 : 23 shows that this is not the case as the density profiles of Triton x - 100 and deoxycholate solubilized synaptic membrane acetylcholinesterase were similar to that for the Lubrol solubilized enzyme. This suggests that the effect of lipid in controlling acetylcholinesterase stability, as shown by sensitivity to detergents, is separate from the difference in stability which has been attributed to the oligomeric size of the molecule.

Far from providing a simple explanation of increased stability of this enzyme in terms of a physical property of the membrane, this work has revealed a complex structural organization between acetylcholinesterase molecules. This relationship is not directly connected with the membrane affinity of the various molecular species and has indicated that caution must be exercised in the interpretation of the kinetic properties of membrane-bound enzymes as the full range of possible lipid-protein relationships have by no means been described.

disrupting the lipoprotein relationship causes increased sensitivity to thermal inactivation. However, this still could not account for the difference in stability between soluble and membrane enzyme, as both enzyme fractions were similarly affected by the detergents.

One possible mechanism to explain the difference in stability may be the oligomeric nature of solubilized acetylcholinesterase. Acetylcholinesterase from many species has been shown to exist in a number of oligomeric aggregates of differing size and dubious physiological significance (Ho & Ellman, 1969; Chan, Shirachi, Bhorgava, Gardner & Trevor, 1972; McIntosh & Plummer, 1973; Wenthold, Mahler & Moore, 1974; Ott & Brodbeck, 1978; Vigny, Gisigier & Masoulie, 1978). It has been demonstrated immunologically that oligomers are produced from a single monomeric protein, in the case of the mouse (Adamson, 1977) although the smallest form usually observed is a dimer. The stability of the membrane form may then be explained if this was composed of one or more higher order oligomers than the soluble form, as it may be expected that a higher order oligomer may be more stable than the monomeric species.

This was examined by sucrose density gradient floation of the Lubrol W - X solubilized soluble and membrane-bound enzyme (Figure 6 : 22). The soluble acetylcholinesterase was localized at a low density range near the top of the 5% - 20% sucrose density gradient, whereas the Lubrol solubilized membrane enzyme was spread across a considerable portion of the gradient, suggesting that several more dense molecular forms exist for this enzyme fraction. This would be unlikely to be an artifact of Lubrol W - X association as the low ionic strength soluble enzyme was not affected in this way and the results presented

If the membrane environment conferred increased stability on the membrane acetylcholinesterase then solubilization with detergents might convert the membrane form to a more labile state similar to the soluble form. Both sodium deoxycholate and Triton x - 100 treatments caused increased temperature sensitivity of not only the membrane enzyme but also the soluble enzyme (Figures 6 : 16 and 6 : 19). This suggests that these detergents act directly on the enzyme to cause instability, rather than by removing the putative stabilising influence of the membrane. However, solubilizing both soluble and membrane acetylcholinesterase with Lubrol W - X had no destabilizing effect in fact the enzyme treated with this detergent was slightly more stable than untreated enzyme (Figures 6 : 20 and 6 : 21).

This latter result strongly suggests that membrane association itself was not responsible for the increased stability of the membrane-bound enzyme. Thus none of the results generated in this study have established any functional connection between the membrane and the properties of rat synaptic membrane acetylcholinesterase.

The effects of these detergents on inactivation of the enzyme were similar to those on the temperature properties presented in chapter 4. In that case a lipid moiety was implicated in the temperature kinetics of acetylcholinesterase which was sensitive to deoxycholate and Triton x - 100 but not to Lubrol W - X, further data as to the lipoprotein identity for this enzyme was presented in chapter 5. Thus the effects of deoxycholate and Triton x - 100 in this study suggest that lipid is involved in the thermal stability of the acetylcholinesterase, and that

The lipid extract of partially purified acetylcholinesterase, as prepared by the conventional Folch-Lees media (Folch-Lees & Sloane-Stanley, 1957) produced no phospholipid spots when separated by thin-layer chromatography. This shows that there was no free phospholipid in the enzyme fractions, nor any phospholipid loosely bound to protein, as this would have been extracted into the hydrophobic chloroform layer. The chloroform: methanol 2.0M ammonia medium, however, extracted a phospholipid class, which separated as a single spot on thin layer chromatography. This spot separated in a similar manner to a commercial preparation of cardiolipin, rather than a manner consistent with the major classes of phospholipid normally obtained for mammalian plasma membrane extracts. The spot was thus tenatively identified as cardiolipin.

This was somewhat unusual as this lipid is characteristic of mitochondrial and bacterial membrane rather than eukaryotic plasma membranes (Maddy 1976). However, this conclusion is similar to that of Beauregard & Roufogalis, (1977) who extracted a lipid, tenatively identified as cardiolipin, from bovine erythrocyte acetylcholinesterase.

The fatty-acid composition of putative cardiolipin from both FI and FII acetylcholinesterase fractions was examined by gas-liquid chromatography, the results of which are presented in Table 5 : 7. The fatty-acid composition of the major phosphoglyceride classes in rat synaptic membranes was similarly examined and presented in Table 5 : 7 for comparison.

The fatty-acid composition of the major rat synaptic membrane phosphoglycerides obtained in this study was similar to that obtained by Cossins, (1977) for phosphoglycerides from the same

tissue source. Now the putative cardiolipin extracted from this enzyme carried a wide range of fatty-acids, similar to those found for the membrane phosphoglycerides, but the unsaturated fatty-acids were all present as a low percentage of the total, with C16 : 0 and C18 : 0 as the major fatty-acids. This provides two possible consequences, firstly, if the acetylcholinesterase carries few cardiolipin phosphoglycerides then it is likely that different enzymes carry lipids of different fatty-acid compositions or alternatively, if acetylcholinesterase carries a larger number of cardiolipin phosphoglycerides then the acetylcholinesterase will be in contact with fatty-acids of lower average unsaturation than for the bulk of the membrane.

It is not possible to directly resolve these alternatives, however, the rat brain enzyme has been shown in this study to have properties in common with the bovine erythrocyte acetylcholinesterase, presented in the studies of Beauregard & Roufogalis, (1977). These authors suggested that their enzyme was a dimer bearing 30 cardiolipin molecules. If this were the case for the rat brain enzyme, then each acetylcholinesterase molecule would be associated with about 60 fatty acyl chains. In this case the enzyme-bound lipid would be likely to contain a representative sample of the fatty acids quoted in Table 5 : 7 hence the latter of the alternatives discussed above would be most likely; that each acetylcholinesterase is in contact with fatty-acids of generally lower unsaturation than is the case for the membrane in general.

If this was the case it is difficult to see how evidence, which would be corroborative or otherwise, may be obtained from existing data. For example, if non-linear Arrhenius plots for acetylcholinesterase and $Na^+ - K^+$ ATPase resulted from state changes in hydrophobic portions of membrane lipids then one would expect differences in the

shape of these plots, as the increased rigidity of the fatty-acids of the enzyme-bound cardiolipin would occur at higher temperatures than for the membrane in general. But, the models developed for the Arrhenius plots of these enzymes in Chapter 3 already show that the Arrhenius plots of acetylcholinesterase and $Na^+ - K^+$ ATPase activity are different and cannot be compared directly.

However, some corroborative evidence comes from the indirect comparison of Arrhenius plots. The variation in the slope of the Arrhenius plots of $Na^+ - K^+$ ATPase, presented in Figure 3 : 9, shows that the slope of these plots was relatively constant between 40^oC and 15^oC and changes sharply below this temperature, whereas in the case of the acetylcholinesterase the slope of the Arrhenius plot was relatively constant between 4^oC and 25^oC, with deviation occurring above this temperature. We may interpret this in terms of the respective models. If increasing rigidity in the hydrophobic portion of the bulk of membrane lipid produces deviation away from the high temperature stable state, in the case of the $Na^+ - K^+$ ATPase, then this becomes significant at temperatures below 15^oC, whereas if similar increase in the rigidity of the fatty-acids of tightly bound cardiolipin constrains the acetylcholinesterase to the low temperature-high activation energy form then this form predominates at temperatures below 25^oC. This suggests that either the physical state of these lipids occur at different temperatures, or that these enzymes are differentially sensitive to membrane changes. The observation that the enzyme-bound cardiolipin contained a higher proportion of saturated fatty-acids than membrane phosphoglycerides in general would tend to suggest that the former was the case.

This work supports the conclusions of Chapter 4, that rat synaptic membrane acetylcholinesterase is a lipoprotein, and that lipid is so tightly bound that high ionic-strength media are necessary to remove it.

This lipid has been tentatively identified as cardiolipin, which is different from the conclusion of Sihotang, (1976) who suggests that human erythrocyte acetylcholinesterase is associated with phosphatidyl-serine, but agrees with the conclusion of Beauregard & Roufogalis, (1977) for bovine erythrocyte acetylcholinesterase.

TABLE 5 : 1

The effect of various concentrations of Triton x - 100 deterrent on the activity of rat synaptic membrane acetylcholinesterase

Triton x - 100 concentration % w/v	Activity after incubation at 20°C			
	0 hrs.	0.5 hrs.	1.0 hrs.	4.0 hrs.
0	25.1	24.9	25.2	24.9
0.2	26.8	27.0	24.5	22.8
2.0	25.0	24.8	21.8	19.1
4.0	24.2	23.8	20.4	-

Specific activity expressed as μ moles thiocholine.mg protein.$^{-1}$ hr^{-1}.

TABLE 5 : 2

The effect of various concentrations of sodium deoxycholate on the activity of the rat synaptic membrane acetylcholinesterase

Aliquots of synaptic membrane were incubated in each concentration of detergent for 20 minutes at 20°C.

sodium deoxycholate conc. (mM)	Enzyme activity (n3)
0	24.6 ± 2.3
10	21.1 ± 4.1
20	24.3 ± 2.5
30	18.4 ± 4.1
40	12.5 ± 2.8
50	7.3 ± 0.7
60	7.5 ± 1.9
70	6.1 ± 2.2
80	6.0 ± 2.4

Specific activity expressed as μ moles thiocholine.mg protein^{-1}. hr^{-1}.

TABLE 5 : 3

The effect of various concentrations of Lubrol W - X on the activity of the rat synaptic membrane acetylcholinesterase

Aliquots of synaptic membrane were incubated in each concentration of Lubrol W - X for 48 hours at 4°C.

Lubrol W - X concentration $mg.cm^{-3}$	Enzyme activity *
0	26.8
0.5	27.0
1.0	25.9
2.0	25.3
3.0	27.6
4.0	27.6
5.0	30.6

* Specific activity expressed as μ moles thiocholine. $mg.protein^{-1}.hr^{-1}$.

TABLE 5 : 4

The specific activities of Lubrol W - X solubilized and column chromatographically purified rat synaptic membrane acetylcholinesterase

		Acetylcholinesterase activity column fractions					
Native Synaptic Membrane (AChEase act)	Supernatant from Lubrol-extraction (AChEase act)	DEAE - A-25 Sephadex		DEAE - A-50 Sephadex	DEAE - A-50 Sephadex (Discontinuous Salt Gradient)		n
		FI	FII		FI	FII	
23.1 ± 2.3	40.8 ± 7.5	122.0 ± 22.5	202.4 ± 31.3	—	—	—	5
24.3 ± 1.7	35.8 ± 7.6	—	—	209.5 ± 54.1	—	—	3
25.4 ± 0.5	32.5 ± 1.4	—	—	—	161.7 ± 1.1	298.1 ± 1.9	3

Specific activity expressed as μ moles thiocholine.mg protein^{-1}. hr^{-1}.

TABLE 5 : 5

Parameters calculated for the Arrhenius profiles of FI and FII fractions of rat synaptic membrane acetylcholinesterase according to the reversible thermal inactivation model

	Activation energy of the low temp. state ($KJ\ mole^{-1}$)	Enthalpy Parameter (ΔH) ($KJ\ mole^{-1}$)	Entropy Parameter (ΔS) ($J^{o}K^{-1}mole^{-1}$)	n
Native membrane preparation AChEase	38.8 ± 9.0	-84.6 ± 1.7	-266 ± 6	4
FI fraction	31.1	-60.8	-188	2
AChEase	34.9	-86.5	-272	
FII fraction	38.3	-86.6	-274	2
AChEase	38.1	-88.1	-279	

TABLE 5 : 6

The apparent activation energies of the temperature plots of high ionic-strength treated soluble, membrane-bound and purified acetylcholinesterase

Values for the apparent activation energy of the low temperature form of the respective untreated enzyme fraction.

	Soluble AChEase	n	Membrane-bound AChEase	n	Partially purified	n
Apparent activation energy of salt treated enzyme	25.1±0.7	3	25.2±0.9	3	27.1 26.4	2
Apparent activation energy of low temp. control enzyme	32.1±1.3	4	33.0±1.6	3	35.6±1.7	4

Percentage fatty-acid composition of rat synaptic membrane phospholipids and putative cardiolipin

	Phosphatidyl choline	Phosphatidyl Serine/Inositol	Phosphatidyl Ethanolamine	Sphingomyelim	Putative Cardiolipin (FI)	Putative Cardiolipin (FII)
16:0	41.3	4.9	8.5	5.3	24.0 ± 5.0	21.0 ± 4.3
16:1w9	1.7	1.0	3.4		3.3 ± 0.6	4.3 ± 0.7
16:2w6				12.5	1.9 ± 0.6	2.2 ± 0.01
18:0	9.6	5.4	11.3	8.8	15.4 ± 1.9	16.3 ± 1.4
18:1w9	25.8	1.2	2.4		8.3 ± 1.8	7.6 ± 1.3
18:2w9	1.0	1.0	4.8	6.4	0.9 ± 0.1	2.4 ± 1.4
18:3w6	0.5	1.5	12.9	5.0	3.0 ± 2.4	0.9 ± 0.3
20:0						
20:2w9				4.2		
20:3w9			6.8		1.9 ± 1.3	1.6 ± 0.2
20:4w6	0.6		5.5	4.3	3.2 ± 2.3	4.6 ± 0.8
20:5w3	0.7	1.0	9.8	4.3	2.9 ± 1.2	2.7 ± 1.4
22:2w9		0.4		2.4		
22:5w6	1.0	0.4	6.2	3.7	2.0 ± 0.4	
22:5w3	1.4	0.3	3.8	3.0	7.0 ± 6.1	4.4 ± 1.3
22:6w3	5.8	69.3	21.6	24.0	1.1 ± 0.2	5.9 ± 1.7
					$n=4 \pm$ 1SE unidentified peaks	$n=4 \pm$ 1SE unidentified peaks

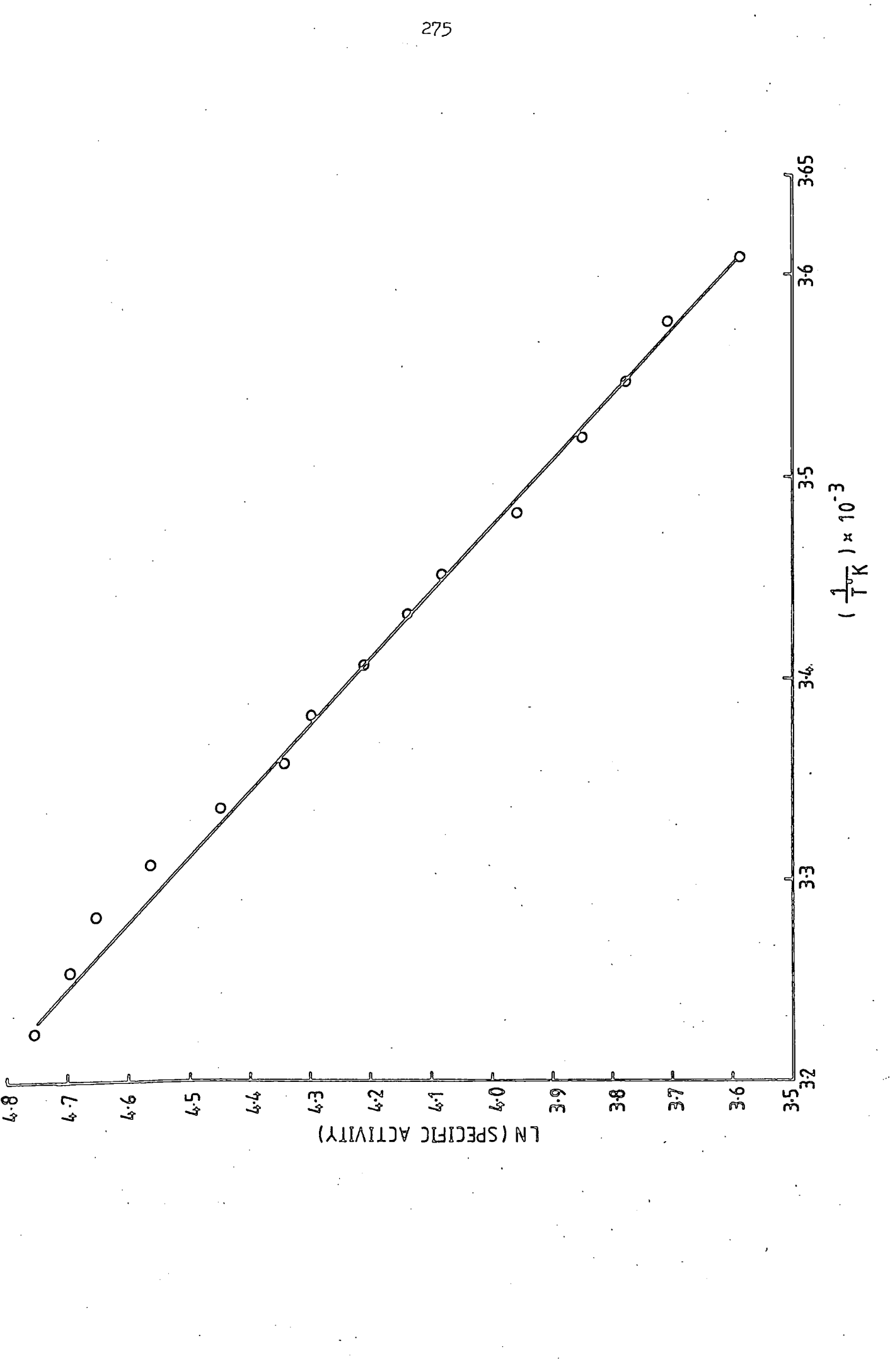

LN (SPECIFIC ACTIVITY)
4·8
4·7
4·6
4·5
4·4
4·3
4·2
4·1
4·0
3·9
3·8
3·7
3·6
3·5
3·2
3·3
3·4
3·5
3·6
3·65
$\left(\frac{1}{T^{\circ}K}\right) \times 10^{-3}$

FIGURE 5 : 7

A typical Arrhenius profile of partially purified rat synaptic membrane acetylcholinesterase in the presence of 2.0×10^{-4}M tetracaine.

This plot gave best fit to linear Arrhenius kinetics when processed according to the reversible thermal inactivation programme.

Specific activity expressed as μ moles thiocholine.mg protein^{-1} hr^{-1}.

Arrhenius μ = 25.3 KJ mole^{-1}

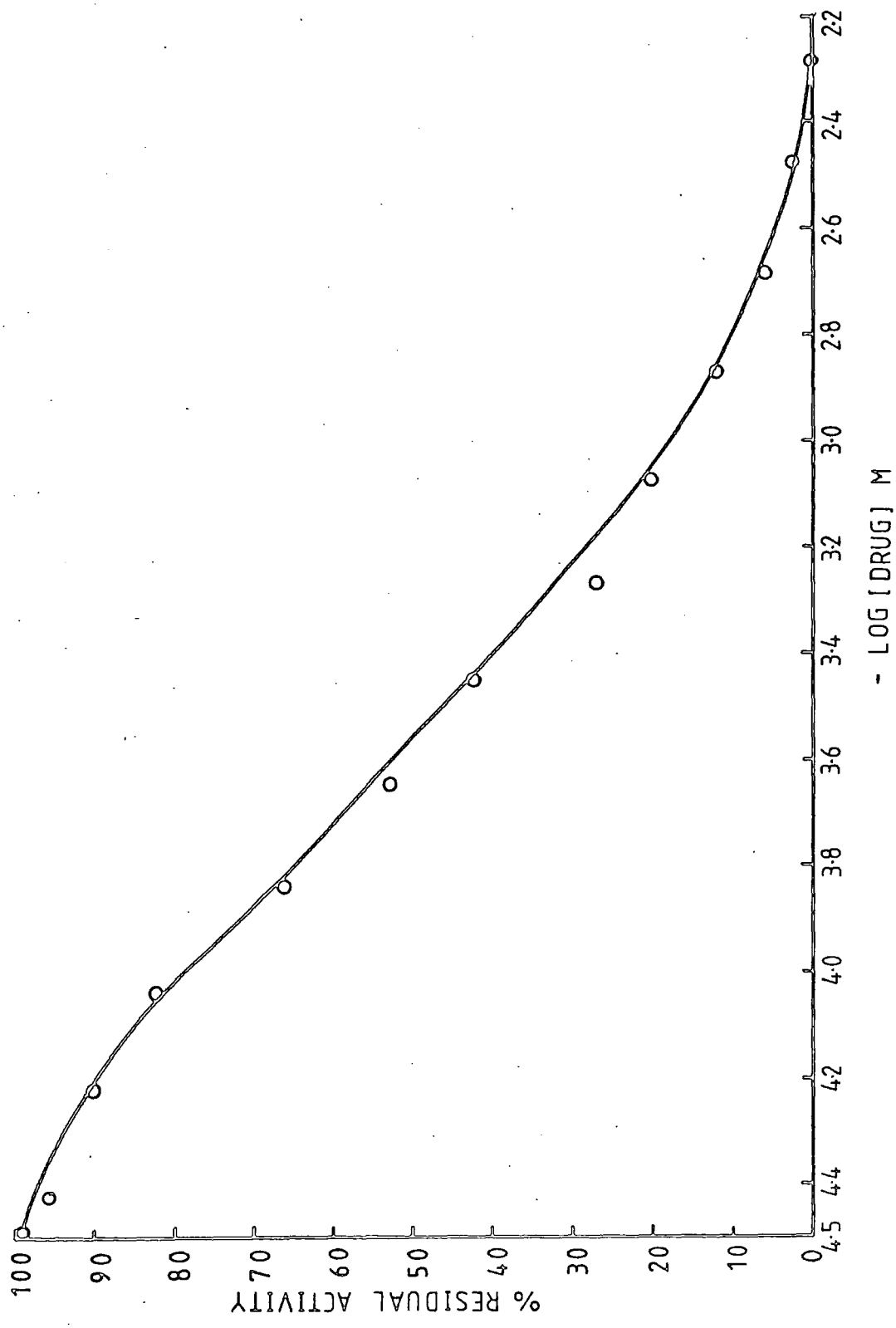

% RESIDUAL ACTIVITY
100
90
80
70
60
50
40
30
20
10
0
4·5
4·4
4·2
4·0
3·8
3·6
3·4
3·2
3·0
2·8
2·6
2·4
2·2
- LOG [DRUG] M

FIGURE 5 : 6

The inhibition of partially purified synaptic membrane acetylcholinesterase by tetracaine

- O - percentage of uninhibited acetylcholinesterase activity

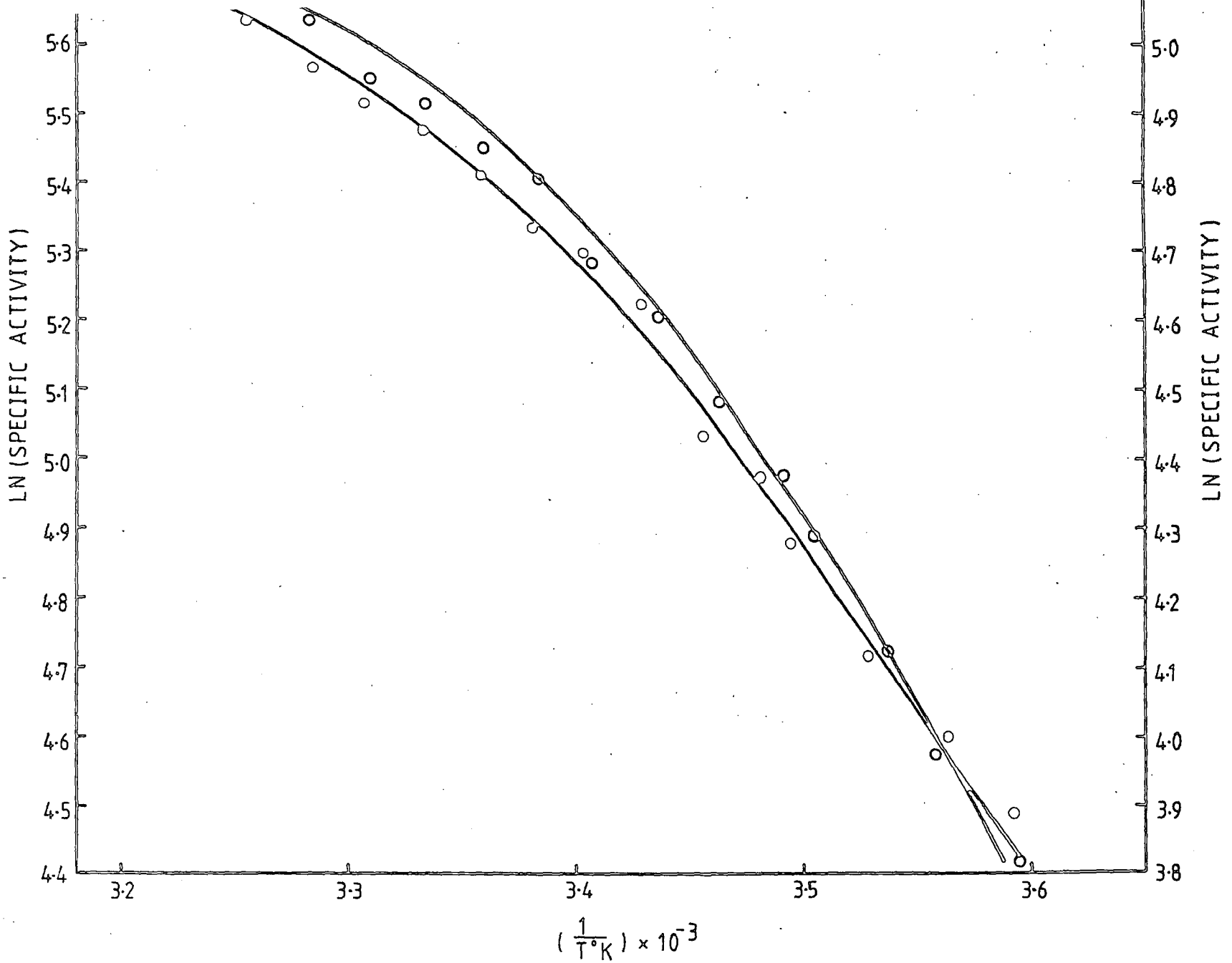

LN (SPECIFIC ACTIVITY)
5·6
5·5
5·4
5·3
5·2
5·1
5·0
4·9
4·8
4·7
4·6
4·5
4·4
LN (SPECIFIC ACTIVITY)
5·0
4·9
4·8
4·7
4·6
4·5
4·4
4·3
4·2
4·1
4·0
3·9
3·8
3·2
3·3
3·4
3·5
3·6
(1/T°K) × 10⁻³

FIGURE 5 : 5

Typical Arrhenius profiles of FI and FII fractions of partially purified acetylcholinesterase

Data was processed according to the reversible thermal inactivation model.

Values expressed as μ moles thiocholine mg.protein^{-1}. hr.$^{-1}$

i) - ● - FI acetylcholinesterase activity fraction

Arrhenius μ at low temperatures = 34.9 KJ mole^{-1}

Enthalpy parameter (Δ H) = -86.5 KJ mole^{-1}

Entropy parameter (Δ S) = -272 J^{o} K^{-1}mole^{-1}

ii) - O - FII acetylcholinesterase fraction

Arrhenius μ at low temperatures = 38.1 KJ mole^{-1}

Enthalpy parameter (Δ H) = -88.1 KJ mole^{-1}

Entropy parameter (Δ S) = -279 J^{o} K^{-1}mole^{-1}

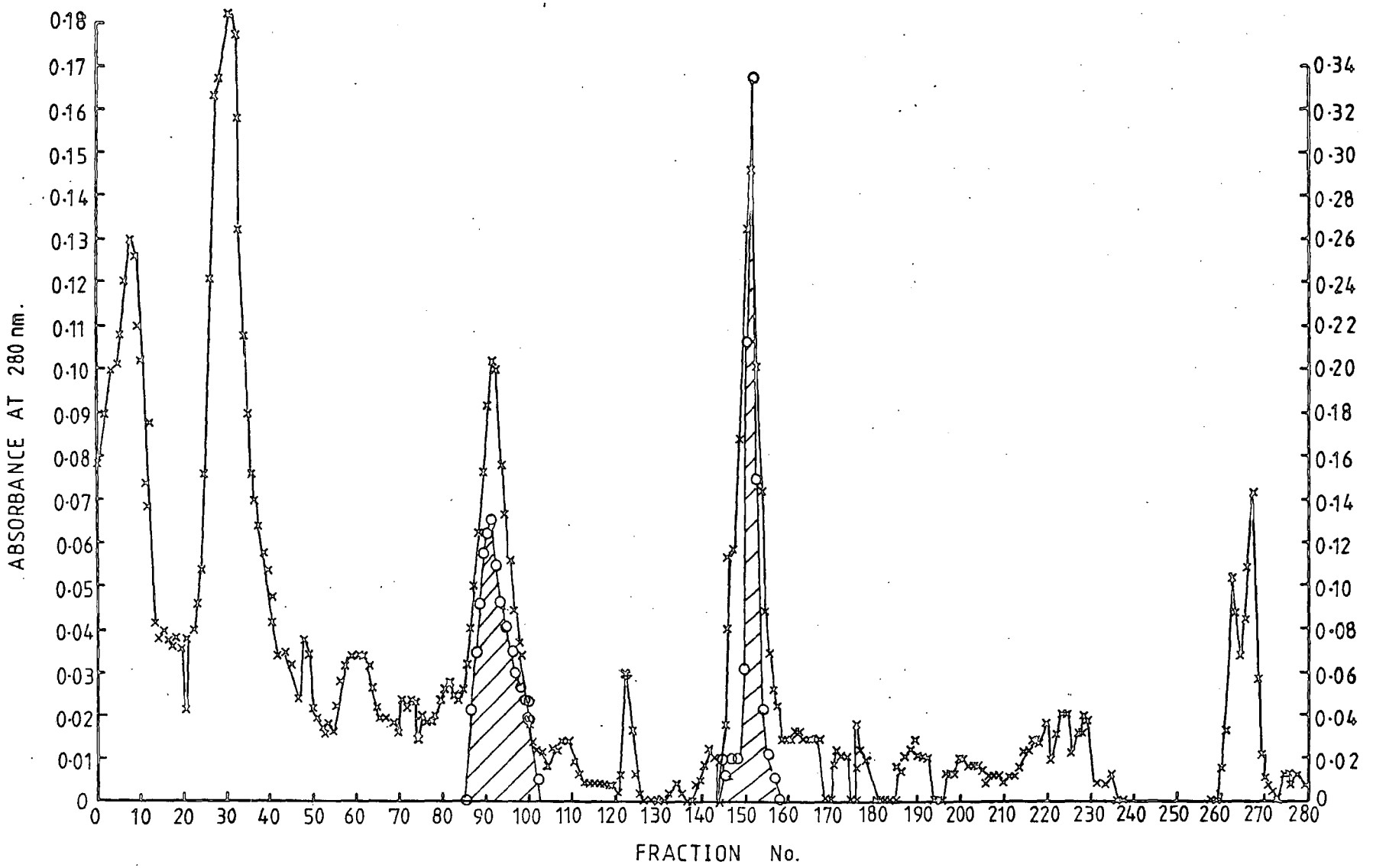

ABSORBANCE AT 280 nm.
ABSORBANCE AT 412 nm.
FRACTION No.
0·18
0·17
0·16
0·15
0·14
0·13
0·12
0·11
0·10
0·09
0·08
0·07
0·06
0·05
0·04
0·03
0·02
0·01
0
0·34
0·32
0·30
0·28
0·26
0·24
0·22
0·20
0·18
0·16
0·14
0·12
0·10
0·08
0·06
0·04
0·02
0
0 10 20 30 40 50 60 70 80 90 100 110 120 130 140 150 160 170 180 190 200 210 220 230 240 250 260 270 280

FIGURE 5 : 4

Ion-exchange chromatography of Lubrol W - X extracted rat synaptic membrane acetylcholinesterase

Column chromatography was carried out on DEAE Sephadex (A-50).

