STUDIES OF THE CHOLINESTERASE OF SHEEP BRAIN AND CONCOMITANT STRUCTURAL PROTEIN

by

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PART I

PURIFICATION OF THE SPECIFIC ACETYLCHOLINESTERASE OF SHEEP-BRAIN.

CHAPTER 1: INTRODUCTION

A. PHYSIOLOGICAL SIGNIFICANCE OF ACETYLCHOLINESTERASE.

Acetylcholine was first synthesized by Baeyer in 1867. At the same time it was only of chemical interest but decades later its powerful pharmacological effects were noted: Hunt and Taveau pointed out the role of acetylcholine in lowering blood-pressure (1). Dale and his co-workers demonstrated that acetylcholine is a chemical mediator of the nerve impulses at parasympathetic endings (2). Thus the notion of chemical mediation that had been introduced by Elliot in respect of adrenalin (3) was extended to the physiology of ganglionic synapses and neuromuscular junctions (4). This hypothesis met with some opposition among neurophysiologists; objections to it were reviewed by Eccles (5). For example, transmission or nerve impulses across the neuromuscular junctions and synapses occurs within milliseconds and chemical mediation by acetylcholine cannot explain this phenomenon. Fulton (6) and Erlanger (7) considered the extremely short interval for the effect of the axon-potential which would preclude the intervention of any process dependent upon substances released at the nerve endings.

Early hypotheses of chemical mediation were based on experiments which employed methods of classical physiology and, according to Nachmansohn and his co-workers, did not permit an interpretation of the precise function of the ester in the physiochemical mechanism of the propogation of nerve impulses. Using suitable methods for recording cellular functions such as conduction of nerve impulses (8,9), Nachmansohn and his school came to the following conclusions(10):

The ester is released at the neuronal surface when a stimulus reaches the nerve cell. By the action of acetylcholine the permeability of the membrane to ions is increased and depolarization occurs. The depolarized point becomes negative to the adjacent region and a flow of current is generated. This flow of current transmits the impulse to a neighbouring point where more acetylcholine is released. The process is repeated and in this way the impulse is propagated.

As the propagation of the nerve impulse hinges on the release and disappearance of the ester, the enzyme responsible for the catalysis of the latter reaction attracted interest.

The aim of the work described in this part of the Thesis was to isolate or purify the acetylcholine hydrolysing enzyme of the sheep brain, a material which had received little attention so far.

The question whether the tissue chosen was suitable for the isolation of the enzyme may be considered through a brief survey of the distribution of the enzyme.

B. DISTRIBUTION AND LOCALIZATION OF CHOLINESTERASES IN CONDUCTIVE TISSUE AND WITHIN THE CELL.

It has been known for more than twenty years that the electric tissue of some fish is rich in the acetylcholine hydrolyzing enzyme. A high concentration of the enzyme is found in the electric organs of ray, Torpedo marmorata, and Gymnotus electricus. Meyerhof and Nachmansohn (11,12) suggested that the potential differences during nerve activity are closely connected with the

metabolism of acetylcholine. They measured acetylcholinterase activity in the region of the surface of the nerve cell and demonstrated that there is a close parallel between E.M.F. and the activity of the enzyme. In the head ganglion of the squid (Loligo paealii) a concentration of cholinesterase has been found which is higher than in any other tissue examined thus far. The giant fibre of squid contains all the enzyme in the sheath, practically none is present in the axoplasm. Nachmansohn et al.(13) measured the concentration of cholinesterase in electric organs (Torpedo, Cymotus electricus) in different sections from the head to the caudal end, and obtained S-shaped curves when the concentration of the enzyme or electric discs per centimeter or E.M.F. per centimeter were plotted against distance. In the muscles adjacent to the electric organ the enzyme concentration is of the order of that in the electric tissue, and much higher than in striated muscle (13). The difference between the concentration of acetycholinesterase in regions where cells and synapses are located and that in the nerve trunk is measurable. The values range from 50-70 mg.hydrolyzed acetylcholine per hour to 400-600 mg.in the superior cervical sympathetic nerve fibres of cats. The lower range refers to the preganglionic fibre and the higher to the ganglionic one (14).