Proteins were eluted with a discontinuous, stepwise, gradient of 0.1M NaCl, 0.2M NaCl, 0.3M NaCl and 0.4M NaCl, in 0.1M Tris - HCl pH 7.4.

- X - protein content of each fraction as given by absorbance at 280 nm

- O - acetylcholinesterase activity

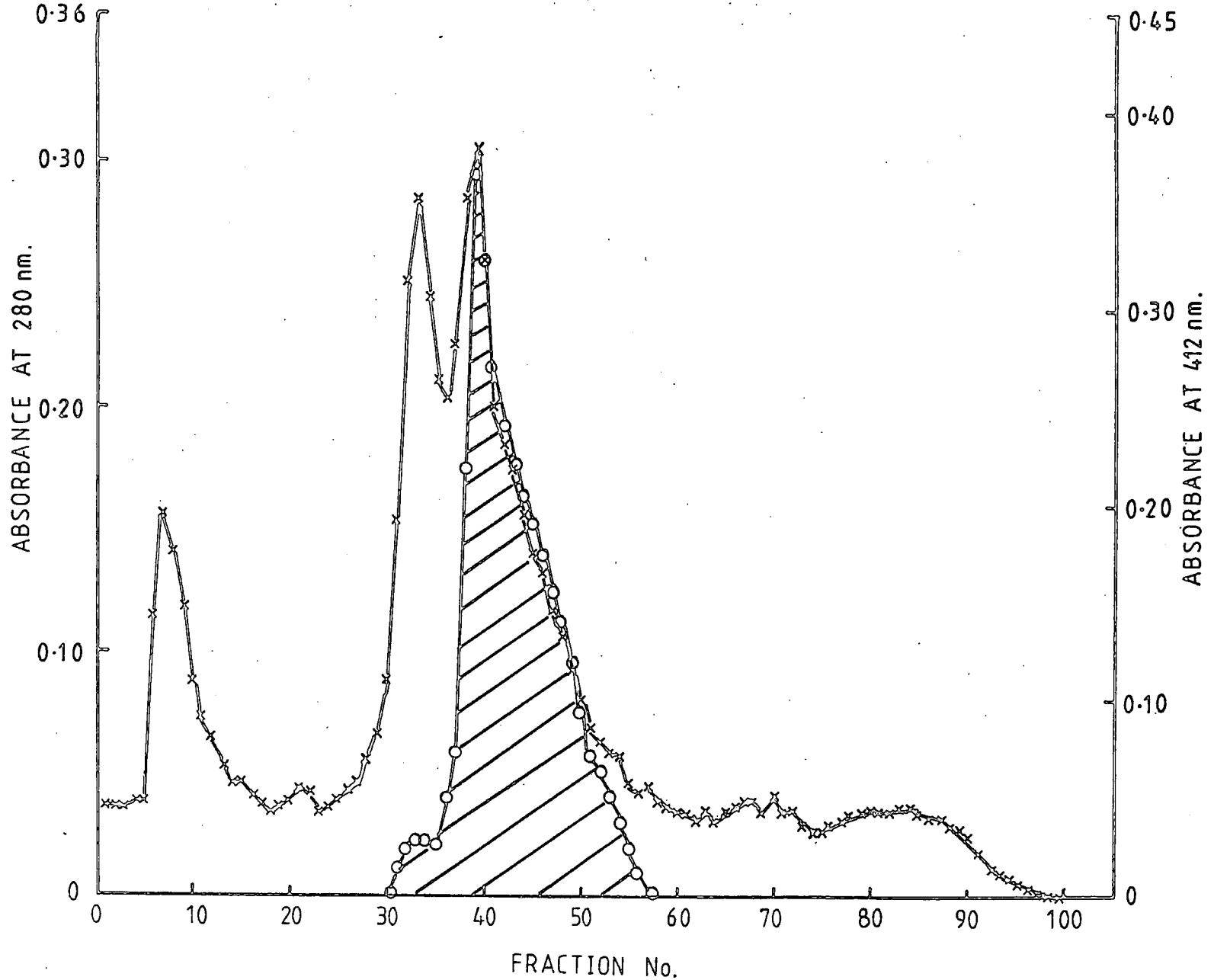

0·36
0·30
0·20
0·10
0
ABSORBANCE AT 280 nm.
0·45
0·40
0·30
0·20
0·10
0
ABSORBANCE AT 412 nm.
0
10
20
30
40
50
60
70
80
90
100
FRACTION No.

FIGURE 5 : 3

Ion-exchange chromatography of Lubrol W - X extracted rat synaptic membrane acetylcholinesterase

Column chromatography was carried out on DEAE Sephadex (A-50).

Proteins were eluted using a continuous NaCl-gradient, of up to 0.5M NaCl in 0.1M Tris - HCl buffer pH 7.2.

- X - protein content of each fraction as given by absorbance at 280 nm.

- O - acetylcholinesterase activity

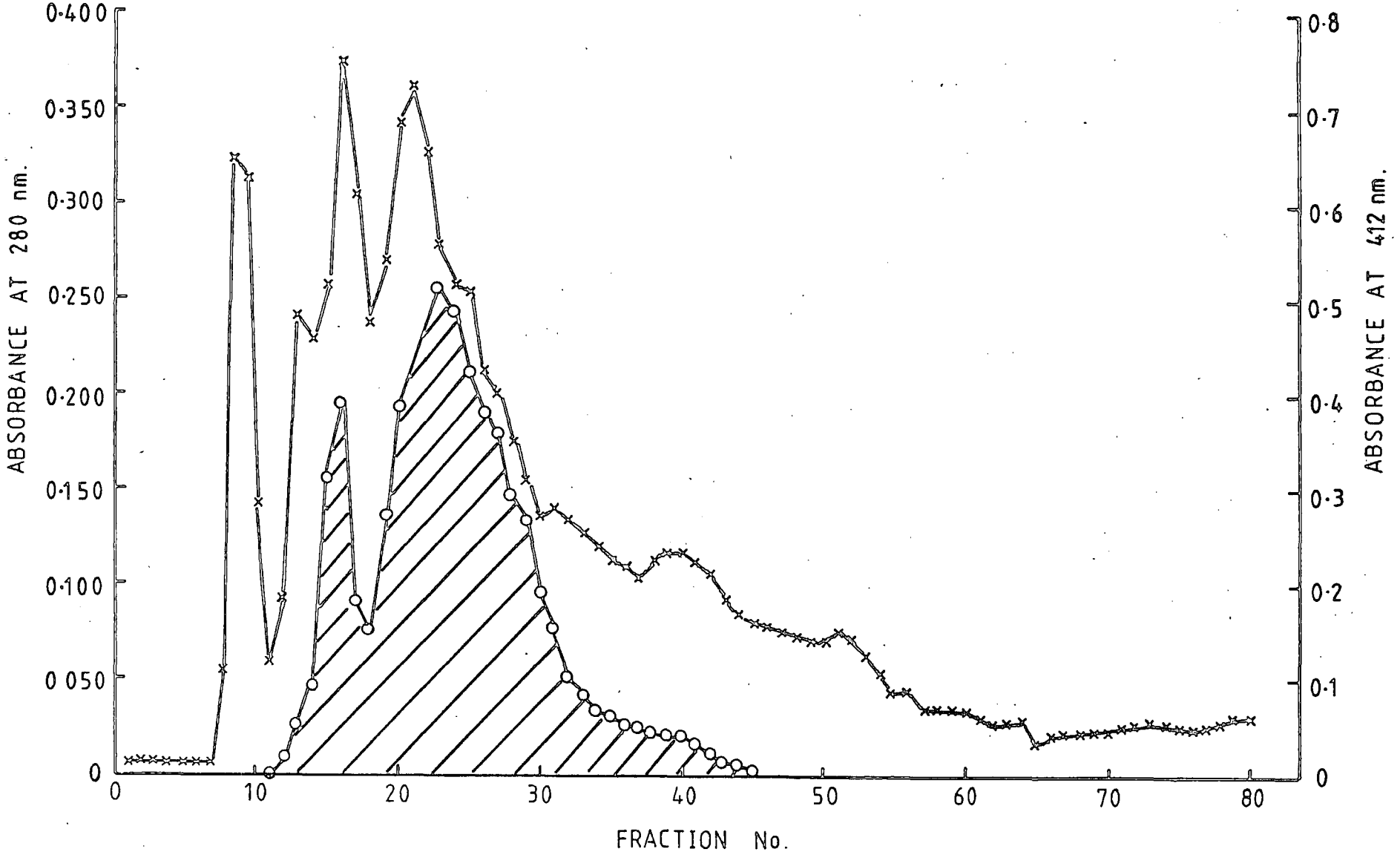

0·400
0·350
0·300
0·250
0·200
0·150
0·100
0 050
0
ABSORBANCE AT 280 nm.
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
0
ABSORBANCE AT 412 nm.
0
10
20
30
40
50
60
70
80
FRACTION No.

FIGURE 5 : 2

Ion-exchange chromatography of Lubrol W - X extracted rat synaptic membrane acetylcholinesterase

Column chromatography was carried out on DEAE Sephadex (A-25).

Proteins were eluted using a continuous NaCl gradient, of up to 0.5M NaCl in 0.1M Tris HCl buffer pH 7.2.

- X - protein content of each fraction as given by absorbance at 280 nm.

- O - acetylcholinesterase activity

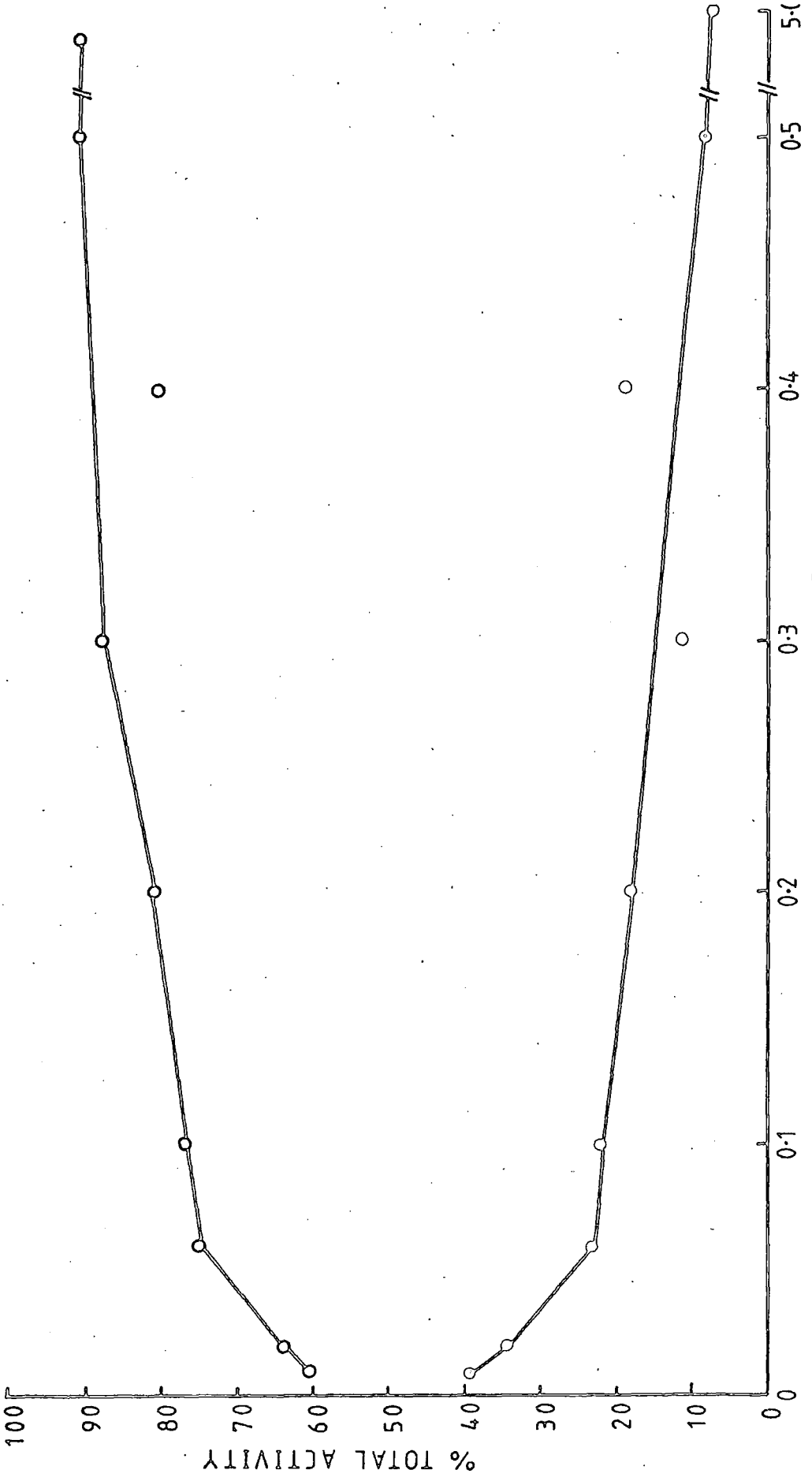

% TOTAL ACTIVITY
100
90
80
70
60
50
40
30
20
10
0
0
0·1
0·2
0·3
0·4
0·5
[LUBROL] mg cm^{-3}

FIGURE 5 : 1

Lubrol W - X solubilization of rat synaptic membrane acetylcholinesterase

Aliquots of rat synaptic membranes were incubated in various concentrations of Lubrol W - X for 24 hours at 4°C. These were then centrifuged at 100,000 xg for 1 hour. The percentage of the original acetylcholinesterase activity in the soluble and particulate fractions was determined.

-○- soluble enzyme

-●- membrane-bound enzyme

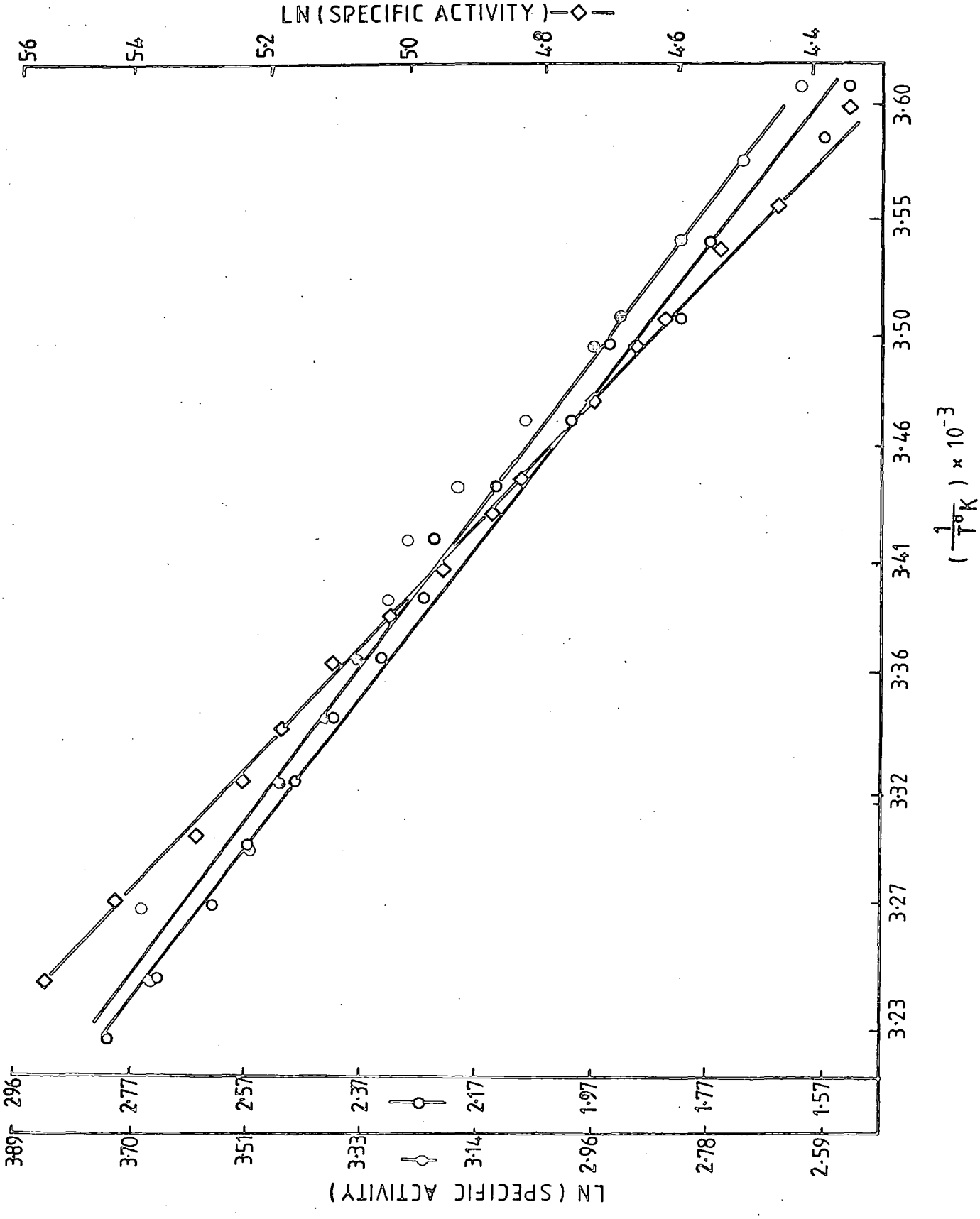

LN (SPECIFIC ACTIVITY)—◇—
5·6
5·4
5·2
5·0
4·8
4·6
4·4
3·89
3·70
3·51
3·33
3·14
2·96
2·78
2·59
2·96
2·77
2·57
2·37
2·17
1·97
1·77
1·57
LN (SPECIFIC ACTIVITY)
3·23
3·27
3·32
3·36
3·41
3·46
3·50
3·55
3·60
$(\frac{1}{T^{\circ}K}) \times 10^{-3}$

FIGURE 5 : 8

Typical Arrhenius profiles of rat synaptic membrane acetylcholinesterase fractions, treated with high-ionic strength media

- ◇ - partially purified acetylcholinesterase

  Arrhenius μ = 27.1 KJ $mole^{-1}$

- ● - coluble acetylcholinesterase

  Arrhenius μ = 24.4 KJ $mole^{-1}$

- ○ - membrane-bound acetylcholinesterase

  Arrhenius μ = 27.0 KJ $mole^{-1}$

Specific activity expressed as μ moles thiocholine.mg $protein^{-1}.hr^{-1}$.

Studies on the influence of membrane association on the thermal stability and kinetic properties of rat synaptic membrane acetylcholinesterase.

Introduction

Studies on the properties of rat synaptic membrane acetylcholinesterase presented in Chapters 4 and 5 have shown that this enzyme could be resolved into two forms by incubation in low-ionic strength media. One of these forms rapidly becomes soluble and a second remains firmly attached to the membrane. These two forms of acetylcholinesterase have been shown to exhibit similar non-linear Arrhenius kinetics and similar sensitivity to tetracaine and detergents. This, together with the observation presented in chapter 4 that the membrane-bound enzyme solubilized with Lubrol W - X detergent had temperature properties and sensitivity to tetracaine similar to that for the original membrane-enzyme, led to the conclusion that these properties were not markedly influenced by membrane association.

It was of interest to examine other properties of this enzyme to determine whether the relationship with the membrane had any consequences for the properties of the rat brain acetylcholinesterase, as several studies have described effects of membrane-association on the properties of acetylcholinesterase from various species. Reavill, Wooster & Plummer,(1978) have shown that the Km for acetylthiocholine of Triton X - 100 solubilized, purified pig brain acetylcholinesterase was influenced by association with liposomes. Association of this enzyme with liposomes made from positively charged phospholipids increased the Km from 44 uM to 150 uM whereas association with liposomes made from negatively charged phospholipids decreased the Km from 44 uM to 29 uM.

Studies of the effect of the membrane lipid fatty acid composition on the co-operativity of fluoride-ion inhibition of acetylcholinesterase have been reviewed by Farias, Bloj, Morero, Sineriz & Trucco, (1975). It has been observed that the fatty acid composition of rat erythrocyte membrane lipids was dependent on the nature of dietary lipid supplement (Guarnieri & Johnson, 1970). Morero, Bloj, Farias & Trucco, (1972) found that essential fatty acid supplement in the diet resulted in an increase in the Hill co-efficient of fluoride-ion inhibition of rat erythrocyte acetylcholinesterase activity from a value of 1.0 in the case of rats on a fat free diet to 1.6 with fat supplement. It was suggested that co-operative interactions between acetylcholinesterase molecules were modified by membrane fatty acid composition.

Cossins & Bowler, (1976) have shown that membrane bound enzymes (e.g. Ca^{2+} - Mg^{2+} ATPase) were more sensitive to irreversible thermal inactivation than soluble enzymes (e.g. pyruvate kinase). They suggested that in the former, the membrane environment contributed significantly to the maintenance of the correct enzyme conformation necessary for activity, and that the disordering of the membrane microenvironment by high temperature caused irreversible enzyme inactivation at temperatures lower than for soluble enzymes.

Thus the studies presented in this chapter examined the thermal stability, substrate kinetics and fluoride-ion inhibition of the low ionic-strength solubilized and membrane-bound rat synaptic membrane acetylcholinesterase. These have been compared in an attempt to attribute kinetic significance to the membrane association of the rat brain acetylcholinesterase.

Materials and methods

Acetylcholinesterase assay

Acetylcholinsterase activity was assayed by the modification of the Ellman, (1961) method as described in the general methods chapter.

Protein assay

Protein was assayed by the modified ninhydrin procedure as described in the general methods chapter.

Preparation of rat synaptic membranes

Rat brain synaptic membranes were prepared according to the bulk extraction method described in detail in the general methods chapter.

The low-ionic strength soluble and membrane-bound forms of acetylcholinesterase were prepared as described in the methods section of chapter 4.

Substrate activation kinetics of the acetylcholinesterase

Substrate activation kinetics of acetylcholinesterase were measured in the conventional assay medium except that enzyme activity was estimated at a range of substrate concentrations.

In a typical experiment 40 test tubes received the conventional 3 cm^3 aliquot of 0.1M Tris -HCl buffer pH 7.4 at reaction temperature ($37^{o}C$), also each received the usual 100 µl of D.T.N.B. solution. The tubes were arranged in 20 pairs. One of each pair received 200 µl of an appropriate dilution of soluble or membrane-bound enzyme (in imidazole/EDTA buffer) and the second tube of each pair received 200 µl of the imidazole/EDTA buffer.

20 different concentrations of acetylthiocholine, between 5.0×10^{-4}M and 7.2×10^{-6}M were prepared and 200 ul of each substrate concentration was used with each pair of tubes. The rate of enzyme activity and the background rate of hydrolysis of substrate were measured in the usual way. Tubes in which the enzyme rate resulted in the consumption of more than 10% of the available substrate were rejected and the data from the remainder, (i.e. 16 or 17 points) were processed for fit to the Michaelis Menten equation using a computer assisted scheme available in the department.

Fluoride-ion inhibition of rat synaptic membrane acetylcholinesterase

Fluoride-ion inhibition of acetylcholinesterase was measured by replacing the 0.1M Tris -HCl reaction buffer with the same buffer containing a range of fluoride-ion concentrations of up to 3×10^{-3}M KF and the assay was carried out in the usual way. The values for residual enzyme activity at each fluoride-ion concentration (v) were converted to percentages of the activity in the absence of fluoride (Vo). These data were represented in the form of the Hill plot in which $\ln \left(\frac{v}{Vo - v} \right)$ is plotted against $\ln \left[F^- \right]$. A line was fitted to these points by eye and the gradient of this line calculated.

Thermal inactivation of rat synaptic membrane acetylcholinesterase

The irreversible thermal inactivation of soluble and membrane-bound acetylcholinesterase activity was estimated in three types of experiment.

i) The temperature range of thermal inactivation was determined by constructing a temperature gradient in a aluminium Forbes' bar. One end of the bar was immersed in a water bath at 30°C and the other was immersed in water maintained at 60°C, and the bar was allowed to equilibrate overnight.

Glass tubes were then placed in holes equally spaced along the bar and equilibrated for at least one hour. Aliquots (500 ul) of enzyme at working dilutions were pipetted into these tubes, and incubated for 10 minutes. After this time the tubes were placed in an ice and water bath ($0^{o}C$). When all the tubes had been incubated and quenched on ice the residual acetylcholinesterase activity was measured in all fractions.

ii) The isothermal inactivation profiles were determined by placing twenty glass tubes in a water bath thermostated to a particular temperature. This was around 44 - $49^{o}C$ for the soluble enzyme and 50 - $58^{o}C$ for the membrane-bound enzyme. Aliquots (500 µl) of soluble or membrane-bound enzyme were placed in each tube and the tubes incubated for between 1 minute and 120 minutes. The enzyme fractions were diluted with 0.1M Tris - HCl buffer brought to pH 7.5 at the temperature of the water bath. Incubations were terminated by placing the tubes in an iced-water bath. The residual enzyme activity was measured in each enzyme aliquot.

The data from these studies was processed by a computer assisted curve fitting procedure, designed to calculate decay constants.

iii) In some experiments isothermal decay of acetylcholinesterase was determined in the presence of various detergents. In these cases both soluble and membrane-bound fractions of acetylcholinesterase were incubated with Triton x - 100, sodium deoxycholate and Lubrol W - X exactly as described in chapter 4 for Arrhenius plot measurements, except that the detergent treated enzyme aliquots in 20mM imidazole/EDTA buffer were diluted with 0.1M Tris - HCl buffer which had been brought to pH 7.5 at the inactivating temperature.

These diluted detergent-treated enzyme fractions were used directly for isothermal inactivation measurements as described above.

Sucrose density gradient centrifugation of rat synaptic membrane acetylcholinesterase

Sucrose density gradients used in this study were prepared in M.S.E. 25cm^3 polycarbonate centrifuge tubes, using a 20cm^3 perspex M.S.E. gradient former. The gradients were of 5% - 20% sucrose buffered with 0.1M Tris -HCl pH 7.5.

The detergent treated acetylcholinesterase fractions were prepared as described above, except that after detergent treatment the membrane-bound fraction was centrifuged at 100,000 xg for 1 hour at 4°C and the supernatant retained. In the case of Lubrol W - X treatment 500 µl aliquots of soluble and membrane fractions of the enzyme were layered over separate gradients. In the case of Triton x -100 and sodium deoxycholate treatment 250 ul aliquots of both soluble and membrane-bound fractions were placed on a single gradient. These gradients were centrifuged at 100,000 xg for 16 hours at 4°C, in an M.S.E. superspeed 40 preparative ultra centrifuge.

The resulting gradients were fractionated by pumping out the contents through a glass capillary tube, held at the bottom of the centrifuge tube with a micromanipulator. Samples of about 1 cm^3 were collected using the drop counter of an L.K.B. fraction collector. The fractions were assayed for acetylcholinesterase activity as described above.

Results

The effect of the membrane association of acetylcholinesterase was investigated by determining the stability of soluble and membrane-bound enzyme fractions to irreversible thermal inactivation. The initial experiments of this type were carried out by incubating aliquots of soluble and membrane-bound enzyme for a fixed time (10 minutes) at a range of temperatures between 30°C and 60°C. The residual activity was then determined in conventional media at 37°C. The effect of this heat treatment on acetylcholinesterase is presented in Figure 6 : 1. From this it can be seen that the membrane-bound enzyme was considerably more stable than the soluble form, with 50% inactivation occurring at about 50°C for the membrane-bound enzyme and 45°C for the soluble enzyme. This represents the first major difference in the properties of soluble and membrane-bound fractions to be detected in this study.

One unusual feature of the data presented in Figure 6 : 1 is that the soluble enzyme undergoes 100% inactivation over an approximately 10°C temperature range, whereas the range is about 20°C for the membrane-bound enzyme. This would suggest that the activation energy of the decay process was different for soluble and membrane-bound enzyme. However, the experiment described in Figure 6 : 1 is a valid representation of the thermal inactivation process only if the decay of enzyme activity at any temperature occurs by a first-order process. It was necessary therefore to test that both enzyme fractions conformed to this requirement.

This was tested by following the isothermal inactivation of the enzyme fractions, that is by incubating aliquots of enzyme at a single temperature for various times between 1 minute and 210 minutes, and determining the residual activity at each time-point.

It was found to be convenient to follow the isothermal decay of soluble acetylcholinesterase at 47°C and that of membrane-bound acetylcholinesterase at 57°C. Typical isothermal inactivation profiles are presented in Figure 6 : 2 for the soluble enzyme and Figure 6 : 3 for the membrane-bound enzyme. If these represented first-order decay processes then the log of residual activity plotted against the duration of incubation would have been linear. Figures 6 : 2 and 6 : 3 show that this was not the case for either form of the enzyme.

These non-first-order decay processes were difficult to interpret. It was clear that the decay in activity in this case represented the decay of more than one state. This could occur by each enzyme molecule undergoing a decay in activity involving more than one intermediate, or by more than one type of enzyme, each with different thermal sensitivities, existing within the total enzyme population. In either case it was not possible to say how many states were involved in the decay process.

The best course of action was to assume the simplest case was true and that two states existed. Again it was not possible to determine whether each enzyme molecule could exist in two states or whether two separate types of acetylcholinesterase existed within the total enzyme population. If the latter were to be the case, then this would mean that four types of acetylcholinesterase had been detected, two within the soluble population and two within the membrane-bound population. However, the isothermal inactivation profiles for both soluble and membrane enzyme were processed according to a computer assisted scheme which calculated the best fit of the data to two decay constants. Typical decay constants presented in Figure 6 : 2

for the soluble enzyme at 47°C were 0.78 x 10^{-1} min^{-1} for the initial thermolabile state, and 0.8018 x 10^{-2} min^{-1} for the thermostable state, which were similar to typical decay constants presented in Figure 6 : 3 for the membrane-bound enzyme at 57°C, namely 0.144 min^{-1} for the thermolabile state and 0.182 x 10^{-1} min^{-1} for the thermostable state.

As stated previously, the two-state decay process may result from each enzyme molecule being capable of existing in a high activity thermolabile state and in a low activity thermostable state, or from two separate enzyme forms. It may be possible to distinguish between those alternatives by extending the substrate-kinetics and fluoride-ion inhibition studies to include a comparison of soluble and membrane enzyme in the labile and stable states. If these states represented different molecular species then kinetic differences might be observed.

This was examined by determining the fluoride-ion inhibition kinetics of both initial soluble and membrane-bound enzyme and also of soluble and membrane enzyme in the thermostable state. Data for fluoride inhibition of soluble and membrane-bound enzyme are presented in the form of Hill plots in Figure 6 : 4 for the soluble enzyme and Figure 6 : 6 for the membrane enzyme. Data for two separate experiments are presented in each plot. The gradient of these plots which represents the Hill co-efficient of co-operativity was determined by linear regression analysis. The gradients of the line fitted to these lines were 1.1 for the soluble enzyme and 0.92 for the membrane-bound enzyme.

The fluoride-ion inhibition kinetics of the thermostable forms of soluble and membrane-bound acetylcholinesterase were determined using partially heat inactivated enzyme. This was produced by heating an aliquot of soluble enzyme for 60 minutes at 47°C and

heating an aliquot of membrane enzyme for 60 minutes at 55°C. The fluoride-ion inhibition kinetics of the residual activity was determined as for the uninactivated enzyme fractions. These data in the form of Hill plots are presented in Figure 6 : 5 for the partially inactivated soluble enzyme and in Figure 6 : 7 for the partially inactivated membrane-bound enzyme. The Hill co-efficients derived from these plots were similar to that of the uninactivated preparations at 0.8 for soluble enzyme and 1.1 for the membrane-bound enzyme.

The substrate activation kinetics of soluble and membrane-bound acetylcholinesterase are presented in Figures 6 : 8 and 6 : 10 respectivel; in the form of typical Lineweaver-Burke plots. The Km values calculated from these plots were 8.8×10^{-5}M for the soluble enzyme and 7.4×10^{-5}M membrane-bound enzyme. The substrate activation of the partially heat inactivated soluble and membrane-bound enzyme are presented as typical Lineweaver-Burke plots in Figures 6 : 9 and 6 : 10 respectively. The Km values calculated from these plots were 1.0×10^{-4}M for the inactivated soluble enzyme and 1.4×10^{-4}M for the partially inactivated membrane-bound enzyme.

Thus membrane association and partial heat-inactivation had no noticeable effect on the kinetic properties of acetylcholinesterase described above.

The temperature dependence of the isothermal decay profiles for soluble and membrane-bound acetylcholinesterase was determined in order to estimate the activation enthalpies of the decay processes for these enzyme fractions. The isothermal inactivation was determined at between 44°C and 49°C for the soluble enzyme (Figure 6 : 12) and between 50°C and 58°C for the membrane-bound

enzyme (Figure 6 : 13). The decay constants of the initial thermolabile states and thermostable state were determined by computer assisted analysis of these data. These decay constants are presented in the form of Arrhenius plots in Figure 6 : 15 for the thermolabile state and in Figure 6 : 16 for the thermostable state. Although these data were somewhat variable an estimate of the activation enthalpy of the decay processes was obtained by fitting straight lines by linear regression analysis.

The gradients of these lines represent $\frac{\mu}{R}$, where μ is the enthalpy factor and R is the gas constant.

From this analysis the activation enthalpy of the decay of the thermolabile species was 158.6 KJ $mole^{-1}$ for the soluble enzyme and 187.2 KJ $mole^{-1}$ for the membrane-bound enzyme. The value for the inactivation of the thermostable species was 135 KJ $mole^{-1}$ for the soluble enzyme and 410 KJ $mole^{-1}$ for the membrane-bound enzyme.

The thermal stability of the membrane-bound form of acetylcholinesterase was further examined, after solubilization in various detergents. This was compared with the effect of these same detergents on the thermal stability of the soluble enzyme. Figure 6 : 16 shows that solubilization with sodium deoxycholate rendered the membrane-bound fraction very much less stable than the untreated preparation. The untreated enzyme activity was reduced to about 10% of the initial activity by incubation at 57°C for 1 hour, whereas the deoxycholate treated enzyme was completely inactivated by 4 minutes at 57°C. Incubation at 52°C for 20 minutes also completely inactivated the deoxycholate treated membrane-enzyme. However, Figure 6 : 17 also

shows that similar deoxycholate treatment of soluble acetylcholinesterase also increases sensitivity to high temperatures. Whereas incubation of soluble enzyme for 90 minutes at 47°C reduced the initial enzyme activity by 90%, the deoxycholate treated soluble enzyme was inactivated by incubation for 15 minutes at 47°C. The inactivation of deoxycholate treated soluble enzyme at 40°C was similar to that of the untreated enzyme at 47°C.

Similar results were obtained with Triton solubilized enzyme. Figure 6 : 18 shows that the Triton-treated membrane enzyme was inactivated after 10 minutes at 57°C, and the Triton-treated soluble enzyme activity was undetectable after 30 minutes at 47°C as shown in Figure 6 : 19.

Somewhat different results were obtained with Lubrol W - X solubilized enzyme fractions. Data for the inactivation of Lubrol W - X treated membrane enzyme at 57°C (Figure 6 : 20) and Lubrol W - X treated soluble enzyme at 47°C (Figure 6 : 21) shows that these enzymes were very slightly more stable to irreversible inactivation than the untreated preparations. Thus the solubilization of membrane-bound enzyme with Lubrol W - X had little effect on the thermal stability of this enzyme fraction.

The sucrose density-gradient analysis of Lubrol W - X solubilized acetylcholinesterase is presented in Figure 6 : 22. This shows that the Lubrol treated soluble enzyme banded at a lower density than the solubilized membrane-bound form, although membrane debris was removed by centrifugation prior to sucrose density-gradient analysis.

The density gradient analysis of deoxycholate-treated and Triton x - 100 treated acetylcholinesterase is presented in Figure 6 : 23. In these gradients both soluble and membrane-enzyme were layered onto a single gradient for each detergent treatment. Figure 6 : 23 shows that the density distribution of activity for sodium deoxycholate and Triton-treated enzyme was similar to that for Lubrol W - X solubilized enzyme (Figure 6 : 22).

Discussion

Initial experiments on the stability of soluble and membrane-bound fractions of rat synaptic membrane acetylcholinesterase showed the first difference between the properties of these fractions, observed in this study, in that soluble enzyme was considerably less stable than the membrane-enzyme (Figures 6 : 1 - 6 : 3). However, these results were not what would have been expected in two major respects.

Firstly it is generally expected that soluble enzymes are more stable than membrane-bound enzymes (Cossins & Bowler, 1976). This is said to be owing to the contribution of the membrane microenvironment to the structure of membrane enzymes in that a particular degree of membrane fluidity is necessary to maintain correct enzyme conformation. In the case of mammalian membranes, temperatures above $40^{\circ}C$ - $45^{\circ}C$ would produce increased membrane fluidity which would not be consistent with correct enzyme conformation. Soluble enzyme conformation is thought to depend not only on disulphide bridges but on the hydrophobicity of some amino-acid side chains which seek to avoid polar environments and on hydrogen bonding within the molecule. Higher temperatures are required to disrupt these hydrophobic and hydrogen bonding forces in proteins than are required to destabilise biomembranes. Thus the increased stability of the membrane form of acetylcholinesterase suggests that either the membrane holds the enzyme in a stable conformation in a manner which is somewhat independent of membrane fluidity or that the membrane association is co-incidental and the stability is a property of the protein itself.

The second unexpected effect was that the isothermal decay of

both soluble and membrane-bound acetylcholinesterase activity was not first-order (Figures 6 : 2 and 6 : 3). A similar effect has been observed for the $Na^+ - K^+$ ATPase (Atkinson, Gatenby & Lowe, 1971; Gladwell, 1975). As described in the results section, this may be owing to a multitude of sequential or overlapping processes, either within an individual molecule or specific to several molecular species, but the simplest method of interpretation is to treat such plots as consisting of two phases; an initial decay of a relatively thermolabile state and the slower decay of a thermostable state. A computer assisted procedure was available in which the theoretical decay constants of these two states could be calculated by fitting data to this model.

These two unexpected results were further examined by testing the effect of various detergents on the thermal stability of both soluble and membrane acetylcholinesterase to see whether solubilising the membrane enzyme could affect this property, and also the kinetic studies were expanded to include the soluble and membrane enzyme in the partially inactivated stable state.

The Hill plots of fluoride-ion inhibition for the soluble and membrane-bound acetylcholinesterase and for the partially inactivated enzyme fractions were similar, and gave Hill co-efficients close to 1.0 in each case (Figures 6 : 4 and 6 : 6). This value of the co-efficient shows that there were no co-operative interactions between the acetylcholinesterase molecules in any of the fractions. This is superficially unlike the data of Morero et al, (1972) who showed that the Hill co-efficient of fluoride inhibition of rat erythrocyte acetylcholinesterase could be correlated with dietary fatty acid supplement, known to alter erythrocyte membrane fatty acid composition. However,

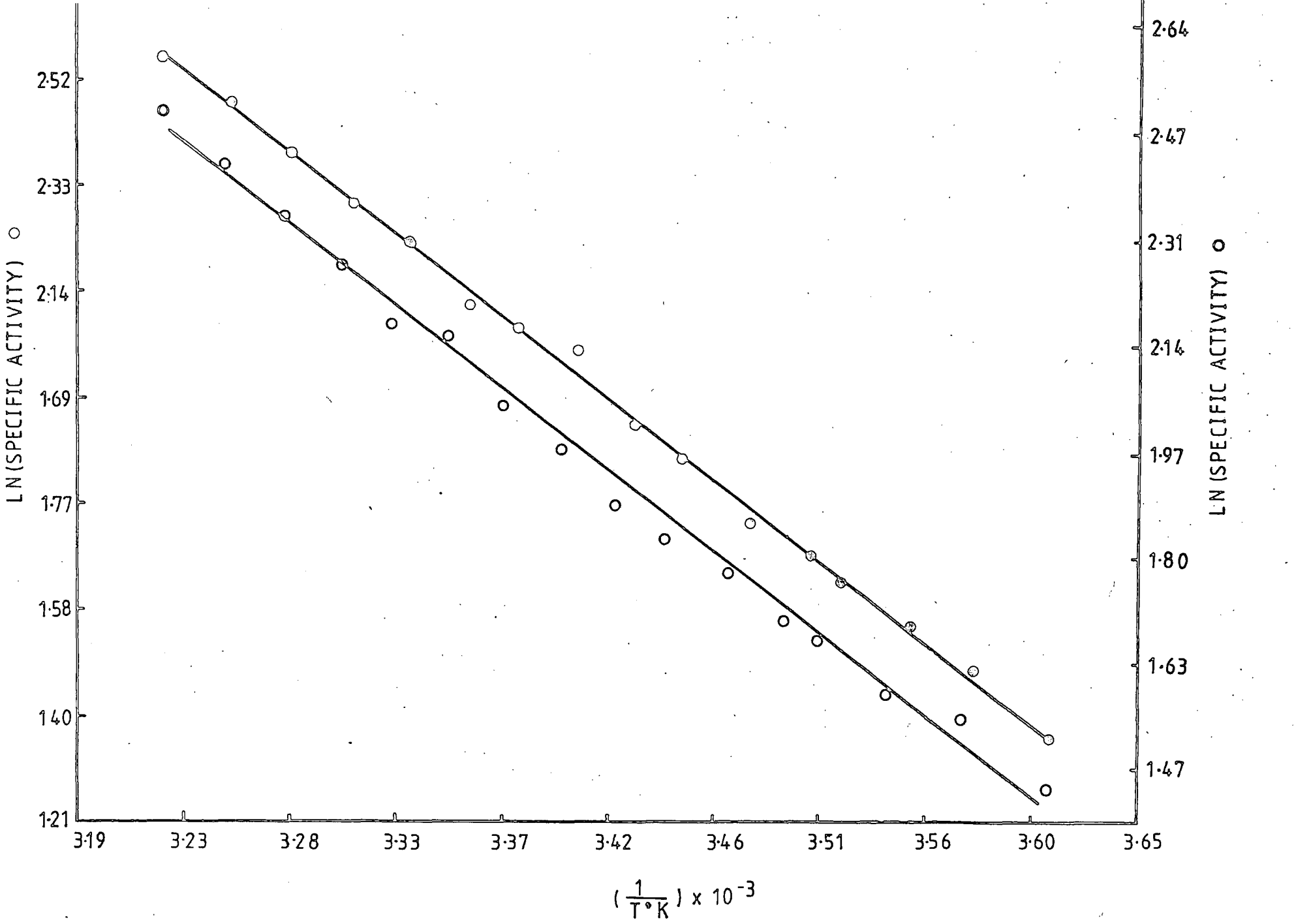
it is also possible that the rat erythrocyte enzyme is a lipoprotein as has been suggested for the brain enzyme in this study (Chapter 4) and that the fatty acid composition of tightly bound lipid affects co-operative properties rather than that of the whole membrane.

The substrate activation of properties of soluble and membrane-bound acetylcholinesterase and their partially inactivated counterparts were very similar, with Km's varying between 7×10^{-5} and 1.5×10^{-4} (Figures 6 : 8 and 6 : 11). These values were similar to those obtained by Goodkin & Howard, (1974) for rat synaptosomal acetylcholinesterase. This is the opposite view to that of Reavill et al, (1977) who detected differences in the Km of pig brain acetylcholinesterase when solubilized by Triton x - 100 and when attached to liposomes. This may be owing to a difference in properties between the pig brain and rat brain enzyme or perhaps the latter result was an artifact of the effect of Triton x - 100 on the enzyme.

The conclusions from this work were that no differences between the properties of soluble and membrane acetylcholinesterase were detectable other than the resistance of the membrane form to reversible inactivation and that this property remains the only candidate for revealing a functional relationship between rat brain acetylcholinesterase and the membrane. Also this study has detected no kinetic differences between the thermolabile and thermostable forms of acetylcholinesterase thus these represent very closely related species.

The difference in the thermal stability of soluble and membrane-bound rat brain acetylcholinesterase was further examined by determining the temperature dependence of the

isothermal inactivation of both enzyme fractions. In both cases this was resolvable across a very narrow temperature range of 44°C - 49°C for the soluble enzyme (Figure 6 : 12) and 50°C - 58°C for the membrane-bound enzyme (Figure 6 : 13). The decay constants calculated for a number of profiles of which those presented above were representatives, were somewhat variable but the activation enthalpy of each decay process was estimated by linear regression from Arrhenius plots of the decay constants presented in Figure 6 : 14 for the decay of the labile states and in Figure 6 : 15 for the stable states. The enthalpies for the labile states, 158.6 KJ $mole^{-1}$ for the soluble enzyme and 187.2 KJ $mole^{-1}$ for the membrane enzyme were consistent with the enthalpy values of 160 - 400 KJ $mole^{-1}$ quoted for the inactivation of catalytic activity (Neilands & Stumpf, 1958). The value for the activation enthalpy of the inactivation of the stable state of the soluble enzyme (135 KJ $mole^{-1}$) was also similar to the value for the decay of the labile states, whereas the activation enthalpy for the decay of the membrane enzyme stable state was much higher at 410 KJ $mole^{-1}$. This latter value is still consistent with protein denaturation but it is not clear why this is so much higher than for the soluble enzyme state. It may be that at the higher temperatures used in the inactivation of the membrane enzyme the estimate of the stable state decay constant becomes less accurate. However, the general conclusion from this work is that the activation enthalpy for the decay of enzyme activity cannot account for the difference in stability between the soluble and membrane-bound forms of the enzyme, as the results for both enzymes were consistent with high temperature protein denaturation.


LN (SPECIFIC ACTIVITY) ○
2·52
2·33
2·14
1·69
1·77
1·58
1·40
1·21
LN (SPECIFIC ACTIVITY) o
2·64
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2·31
2·14
1·97
1·80
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1·47
3·19
3·23
3·28
3·33
3·37
3·42
3·46
3·51
3·56
3·60
3·65
$(\frac{1}{T°K}) \times 10^{-3}$

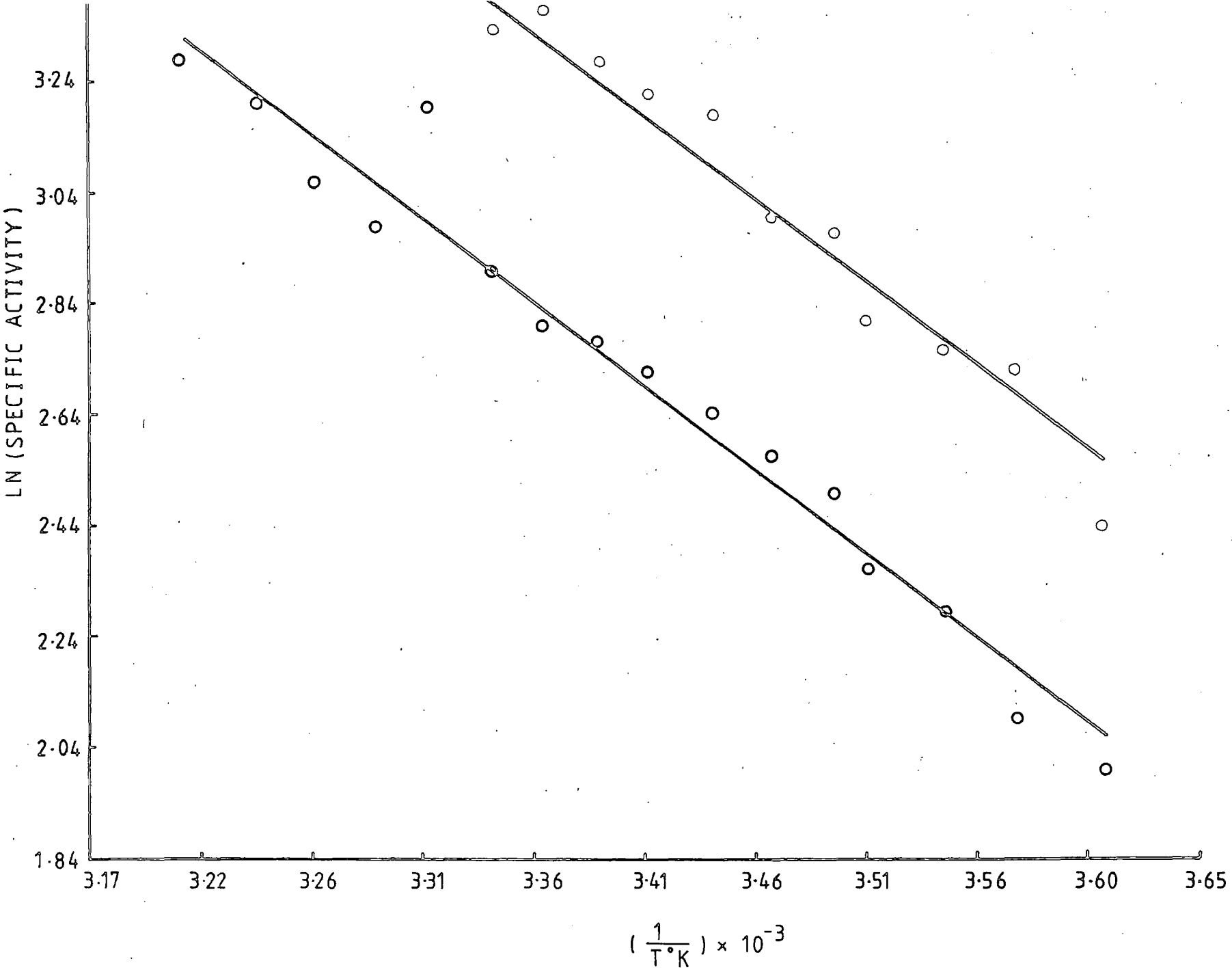
FIGURE 4 : 12

Typical Arrhenius plots of soluble and membrane-bound fractions of rat synaptic membrane acetylcholinesterase activity in the presence of 1mM n-octanol.

- ● - soluble acetylcholinesterase (μ = 23.6 KJ $mole^{-1}$)

- ○ - membrane-bound acetylcholinesterase (μ = 25.7 KJ $mole^{-1}$)

Specific activity expressed as μ moles thiocholine $mg.protein^{-1}$ hr^{-1}.


LN (SPECIFIC ACTIVITY)
3·24
3·04
2·84
2·64
2·44
2·24
2·04
1·84
3·17
3·22
3·26
3·31
3·36
3·41
3·46
3·51
3·56
3·60
3·65
(1 / T°K) × 10⁻³

Isolation and characterisation of lipid molecules associated with rat synaptic membrane acetylcholinesterase

Introduction

Data presented in chapter 4 strongly suggested that acetylcholinesterase was a lipoprotein, in that it contained tightly-bound lipid molecules distinct from bilayer lipid. It was also suggested that thermal changes in the physical state of this lipid were responsible for the non-linear temperature plots found for this enzyme. It was considered possible therefore to confirm this view by extracting acetylcholinsterase from the membrane, purifying the enzyme free of solubilised lipids and subjecting the enzyme to lipid extraction.

The purification and lipid extraction of a membrane-bound enzyme is in itself an unusual approach to the identification of the nature of lipid-protein relationships, and is only feasible owing to the suspected lipoprotein nature of acetylcholinesterase. In the past it has often not proven possible to identify directly lipids associated with membrane-enzymes, but merely to infer the involvement of a particular lipid class. Wheeler & Whittam, (1970) produced a lipid depleted $Na^+ - K^+$ ATPase preparation which was found to be reactivated by phosphatidylserine. Thus it was suggested that this lipid was an absolute requirement for $Na^+ - K^+$ ATPase activity. Sihotang, (1976) similarly showed that a lipid depleted human erythrocyte acetylcholinesterase preparation could be reactivated by phosphatidylserine. This also suggested that the human enzyme was absolutely dependent on this lipid.

However, in the case of the rat brain acetylcholinesterase

the form of the Arrhenius plot and the effects of lipophilic agents suggested that perturbation of the lipoprotein relationship in this enzyme did not abolish activity. The suggestion that any lipid bound to acetylcholinesterase from some species was not an absolute requirement for activity was supported by work on bovine erythrocytes (Beauregard & Roufogalis, 1977) and pig brain (Reavill, Wooster & Plummer, 1978).

It is clear that the most efficient extraction of rat synaptic membrane acetylcholinesterase would involve the use of detergents which in some cases have been shown to modify the temperature properties of the enzyme. It would be necessary to verify the stability of the enzyme activity during extraction and subsequent manipulation but also to verify the persistence of the non-ideal Arrhenius behaviour. However, it was considered that an attempt at such an extraction might provide useful corroboration of the conclusions drawn from the work of previous chapters.

MATERIALS AND METHODS

Acetylcholinesterase assay

Acetylcholinesterase was assayed using the modification of the Ellman, (1961) colourimetric method, described in the general methods section.

Protein assay

Protein was assayed by the modified ninhydrin technique described in detail in the general methods section.

Rat synaptic membrane isolation procedure

Rat synaptic membranes were extracted from cerebral cortex by the bulk extraction method, modified to avoid the use of a sucrose density gradient, described in detail in the general methods section. Stock membrane preparations, used as a source of acetylcholinesterase were stored at $4^{o}C$ packed in ice, suspended in 20mM imidazole buffer pH 7.2 containing 2mM EDTA at a protein concentration of above 1 mg. cm^{-3}.

For experiments involving soluble and membrane-bound enzymes, these were resolved by incubating membrane suspensions in the conditions described above for at least three days. After this time they were centrifuged at 100,000 xg for 1 hour at $4^{o}C$. The supernatant was retained and the membrane pellet was resuspended in a volume of imidazole/EDTA buffer approximately equal to that of the supernatant. These represent the soluble and membrane-bound acetylcholinesterase fractions.

The effect of detergents on rat synaptic membrane acetylcholinesterase

The detergents used in this study were made up fresh for each

experiment. Each was dissolved at twice working concentration in 20 mM imidazole, 2mM EDTA pH 7.2, and diluted with an equal volume of enzyme solution for use.

Although Triton X - 100 was supplied as a liquid, it did not mix readily with aqueous buffers. It was necessary to warm gently the suspension of Triton and buffer to ensure rapid and thorough dissolution. In an individual experiment a 2 cm^3 aliquot of a synaptic membrane suspension at about 500 µg cm^{-3} protein concentration was added to each of 4 test tubes. To 3 of the test tubes 2 cm^3 of Triton solution was added to give final Triton concentrations of 4.0% w/v, 2.0% w/v and 0.2%. w/v. The fourth tube received 2 cm^3 of imidazole/EDTA buffer as a control.

The suspensions were incubated at 20°C and 1 cm^3 aliquots were taken from each tube after 30 minutes, 1 hour and 4 hours. These 1 cm^3 samples were chilled on ice and assayed for acetylcholinesterase activity immediately. After the final aliquot had been recovered and assayed for enzyme activity, the remainder of each sample was measured for protein concentration.

In a typical experiment with sodium deoxycholate, 0.5 cm^3 aliquots of a synaptic membrane suspension at about 500 µg. cm^3 protein concentration were added to each of 9 test tubes. Then 8 of the tubes received 0.5 cm^3 aliquots of detergent solution, to give a final concentration of between 10 mM and 80 mM deoxycholate. The ninth tube received 0.5 cm^3 of buffer as a control. The tubes were incubated at 20°C for 20 minutes after which time 200 µl samples were taken for assay of

acetylcholinesterase activity. When all enzyme assays had been completed, the samples were taken from each tube for protein assay as described previously.

In a typical experiment with Lubrol W - X, 0.5 cm^3 of synaptic membrane suspension, at a protein concentration of about 500 $\mu g.cm^{-3}$ was added to each of 7 test tubes. 6 of these tubes received 0.5 cm^3 of Lubrol solution to give final concentrations of between 0.5 and 5.0 $mg.cm^3$. These were incubated at 5°C for 48 hours. After this time 200 µl aliquots were taken from each tube and assayed for acetylcholinesterase activity. The protein concentration of each of the 7 fractions was measured as described above.

Lubrol W - X solubilization of rat synaptic membrane acetylcholinesterase

In a single experiment 2 cm^3 of a synaptic membrane suspension at 500 $\mu g\ cm^{-3}$ protein concentration in imidazole/EDTA buffer was pipetted into each of 9 test tubes. A 2 cm^3 aliquot of Lubrol W - X solution at a concentration of between 0.0125 and 5 $mg.cm^{-3}$ was added to each tube. The 4 cm^3 aliquots were incubated for 24 hours at 4°C. The 4 cm^3 aliquots were then centrifuged at 100,000 xg for 1 hour at 4°C in a 10 x 10 titanium rotor in an MSE Prepspin 50 ultracentrifuge. The supernatants were retained and the pellets were resuspended in 4 cm^3 of imidazole/EDTA buffer. The acetylcholinesterase activity of the supernatant and membrane debris fractions was determined as described above.

High ionic strength treatment of rat synaptic membrane acetylcholinesterase

High ionic strength treatment was achieved by taking 200 µl aliquot of the stock soluble and membrane-bound acetylcholinesterase,

prepared as described above, and diluting with 200 ul of cold 2.0 M NaCl in imidazole/EDTA buffer. This is incubated at 4°C for 30 minutes, before being diluted to 2.0 cm^3 with the addition of 1.4 cm^3 of imidazole/EDTA buffer. These diluted enzyme fractions were then used directly in Arrhenius kinetics measurements as described below.

Arrhenius plots of acetylcholinesterase activity

Temperature kinetics measurements for partially purified acetylcholinesterase and salt-treated acetylcholinesterase were carried out as described in the methods section of chapter 3. Also the temperature kinetics of partially purified acetylcholinesterase in the presence of 2.0×10^{-4} tetracaine was carried out as described in the methods section of chapter 4.

Tetracaine inhibition of acetylcholinesterase

Tetracaine inhibition of partially purified acetylcholinesterase was carried out as described in the methods section of chapter 4 for the soluble and membrane-bound enzyme.

Extraction and partial purification of rat synaptic membrane acetylcholinesterase

In a typical experiment 20 - 30 rats were killed and processed for the bulk extraction of rat synaptic membranes as described above. The synaptic membrane suspension (4 cm^3 in imidazole/EDTA buffer) was diluted with 4 cm^3 of 8 $mgs.cm^{-3}$ Lubrol W - X in imidazole/EDTA buffer and incubated for 24 hours at 4°C. The suspension was then centrifuged at 100,000 xg for 1 hour at 4°C in an MSE prepspin 50 ultracentrifuge. The pellet was discarded, the supernatant was used directly for column chromatography.

The chromatography media used was diethylaminoethyl-Sephadex (Sigma Chemical Company). Both A-25 and A-50 Sephadex types

were used. The dry gel was swollen by suspending in 0.1 M Tris HCl buffer pH 8.0 and heating in a boiling water bath for 5 hours. The swollen beads were then poured into a 1.6 cm x 48 cm glass chromatography column (Wright). Sufficient beads were swollen to fill the column (100 cm^3 bed volume). The column was equilibrated with 0.1 M Tris - HCl pH 7.0. The sample was applied to the column and one bed volume run through. Proteins were eluted by passing a continuous and discontinuous salt gradient through the column. The salt gradient was in 0.1 M Tris - HCl buffer pH 7.0 with up to 0.5M NaCl. 3 cm^3 fractions were collected by drop counting in a fraction collector (LKB). All manipulations were carried out at 4^oC in a cold room.

The absorbance of fractions was measured at 280 nm and fractions containing protein peaks were assayed for acetylcholinesterase activity as described above. The most active fractions were pooled and used directly for kinetic analyses. For lipid extractions, enzyme was concentrated by dialysis against solid methyl cellulose powder.

Lipid extraction procedures.

i) Extraction of rat synaptic membrane lipids

In a typical experiment the synaptic membranes derived from the bulk extraction of 20 rat brains were pelleted by centrifuging at 100,000 xg for 1 hour at 4^oC. The pellet was carefully lifted from the polycarbonate centrifuge tube with a clean spatula and placed in 10 cm^3 of chloroform/methanol (2 : 1) containing 0.01% ditertiary butyl-p-cresol as an anti-oxidant in a glass homogenization tube, and the suspension was homogenized with several passes of a teflon pestle. The homogenate was decanted into a polypropylene centrifuge tube and centrifuged at 4,000 xg for 30 minutes at 4^oC in an MSE

Mistral 2L centrifuge. The chloroform/methanol supernatant was retained and the pellet was extracted twice more in this medium. The supernatants were retained in each case and pooled. The pellet was further extracted 3 times as above in chloroform/methanol (2 : 1) containing 1% hydrochloric acid. The supernatants were pooled with those from the chloroform/methanol extract. The pellet was then extracted 3 times in chloroform/methanol (2 : 1) containing 1% ammonia, as described previously. The supernatants were pooled with those from previous extractions.

The pooled supernatant was shaken in a 'quick-fit' flask with an equal volume (80cm^3) of 0.79% potassium chloride. The resulting mixture was centrifuged in an MSE Mistral 2L centrifuge at 3,000 xg for 30 minutes in a 4 x 50 cm^3 swing out rotor. This separated the chloroform and aqueous phases. The chloroform phase was re-extracted twice more with a volume of 0.79% KCl to that of the chlorform phase. The final chloroform phase was dried by standing with anhydrous sodium sulphate overnight. The dry chloroform was concentrated in a rotary evaporator and sealed into glass ampoules and stored at - 20°C until use.

ii) Extraction of acetylcholinesterase lipid

The scheme employed for the extraction of lipid from partially purified acetylcholinesterase was similar to that described above, but with the following modifications. The concentrated enzyme solution (10 cm^3) was extracted by mixing with an equal volume of each of the chloroform/methanol (2 : 1) phases. This was homogenized with several passes of a teflon glass homogenizer. The mixture was centrifuged at 3,000 xg for 30 minutes to separate the phases. The lower chloroform phase was retained and the aqueous phase re-extracted twice with chloroform/methanol (2 : 1). The a queous phase was then extracted three times with

chloroform/methanol (2 : 1) with 1% ammonia. The chloroform phases were pooled and subjected to the 'salt cut' with 0.79% KCl as described above, dried over sodium sulphate, concentrated to a small volume (50 µl) in a rotary evaporator. The concentrated lipid solution was then sealed into a glass ampoule and stored at - 20°C.