80-140 mg.acetylcholine is split per hour in the abdominal chain of lobster in the trunk and 180-300mg.where cells and synapses are present. In the grey matter of brain the figures range between 30 and 400 mg, in the white matter

they are below 10. The values for brain also vary from one species to another, e.g. in the increasing order man < ox < rat < rabbit (14).

Marnay and Nachmansohn (15-17) investigated the concentration of acetylcholine in different parts of the muscle; they found higher concentrations in those parts of the frog's sartorius which are rich in nerve endings than in the nerveless pelvis end. In the dog's gastrocnemius also the cholinesterase is unevenly distributed in muscle tissue. The rate of hydrolysis of acetylcholine by lizard's muscle is three to fivefold that produced by frog's muscle and two to threefold that produced by mammalian muscle at 37°.

The distribution of acetylcholinesterase in different mammalian tissues has been studied (18-22), but only Ord and Thompson attempted to compare the different types of cholinesterase in different tissues (19).

Zeller found that the cobra venom contains a cholinesterase a hundred times more active than the enzyme of the electric organs of fish (23). The venoms of viperids, however, do not contain the enzyme (24); this is a characteristic difference between the venom of Colubridae and Viperidae.

C. PHYSICO-CHEMICAL PROPERTIES OF CHOLINESTERASES

Once the attention of nerve physiologists had turned towards cholinesterases, extensive investigations were undertaken to characterise the enzyme. At first two types of enzyme were demonstrated in blood by Glick who reported that horse serum

enzyme is not specific toward acetylcholine (26-28). Alles and Hawes (29) and Richter and Croft (31) have shown the same for human serum enzyme. Enzymatic scission of n-acetylcholine esters increases with the lengthening of the hydrocarbon chain to the butyryl compound, and decreases thereafter; the esters of the dicarboxylic acids are split relatively slowly (28).

The red blood cell esterase differs remarkably from serum esterase (28,29,31). The former is specific towards acetylcholine and has a well defined substrate optimum. Acetyl-β-methylcholine is split at a lower rate; it is not split by serum esterase(20). Adams arrived at the same conclusion, although he criticised the term "specificity" (32). He claimed that human erythrocyte cholinesterase catalyses the hydrolysis of a number of non-choline esters, although at a much lower rate than those derived from choline, and does not agree with the opinion of those who stated a qualitative specificity difference between serum and erythrocyte cholinesterases (28, 29).

Adams and co-workers tested plasma cholinesterase against some 40 choline and non-choline esters (33). The plasma enzyme hydrolyses butyrates most rapidly in any series of esters. The "alcohol specificity", however, is similar to that of the erythrocyte enzyme. The more similar the alcohol is to choline the higher the rate of hydrolysis. Only the substitution in the carbon atom adjacent to the ester link makes a real difference for the two types of esterases.

Zeller and Bissegger studied the esterase of human brain(30) and found it to be fundamentally similar to red blood cell esterase.

Both have a substrate optimum are only slightly inhibited by procain, irgamid and isopropyl-antipyrin, while serum cholinesterase is strongly inhibited. These authors distinguish in their work between e-Type and s-Type, giving a model of the active surfaces of the two different kinds of enzymes.

Nachmansohn and Rothenberg studied the specificity of the enzyme in all types of conductive tissues (35). They found that acetylcholinesterases of the brain of the rat and mouse, of the nucleus caudatus of ox and cat, and that of the cortex of ox would all split tributyrin to a small extent, but do not split benzoylcholine. However, acetylcholine, propionylcholine, butyrylcholine, acetyl-β-methylcholine are split in the order given.