The aqueous phase from the extraction scheme described above was retained and subjected to one further cycle of extraction. The acqueous phase was thus extracted three times in an equal volume of chloroform/methanol with 2.0M ammonia. The chloroform phase was separated by centrifugation at 3,00 xg for 30 minutes at 4°C. The pooled organic phases were extracted with 0.79% potassium chloride as above, dried over anhydrous sodium sulphate and concentrated on a rotary evaporator. This was then sealed into a glass ampoule and stored at - 20°C until needed.

Thin layer chromatography

Thin layer chromatography (TLC) plates, for the separation of polar lipids were prepared as follows. 45 gms of Kieselguhr (type H) was placed in a 250 cm^3 plastic beaker. 100 cm^3 of 0.1% sodium acetate solution were slowly added to the silica gel with constant stirring with a clean glass rod, to give a smooth paste. The thin paste so produced was sufficient to coat five glass chromatography plates. The plates were poured using a Shandon uniplan spreader. The slurry was poured into the spreading device, which was then pulled rapidly over five plates. The plates were poured at a thickness of 0.5 mm. Immediately after pouring, the plates were removed and gently vibrated on a rota-mixer to produce a smooth surface, and placed in a rack and air dried overnight. Before use the plates were 'activated' by drying in an oven at 110°C for 1 hour.

The phospholipids were separated in the following way. The samples, in chloroform, were spotted onto the corner of one of the activated plates about 2cm^3 from each face. The spotted plate was dried for a few minutes at 110^{o}C. The phospholipid classes were resolved by a two dimensional solvent system. The first dimension solvent was chloroform/methanol/7M ammonia (65:25:4). The second dimension solvent was chloroform/methanol/acetic-acid/water (135:65:18:3).

This resolved the major phospholipid classes. The presence of a phospholipid on the plate was revealed by spraying the plate with a 0.1% aqueous solution of 8 analino-1 naphthalene sulphonic acid (ANS). When dried, phospholipid spots can be seen as blue-green spots under a U.V. lamp.

Preparation of Fatty-acid methyl esters

Areas of silica gel powder on these TLC plates corresponding to phospholipid spots were scraped from the plate with a spatula and placed in a 2cm^3 bijou bottle. The rubber seal in these bottles was replaced with a teflon faced seal. A volume of boron-trifluoride methanol reagent was placed in the bottle, such that the silica gel was just covered. The bottle was sealed and heated in an oven at 60^{o}C for 15 minutes. After this time the bottle was cooled and 0.5 cm^3 of n-hexane was added and thoroughly mixed. The contents of the vial were filtered through a small glass scinter and concentrated under a stream of nitrogen.

Gas-chromatography of fatty-acid methyl esters

The column used for the gas-chromatographic separation of fatty-acid methyl esters was 3% polyethyleneglycol adipate on Gas Chrom Q, packed into a 3 ft. Pye-Unicam gas chromatography column. The column was fitted into a Pye-Unicam 104 chromatograph

the oven was set at 180°C with a carrier gas (N_2) flow rate of 45cm^3 min^{-1}. A flame-ionisation detector was used to detect the separated esters. The methanolysed sample was injected in a total volume of 5 µl using a 10 µl Hamilton 701 N glass microsyringe with an aluminium Chaney adaptor. The amplified output was displayed on a Servoscribe potentiometric chart recorder at 10 mV full scale.

The retention times were recorded and processed as described in the general methods section.

Results

It was clear that the low ionic-strength solubilized fraction of rat synaptic membrane acetylcholinesterase, described in Chapter 4, representing only a 50% yield of soluble acetylcholinesterase, was an insufficient percentage of the total acetylcholinesterase to permit this to be used as an initial step in any purification scheme. It was, therefore necessary to employ a detergent treatment to solubilize a larger proportion of the total activity. With this in mind synaptic membrane preparations were treated with detergents which have been used in previous studies on acetylcholinesterase, namely sodium deoxycholate, Triton x - 100 and Lubrol W - X. Initial experiments were concerned with the stability of acetylcholinesterase in these detergents.

Data concerning the stability of acetylcholinesterase in Triton x - 100 at 20°C are presented in Table 5 : 1. From this, it can be seen that 0.2% Triton x - 100 began to inactivate the enzyme after 4 hours, whereas with higher concentrations of Triton x - 100 this was noticable after only 1 hour. Similarly the stability of acetylcholinesterase in various concentrations of sodium deoxycholate, is presented in Table 5 : 2. This shows that 30 mM deoxycholate caused significant inactivation in only 20 minutes at 20°C. Higher concentrations produced further inactivation. Conversely, data presented in Table 5 : 3 shows that concentrations of Lubrol W - X of up to 5 mg. cm^{-3} caused no inactivation of acetylcholinesterase after incubation for 48 hours at 5°C.

Thus Lubrol W - X was suitable for further study. However, published work has shown that both Triton x - 100 and sodium deoxycholate solubilize acetylcholinesterase very quickly at low

concentrations (Riger & Vigny, 1976; Sihotang, 1976) whereas Lubrol W - X acts more slowly as a solubilizer of acetylcholinesterase (Beauregard & Roufogalis, 1977). It was therefore necessary to test the time course of solubilization of the acetylcholinesterase by Lubrol W - X. Data presented in Figure 5 : 1 shows that concentrations of Lubrol W - X above 0.5 mg. cm^{-3} were suitable for the solubilization of over 90% of enzyme activity, under the conditions described in the methods section. It was concluded that 4 mgs. cm^{-3} represented the best compromise between efficacy as a detergent and excessive detergent concentration, and this concentration was used in further experiments.

Thus Lubrol extracted rat synaptic membranes were subjected to a column chromatographic procedure in order to effect further purification of the enzyme. Most conventional enzyme isolation procedures involve an ion-exchange column chromatography step followed by molecular exclusion chromatography. However, no method of concentrating the enzyme between chromatographic procedures was readily available. Consequently the chromatographic methods were combined with the use of diethyl amino-ethyl-Sephadex.

Two forms of this chromatographic medium exist, A-25 and A-50. The Sephadex type of A-25 resolves proteins in the molecular weight range 10,000 - 40,000, whereas A-50 resolves proteins in the molecular weight range 40,000 - 200,000. It was necessary to test both of these forms of ion-exchange Sephadex for their ability to resolve acetylcholinesterase from other proteins. Acetylcholinesterase from mammalian sources had been shown to be oligomeric when solubilized with detergent, thus it was not possible to predict the molecular weight of the Lubrol-isolated complex. Also proteins can be eluted from the column by either a continuous or discontinuous gradient. It was also necessary

to test these alternative methods of eluting proteins.

Typical results of eluting Lubrol extracted rat synaptic membrane proteins with a continuous sodium chloride gradient of up to 0.5M NaCl in 0.1M Tris - HCl pH 7.4 buffer are presented in Figure 5 : 2 for A-25 and Figure 5: 3 for A-50. From this it can be seen that A-25 type DEAE Sephadex resolved acetylcholinesterase into two fractions whereas A-50 type DEAE-Sephadex did not resolve these fractions, however, in both cases the acetylcholinesterase was poorly resolved from other extracted membrane proteins. This is confirmed by the specific activity of the pooled acetylcholinesterase fractions presented in Table 5 : 4. The specific activity of the first, smaller peak of acetylcholinesterase activity was about 120 u moles thiocholine/mg protein/hr whereas that of the second greater peak was nearer 200 u moles thiocholine/mg protein/hr.

A typical separation of Lubrol W - X solubilized rat synaptic membrane proteins on A-50 DEAE-Sephadex, using a discontinuous salt gradient of five concentrations between 0.1M and 0.5M NaCl is presented in Figure 5 : 4. This gave a more satisfactory separation in that acetylcholinesterase activity was better resolved from other proteins and the two peaks of acetylcholinesterase activity were completely resolved. Also, it can be seen from data presented in Tables 5 : 4, the specific activities of these fractions was higher than those obtained by the continuous gradient method. The two fractions were thus designated FI and FII and will be referred to as such.

It was necessary to characterise the acetylcholinesterase separated on this column, in terms of the properties found for this enzyme and reported in previous chapters. Namely, non-ideal

temperature kinetics, inhibition by tetracaine and the effect of tetracaine on acetylcholinesterase Arrhenius plots. Representative Arrhenius plots for FI and FII acetylcholinesterase activities are presented in Figure 5 : 5. The computer-calculated thermodynamic parameters from these and Arrhenius plots for one other column run are presented in Table 5 : 5 along with similar data for native synaptic membrane acetylcholinesterase. This shows that the temperature kinetics of the partially purified acetylcholinesterase fractions were similar to that for the native membrane-enzyme.

Figure 5 : 6 shows the effect of various concentrations of tetracaine on partially purified acetylcholinesterase activity. This enzyme was obtained from an early column chromatographic experiment as described in Figure 5 : 3, thus FI and FII fractions were not obtained. The tetracaine inhibition profile in this case was similar to that obtained for native membrane acetylcholinesterase (Figure 4 : 10), with 50% inhibition occurring at about 2.0×10^{-4}M for the native enzyme and 2.5×10^{-4}M for the partially purified enzyme. This Arrhenius profile of this preparation in the presence of 2.5×10^{-4}M tetracaine is presented in Figure 5 : 6. This plot was estimated to be best described in terms of linear Arrhenius kinetics by the computer assisted reversible inactivation model. This was similar to that obtained for the native preparation and the apparent activation energy of this plot (25.3 KJ $mole^{-1}$) was similar to that obtained for tetracaine treated native preparations (Figure 4 : 11). Thus, this shows that the partially purified acetylcholinesterase was similar to native preparations with respect to kinetic properties.

This suggested that this preparation of acetylcholinesterase was suitable for lipid extractions. However, it was necessary to

examine the effect of high ionic-strength on the temperature kinetics of acetylcholinesterase because Beauregard & Roufogalis, (1977) showed that not only could such treatment produce linear temperature plots but was also necessary to disturb the lipoprotein relationship suggested to explain temperature kinetics of bovine erythrocyte acetylcholinesterase. This was considered necessary in order to test whether such was the case for the rat synaptic membrane enzyme and whether such rigorous lipid extraction techniques would be necessary in further work.

This was investigated by incubating aliquots of stock acetylcholinesterase in 1.0M NaCl for 30 minutes at $4^{o}C$. This was diluted 10 fold, with imidazole/EDTA buffer, and used directly for temperature plot measurements. Typical plots for salt treated soluble membrane-bound and partially purified acetylcholinesterase are presented in Figures 5 : 8 and 5 : 9. It can be seen that these plots were linear in each case. Data for apparent activation energies of several salt-treated soluble, membrane-bound and partially purified acetylcholinesterase are presented in Table 5 : 6. The apparent activation energies of the low temperature state of untreated enzyme are also presented for comparison. The values for the apparent activation energies were about 25 KJ $mole^{-1}$ for the salt treated enzyme which was significantly lower than that obtained for untreated preparations which were between 32 and 36 KJ $mole^{-1}$.

As a result of this, it was reasonable to attempt to extract lipid from the partially purified preparation. The FI and FII enzyme preparations were concentrated as described in the methods section and subjected to lipid extraction as described. This gave two extracts, that extracted in conventional chloroform/ methanol media and that extracted with 2.0M ammonia.

These were both subjected to two dimensional thin-layer chromatography, and phospholipid spots were revealed by spraying with 0.1% acqueous ANS. The conventional extract failed to produce any phospholipid spots whereas the high ionic-strength extraction medium produced one spot with an rf of about 0.5 in the first dimension and an rf of about 0.9 in the second dimension in the case of both FI and FII fractions.

The lipids were extracted from the native synaptic membranes in the conventional media and separated by the same thin layer chromatography procedure as for enzyme-lipid extracts. The spots corresponding to each phospholipid class was identified by running test plates with commercial phospholipid standards. However, none of the major phospholipid classes separated with rf values consistent with that observed for the phospholipid extracted from acetylcholinesterase. This spot was found to separate in a similar manner to that of a commercial preparation of cardiolipin (diphosphatidylglycerol). Thus the lipid extracted from FI and FII fractions was tentatively identified as cardiolipin.

This lipid fraction was further characterised by gas-liquid chromatography i.e. analysis of fatty acid methyl esters, prepared from this lipid. The techniques used in this respect were calibrated by preparing fatty acid methyl esters from phospholipid classes extracted from rat synaptic membrane and separated by 2 dimensional thin-layer chromatography.

The fatty acids were tentatively identified according to the relative retention times as described in the general methods chapter. The relative quantity of each fatty acid methyl

ester was estimated by calculating the peak height x retention time for each peak. These values are expressed as percentage composition of each fatty acid class in Table 5 : 7. The fatty acid composition of the major phospholipid classes of synaptic membrane lipids and the putative cardiolipin from FI and FII fractions are both thus presented in Table 5 : 7.

These data show that cardiolipin contained a range of fatty acids similar to that for the major membrane phosphoglyceride classes. However, cardiolipin contained only small amounts of many fatty acids with C16 : 0 (21 - 24%) and C18 : 0 (15 - 16%) accounting for most of the resolved fatty acyl chains. Each of the major membrane phospholipids carried a significant proportion of some unsaturated fatty acid such as 18 : 1 w 9 for phosphatidyl choline, and 22 : 6 w 3 for the other phospholipids. Thus the lipid bound to acetylcholinesterase contained a higher proportion of saturated fatty acids than was the case for the membrane in general.

Discussion

In the few published studies which have attempted a purification of mammalian acetylcholinesterase, detergent solubilization of the enzyme was commonly employed at an initial step (Sihotang, 1974; Rieger & Vigny, 1976; Adamson, 1977; Beauregard & Roufogalis, 1977; Reavill, Wooster & Plummer, 1978). In the case of the rat synaptic membrane acetylcholinesterase, it was necessary to ascertain which, if any, of the commonly used detergents would best maintain the stability of the enzyme as well as dissociate it from the membrane.

Data presented in Table 5 : 1 shows that the acetylcholinesterase was somewhat unstable in Triton x - 100 concentrations of 2.0% and 4.0% w/v when incubated for a few hours at 20°C. It may be possible to increase the stability of the enzyme by using lower concentrations of Triton and maintaining the preparations at 4°C. Indeed Goodkin & Howard, (1974) used 0.1% Triton x - 100 to solubilize the rat synaptic membrane acetylcholinesterase and found the soluble enzyme was sufficiently stable for some limited kinetic analyses. Triton x - 100 has been used in the preparation of pure or partially purified acetylcholinesterase from mouse tissues (Adamson, 1977), pig-brain (Reavill, Wooster & Plummer, 1978) and rat-brain (Rieger & Vigny, 1976). Thus it may have been possible to use this detergent under carefully controlled conditions and maintain enzyme activity but as Triton x - 100 perturbed the non-linear temperature properties of the rat synaptic membrane acetylcholinesterase (Tables 4 : 3 and 4 : 4) it was considered advisable to avoid Triton if possible.

In studies on human erythrocyte acetylcholinesterase, Sihotang, (1974) showed that 40 mM sodium deoxycholate was necessary to solubilize the enzyme. Data presented in Table 5 : 2 shows that concentrations

of sodium deoxycholate in this range caused considerable inactivation of the rat brain enzyme in 20 minutes at 20°C. This together with the data presented in Chapter 4 (Tables 4 : 3 and 4 : 4) which shows the temperature properties of acetylcholinesterase to be significantly affected by sodium deoxycholate treatment suggested that sodium deoxycholate was unsuitable for purification of the rat synaptic membrane enzyme in this study.

Lubrol W - X, however, caused no inactivation of acetylcholinesterase, with prolonged incubation (48 hours) in detergent concentrations of up to 5 mgs. cm^{-3} at 4°C (Table 5 : 3) Also, this detergent had no effect on the temperature properties of acetylcholinesterase, in the short term (Tables 4 : 3 and 4 : 4). This was similar to the observations of Beauregard & Roufogalis, (1977). It was clear from this that Lubrol W - X was the most suitable of these three detergents for use in the purification of rat brain acetylcholinesterase in this study. Also Figure 5 : 1 shows that concentrations of Lubrol W - X of 0.5 mg. cm^{-3} were adequate to solubilize 90% of rat synaptic membrane acetylcholinesterase by incubation at 5°C for 24 hours. This was also similar to the observation of Beauregard & Roufogalis, (1977). A concentration of 4 mgs. cm^{-3} Lubrol was used in subsequent experiments.

Having obtained a soluble enzyme, it was now necessary to affect a purification of the enzyme free of excess detergent and any lipds released during the extraction procedure. Much attention has focussed on the purification of acetylcholinesterase from Electrophorus electricus and Torpedo californica (Massoulie, Rieger & Silman, 1971; Dudai, Silman, Shinitzky & Blomberg, 1973; Dudai & Silman, 1974; Rosenberry, 1976; Rosenberry & Richardson, 1977), but few workers have reported purification of acetylcholinesterase

from mammalian tissues. Thus no convention exists for mammalian acetylcholinesterase purification.

Sihotang, (1976) prepared electrophoretically purified human erythrocyte acetylcholinesterase using ammonium sulphate fractionation and a single Sephadex - G200 column chromatographic step. This would be unsuitable for the rat synaptic membrane enzyme as the quantity of protein isolated from the synaptic membranes from even a large number of rats would be insufficient for efficient ammonium sulphate precipitation.

Adamson, (1977) and Reavill, Wooster & Plummer, (1978) used affinity chromatography to purify Triton x - 100 extracted acetylcholinesterase. This technique produced an enzyme of high specific activity but the columns used were reported to be inefficient and only effective for one or two preparations.

Enzymes are usually purified using a combination of ion-exchange chromatography and molecular exclusion chromatography. However, in this study, the lyophilizing equipment necessary for concentrating proteins between column steps was not available, thus it was important to achieve as much purification as possible in one column step.

Ion exchange groups covalently linked to Sephadex (DEAE Sephadex) is available as a column chromatographic medium, and has been used in a batch purification step for bovine erythrocyte acetylcholinesterase (Beauregard & Roufogalis, 1977). This column material was used for the separation of Lubrol W - X extracted synaptic membrane proteins. Both A-25 (10,000 - 40,000 molecular weight range) and A-50 (40,000 - 200,000 molecular weight range) types of DEAE-Sephadex were tested, with a continuous salt gradient. Typical separations are shown

in Figure 5 : 2 for A-25 type and in Figure 5 :3 for A-50 type. This shows that the A-25 type resolved two fractions of acetylcholinesterase activity, whereas these were incompletely resolved by the A-50 form (Figure 5 : 3). This brought about a 10 fold purification of acetylcholinesterase, as can be seen from data presented in Table 5 : 4. The pooled column fractions containing acetylcholinesterase activity from these experiments were used for some kinetic characterizations of the enzyme, discussed below.

The discontinuous salt gradient separation on A-50 type DEAE - Sephadex was superior to that for a continuous salt gradient (Figure 5 : 4). Two fractions of acetylcholinesterase were clearly resolved (FI and FII fractions). Also the specific activities of the enzyme prepared in this way were slightly higher than those obtained by the continuous salt gradient elution (Table 5 : 4). These latter preparations were used particularly for lipid extractions as well as for kinetic characterization.

The precise significance of the two forms of acetylcholinesterase FI and FII separated by this ion-exchange procedure is not clear. Figure 5 : 3 shows that these forms have different affinities for DEAE groups, as higher concentrations of salt were required to dislodge the FII fraction. One possible explanation is that these represent oligomeric forms of the enzyme. Oligomers of this enzyme have been observed from rat tissue extracts (Rieger & Vigny, 1976; McLaughlin, Engel & Reddy, 1978) mouse tissue extracts (Adamson, 1977) and human erythrocyte membrane extracts (Ott & Brodbeck, 1978), and it may be that higher order oligomers, containing more monomer units bind more tightly to the ion-exchange column.

It was necessary to ascertain whether the properties of the acetylcholinesterase, of particular interest in this study, namely non-linear temperature kinetics and sensitivity to tetracaine, had been affected by the isolation procedure. Figure 5 : 5 shows that the Arrhenius plots of both FI and FII fractions were non-linear and data from plots for two separate preparations, presented in Table 5 : 5, showed that the thermodynamic parameters, calculated for these plots using the reversible thermal inactivation model were similar to those calculated for native synaptic membrane acetylcholinesterase Arrhenius plots. Also the inhibition of partially purified acetylcholinesterase by tetracaine (Figure 5 : 6) was similar to that for soluble and membrane-bound acetylcholinesterase preparations presented in Chapter 4 (Figure 4 : 10). Similarly the Arrhenius plot of partially purified acetylcholinesterase in the presence of 2.5×10^{-4}M tetracaine (Figure 5 : 7) was linear, with an apparent activation energy of 25 KJ $mole^{-1}$, which was similar to such plots for low ionic-strength soluble and membrane-bound acetylcholinesterase in the presence of tetracaine (Figure 4 : 11).

The properties of the partially purified enzyme were clearly similar to those of the enzyme from native preparations and the former were thus considered suitable subjects for an attempt at lipid extraction. However, Beauregard & Roufogalis, (1977) showed that the non-linear temperature kinetics of bovine acetylcholinesterase could be affected by high ionic strength (1.0M NaCl) to produce linear plots, and that this ionic strength was necessary to displace lipid from the enzyme. It was thus necessary to test the response of the rat enzyme to high ionic strength, and indeed Figures 5 : 8 and 5 : 9 show that this salt treatment also produced linear Arrhenius plots for low ionic strength soluble, membrane-bound and partially purified acetylcholinesterase. As a result of this an extra lipid extraction step containing a high ammonia concentration (2.0M ammonia) was employed.

FIGURE 4 : 11

Typical Arrhenius plots of soluble and membrane-bound fractions of rat synaptic membrane acetylcholinesterase activity in the presence of 2.0×10^{-4} M tetracaine.

- ● - soluble acetylcholinesterase (μ = 25.9 KJ $mole^{-1}$)

- O - membrane-bound acetylcholinesterase (μ = 23.8 KJ $mole^{-1}$)

Specific activity values expressed as μ moles thiocholine $mg.protein^{-1}h$

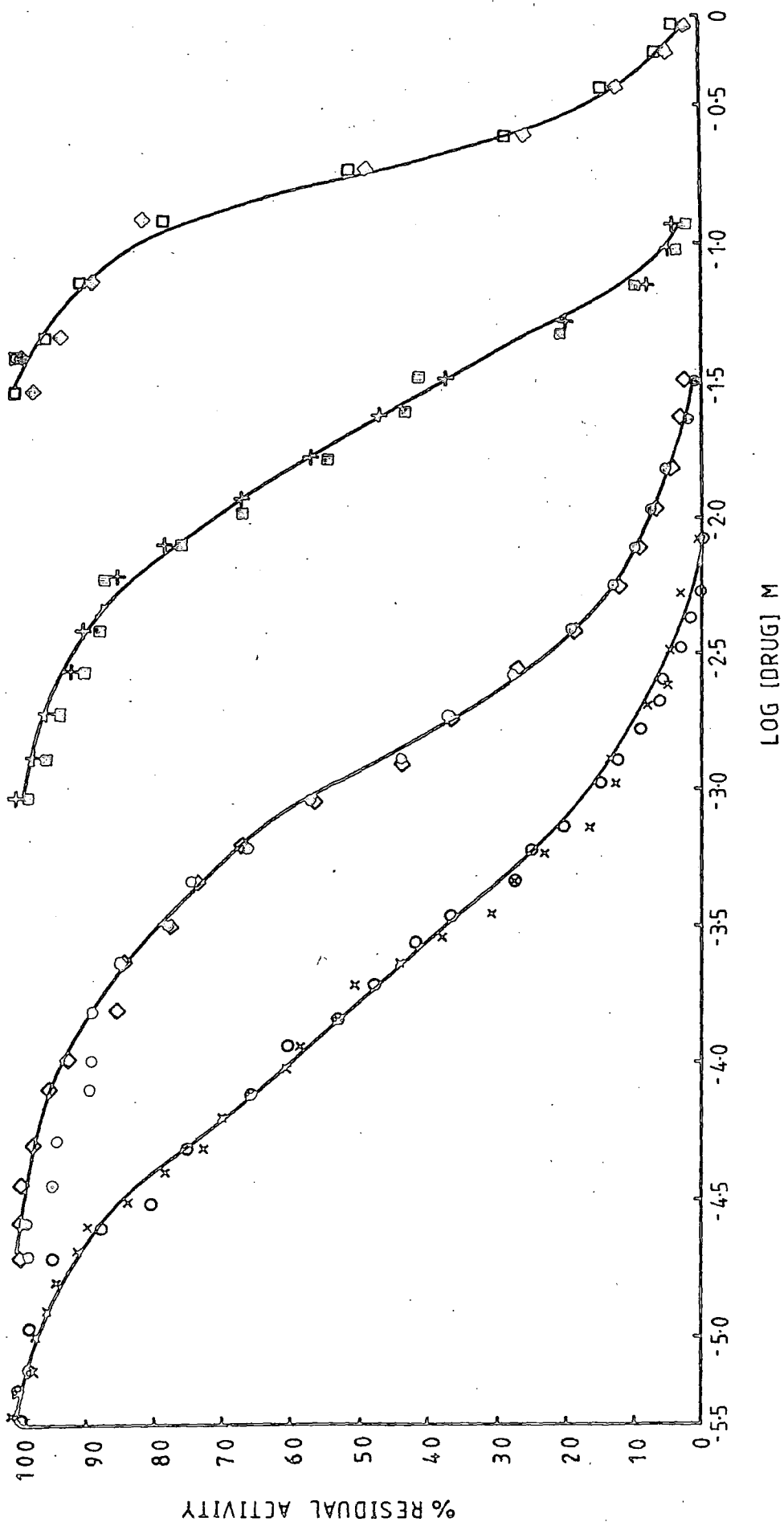

% RESIDUAL ACTIVITY
100
90
80
70
60
50
40
30
20
10
0
LOG [DRUG] M
0
-0·5
-1·0
-1·5
-2·0
-2·5
-3·0
-3·5
-4·0
-4·5
-5·0
-5·5

FIGURE 4 : 10

The inhibition of soluble and membrane-bound acetylcholinesterase fractions by local anaesthetics.

i)	Tetracaine	- O -	soluble acetylcholinesterase
		- X -	membrane-bound acetylcholinesterase
ii)	Procaine	- ◇ -	soluble acetylcholinesterase
		- ● -	membrane-bound acetylcholinesterase
iii)	Mepivicaine	- ✚ -	soluble acetylcholinesterase
		- ■ -	membrane-bound acetylcholinesterase
iv)	Lidocaine	- □ -	soluble acetylcholinesterase
		- ◆ -	membrane-bound acetylcholinesterase

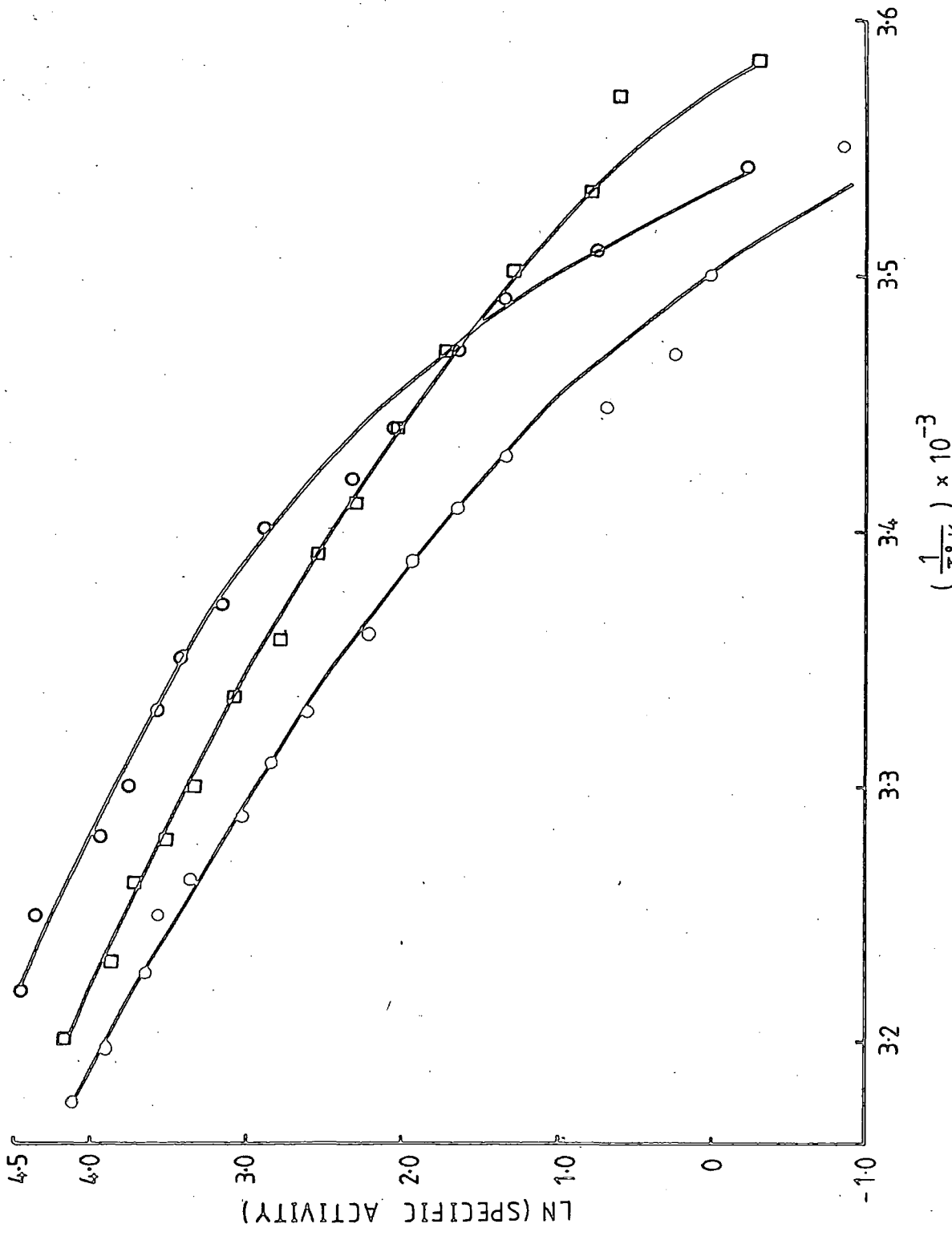

LN (SPECIFIC ACTIVITY)
4·5
4·0
3·0
2·0
1·0
0
-1·0
3·2
3·3
3·4
3·5
3·6
$(\frac{1}{T°K}) \times 10^{-3}$

FIGURE 4 : 9

Typical Arrhenius plots of $Na^+ - K^+$ ATPase assayed in the presence of lipophilic agents.

- O - Control $Na^+ - K^+$ ATPase profile

- □ - $Na^+ - K^+$ ATPase assayed in the presence of 4.0×10^{-3} M tetracaine.

- ● - $Na^+ - K^+$ ATPase assayed in the presence of 1.6×10^{-4} M chlorpromazine

Activity expressed as μ moles Pi mg. $protein^{-1}$ hr^{-1}

Lines fitted by reversible thermal inactivation model.

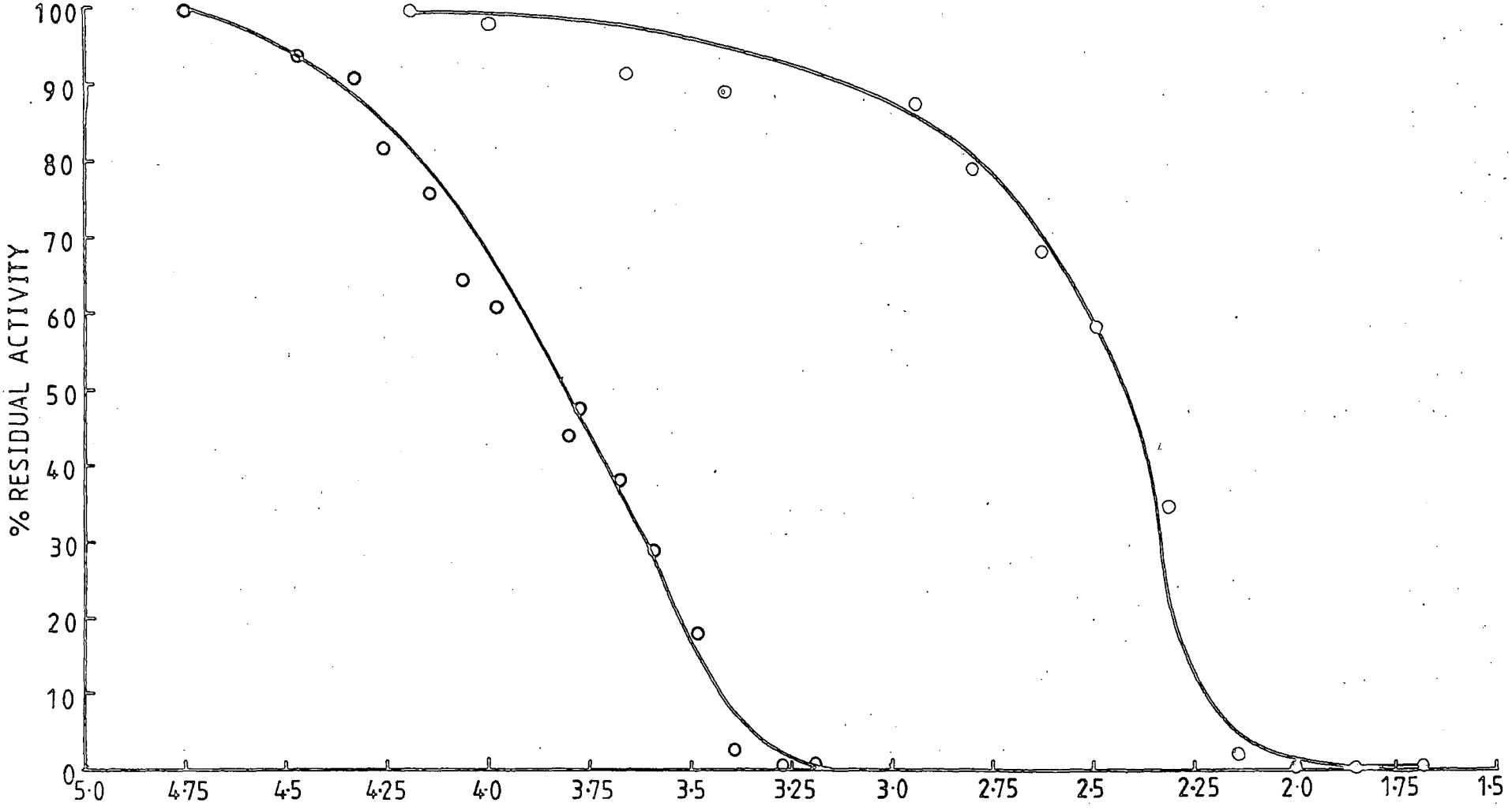

% RESIDUAL ACTIVITY
100
90
80
70
60
50
40
30
20
10
0
5·0
4·75
4·5
4·25
4·0
3·75
3·5
3·25
3·0
2·75
2·5
2·25
2·0
1·75
1·5
- LOG [DRUG] M

FIGURE 4 : 8

The inhibition of rat synaptic membrane $Na^+ - K^+$ ATPase activity with chlorpromazine and tetracaine.

- O - $Na^+ - K^+$ ATPase activity in the presence of chlorpromazine

- O - $Na^+ - K^+$ ATPase activity in the presence of tetracaine.

Specific activity expressed in terms of μ moles Pi. mg. protein^{-1} hr.

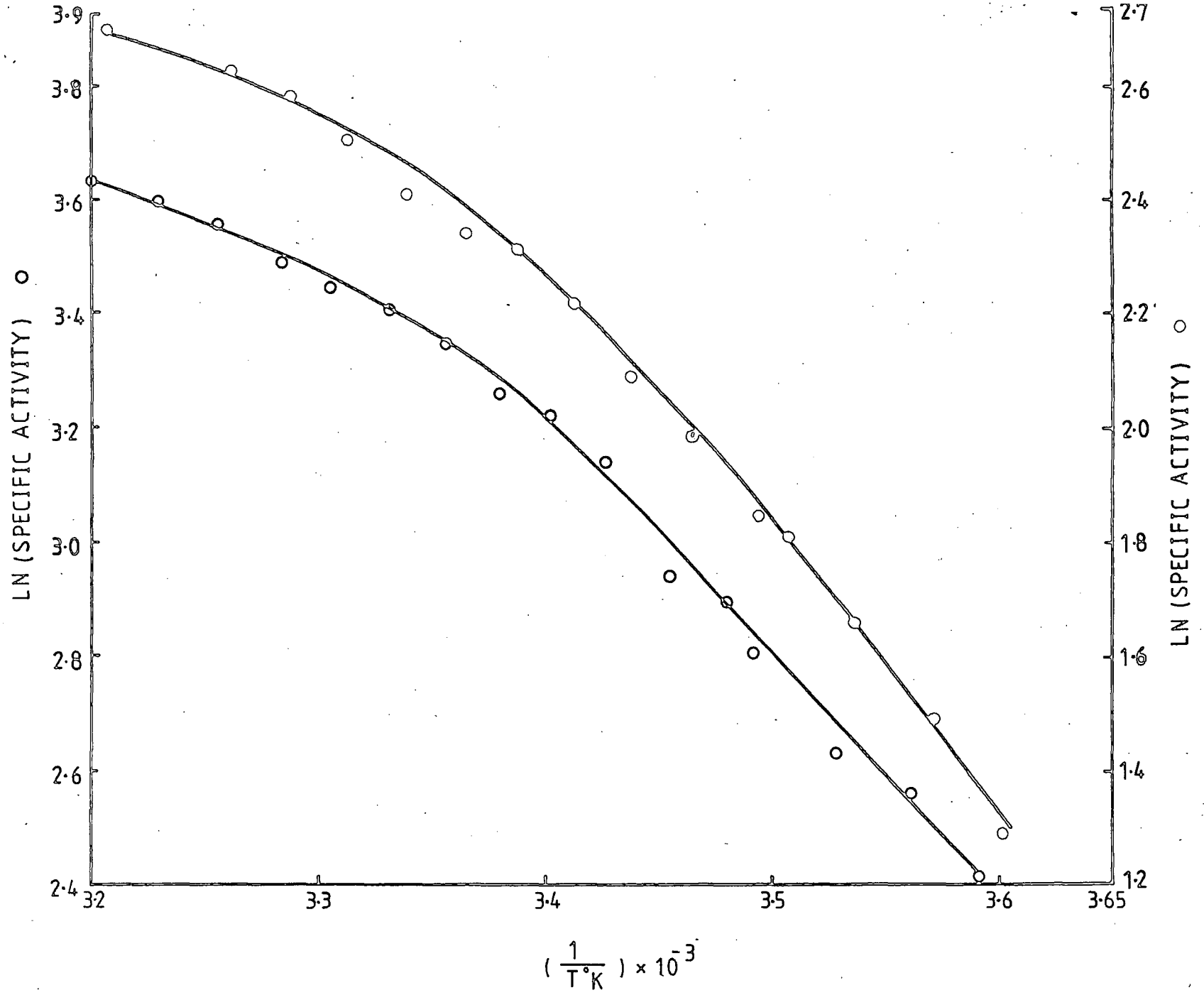

LN (SPECIFIC ACTIVITY) ○
3·9
3·8
3·6
3·4
3·2
3·0
2·8
2·6
2·4
3·2
3·3
3·4
3·5
3·6
3·65
2·7
2·6
2·4
2·2
2·0
1·8
1·6
1·4
1·2
LN (SPECIFIC ACTIVITY) ○
$(\frac{1}{T°K}) \times 10^{-3}$

FIGURE 4 : 7

Typical Arrhenius plots of low ionic-strength soluble and membrane-bound acetylcholinesterase, treated with 5 mg cm^{-3} Lubrol W - X, diluted to 0.5 mg.cm^{-3} before use.

Specific activity expressed as μ moles thiocholine mg. $protein^{-1}$ hr^{-1}

Lines were fitted according to the reversible thermal inactivation model.

i) - ● - membrane-bound acetylcholinesterase

Arrhenius μ at low temperatures = 38.3 KJ $mole^{-1}$

Enthalpy parameter = -86.6 KJ $mole^{-1}$

Entropy parameter = -274 J^{o} K^{-1} $mole^{-1}$

ii) - O - soluble acetylcholinesterase

Arrhenius μ at low temperatures = 34.9 KJ $mole^{-1}$

Enthalpy parameter = -86.5 KJ $mole^{-1}$

Entropy parameter = -272 J^{o} K^{-1} $mole^{-1}$

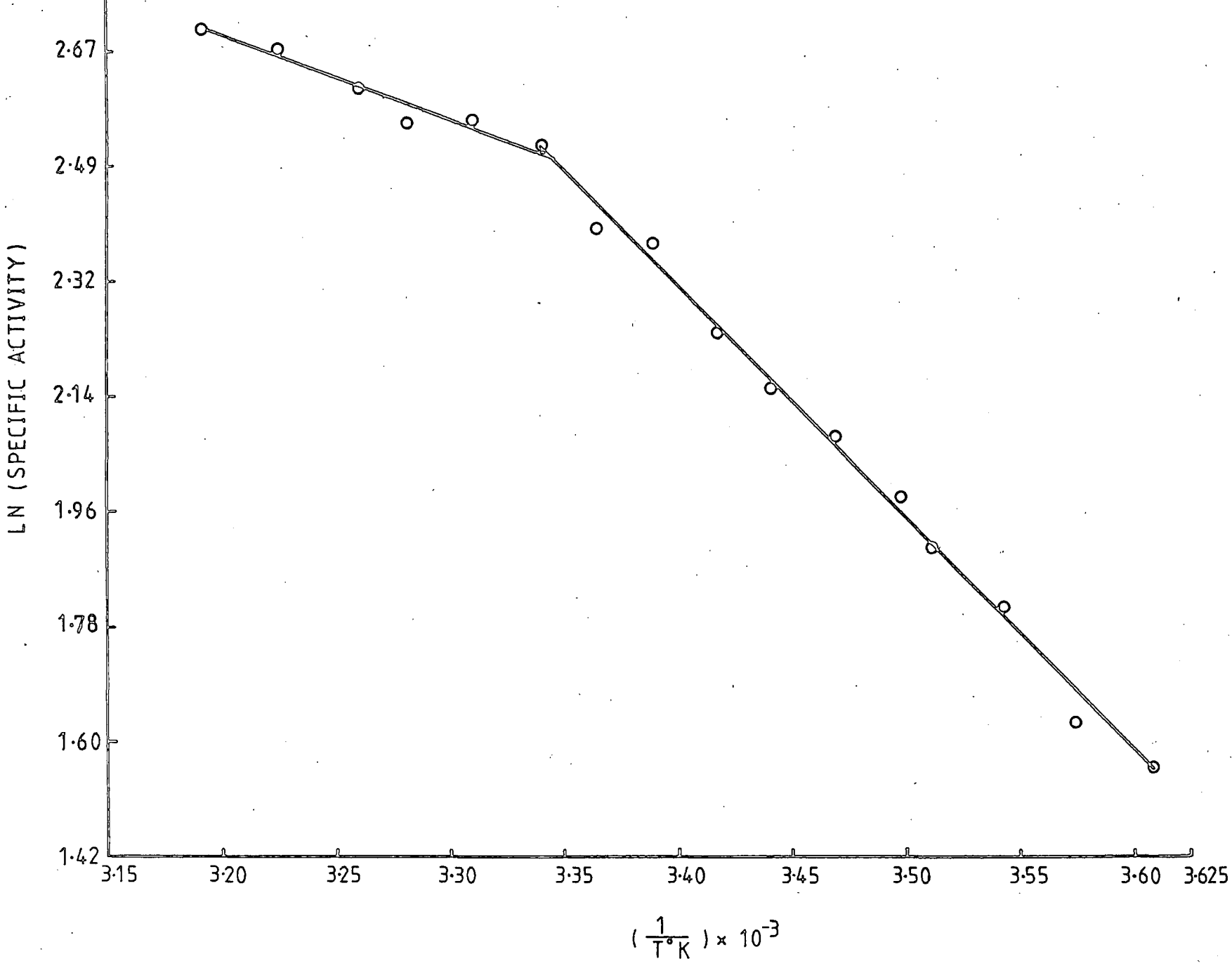

LN (SPECIFIC ACTIVITY)
2·67
2·49
2·32
2·14
1·96
1·78
1·60
1·42
3·15
3·20
3·25
3·30
3·35
3·40
3·45
3·50
3·55
3·60
3·625
(1/T°K) × 10⁻³

FIGURE 4 : 6

Typical Arrhenius plot of the membrane-bound fraction of rat synaptic membrane acetylcholinesterase, treated with 10mM sodium deoxycholate, diluted to 1.0mM sodium deoxycholate before use

Specific activity expressed as μ moles thiocholine mg.protein^{-1}. hr^{-1}.

The lines were fitted to this plot according to the phase change effect.

Arrhenius μ at high temperatures	=	11.4 KJ mole^{-1}
Critical temperatures	=	27.5^{o}C
Arrhenius μ at low temperatures	=	30.2 KJ mole^{-1}

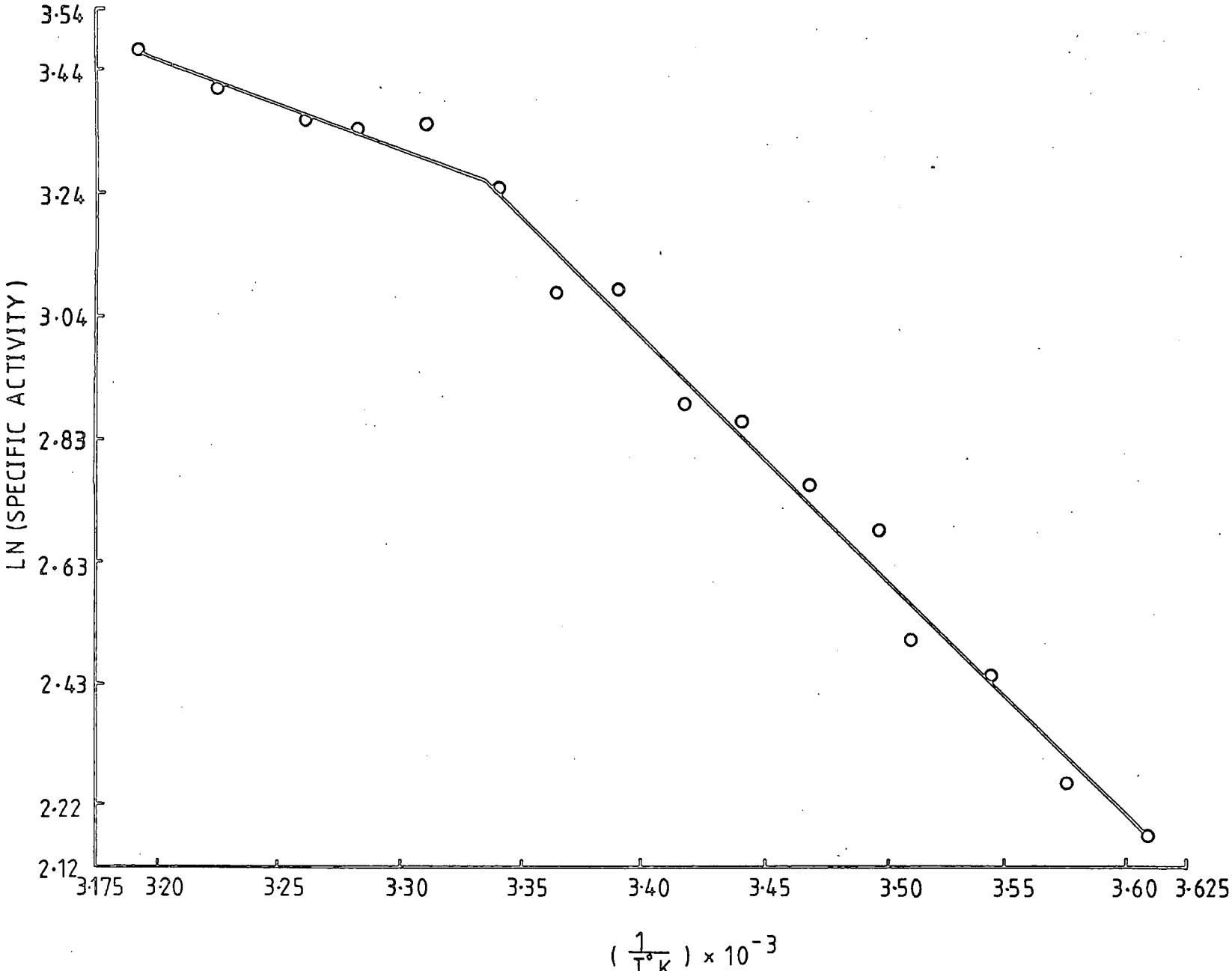

3·54
3·44
3·24
3·04
2·83
2·63
2·43
2·22
2·12
LN (SPECIFIC ACTIVITY)
3·175
3·20
3·25
3·30
3·35
3·40
3·45
3·50
3·55
3·60
3·625
$(\frac{1}{T°K}) \times 10^{-3}$

FIGURE 4 : 5

Typical Arrhenius plot of the low ionic-strength soluble rat synaptic membrane acetylcholinesterase, treated with 10mM sodium deoxycholate, diluted to 1.0mM sodium deoxycholate before use.

Specific activity expressed as μ moles thiocholine mg. protein^{-1} hr^{-1}.

The lines were fitted to this plot according to the phase change effect.

Arrhenius μ at high temperatures	=	12.3 KJ mole^{-1}
Critical temperature	=	30.1^{o}C
Arrhenius μ at low temperatures	=	32.9 KJ mole^{-1}

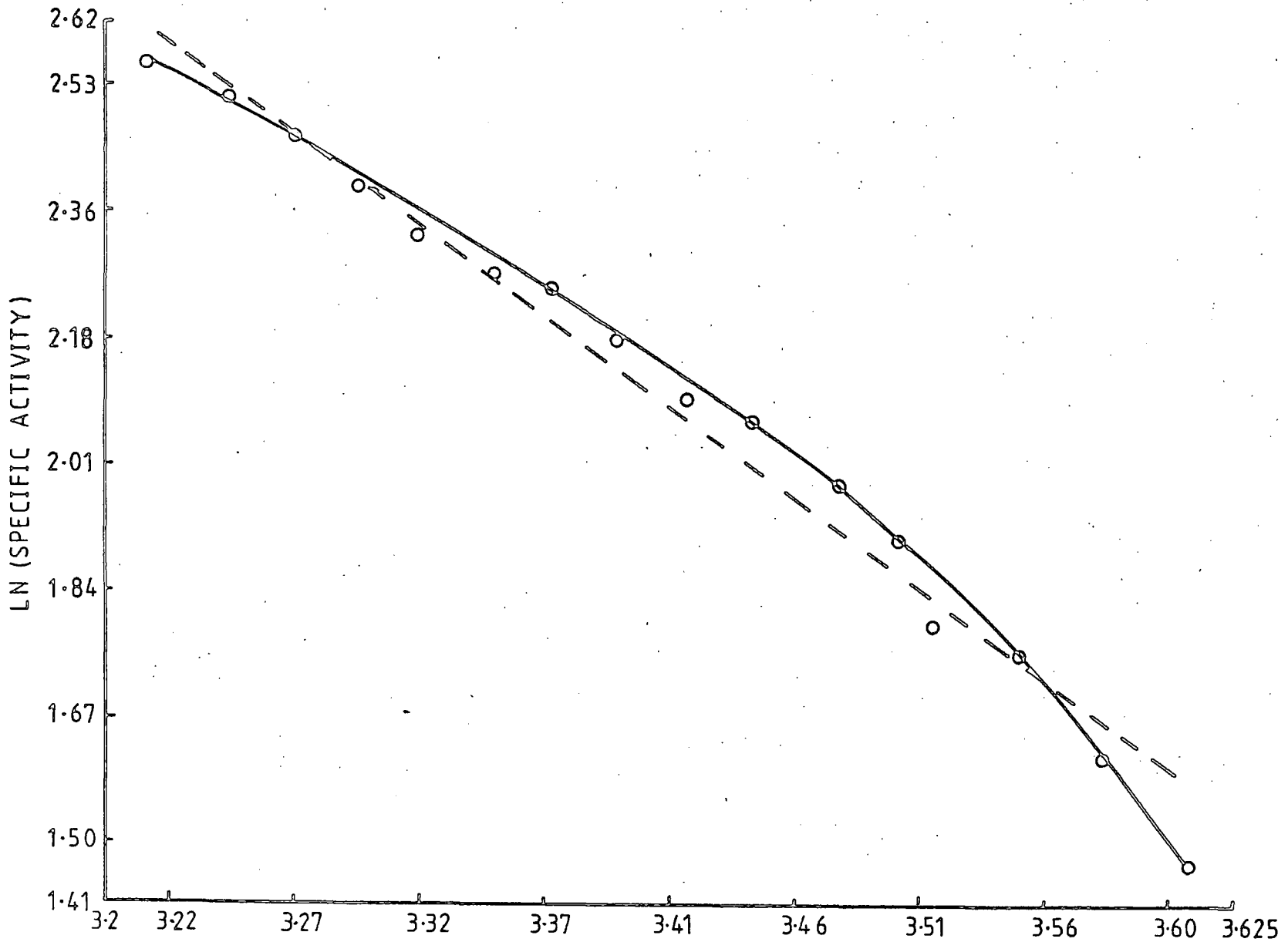

2·62
2·53
2·36
2·18
2·01
1·84
1·67
1·50
1·41
LN (SPECIFIC ACTIVITY)
3·2
3·22
3·27
3·32
3·37
3·41
3·46
3·51
3·56
3·60
3·625
a
b

FIGURE 4 : 4

Typical Arrhenius plot of the membrane-bound fraction of rat synaptic membrane acetylcholinsterase, treated with 1% Triton x - 100, and diluted to give 0.1% Triton x - 100 before use.

Specific activity expressed as μ moles thiocholine mg. protein^{-1} hr^{-1}

This plot gave best fit to linear temperature kinetics according to the reversible thermal inactivation model.

— — — line fitted according to the computer programme (μ = 21.5 KJ mole^{-1})

——————— line fitted by eye

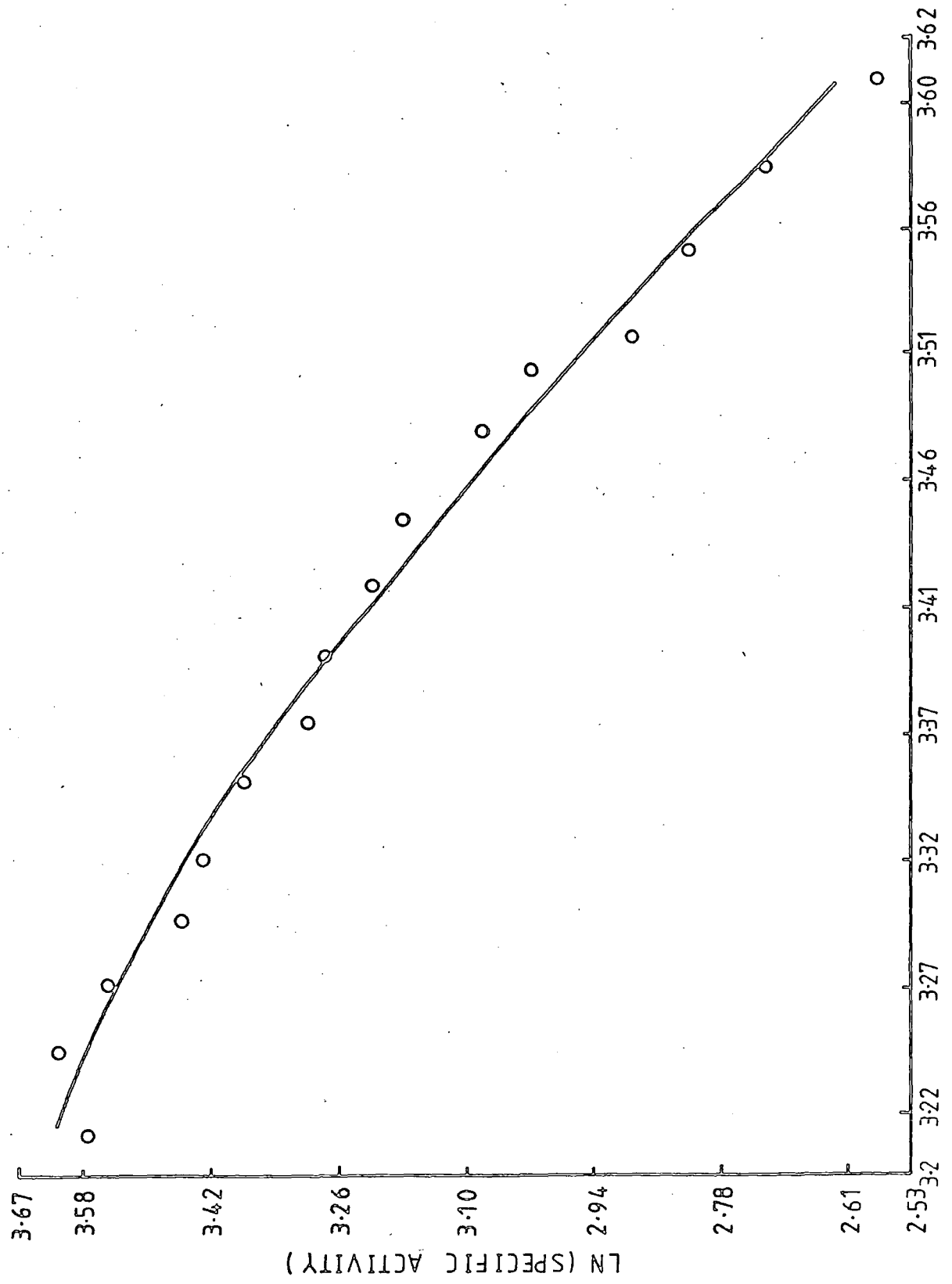

LN (SPECIFIC ACTIVITY)
3·67
3·58
3·42
3·26
3·10
2·94
2·78
2·61
2·53
3·2
3·22
3·27
3·32
3·37
3·41
3·46
3·51
3·56
3·60
3·62
$(\frac{1}{T}) \times 10^{-3}$

FIGURE 4 : 3

Typical Arrhenius plot of the low ionic-strength soluble fraction of rat synaptic membrane acetylcholinesterase, treated with 1% Triton x - 100 diluted to 0.1% Triton x 100 before use.

Specific activity expressed as μ moles thiocholine. mg.protein^{-1}. hr^{-1}

The line was fitted according to the reversible thermal inactivation model.

The parameters calculated for this plot are;

Arrhenius μ at low temperature = 27.4 KJ mole^{-1}

Enthalpy parameter = -62.7 KJ mole^{-1}

Entropy parameter = -194 J^{o} K^{-1} mole^{-1}

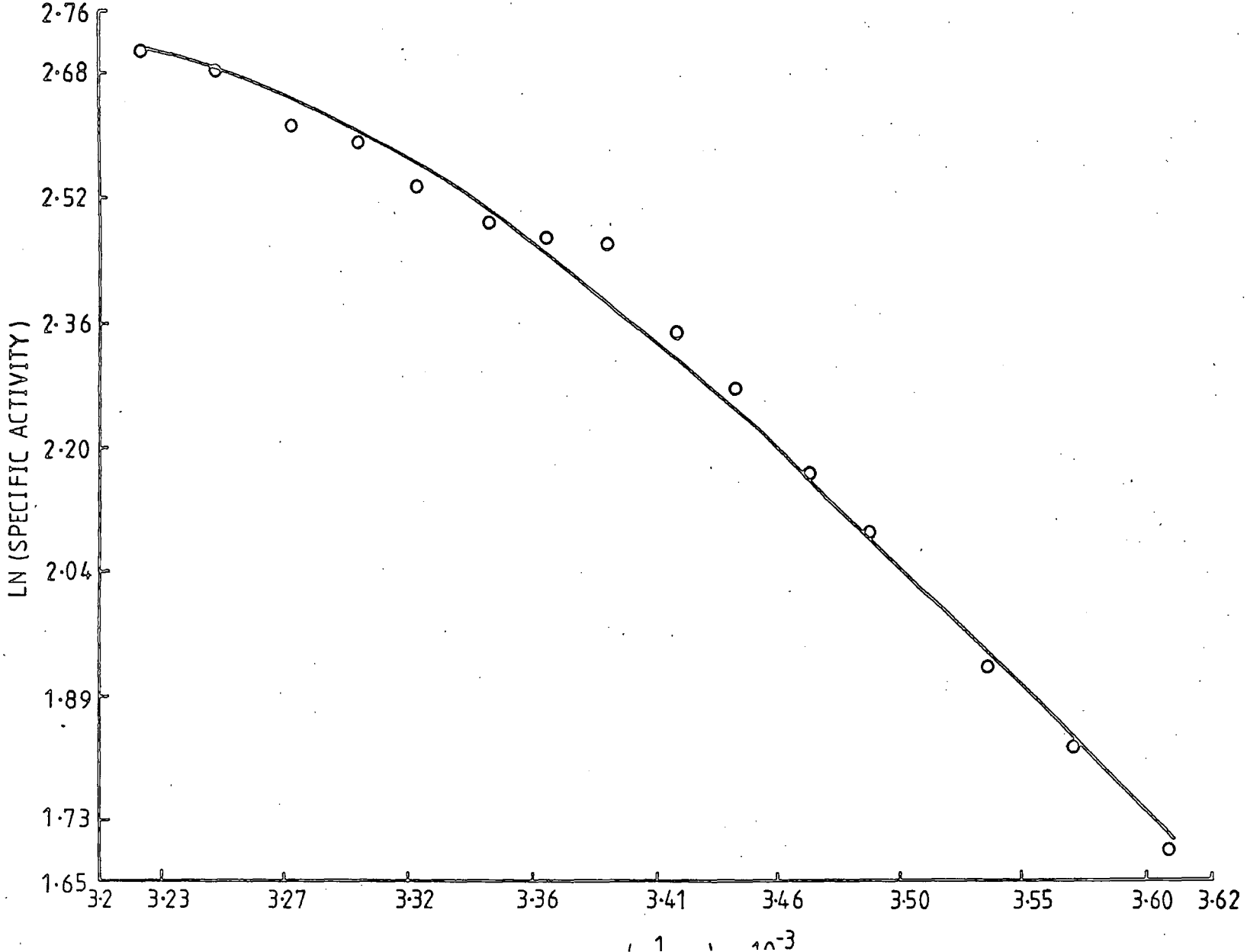

2·76
2·68
2·52
2·36
2·20
2·04
1·89
1·73
1·65
LN (SPECIFIC ACTIVITY)
3·2
3·23
3·27
3·32
3·36
3·41
3·46
3·50
3·55
3·60
3·62
$\left(\frac{1}{T}\right) \times 10^{-3}$

FIGURE 4 : 2

A typical Arrhenius plot of the membrane-bound acetylcholinesterase activity from rat synaptic membrane preparations.