From these and other data (24,36,37) one may conclude that the main characteristics of the enzyme found in all conductive tissue are:-

1. a high affinity for acetylcholine;
2. well-defined substrate optimum;
3. decreased rate of hydrolysis with increased length of acyl chain (acetyl > propionyl > butyryl).

Red blood cells contain an acetylcholinesterase with the same properties as that in conductive tissue. The function of acetylcholine, however, in red blood cells is not yet established.

The enzyme of the electric organ of Torpedo, that of the Electrophorus electricus, also the enzymes present in the abdominal chain of lobster and in the ganglion and fibre of

squid show a specificity similar to the conductive tissue (35).

The question naturally arose whether the specificity of a purified enzyme sample would be the same as that of the crude preparation.

Augustinsson compared the crude extract of electric tissue of Electrophorus electricus with a highly purified enzyme preparation of the same origin and found no difference from the point of view of specificity and optimum substrate concentration(38).

Physico-chemical data on the specific acetylcholinesterase were reported early (31,39,40). The isoelectric point of erythrocyte cholinesterase is given as 4.65 - 4.70.

Specific acetylcholinesterase is inactivated by dialysis and reactivated on addition of the dialysate or metal ions (42-46). The enzyme of the electric tissue is inhibited by SH reagents, so presumably has a free SH group at the active centre (42, 47). Barnard has assumed a possible haem nature of cholinesterase(48,49).

The normal substrate of the specific enzyme has a positive electrical charge. This indicates the presence of a negatively charged region on the surface of the enzyme (24). Wilson and Bergmann measured the velocity of acetylcholine hydrolysis as a function of pH and found the optimum between pH 8 and 9 (52). A lower value is also given in the literature in the range of 7.5 - 8.0 (51). The changes in activity by pH changes are due to changes in the protein structure and may be interpreted in terms of the dissociation of acidic and basic groups (52,53). This may be represented schematically as follows:

$$\underset{\text{inactive}}{EH_2} + \overset{H+}{\rightleftharpoons} \underset{\text{active}}{EH} \overset{+OH}{\rightleftharpoons} \underset{\text{inactive}}{E^-} + H_2O$$

where EH_2 and E^- are inactive forms of the enzyme and EH is the active form, being able to form complexes with the substrate. The rate equation is:

$$EH + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EHS \xrightarrow{k_2} \text{products}$$

where S is the substrate and EHS is the active complex.

It appears that the basic group of the enzyme is a direct reactant (54).

The basic group of the enzyme forms a bond with the acyl carbon of the ester and this binding and ionic and dispersion binding at the ionic site constitute the main forces which stabilise the enzyme-substrate complexes (52).

Experiments with different substrates have shown that those substrates with greater electrophilic acyl carbons have a lower Michaelis-Menten constant. K_m values were obtained ranging from 5×10^{-1} to 1.2×10^{-4} using different substrates. Acetylcholine gave a value of 4.5×10^{-4}. The following equation was used to calculate the Michaelis constant for acetylcholinesterase:-