Data was obtained as described in the methods section.

Specific activity units = μ moles thiocholine.mg.protein^{-1}. hr^{-1}

The curve was fitted by a computer assisted scheme, according to the reversible thermal inactivation model.

Arrhenius μ at the low temperature state = 29.6 KJ mole^{-1}

Enthalpy parameter = -75.1 KJ mole^{-1}

Entropy parameter = -236 J^{o} K^{-1} mole^{-1}

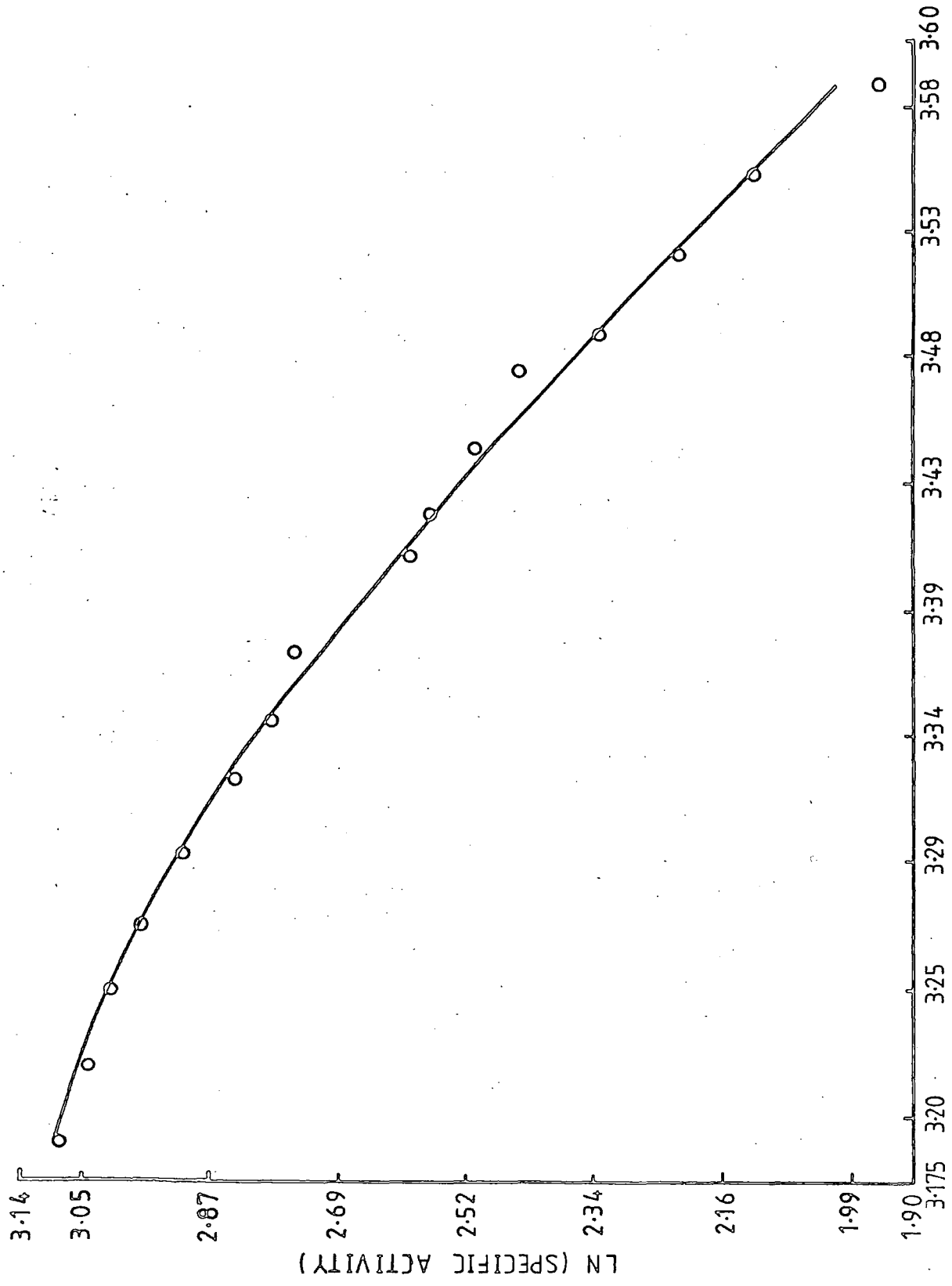

LN (SPECIFIC ACTIVITY)
3·14
3·05
2·87
2·69
2·52
2·34
2·16
1·99
1·90
3·175
3·20
3·25
3·29
3·34
3·39
3·43
3·48
3·53
3·58
3·60
$(\frac{1}{T}) \times 10^{-3}$

FIGURE 4 : 1

A typical Arrhenius plot of soluble acetylcholinesterase activity dissociated from rat synaptic membranes.

Data was obtained as described in the methods section. Specific activity units = μ moles thiocholine.mg.protein^{-1}. hr^{-1}. Curve was fitted by a computer assisted scheme according to the reversible thermal inactivation model.

The parameters calculated for this particular preparation were:

Arrhenius μ of the low temperature state = 30.7 KJ mole^{-1}

Enthalpy parameter = -75.7 KJ mole^{-1}

Entropy parameter = -235 J^{o} k^{-1} mole^{-1}

TABLE 4 : 8

Effect of 1mM n-octanol on the temperature properties of rat synaptic membrane acetylcholinesterase

	Activation energy of the low temp. form of AChEase ($KJ\ mole^{-1}$)	n	Activation energy of the low temp. form of AChEase (+1mM octanol) ($KJ\ mole^{-1}$)	n
Soluble Acetylcholinesterase	32.1 ± 1.3	4	24.8 ± 0.5 P = 0.02	3
Membrane-bound Acetylcholinesterase	33.0 ± 1.6	3	25.0 ± 0.6 P = 0.02	3

Values expressed at $\pm$ 1 x S.E.

TABLE 4 : 7

Effect of 2.0×10^{-4} M tetracaine on the temperature properties of rat synaptic membrane acetylcholinesterase

	Activation energy of the low temp.form of AChEase (KJ $mole^{-1}$)	n	Activation energy of the low temp.form of Tetracaine treated AChEase (KJ $mole^{-1}$)	n
Soluble Acetylcholinesterase	32.1 ± 1.3	4	29.8 ± 1.9	3
Membrane-bound Acetylcholinesterase	33.0 ± 1.6	3	29.1 ± 2.4	3

Values expressed at $\pm$ 1 x S.E.

This also suggests that Lubrol would be suitable for use in the further purification of this enzyme in a state which would preserve the non-ideal Arrhenius kinetics of this enzyme.

The surfactant properties of these detergents being somewhat non-specific, it was of interest to extend this study to the investigation of compounds which associate with phospholipids in a more predictable fashion, namely the phenothiazines, quaternary ammonium-ion aromatic hydrocarbons and the short chain n-alkanols.

It has been shown that the phenothiazines, of which chlorpromazine is one, and quaternary ammonium ion anaesthetics of which tetracaine is one,can inhibit $Na^+ - K^+$ ATPase, but with a potency which correlated with their lipophilicity (Roufogalis, 1975). Figure 4 : 8 shows that chlorpromazine and tetracaine inhibited the Na^+ K^+ ATPase activity of the synaptic membrane preparations, used as a source of acetylcholinesterase, in a similar manner to that reported by Roufogalis, (1975).

It has been shown that these compounds have membrane effects in common in that they bind to membranes in an exothermic reaction (Pauling, 1961; Kwant & Seeman, 1969 (a)), they can protect red blood cells from hypotonic lysis (Roth & Seeman, 1971), they block nervous transmission (Taylor, 1959; Inove & Frank, 1962; Aoki & Yata, 1962; Hille, 1966), they expand biological membranes to a greater extent than can be accounted for in terms of anaesthetic molecular volume (Skov, 1954 (a) and (b); Seeman & Kwant, 1969; Seeman, Kwant & Sauks, 1969; Seeman, Kwant, Sauks & Argent, 1969; Roth & Seeman, 1972; Seeman & Roth, 1972), they displace membrane-bound calcium (Hauser & Dawson, 1968; Kwant & Seeman, 1969 (b); Ohki, 1970), and they disorder the membrane microenvironment (Feinstein, Spero & Falsenfield, 1970). However, Figure 4 : 9 shows that both chlorpromazine and tetracaine changed

the temperature properties of the $Na^+ - K^+$ ATPase. This enzyme was used as it is well characterised with respect to its lipid dependence (Wheeler & Whittam, 1970). The effects of both of these compounds was to significantly reduce the broadness of the transition between active and inactive species and reducing the activation energy of the high temperature active form of the enzyme, when data were processed according to the reversible inactivation model (Table 4 : 5). The phase change model was not so sensitive in detecting changes in the curvature of Arrhenius plots as the only effect of the lipophilic drugs detected in this system was that the apparent activation energy of tetracaine treated enzyme was significantly lower than for control preparations (Table 4 : 6). This emphasises the usefulness of a realistic model for the temperature kinetics of this enzyme.

The effects of chlorpromazine and tetracaine in this case were also consistent with a "fluidising" of membrane lipid around the $Na^+ - K^+$ ATPase, although the magnitude of this effect was greater in the case of tetracaine, as the thermodynamic parameters defining the breadth of the transition were almost halved, as compared with untreated preparations. This, and the fact that tetracaine and other such anaesthetics were inhibitors of acetylcholinesterase, led to further experiments concentrating on the effects of these compounds on acetylcholinesterase.

Figure 4 : 10 shows that, as with the $Na^+ - K^+$ ATPase (Roufogalis, 1977) there was a considerable variation in the potency of these anaesthetics as acetylcholinesterase inhibitors, with tetracaine being the most potent followed by procaine, then mepivicaine, then lidocaine. It is interesting that this order is the same as for the lipophilicity of these compounds as defined by the octanol/water partition co-efficient (Leo, Hansch & Elkins, 1971; Seeman, 1972)

correlation between lipophilicity and inhibition. Thus considering the large literature connecting the lipophilic nature of these compounds with their membrane effects, it is reasonable to suggest that the major factor governing the access of the quaternary ammonium ion portion of the anaesthetic molecule to the active site would be the affinity of each of these compounds for phospholipid molecules. However, as Figure 4 : 10 shows that both soluble and membrane-bound acetylcholinesterase were equally sensitive to each anaesthetic, it is also reasonable to suggest that the lipid molecules which govern the binding of anaesthetic molecules were equally associated with both soluble and membrane fractions of acetylcholinesterase.

Several studies have described in some detail the nature of the association of local anaesthetics with membranes. This work has centred on the use of various magnetic resonance techniques and pure phospholipid liposomes to measure the motional environment of the anaesthetic carbon atoms, and thus to infer the polarity of that environment. It was found that all of the compounds used in this study would preferentially associate with liposomes of acidic lipids, such as phosphatidyl serine, but only tetracaine would associate with liposomes of zwitterionic lipids like phosphatidylcholine (Cerbon, 1972). Thus the majority of local anaesthetics bound to membranes primarily by electrostatic attraction, but tetracaine was somewhat unusual in its ability to bind to lipids purely by hydrophobic attraction. This was shown to be due to the butylene carbon atoms located in the para position to the quaternary ammonium ion group on the benzene ring. This hydrophobic tail could penetrate the bilayer up to the 7th or 8th carbon atom of the fatty acyl chains, whereas the benzene ring was level with the glycerol region of the bilayer.

The amino group interacted with the phosphate groups (Hauser, Penkett & Chapman, 1969; Cerbon, 1972; Fernandez & Cerbon, 1973; Yeagle, Hutton & Martin, 1977; Smith, 1978).

In view of this specific association with a range of phospholipid molecules and the membrane fluidizing properties of tetracaine (Feinstein, Spero & Falsenfield, 1970) it was of interest to investigate its effect on the temperature kinetics of acetylcholinesterase.

As can be seen from Figure 4 : 11, 2.0×10^{-4}M tetracaine abolished the non-linearity of the temperature plot of both soluble and membrane-bound acetylcholinesterase, resulting in a completely linear plot. As with the effect of detergents, this suggests that phospholipid molecules are associated with both soluble and membrane acetylcholinesterase and that they are responsible for the observed non-linear temperature plots. Also data presented in Table 4 : 7 shows that not only was the curvature abolished but also the apparent activation energy, calculated for the tetracaine-treated enzyme was lower than that calculated for the low temperature form of the enzyme. This suggests that the apparent activation energy of the low temperature form of the enzyme may also be influenced by the fluidity of the membrane microenvironment, as well as the degree of curvature of the Arrhenius plot.

A similar effect was produced by including 1mM n octanol in assay media for the Arrhenius plot measurements. Figure 4 : 12 shows that both soluble and membrane-bound acetylcholinesterase assayed in the presence of 1mM octanol gave linear Arrhenius plots. The apparent activation energy calculated from these plots was, as was the case with tetracaine, lower in each case

than that calculated for the low temperature, stable state for control preparations (Table 4 : 8). This confirms the conclusions drawn for the effect of tetracaine, namely that the fluidity of the fatty acyl chains of phospholipids associated with both soluble and membrane-bound acetylcholinesterase controls the curvature of these plots and the apparent activation energy of the "ground state" of the enzyme.

In conclusion, this work has shown that acetylcholinesterase of rat synaptic membranes exists in two forms, of differing degrees of membrane association. Both of these forms display the non-ideal Arrhenius plots characteristic of the native membrane preparation. The temperature properties of both forms of this enzyme can be modified by membrane lipid perturbing agents to a similar extent. Although none of the experimental results described in this chapter constitutes direct proof that the temperature properties of this enzyme are dependent on the physical state of phospholipid, they are consistent in that they strongly suggest this to be the most likely explanation.

TABLE 4 : 1

The effect of ionic strength, pH and calcium ion on the membrane association of rat synaptic membrane acetylcholinesterase

Composition of Incubation Medium	Specific Activity of AChEase at day 1 +	Specific Activity of AChEase at day 3 +	Specific Activity of Soluble AChEase (day 3) +	Specific Activity of Membrane Bound AChEase (day 3) +	Total Acitivity of the Soluble Fraction *	Total Activity of the Membrane Fraction *
10mM Phosphate buffer pH 7.4	26.3	25.8	43.6	17.7	65.3	52.8
10mM Phosphate buffer pH 5.8	25.3	25.1	30.7	24.0	17.4	69.5
10mM Phosphate buffer pH 7.4 +3mM CaCl	24.8	23.6	13.2	26.6	4.8	61.6
10mM Phosphate buffer pH 7.4 +1.2M NaCl	24.4	25.1	36.9	24.4	17.2	79.2
20mM Imidazole buffer pH 7.4	24.9	24.8	37.6	14.0	55.9	59.8
20mM Imidazole buffer pH 7.4 1mM EDTA	25.7	26.1	39.0	13.0	49.2	54.4

\+ Specific activity units = μ moles thiocholine/mg protein/hr.

* Total activity units = μ moles thiocholine/3cm^3 sample/hr.

TABLE 4 : 2

Thermodynamic parameters calculated from Arrhenius plots of native synaptic membrane acetylcholinesterase and soluble and membrane-associated acetylcholinesterase activity.

These plots were processed according to the reversible inactivation model.

	Activation Energy of the low temp. form of AChEase (KJ $mole^{-1}$)	Enthalpy Parameter (KJ $mole^{-1}$)	Entropy Parameter (J^{o} K^{-1} $mole^{-1}$	n
Native synaptic membrane AChEase	38.8 ± 9.0	-84.6 ± 1.7	-266.0 ± 5.9	4
Membrane-bound AChEase	33.0 ± 2.0	-78.8 ± 1.9	-246.3 ± 5.4	3
Soluble AChEase	32.1 ± 1.5	-86.7 ± 4.8	-272.5 ± 16.0	4

TABLE 4 : 3

Effect of detergent treatment on the temperature properties of soluble and membrane-bound acetylcholinesterase fractions

Data were processed according to the reversible thermal inactivation model.

* P = 0.01, ** P = 0.05

	Activation Energy of the low temp.state (KJ $mole^{-1}$)	Enthalpy Parameter ΔH (KJ $mole^{-1}$)	Entropy Parameter ΔS ($J^{o}K^{-1}mole^{-1}$)	n
Triton-treated soluble enzyme	27.3 ± 0.4 **	-61.1 ± 1.0 *	-191.7 ± 1.9 *	3
Triton-treated membrane-bound enzyme	22.5 ± 0.6 *			3
Deoxycholate-treated soluble enzyme	35.7 32.7	-72.1 -80.7	-226 -254	2
Deoxycholate-treated membrane-bound enzyme	31.9 34.9	-83.0 -79.3	-261 -236	2
Lubrol-treated soluble enzyme	36.6 ± 1.0	-84.6 ± 2.0	-265 ± 8	3
Lubrol-treated Membrane-bound enzyme	36.1 ± 1.7	-83.2 ± 2.4	-262 ± 8	3
Control soluble-enzyme	32.1 ± 1.3	-86.7 ± 5.0	-273 ± 16	4
Control membrane-bound enzyme	33.0 ± 1.6	-78.8 ± 1.9	-246 ± 5	3

TABLE 4 : 4

Effect of detergent treatment on the temperature properties of soluble and membrane-bound acetylcholinesterase fractions

Data were processed according to the phase change model.

	Activation Energy of the high temp.range (KJ mole^{-1})	Critical Temperature (°C)	Activation Energy of the low temp.range (KJ mole^{-1})	n
Triton-treated soluble enzyme	17.6 ± 0.4 **	13.4 ± 0.5 **	26.9 ± 0.8 **	3
Triton-treated membrane-bound enzyme	17.4 ± 0.6 n.s.	13.3 ± 0.8 *	28.2 ± 0.4 *	3
Deoxycholate-treated soluble enzyme	12.3 11.7	31.4 28.8	32.9 31.3	2
Deoxycholate-treated membrane-bound enzyme	11.4 13.4	28.8 30.7	30.2 32.0	2
Lubrol-treated soluble enzyme	18.5 ± 1.7	20.5 ± 2.7	35.8 ± 1.6	3
Lubrol-treated membrane-bound enzyme	17.9 ± 1.8	22.5 ± 0.4	35.7 ± 2.5	3
Control soluble-enzyme	13.9 ± 1.0	22.7 ± 1.2	31.0 ± 1.1	4
Control membrane-bound enzyme	17.2 ± 2.1	20.4 ± 2.0	35.1 ± 2.3	3

*P = 0.05, ** P = 0.02

TABLE 4 : 5

Effect of lipophilic agents on the temperature kinetics of the rat synaptic membrane $Na^+ - K^+$ ATPase

Data processed according to the reversible thermal inactivation model.

	Activation energy of the high temp.state (KJ $mole^{-1}$)	Enthalpy Parameter (KJ $mole^{-1}$)	Entropy Parameter ($J^oK^{-1}mole^{-1}$)	n
Control $Na^+ - K^+$ATPase	67.3 ± 1.5	-196.0 ± 14.0	-683.2 ± 50.3	5
$Na^+ - K^+$ATPase + 1.6×10^{-4}M Chlorpromazine	53.3 ± 3.5 P = 0.02	-120.4 ± 8.2 P = 0.01	-404.8 ± 27.4 P = 0.01	4
$Na^+ - K^+$ATPase + 4.0×10^{-3}M Tetracaine	45.2 ± 9.2 P = 0.05	-96.7 ± 6.8 P = 0.01	-324.7 ± 26.0 P = 0.01	3

Values expressed as $\pm$ 1 x SE

TABLE 4 : 6

Effect of lipophilic agents on the temperature kinetics of the rat synaptic membrane $Na^+ - K^+$ ATPase

Data processed according to the phase change model.

	Activation Energy of the high temp.range (KJ $mole^{-1}$)	Critical Temperature (^oC)	Activation Energy of the low temp.range (KJ $mole^{-1}$)	n
Control $Na^+ - K^+$ ATPase	72.2 ± 1.1	18.8 ± 1.0	172.9 ± 7.6	5
$Na^+ - K^+$ ATPase $+1.6 \times 10^{-4}$M Chlorpromazine	73.1 ± 2.8 N.S.	22.8 ± 2.5 N.S.	152.3 ± 7.0 N.S.	4
$Na^+ - K^+$ ATPase $+4.0 \times 10^{-3}$M Tetracaine	68.5 ± 1.0 N.S.	20.9 ± 1.4 N.S.	115.9 ± 8.8 P = 0.01	3

Values expressed as $\pm$ 1 x S.E.

FIGURE 3 : 8

Typical Arrhenius plot of rat synaptic membrane $Na^+ - K^+$ ATP ase fitted to the phase change effect

i) Specific activity expressed as μ moles Pi/ mg. protein/hr.

ii) Parameters obtained for this plot.

Arrhenius μ at high temp range = 71.9 KJ $mole^{-1}$

Arrhenius μ at low temp range = 148.7 KJ $mole^{-1}$

Critical temperature = 22.6°C

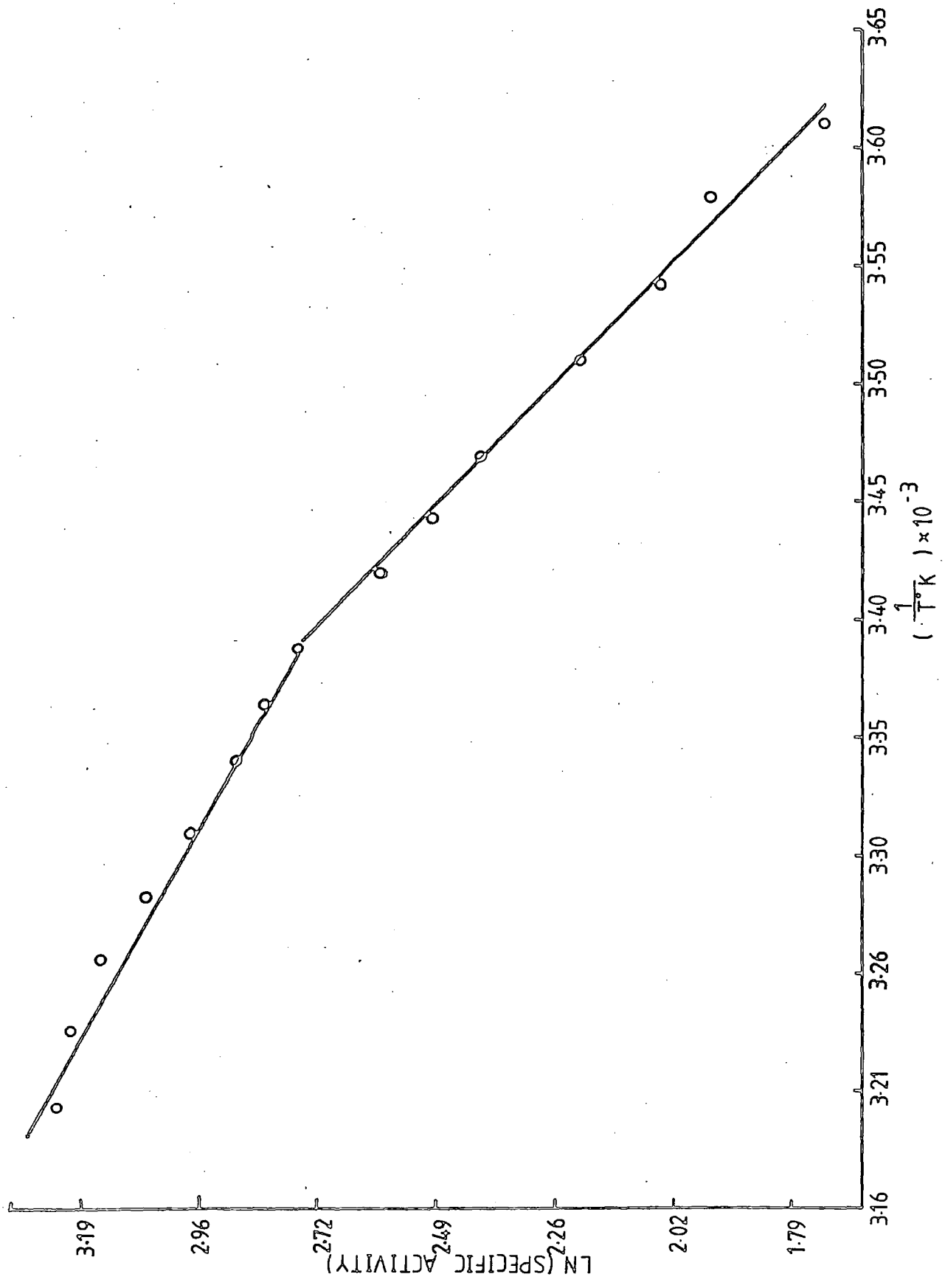

LN (SPECIFIC ACTIVITY)
3·19
2·96
2·72
2·49
2·26
2·02
1·79
3·16
3·21
3·26
3·30
3·35
3·40
3·45
3·50
3·55
3·60
3·65
$\left(\frac{1}{T°K}\right) \times 10^{-3}$

FIGURE 3 : 7

Typical Arrhenius plot of rat synaptic membrane acetylcholinesterase fitted to the phase change effect

i) specific activity expressed as μ moles thiocholine/ mg. protein/hr.

ii) Parameters obtained from this plot

Arrhenius μ at high temp. range	=	16.8 KJ $mole^{-1}$
Arrhenius μ at low temp. range	=	39.1 KJ $mole^{-1}$
Critical temperature	=	21.8°C

SPECIFIC ACTIVITY
0
10
20
30
40
50
60
70
80
90
6·4
6·6
6·8
7·0
7·2
7·4
7·6
7·8
8·0
8·2
pH

FIGURE 3 : 6

pH profile of $Na^+ - K^+$ ATPase activity from rat synaptic membranes.

i) specific activities expressed as μ moles Pi/ mg. protein/hr.

ii) Buffers prepared as described in methods

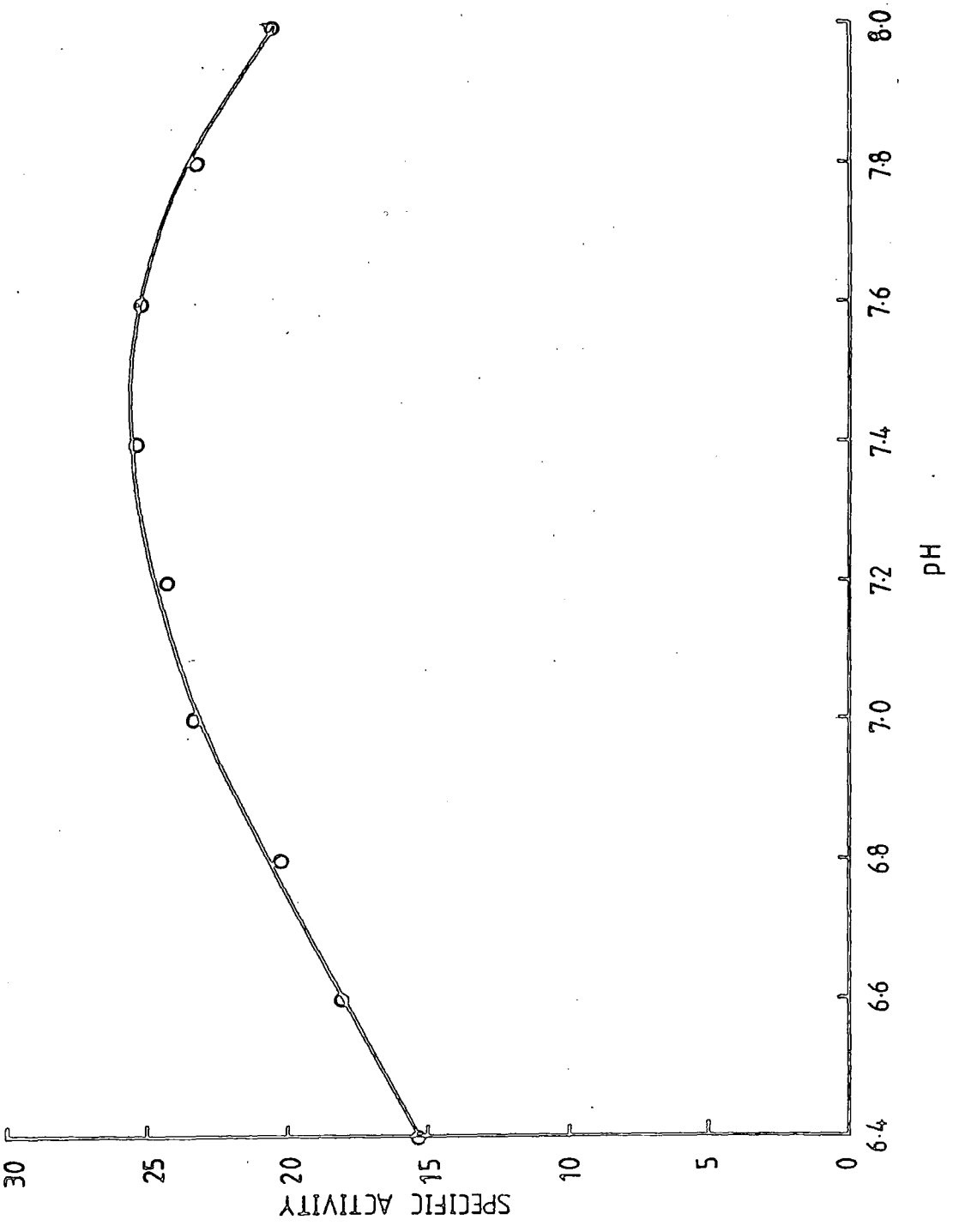

SPECIFIC ACTIVITY
30
25
20
15
10
5
0
6·4
6·6
6·8
7·0
7·2
7·4
7·6
7·8
8·0
pH

FIGURE 3 : 5

pH profile of rat synaptic membrane acetylcholinesterase

i) - O - specific activity expressed as μ moles thiocholine/mg protein/hr.

ii) Buffers prepared as described in methods section

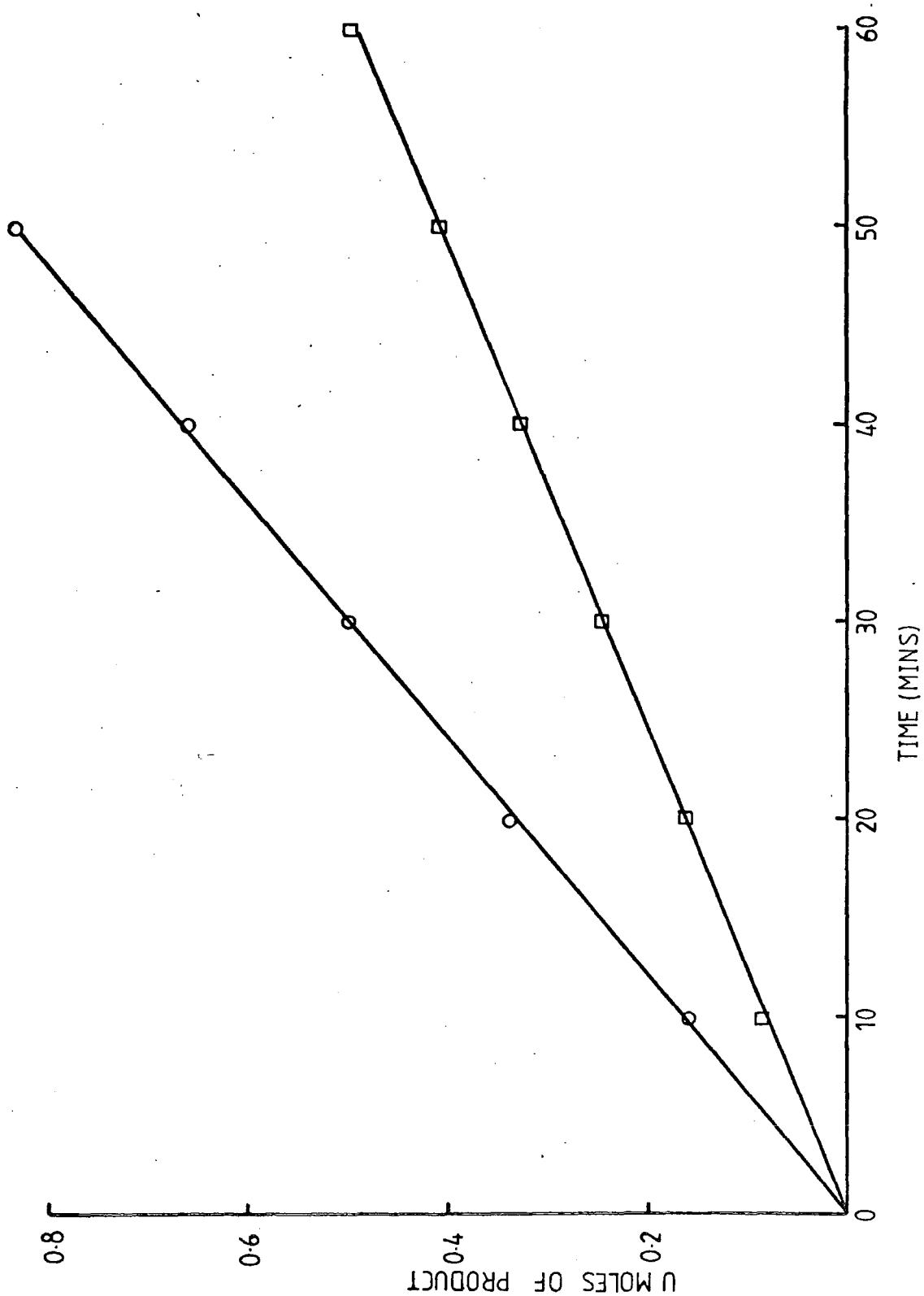

0·8
0·6
0·4
0·2
U MOLES OF PRODUCT
0
10
20
30
40
50
60
TIME (MINS)

FIGURE 3 : 14

Typical Arrhenius plot of guinea-pig acetylcholinesterase from synaptic membranes

i) -O- specific activity expressed as μ moles thiocholine/mg. protein/hr

ii) activation energy of low temperature form of the enzyme = 41.4 KJ $mole^{-1}$

enthalpy parameter Δ H = -99.4 KJ $mole^{-1}$

entropy parameter Δ S = -319 $J^{o} K^{-1}$ mole

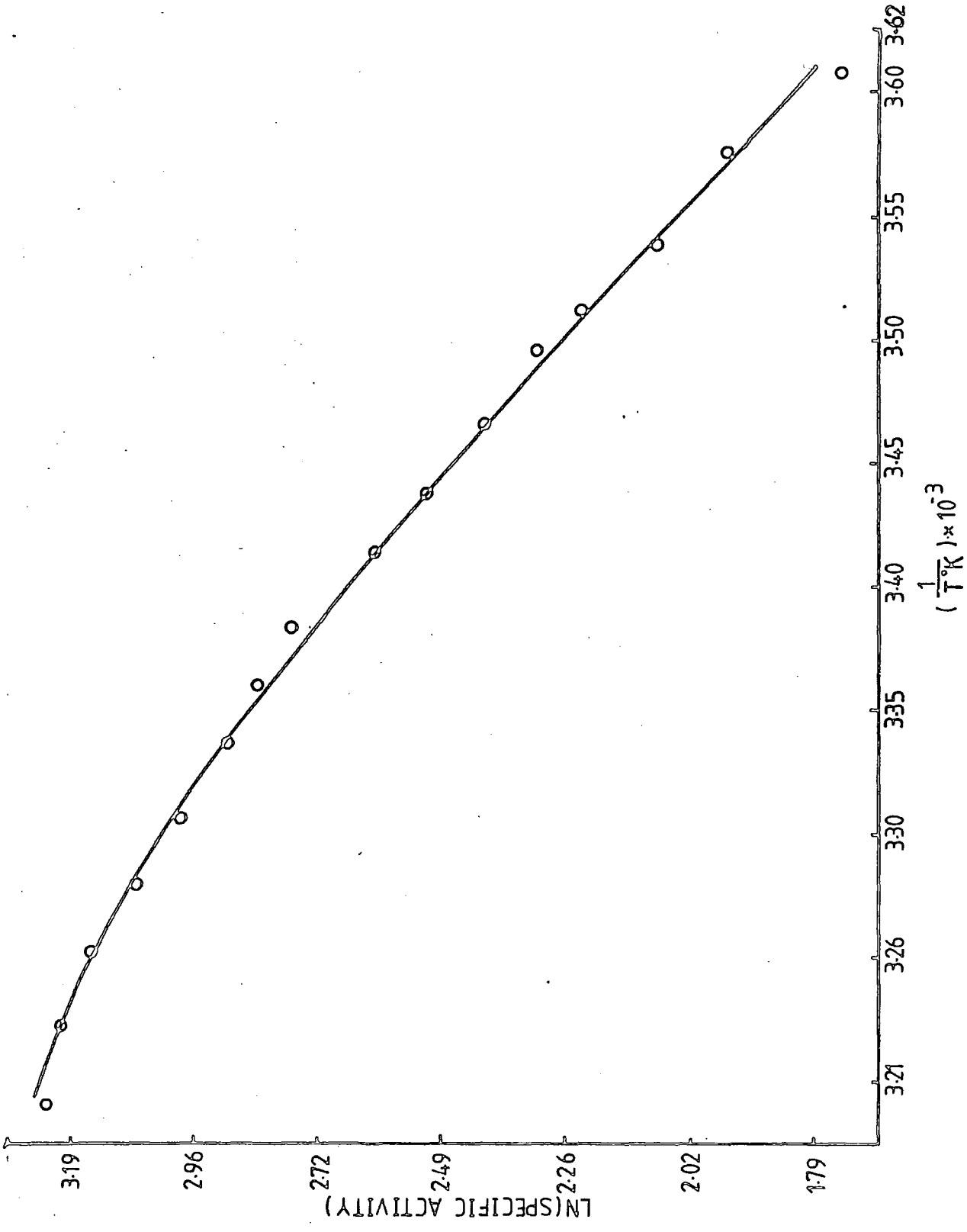

LN(SPECIFIC ACTIVITY)
3·19
2·96
2·72
2·49
2·26
2·02
1·79
3·21
3·26
3·30
3·35
3·40
3·45
3·50
3·55
3·60
3·62
$(\frac{1}{T°K})\times 10^{-3}$

FIGURE 3 : 13

Typical Arrhenius plot of acetylcholinesterase activity processed according to the reversible thermal inactivation model

i) -O- = specific activity expressed as μ moles thiocholine/mg.protein/hr

ii) parameters calculated for this preparation

Activation energy of low temperature state		= 37.6 KJ $mole^{-1}$
enthaply parameter	Δ H	=-81.0 KJ $mole^{-1}$
entropy parameter	Δ S	=-253 J^{o} K^{-1} $mole^{-1}$

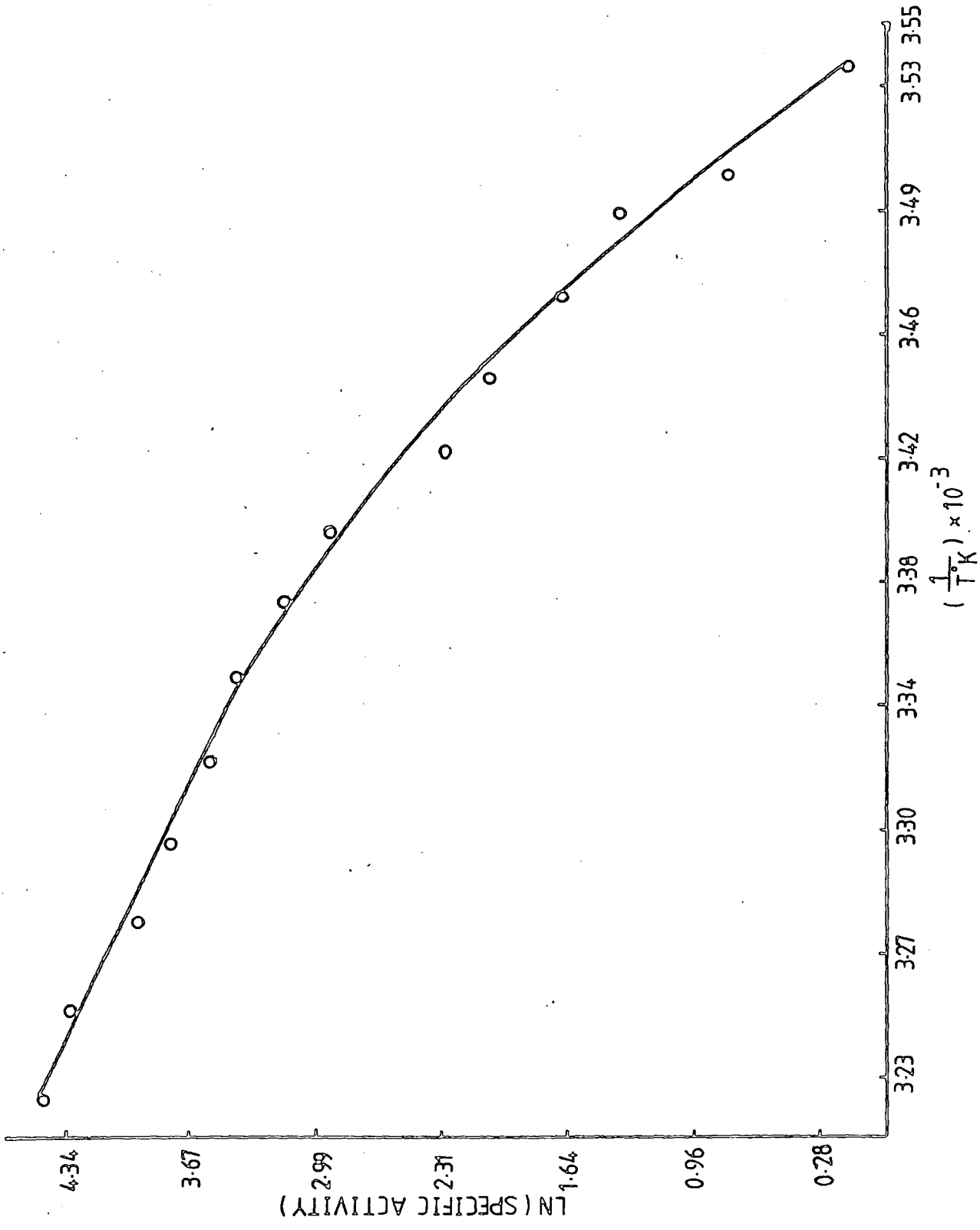

LN (SPECIFIC ACTIVITY)
4·34
3·67
2·99
2·31
1·64
0·96
0·28
3·23
3·27
3·30
3·34
3·38
3·42
3·46
3·49
3·53
3·55
$(\frac{1}{T°K}) \times 10^{-3}$

FIGURE 3 : 12

Typical Arrhenius plot of $Na^+ - K^+$ ATPase processed according to the reversible thermal inactivation model

i) -O- specific activity expressed as μ moles Pi/mg.protein/hr.

ii) Parameters calculated for this preparation

Activation energy of high temperature state = 63.9 KJ $mole^{-1}$

Enthaply parameter Δ H = -159.5 KJ $mole^{-1}$

Entropy parameter Δ J = -550 J^{o} K^{-1} $mole^{-1}$

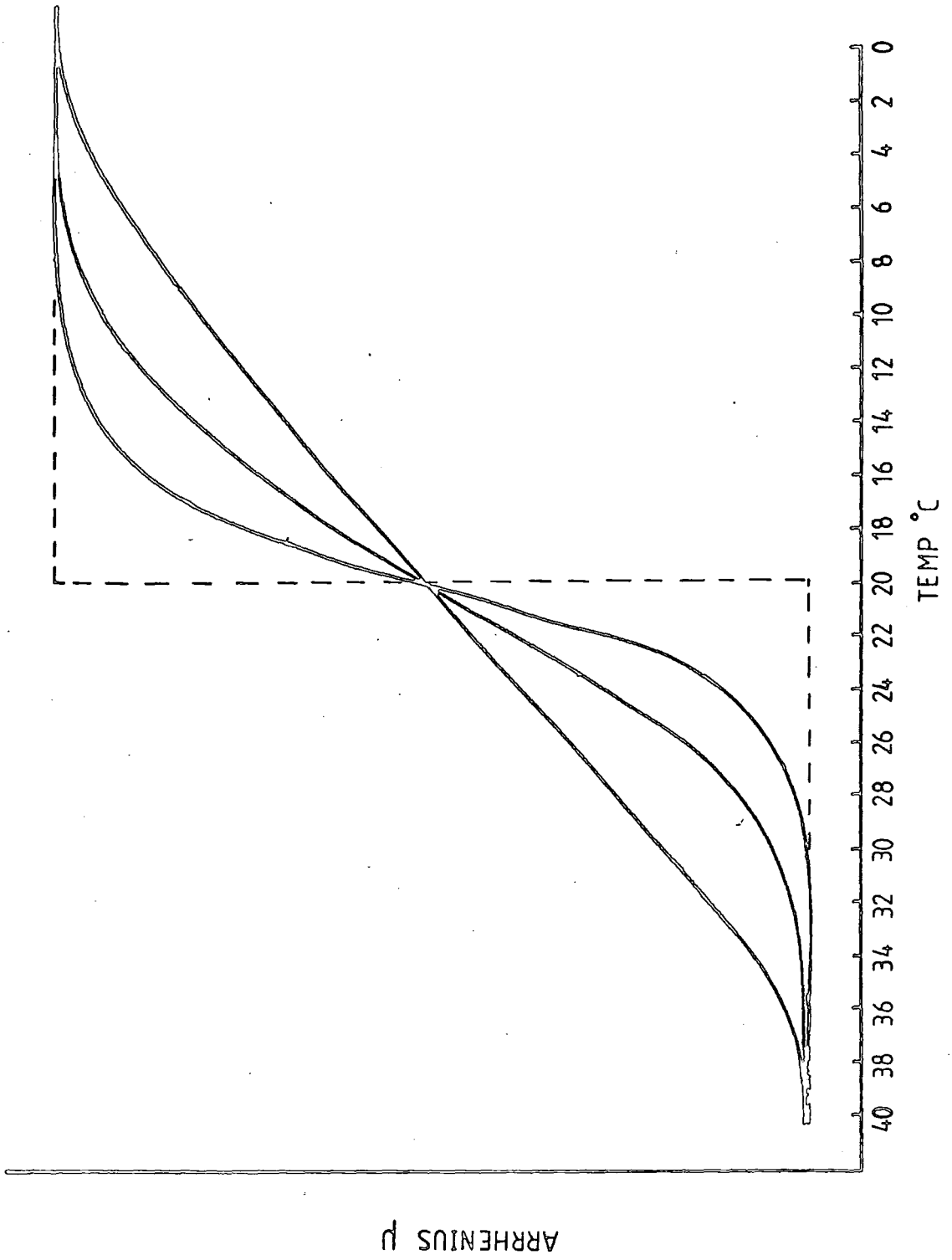

ARRHENIUS μ
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34
36
38
40
TEMP °C

FIGURE 3 : 11

Temperature dependence of Arrhenius μ for Arrhenius plots predicted by the phase change effect or for a thermal equilibrium between active species, extracted from Han (1972).

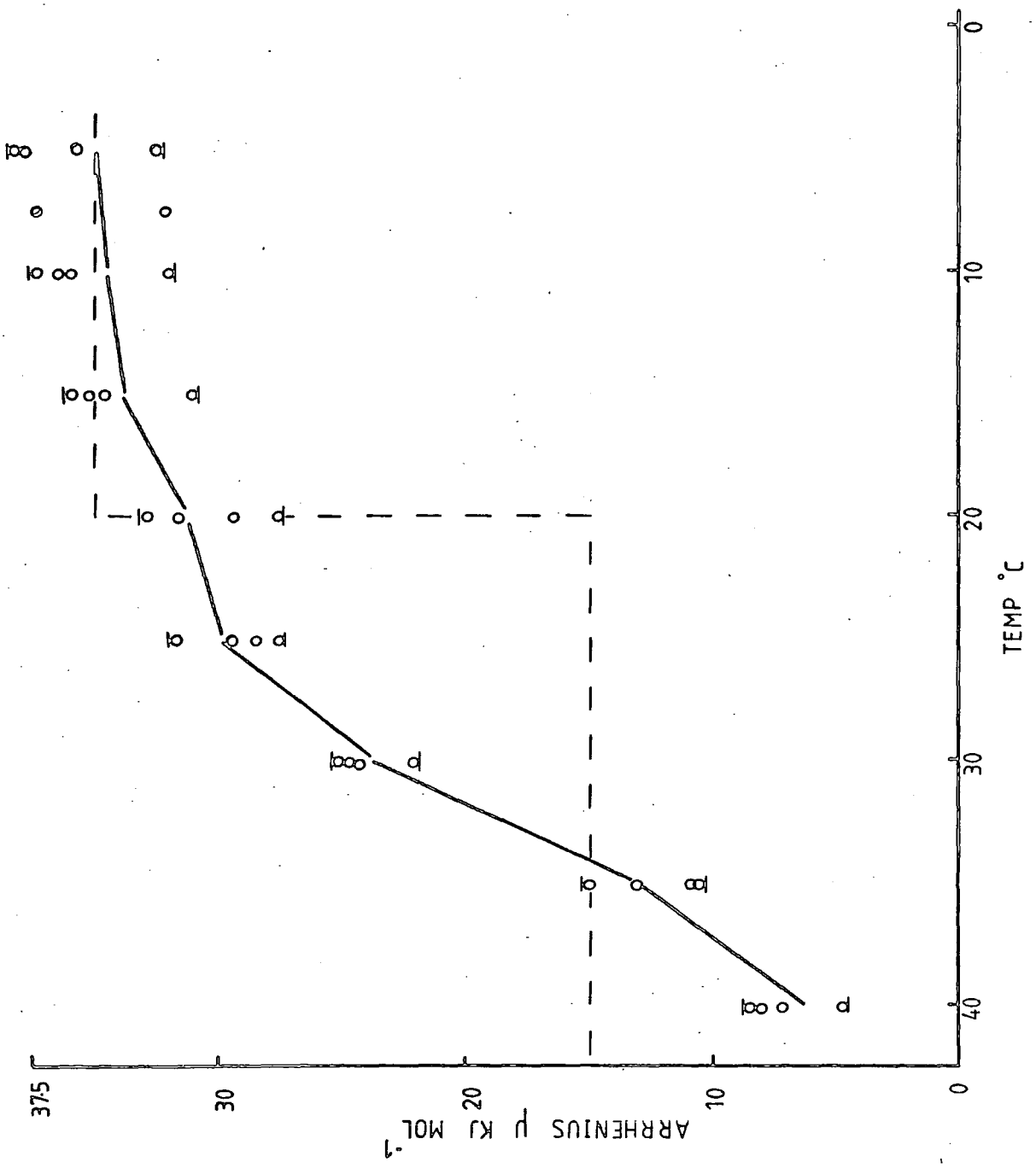

ARRHENIUS μ KJ MOL⁻¹
37·5
30
20
10
0
0
10
20
30
40
TEMP °C

FIGURE 3 : 10

Change in Arrhenius μ with temperature for Arrhenius plots of rat synaptic membrane acetylcholinesterase

- o - = observed values of μ (KJ $mole^{-1}$)

— — — — = change in μ predicted by phase change effect

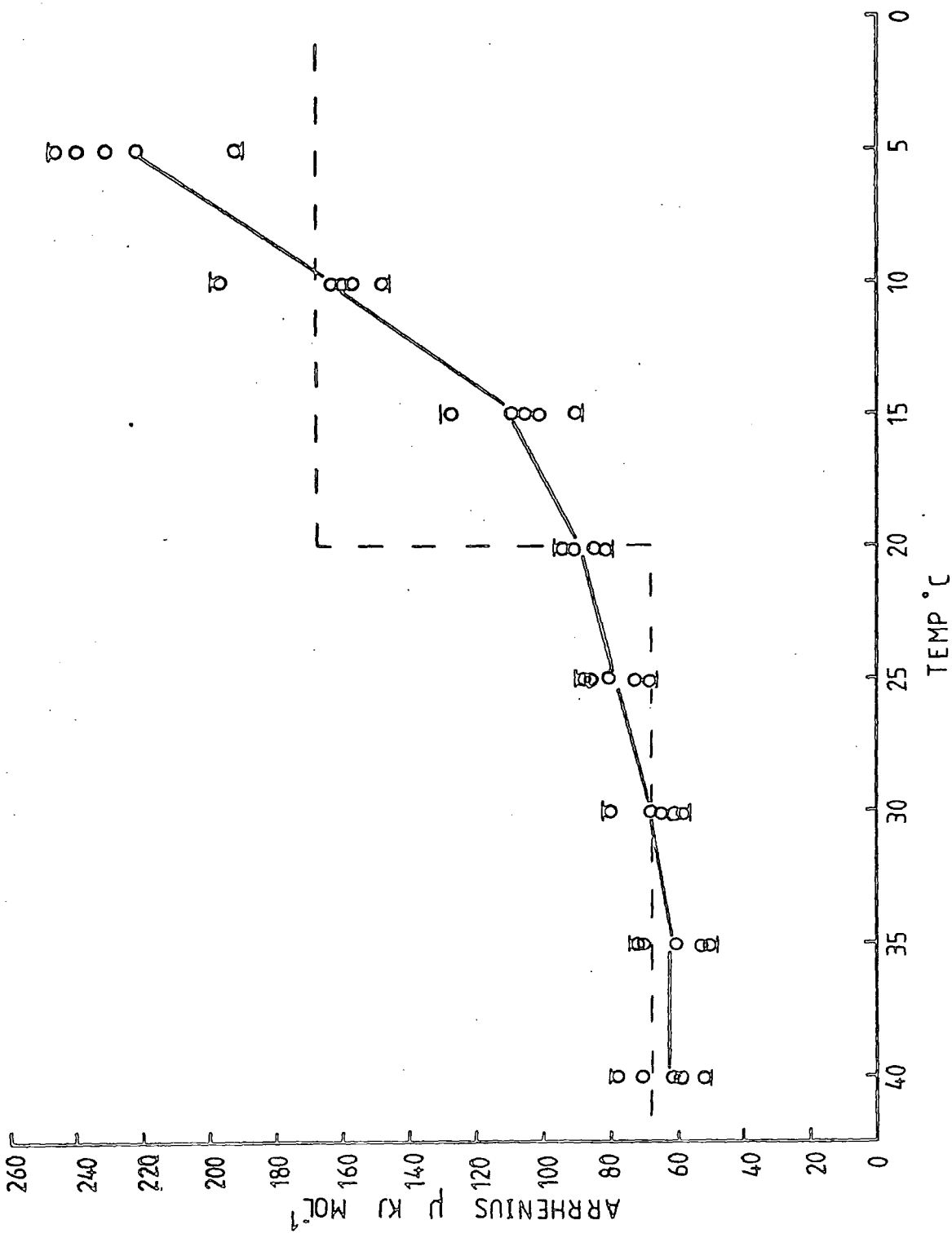
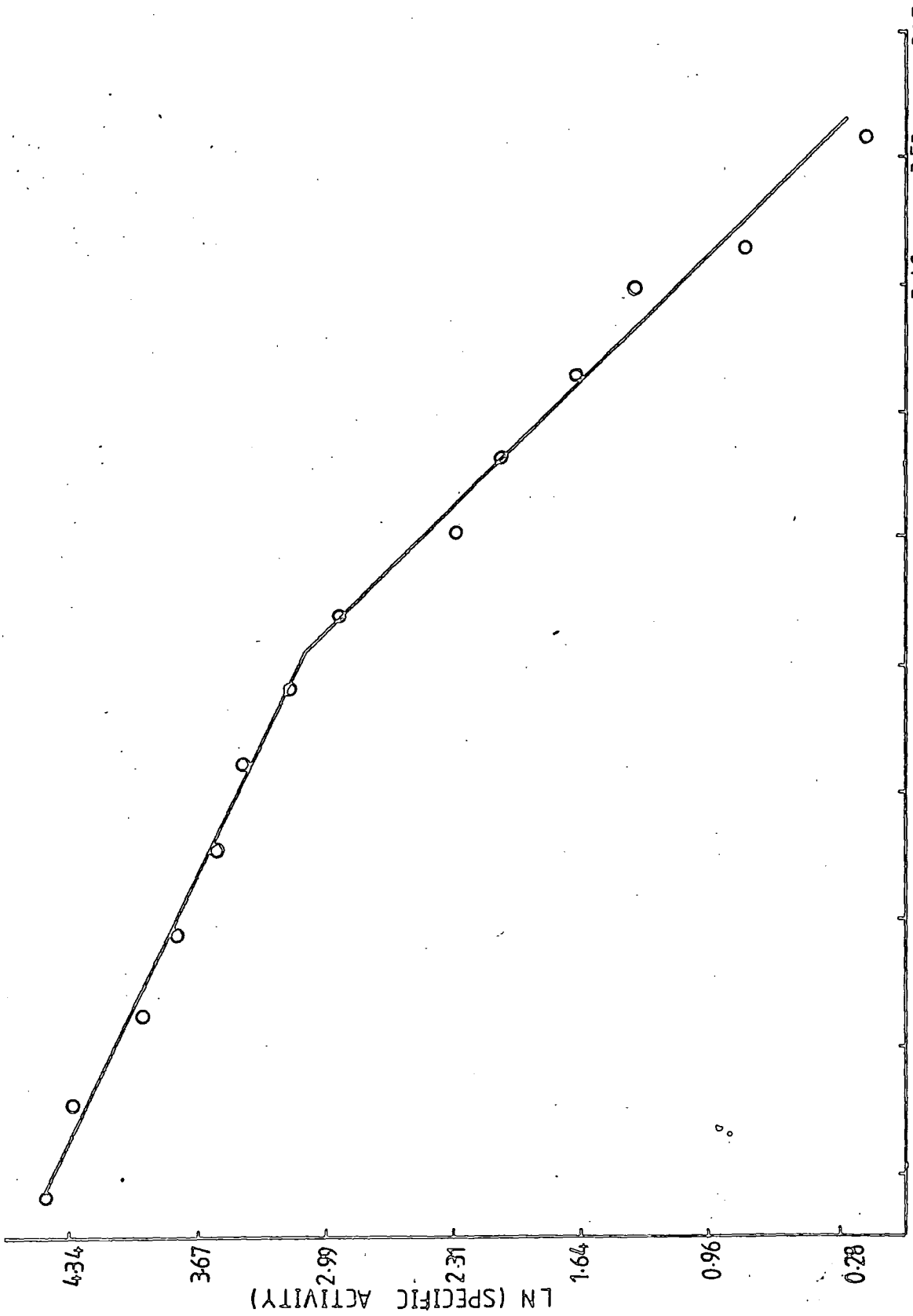

ARRHENIUS μ KJ MOL⁻¹
260
240
220
200
180
160
140
120
100
80
60
40
20
0
0
5
10
15
20
25
30
35
40
TEMP °C

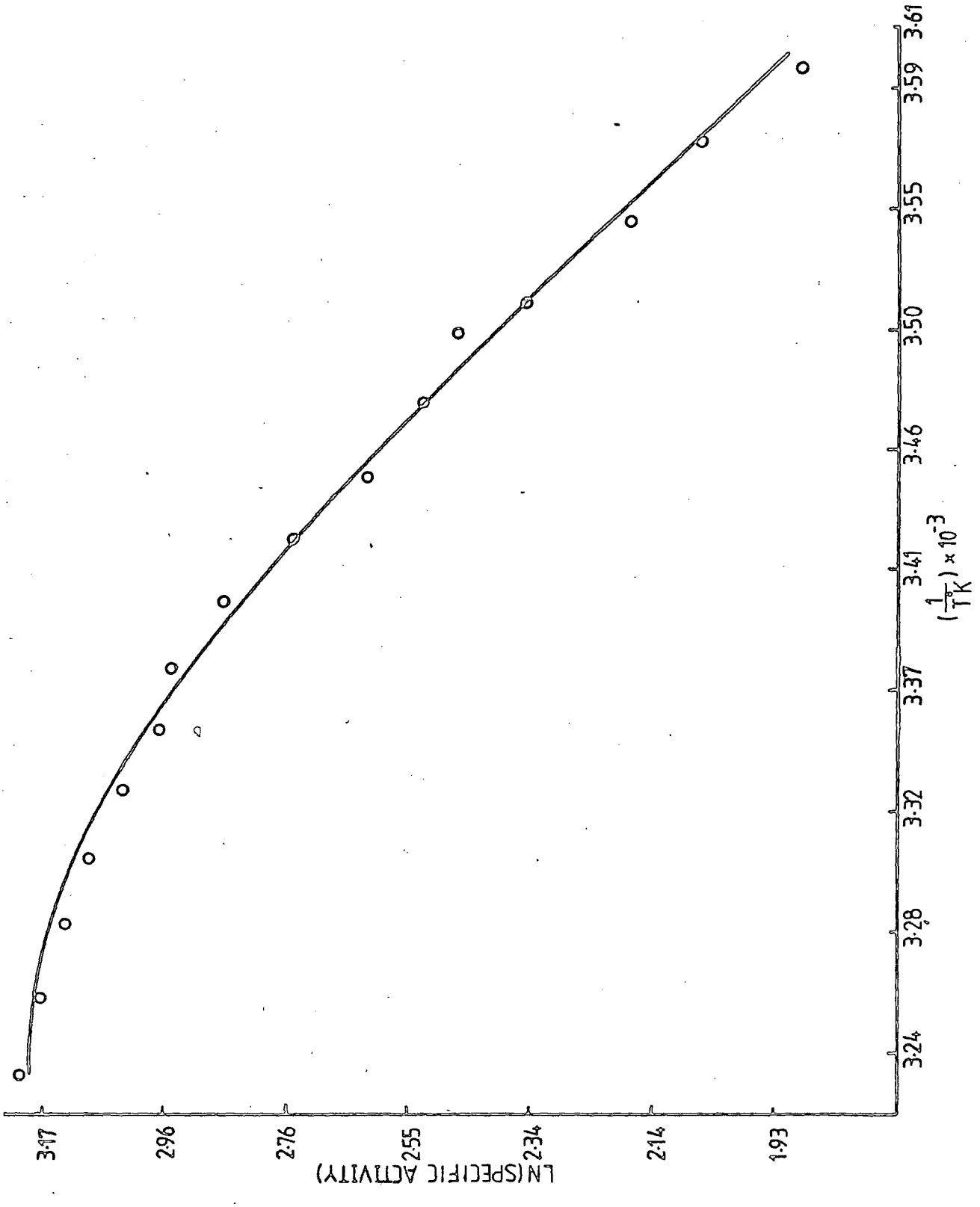
FIGURE 3 : 9

Change of Arrhenius μ with temperature for Arrhenius plots of rat synaptic membrane $Na^+ - K^+$ ATPase

- o - observed values of μ = KJ $mole^{-1}$

— — — — change in μ predicted by phase change effect


LN (SPECIFIC ACTIVITY)
4·34
3·67
2·99
2·31
1·64
0·96
0·28
3·23
3·27
3·30
3·34
3·38
3·42
3·46
3·49
3·53
3·57
$\left(\frac{1}{T°K}\right) \times 10^{-3}$


LN(SPECIFIC ACTIVITY)
3·17
2·96
2·76
2·55
2·34
2·14
1·93
3·24
3·28
3·32
3·37
3·41
3·46
3·50
3·55
3·59
3·61
$(\frac{1}{T^\circ K}) \times 10^{-3}$

The effect of membrane association and lipophilic agents on the temperature kinetics of the acetylcholinesterase from rat brain synaptic membranes.