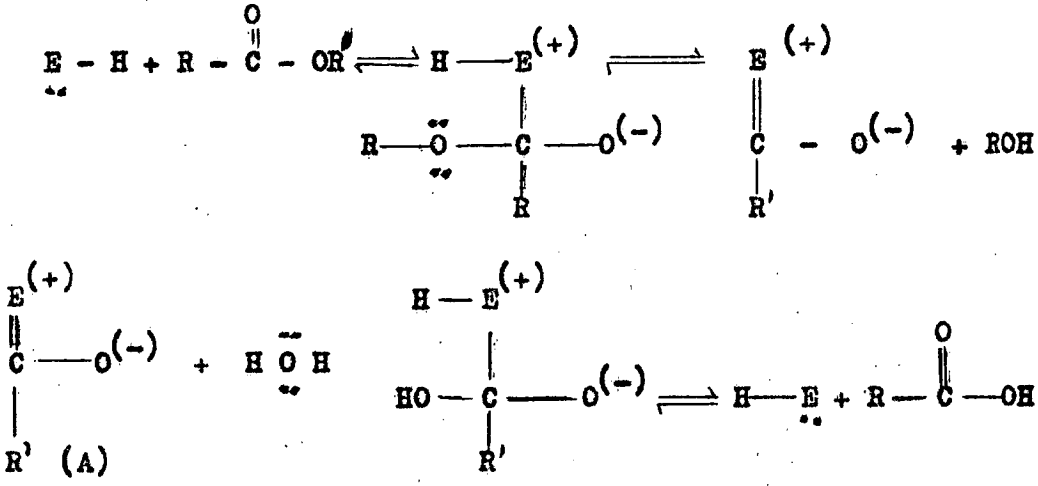
$$K_m = \frac{k_2 + k_3}{k_1} \left[1 + \frac{[H+]}{K_{EH_2}} + \frac{K_{EH}}{[H^+]} \right]$$

where $K_{EH_2}^+$ is the acid dissociation constant of the basic

group and K_{EH} is the dissociation constant of the acid group of the enzyme-substrate complex (55).

The nature of the enzyme-substrate complex having been established, attention was turned towards the hydrolytic process(56).

The mechanism of hydrolysis of an ester (RCO_2R') by the active enzyme (EH) has been formulated as follows:-



Form A is a highly reactive intermediate capable of reacting with nuleophilic reagents. The formation of A is the rate determining step.

Wilson and Cabib have measured the maximum velocities and Michaelis-Menten constants as a function of temperature for acetylcholinesterase and a series of acetyl esters of ethanolamine. The Michaelis-Menten constants do not change with temperature. Linear Arrhenius plots of the maximum velocities were obtained for the poorer substrate, but acetylcholine yielded a smooth curve. These data were interpreted in terms of a two step hydrolytic process involving an acetyl enzyme (57).

enzyme-ester complex ⟶ amino alcohol + acetyl enzyme

acetyl enzyme + H_2O ⟶ acetic acid + enzyme

Krupka and Laidler investigated the influence of pH on the rates of reactions catalyzed by acetylcholinesterase (58). They found the pK values for the ionizing groups in the active centre of the free enzyme to be 6.5 and 9.35. The ionization of these groups is suppressed completely in the Michaelis complex; the complex EHS is formed, but EH_2S and EHS are non existent.

In the acetylated enzyme these ionizations appear again, pK values being 6.27 and 10.03. These results are consistent with the hypothesis that the Michaelis complex involves reaction of the substrate with an imidazole group and an acid group.

As the enzyme was available in a highly purified form it was possible to determine the turnover number. The earliest value was given by Rothenberg and Nachmansohn (75) taking the molecular weight of the enzyme from electric eel as 3×10^6. The turnover number then is approximately 20×10^6 min^{-1}.

Other workers using an irreversible inhibitor (diisopropyl fluorophosphate containing radioactive phosphorus to label the active sites) gave a value in the range of $4.9-7.2 \times 10^5$ per minute (59). A similar value of 3×10^5 min^{-1} was obtained by Cohen and Warringa (60). Wilson and Harrison recently determined the turnover number using dimethylcarbamyl fluoride as inhibitor of acetylcholinesterase. The value obtained at 25°, pH 7.0, acetylcholine concentration 2.5×10^{-3}M is 7.4×10^5 min^{-1} (61)

It is evident that the enzyme is extremely fast as it has been formerly stated by Nachmansohn (62), and this is an absolute requirement to fulfil its role in nerve activity.

D. PURIFICATION OF THE CHOLINESTERASES FROM DIFFERENT SOURCES

Extensive work has been done on the isolation and purification of both the specific and the unspecific enzyme. The purification of the unspecific enzyme from serum seems to be a fairly easy task.