Introduction

The work presented in this chapter seeks to use the model of non-linear Arrhenius plots for acetylcholinesterase, devised in the previous chapter, to analyse the possible involvement of lipid in this phenomenon. Non-linear Arrhenius kinetics observed for a membrane-bound enzyme are invariably explained in terms of a thermotropic phase change in membrane lipids similar to that observed in pure phospholipid dispersions. This view is contradicted by the observation that sharp phase changes of this form do not occur in biological membranes (Pagano, Cherry & Chapman, 1973; Feinstein, Fernandez & Sha'afi, 1973) and that the apparent activation energy at low temperatures calculated for intrinsic enzymes, such as the $Na^+ - K^+$ ATPase are outside the range normally expected for biological rate processes (20 - 80 KJ $mole^{-1}$). Thus the slope of the Arrhenius plot at low temperatures is unlikely to represent a true activation energy under the constraint of the gel phase of membrane lipid. Although a phase change effect is unlikely to produce non-linear temperature plots, intrinsic trans-bilayer proteins such as $Na^+ - K^+$ ATPase, $Ca^{2+} - Mg^{2+}$ ATPase and membrane transport processes certainly depend on membrane integrity for activity (Schatzmann, 1962; Roufogalis, 1973) thus it is reasonable to assume that the properties of these enzymes may be regulated or determined in some way by the physical state of membrane lipids.

However, an enzyme such as acetylcholinesterase is thought to be a peripheral protein, bound to the membrane by salt linkages

or by weak hydrophobic bonds, such that it may be solubilized by low detergent concentrations which do not disrupt bilayer integrity (Mitchell & Hanahan, 1966; Burger, Fujii & Hanahan, 1968; Aloni & Livne, 1974). Hence it is difficult to conceive how non-linear Arrhenius kinetics of such a peripheral enzyme could result from changes in the physical state of the bulk membrane lipid.

Some evidence exists to show that the properties of acetylcholinesterase may be modified by membrane lipid composition. Studies have shown that rats fed on corn oil and lard have erythrocyte membranes with a lesser or greater proportion of unsaturated fatty acids respectively, and that this change in membrane composition alters the Hill co-efficient for fluoride inhibition of membrane-bound enzymes including the acetylcholinesterase (Moreno, Bloj, Farias & Trucco, 1972; Farias, Bloj, Moreno, Vineriz & Trucco, 1975). It has also been shown that the final specific activity of purified human erythrocyte acetylcholinesterase depended on the deoxycholate concentration initially used to solubilise the enzyme. This was interpreted as being caused by lipid depletion of the enzyme, which suggested that this enzyme was absolutely dependent on lipid for activity. It was also found in this study that the lipid-depleted enzyme could be optimally reactivated by acidic lipids such as phosphatidyl serine (Sihotang, 1976).

No existing work suggested a lipid involvement in the properties of the rat synaptic membrane acetylcholinesterase, nor could it be certain that non-linear temperature kinetics necessarily involved lipid dependence, as this phenomenon could conceivably by due to a thermal transition in the protein molecule.

The experiments designed to resolve this problem and reported

in this chapter, have followed three lines of approach;

(1) As the acetylcholinesterase is a peripheral protein it should be possible to prepare a soluble form of this enzyme. It would then be possible to evaluate the effect of membrane association on the acetylcholinesterase temperature kinetics.

(2) Acetylcholinesterase from a variety of sources has been solubilised in a stable form using detergents (Mitchell & Hanahan,1966; Sihotang, 1976; Beauregard & Roufogalis, 1977). The surfactant properties of these compounds may disturb the non-linear temperature kinetics of the rat brain enzyme, in the event of lipid dependence.

(3) Certain lipophilic agents can fluidise the hydrophobic portion of phospholipids (Seeman, 1972). If phospholipids are involved in non-linear temperature kinetics, then these lipophilic compounds should be able to bind to the relevant lipid molecules and disturb temperature kinetics of the acetylcholinesterase.

If the information from experiments of the form suggested above tend to confirm the involvement of lipid in the acetylcholinesterase temperature kinetics, then it may be possible to continue the study by identifying and characterising the relevant lipid molecules from partially purified acetyl-colinesterase.

Materials and methods

Enzyme assays

Acetylcholinesterase and $Na^+ - K^+$ ATPase were assayed as described in the general methods section.

Arrhenius plots of acetylcholinesterase and $Na^+ - K^+$ ATPase

The Arrhenius plots for acetylcholinesterase were performed in an aluminium Forbes' bar using the reaction media and equilibration schedule as described in Chapter 3.

Data for the rate of enzyme reactions across the temperature range produced on this bar, were processed by computer assisted error minimisation scheme. These data were fitted either to two straight lines or to the reversible thermal-inactivation model.

Synaptic membrane isolation

Rat brains were processed for synaptic membrane isolation according to the bulk-extraction method described in the general methods sections.

Protein assay

Protein was assayed according to the ninhydrin method as described in the general methods section.

Effect of ions, pH and ionic strength on the membrane association of rat synaptic membrane acetylcholinesterase

For these experiments synaptic membranes were prepared as described above. In this case a synaptic membrane preparation from 24 animals was divided into 6 aliquots, each containing about 3 - 4 mg of protein. These aliquots were centrifuged at 100,000 xg at 4^oC for 1 hour. The pellets were resuspended by homogenization in 3 cm^3 of the following media;

1. 10mM phosphate buffer, pH 7.4
2. 10mM phosphate buffer, pH 5.8
3. 10mM phosphate buffer, pH 7.4 + 3mM CaCl
4. 10mM phosphate buffer, pH 7.4 + 1.2M NaCl
5. 20mM imidazole buffer, pH 7.4
6. 20mM imidazole buffer, pH 7.4 + 1 mM EDTA

These membrane suspensions were stored, packed in ice ($4^{o}C$), for 3 days. After this time the suspension was centrifuged at 100,000 xg for 1 hour at $4^{o}C$. The supernatants were retained and the pellets were resuspended in 3 cm^{3} of the relevant medium (that in which each was stored).

The acetylcholinesterase activity and protein composition of the initial homogenates, resuspended pellets and supernatants were measured, as described above.

Effect of detergents on the temperature properties of rat synaptic membrane acetycholinesterase

Soluble and membrane-bound forms of acetylcholinesterase were prepared by incubating freshly prepared rat synaptic membranes at a protein concentration of about 1 mg. cm^{-3} in 20mM imidazole 1mM EDTA at pH 7.2 for 3 days at $4^{o}C$. After this time the suspension was centrifuged at 100,000 xg at $4^{o}C$ for 1 hour. The supernatant was retained and the pellet was resuspended in the same volume of 20mM imidazole 1mM EDTA pH 7.2 as the volume of the supernatant. Both fractions were found to be stable for several weeks when stored packed in ice ($4^{o}C$).

The detergents used in this study were sodium deoxycholate, Triton x - 100 and Lubrol W - X. These were always freshly prepared by dissolving in 20mM imidazole 1mM EDTA pH 7.2 at twice the working concentration. Working concentrations were 10mM sodium deoxycholate, 1.0% (w/v) Triton x - 100 and 5 mgs. cm^{-3} Lubrol W - X.

Aliquots of soluble and membrane-bound acetylcholinesterase fractions were diluted with equal volumes of detergent solution to give the concentrations shown above. These are incubated at 4°C for 30 minutes in the case of sodium deoxycholate and Triton x - 100 and 24 hours for Lubrol W - X, as these have been found to solubilize acetylcholinesterase. After incubation the enzyme fractions were diluted with nine volumes of 20mM imidazole 1mM EDTA pH 7.2 and these were used directly for temperature kinetic measurements.

The effect of lipophilic agents on synaptic membrane enzymes

The lipophilic agents used in this study were n-octanol, the tranquillizer chlorpromazine, the local anaesthetics tetracaine, procaine, mepivicaine and lidocaine. These drugs were found to be more soluble at pH 7.0 rather than pH 7.5 which was the usual pH of the reaction media, consequently all reaction media were brought to pH 7.0 when used in conjunction with lipophilic drugs. Also it was necessary to incubate the lipophilic agents with the enzyme fractions for 15 minutes at 37°C, this incubation was found to be necessary to permit equilibrium between enzyme and lipophilic agents.

In this case the normal assay procedure was modified in the following way. In the case of the $Na^+ - K^+$ ATPase, reaction media, containing lipophilic agent, were incubated with an aliquot (200 ul) of enzyme, at 37°C for 15 minutes. Then reactions were initiated with 0.5 cm^3 of ATP, pre-warmed to 37°C, rather than by the addition of enzyme. Similarly the acetylcholinesterase was assayed in the presence of lipophilic agents by incubating media containing the agent with an aliquot of enzyme (200 ul) and incubated at 37°C for 15 minutes and the reaction was initiated with 200 ul of acetylthiocholine.

Results

Data presented in Table 4 : 1 show the effect of various incubation conditions on the membrane association and stability of synaptic membrane acetylcholinesterase. Details of these conditions are presented in the materials and methods section of this chapter. It is clear that this enzyme was quite stable in each of these media as the specific activities of the fresh preparations (day 1) and after 3 days at $4^{o}C$ were similar. Also in each case, centrifugation of 3 day old preparations at 100,000 xg for 1 hour, which pelleted all membranous material, did not pellet all of the acetylcholinesterase activity. In the case of phosphate buffer at pH 7.4, imidazole buffer at pH 7.4 and imidazole EDTA buffer at pH 7.4; about 50% of the total acetylcholinesterase activity in each $3cm^{3}$ fraction remained soluble after centrifugation, whereas with phosphate buffer at pH 5.8, phosphate buffer with 3mM CaCl and phosphate buffer with 1.2M NaCl, a much lower fraction of the total acetylcholinesterase activity remained soluble, between 7 and 24%.

The specific activities of the soluble and membrane fractions are also presented in Table 4 : 1. These values reflect not only the quantity of acetylcholinesterase which dissociated from the membrane but also the quantity of other proteins which have dissociated. The buffers which brought about most soluble acetylcholinesterase, caused about 30% of membrane protein to become soluble, hence the original synaptic membrane preparation of acetylcholinesterase activity of about 25 μ moles thiocholine/mg. protein/hr gives a soluble fraction of about 40 μ moles thiocholine/mg. protein/hr and a membrane fraction of 13 - 17 μ moles thiocholine/mg. protein/hr. In the case of the pH 5.8

buffer, about 25% of membrane protein became soluble. With calcium containing and 1.2M NaCl containing buffers only 14 - 15% of membrane protein became soluble after three days.

At this stage the preparations suspended in phosphate buffer were discarded, as this buffer is particularly susceptible to bacterial contamination. However, the membrane fractions in imidazole buffers were retained and stored on ice for a further 2 weeks. After this time both suspensions were centrifuged at 100,000 xg for 1 hour. The supernatants were assayed for acetylcholinesterase activity, however none was detected in either case.

The ability to isolate stable membrane-associated and soluble forms of this enzyme raises the question of whether both exhibit the anomalous Arrhenius kinetics found for the acetylcholinesterase of native synaptic membrane preparations. Thus the temperature kinetics of soluble and membrane-bound acetylcholinesterase were evaluated, as for the native preparations reported in Chapter 3. Typical examples of the resulting Arrhenius plots are presented in Figures 4 : 1 and 4 : 2. These Arrhenius plots were processed according to the reversible inactivation model, devised for acetylcholinesterase, presented in Chapter 3. The values for the Arrhenius u at low temperatures, enthalpy and entropy parameters calculated for these data, according to this model are presented in Table 4 : 2, along with similar values for native preparations presented in Chapter 3, for purposes of comparison.

From this data it can be seen that both soluble and membrane-bound acetylcholinesterase exhibited non-linear Arrhenius plots. Data presented in Table 4 : 2 shows that the form of these non-

linear plots are similar to those obtained for native synaptic membrane acetylcholinesterase. This suggests that the non-ideal Arrhenius kinetics of the enzyme in the membrane-bound form is not caused by its association with the bulk of membrane lipid.

This can be further tested by introducing known membrane lipid perturbants detergents, tranquillizers and anaesthetics and assessing the effects of these compounds on the temperature properties of the enzyme fractions.

Detergents were chosen which had been used by other workers to solubilize acetylcholinesterase. These were the ionic sodium deoxycholate and the non-ionic detergents Triton x - 100 and Lubrol W - X (Mitchell & Hanahan, 1966; Sihotang, 1976; Beauregard & Roufogalis, 1977). Both soluble and membrane-bound fractions of acetylcholinesterase were treated with these detergents as described in the methods section. These detergent treated enzyme fractions were used for temperature kinetics measurements as described previously.

Arrhenius plots of soluble and membrane-bound acetylcholinesterase treated with detergent were processed on the computer using both reversible inactivation and phase-change models. The parameters generated in this way are presented in Table 4 : 3 for the reversible inactivation model and in Table 4 : 4 for the phase change model. Similar parameters, calculated for Arrhenius plots in the absence of detergent are also presented, for comparison.

An Arrhenius profile of the soluble form of acetylcholinesterase treated with Triton x - 100 is presented in Figure 4 : 3. Temperature kinetics are still non-linear in this case, but

much less so than for the untreated preparations. This fact is reflected in the enthalpy and entropy factors calculated by fitting data for the Triton-treated soluble fraction to the reversible inactivation programme, which are lower than those calculated for untreated preparations. Also the Triton x - 100 treatment has significantly reduced the Arrhenius u at low temperatures (Table 4 : 3). This suggests that in the case of the soluble enzyme, Triton treatment has not only altered the nature of the transition between the high temperature and low temperature forms but has also reduced the activation energy of the low temperature form of the enzyme.

When the data for the Triton-treated soluble acetylcholinesterase were processed according to the phase change model, in which two straight lines are fitted to this data, the Arrhenius u at high temperatures was slightly higher than for untreated soluble preparations, the Arrhenius u at low temperature was significantly lower than untreated preparations and the "critical" temperature was significantly reduced (Table 4 : 4). These parameters confirm the interpretation of the shape of the Arrhenius plot of Triton-treated soluble acetylcholinesterase stated above.

A typical Arrhenius plot of Triton x - 100 treated membrane-associated acetylcholinesterase is presented in Figure 4 : 4. Although it appears that the data points fit a curve, and have been fitted to such by eye, the curvature was less than the minimum which can be detected by the reversible inactivation programme. Hence computer analysis resulted in a straight line being fitted to the points. This was the case for three separate preparations (Table 4 : 3). However, the trend is clear, in that, as with the soluble enzyme, Triton-treatment

tended to abolish the non-linearity of the Arrhenius plot of membrane-associated acetylcholinesterase.

When these data were processed according to the phase-change model, the values for the Arrhenius u at high and low temperature and the values for the critical temperature were similar to those for the Triton-treated soluble preparations. When these were compared with similar parameters for untreated membrane-associated acetylcholinesterase also processed according to the phase change model, it can be seen that the Arrhenius u at high temperatures was similar but that the critical temperature and Arrhenius u at low temperatures were both lower for the Triton treated preparations. Thus unlike the soluble preparation, Triton treatment has had no effect on the Arrhenius u at high temperatures, but as with the soluble preparation the critical temperature was reduced by $7^{o}C$ and the Arrhenius u in the low temperature range was reduced by about 7 KJ $mole^{-1}$. This suggests that the temperature kinetics of the membrane-bound acetylcholinesterase were altered in a manner similar to that of the effect on the soluble acetylcholinesterase.

Typical Arrhenius plots for sodium deoxycholate treated soluble and membrane-bound acetylcholinesterase are presented in Figures 4 : 5 and 4 : 6 respectively. Data for two such preparations was processed according to the phase change model (Table 4 : 4) and reversible inactivation model (Table 4 : 3). The parameters calculated for these data from the reversible inactivation model are similar to those for untreated preparations for both soluble and membrane-bound acetylcholinesterase (Table 4 : 3). However, the procedure which fits two straight lines to these data detected a considerable difference between deoxycholate treated and untreated preparations, in that the critical temperature

was raised from close to 20°C to nearer 30°C by preincubation with deoxycholate (Table 4 : 4).

Lubrol W - X treated soluble and membrane-bound acetylcholinesterase showed temperature plots similar to those for untreated preparations. Typical plots are shown in Figure 4 : 7. Overnight incubation with Lubrol W - X had no effect on the parameters calculated for soluble and membrane-bound enzyme according to either the reversible inactivation model or the phase change model (Tables 4 : 3 and 4 : 4). Thus Lubrol W - X had no effect on the temperature kinetics of the acetylcholinesterase fractions.

Various phenothiazine tranquilizers and local anaesthetics have been shown to interact with biological membranes, altering the state of membrane lipids (Skov, 1954 (a) and (b); Seeman & Kwant, 1969; Seeman, Kwant & Sauks, 1969; Feinstein, Spero & Falsonfield, 1970; Roth & Seeman, 1972; Seeman & Roth, 1972). However, the effect of these compounds on the temperature kinetics of membrane enzymes is not well characterized. For this reason, the effect of a representative phenothiazine (chlorpromazine) and anaesthetic (tetracaine) on the temperature properties of synaptic membrane $Na^+ - K^+$ ATPase were each examined. This enzyme was chosen because it is a integral membrane protein, well characterized and known to be inhibited by both chlorpromazine and tetracaine (Roufogalis, 1975). The effects of these compounds on the temperature properties of the $Na^+ - K^+$ ATPase were examined with a view to extending the use of these compounds as lipophilic probes of a possible lipid involvement in the non-ideal temperature properties of acetylcholinesterase.

Data presented in Figure 4 : 8 shows that both chlorpromazine and tetracaine were inhibitors of synaptic membrane $Na^+ - K^+$ ATPase, although chlorpromazine was the most potent inhibitor, with half maximal inhibition at a concentration of about 1.6×10^{-4}M. Tetracaine was somewhat less potent with half maximal inhibition occurring at about 4×10^{-3}M. Typical Arrhenius plots of $Na^+ - K^+$ ATPase assayed in half maximal inhibitory concentrations of chlorpromazine and tetracaine are presented in Figure 4 : 9, along with a typical control profile for comparison. It is clear that chlorpromazine, reduced the curvature of the Arrhenius plot. This view is confirmed by the parameters calculated, according to the reversible thermal inactivation model, for several preparations (Table 4 : 5). In this case the enthalpy and entropy factors were significantly lower than that for the control profiles, also the Arrhenius u value for the high temperature form of the enzyme was significantly lower than for control profiles. Data for Na^+ K^+ ATPase temperature plots, assayed in the presence of chlorpromazine were also processed according to the phase change model gave parameters for Arrhenius u at both high and low temperature range and for the critical temperature were not significantly different from those for control profiles (Table 4 : 6). Thus the changes in the Arrhenius profile induced by chlorpromazine were too subtle to be detected by fitting two straight lines.

A typical Arrhenius plot of $Na^+ - K^+$ ATPase, assayed in the presence of half maximal inhibitory concentrations of tetracaine, is also presented in Figure 4 : 9. This produces a broader curve than that for controls. However, this effect was even more marked than that for chlorpromazine. This is reflected in the parameters calculated for several preparations, according to the reversible inactivation model, in this case the apparent

activation energy of the high temperature state was only 45.2 KJ $mole^{-1}$, as opposed to 67.3 KJ $mole^{-1}$ for controls. The ΔH terms in the transition at -96.7 KJ $mole^{-1}$ and for the ΔS term at -324.7 J^{o} K^{-1} $mole^{-1}$ were almost half that for the control profiles (Table 4 : 5). Even processing these data according to the phase change model detected a difference from controls, in that the Arrhenius u at low temperatures for the Na^{+} - K^{+} ATPase in the presence of tetracaine was significantly lower than controls (Table 4 : 6).

It is clear then that tetracaine had a more marked effect on the temperature kinetics of Na^{+} - K^{+} ATPase than chlorpromazine. Tetracaine was also found, in this study, (see below) to be a potent inhibitor of acetylcholinesterase whereas chlorpromazine was not an inhibitor of this enzyme. Thus it was decided to concentrate on the effect of the group of anaesthetics, of which tetracaine is one, on the soluble and membrane-bound acetylcholinesterase.

This was investigated in two ways; firstly by measuring the potency with which a range of anaesthetics, of differing lipophilicity, inhibited both soluble and membrane-bound acetylcholinesterase, and secondly by evaluating the effect of the most potent of these inhibitions on the temperature properties of soluble and membrane-bound acetylcholinesterase.

Data for the inhibition of both soluble and membrane-bound acetylcholinesterase fractions by lidocaine, mepivicaine, procaine and tetracaine are presented in Figure 4 : 10. From this it can be seen that each anaesthetic had a characteristic potency as an acetylcholinesterase inhibitor and also that both soluble and membrane-bound forms of acetylcholinesterase were equally sensitive to each anaesthetic. Tetracaine was

the most potent inhibitor, with the concentration giving 50% inhibition (I 50) of about 1.78×10^{-4}M, followed by procaine with I 50 of 1.26×10^{-3}M than mepivicaine with I 50 of 2.0×10^{-2}M and lastly lidocaine with I 50 of 2.0×10^{-1}M. These were correlated with lipid solubility as defined by octanol/water co-efficient.

As tetracaine changed the Arrhenius kinetics of $Na^+ - K^+$ ATPase then it seemed reasonable to investigate the effect of this drug on acetylcholinesterase temperature properties. Typical Arrhenius profiles of the low ionic-strength solubilized and membrane-associated acetylcholinesterase assayed in the presence of 2.0×10^{-4}M tetracaine are presented in Figure 4 : 11. These both represent linear plots according to the reversible inactivation model, with Arrhenius u of 24 - 26 KJ $mole^{-1}$ in both cases. Data for several such plots are presented in Table 4 : 7, along with the activation energies of the low temperature forms of the relevant enzyme fractions calculated for the untreated enzyme by the reversible inactivation programme. It is clear from this that the linear plots produced by the inclusion of tetracaine in the reaction medium exhibit apparent activation energies which are not significantly different from those calculated for the theoretical stable low temperature species for untreated preparations. It is clear from this that tetracaine interacts with the soluble form of acetylcholinesterase in a similar way to the membrane-associated enzyme.

The aliphatic, short chain alcohols are recognised as having anaesthetic action in that they cause disordering or "fluidising" of biological membranes (Seeman, 1972). It is clear that these compounds cannot interact with membranes by any ionic or free-radical reaction but simply because of their hydrophobic nature. Thus it was considered interesting to test the effect of one of these

compounds on the temperature kinetics of the acetylcholinesterase enzyme fractions.

The effects of several n-alkanols were tested on the activity of acetylcholinesterase and none were found to be inhibitors of this enzyme. Thus the most potent membrane disordering n-alkanol, n-octanol (Seeman, 1972) was examined for its possible effect on the temperature properties of both forms of acetylcholinesterase. Typical Arrhenius plots of soluble and membrane-bound acetylcholinesterase assayed in the presence of 1mM octanol are presented in Figure 4 : 12. As with the case of tetracaine, both of these represent linear profiles, however, unlike the case of tetracaine the computer calculated apparent activation energies of several membrane associated and soluble preparations assayed in the presence of n-octanol were significantly lower than the apparent activation energies of the low temperature state of this enzyme calculated according to the reversible thermal inactivation model. Thus octanol causes significant perturbation of the temperature properties of both soluble and membrane-bound forms of rat brain acetylcholinesterase.

Discussion

The usual interpretation of non-linear Arrhenius kinetics for membrane enzymes, has been to assume that this resulted from a temperature induced change in the state of membrane lipids in the proximity of the enzyme (Raison, Lyons & Thomson, 1970). In the case of the acetylcholinesterase however, a peripheral rather than an integral protein like $Na^+ - K^+$ ATPase, it might be expected that Arrhenius kinetics would be less constrained by the lipid environment. The aim of the work presented in this chapter was to examine the apparent paradox of non-linear Arrhenius kinetics being found for the acetylcholinesterase.

Initial experiments, to this end, sought to prepare a soluble form of rat synaptic membrane acetylcholinesterase, in order to compare the temperature kinetics of this and the original membrane preparation. Data presented in Table 4 : 1 show that incubating synaptic membranes in low ionic-strength media for 3 days at 4^oC caused the dissociation from the membrane of about 50% of the total acetylcholinesterase activity. The remainder of this enzyme remained firmly attached to the membrane. Similar low ionic-strength solubilization of acetylcholinesterase has been reported for brain and muscle preparations of rat (Rieger & Vigny, 1976), for brain and muscle and erythrocyte from mouse (Adamson, 1977) and for bovine erythrocytes (Burger, Fujii & Hanahan, 1968).

It is clear from the specific activities of the low ionic-strength solubilized acetylcholinesterase (Table 4 : 1) that although 50% of the activity became soluble, other proteins were also solubilized. In this case about 30% of membrane protein became soluble in this way. Thus the soluble fraction is somewhat enriched in acetylcholinesterase.

Mitchell & Hanahan, (1966) reported that low ionic-strength was ineffective in solubilizing human erythrocyte acetylcholinesterase, but that incubating these membranes with 1.2M NaCl extracted up to 80% of the acetylcholinesterase in a soluble form. This was not the case for the rat enzyme, as Table 4 : 1 shows that 1.2M NaCl was less effective in solubilizing acetylcholinesterase. Only 12% of acetylcholinesterase was solubilized in this case. Burger, Fujii & Hanahan, (1968) found that calcium and other divalent cations could prevent dissociation of this enzyme from bovine erythrocytes. This was also the case for the membrane preparations used in this study. Data presented in Table 4 : 1 shows that incubation with 1mM CaCl caused the dissociation of only 7% of acetylcholinesterase. However, chelating agents were no more efficient than low ionic-strength media at extracting acetylcholinesterase as incubation with 1mM EDTA caused no more soluble acetylcholinesterase than low ionic-strength media without EDTA (Table 4 : 1).

This work shows that, although a portion of the rat synaptic membrane acetylcholinesterase can be made soluble by manipulating the ionic composition of the membrane suspension medium, no single definition of a "peripheral" protein, according to the Singer-Nicholson model of membrane structure (Singer & Nicholson, 1972) could account for the membrane relationship of acetylcholinesterase for this and other species. The human erythrocyte acetylcholinesterase cannot be removed by incubation in low ionic-strength media (Mitchell & Hanahan, 1966), rat acetylcholinesterase can be partially solubilized in this way (Riger & Vigny, 1976; Table 4 : 1) and up to 80% of bovine erythrocyte acetylcholinesterase can be removed in this way (Burger, Fujii & Hanahan, 1968).

The choice of the rat as a subject facilitated the examination of the role of membrane association in the non-linear Arrhenius kinetics

of the enzyme, as stable soluble and membrane-associated forms can be produced. Non-linear Arrhenius kinetics were observed for both forms of acetylcholinesterase (Figures 4 : 1 and 4 : 2) which was very similar to that observed for native preparations. The values for the apparent activation energy of the low temperature form of this enzyme (about 33 KJ $mole^{-1}$) and the values for enthalpy (-80 KJ $mole^{-1}$) and entropy (-250 J^{o} $K^{-1}mole^{-1}$) of the transition were similar. This shows that both sets of data gave similar fit to the reversible inactivation model (Table 4 : 2).

Clearly membrane association itself cannot be responsible for, nor influence the Arrhenius kinetics of rat brain acetylcholinesterase as the soluble and membrane forms closely resemble the native preparation in this respect. Two alternative interpretations can be placed upon this; either non-linear Arrhenius kinetics of acetylcholinesterase may result from a thermal transition in the enzyme molecule, or a small quantity of phospholipid is associated with this enzyme, maintaining the conformation of the enzyme at physiological temperatures, may confer cold-sensitivity on this enzyme. These possibilities were examined by treating soluble and membrane-associated acetylcholinesterase with chemical agents, known to disorder membrane lipids.

Treatment of both forms of acetylcholinesterase with Triton x - 100 did produce detectable changes in the Arrhenius profiles, in that Triton-treatment tended to reduce the curvature of these plots (Figure 4 : 3 and 4 : 4). Data for the Triton-treated soluble form gave values for transition enthalpy, entropy and apparent activation energy of low temperature species which were significantly lower than for control preparations (Table 4 : 3). Data for the Triton-treated membrane-bound form of the enzyme gave linear temperature kinetics in terms of the reversible thermal inactivation model, although these plots are detectably non-linear when fitted

to two straight lines (Tables 4 : 3 and 4 : 4). The membrane-bound plot does appear to be slightly non-linear by inspection although outside the limits detectable by the reversible inactivation model.

Primarily this work suggested that the detergent properties of Triton x - 100 interfered with the non-linear Arrhenius kinetics of acetylcholinesterase. This was similar to the observation of Wright & Plummer, (1970).

The effect of sodium deoxycholate was less clear (Figures 4 : 5 and 4 : 6) in that the values for the thermodynamic parameters calculated for deoxycholate treated soluble and membrane enzymes were similar to those calculated for untreated preparations (Table 4 : 3). However, when these data were processed according to the phase change model the critical temperature calculated for deoxycholate treated enzyme was nearer 30°C than 20°C as in control preparations (Table 4 : 4).

In contrast to these detergents, Lubrol W - X did not alter the temperature kinetics of either form of acetylcholinesterase (Figure 4 : 7, Tables 4 : 3 and 4 : 4). Thus any lipid-protein relationship involved in the temperature kinetics of acetylcholinesterase was resistant to Lubrol W - X treatment. This is similar to the observation of Beauregard and Roufogalis, (1977) who reported non-linear temperature plots of bovine erythrocyte acetylcholinesterase to be unaffected by Lubrol W - X.

The observation that Triton x - 100 treatment caused more pronounced perturbation of the temperature kinetics of both soluble and membrane-bound forms of acetylcholinesterase than sodium deoxycholate is surprising in that the latter is thought to be more able to remove lipid from protein (Martonosi, 1968;

Helenius & Simons, 1971; Hardwicke & Green, 1974). This can be explained with reference to data presented in Chapter 5 (Table 5 : 2) which shows that acetylcholinesterase was somewhat unstable in deoxycholate. Thus the form of the plots shown in Figures 4 : 5 and 4 : 6 for deoxycholate treated enzyme could result from linear kinetics at temperature below 30°C where the enzyme is stable with irreversible inactivation becoming significant at temperatures above 30°C.

The effects of Triton x - 100 discussed above suggest that lipid is involved in the non-linear temperature plots of acetylcholinesterase, as the broader transition and reduction in activation energy produced are consistent with the membrane "fluidising" or disordering effect of this detergent. However, as the soluble form of the enzyme, which was extracted under conditions which did not disrupt the integrity of the membrane, exhibited non-linear temperature plots, which were sensitive to Triton treatment, then it is likely that acetylcholinesterase is a lipoprotein, as has been suggested for bovine erythrocyte acetylcholinesterase by Beauregard & Roufogalis, (1977).

If this is true, then it would be necessary to account for the lack of effect of Lubrol W - X on the temperature properties of acetylcholinesterase. No definitive explanation can be advanced but, as Triton extracts acetylcholinesterase from membranes within a few minutes (Vigny, Gisigier & Massoulie, 1978) and Lubrol W - X requires many hours to accomplish this (Beauregard & Roufogalis, 1977) then it is reasonable to assume that Triton is more effective at disrupting lipid-lipid bonds than Lubrol W - X. Similarly Triton would disturb the possible lipoprotein state of acetylcholinesterase, in a manner in which Lubrol W - X might not.