Stedman and co-workers attempted the purification of cholinesterase from serum in 1932 (63). McMeekin prepared highly purified cholinesterase from serum (64). A sample of even higher activity has been obtained by low-temperature alcohol fractionation by Surgenor and Ellis (65). Malmstrom et al. achieved a further purification of the enzyme by chromatography on calcium phosphate and on an anion exchange resin (Dowex 2) with an enrichment about 50 times (66).

Heilbronn obtained a preparation of serum cholinesterase that was able to split 14×10^{-4} moles of acetylcholine/h/mg.dry wt. (67), from a sample previously purified by Strelitz's method (68) using DEAE-Sephadex as ion exchanger.

Serum cholinesterase shows a marked heterogeneity when investigated by electrophoresis in starch gel (69,70): as many as 7 bands of enzymatic activity against choline esters have been demonstrated in human, rat and cat sera. The evidence suggests that these bands derive from distinct molecular species of enzyme.

In order to isolate and purify the specific acetylcholinesterase of the red blood cell, Cohen and Warringa prepared freeze dried powder from haemolysed blood, then extracted it with butanol in the cold (71). Extraction of the dry powder with buffer followed by an elaborate fractionation with sulphate afforded a 368-fold purification of the enzyme.

Zittle et al achieved a 250-fold purification of human red cell acetylcholinesterase (72). The acetylcholinesterase in human red blood cells was extracted from the stromata with Tween 20 (polyoxyethylene sorbitan monolaurate). After a number of steps, preparations dried in the frozen state were freed of Tween by extraction with acetone, then freed of lipid by extraction with n-butanol or ethanol. Electrophoresis on paper, ultracentrifugation, and gradient extraction all furthered the purification of cholinesterase but did not completely purify it. In most cases increase in purification on repetition of the procedure diminishes rapidly at a level of specific activity of 3000 units/mg. protein (73).

Specific acetycholinesterase has been isolated from snake venom (40,41). The enzyme of venom from Naja tripudians and Bungarus fasciatus were purified by successive precipitation with sodium and ammonium sulphate. The degree of purification in the former case was approximately twice that of attained in the latter, but adsorption and elution experiments did not detect any impurity in either of the enzyme preparations.

Work described in this Thesis was directed towards the isolation and purification of the specific acetylcholine splitting enzyme of conductive tissue.

There is a great gap between the results achieved in the isolation and purification of the enzymes from erythrocytes(71-73) or electric tissue (36,47,75-79) on one hand, and that obtained from mammalian brain (81,83,85-87) on the other. Nachmansohn explains this by the difficulty of separating enzymatic protein from lipids present in high concentrations in the brain. At the same time he adduces evidence for the similarity of the enzymes obtained from electric organs and brain; thus the properties of the enzyme may be studied on samples from the more convenient source (24,25).

A fruitful series of studies had been started in 1939 by Nachmansohn and Lederer who obtained a cell from extract from the homogenate of the electric tissue of the Electrophorus electricus by centrifugation. The supernatant contained most of the enzyme activity that was present in the whole organ (47). As the enzyme could be extracted with water further purification of the specific protein by classical methods became possible.

One of the remaining difficulties is that the extracted protein contains a large amount of mucin associated with lipoids(88). Before any substantial purification can be expected the mucin must be removed, e.g. by incubation of the electric organs with toluene at 0° for six weeks (36,75,88).

The mucin-free tissue is fractionated by means of ammonium sulphate (36,75). A great part of the protein other than

cholinesterase may be precipitated by ammonium sulphate concentrations up to 21 per cent; most of the enzyme protein remains in solution and can be precipitated at 27 per cent concentration. Fractionation has to be carried out in several steps in order to avoid great losses of the specific protein and to achieve a high purification level. A slight degree of acidification (pH 5.6-5.9) furthers purification but the enzyme is apparently sensitive to greater pH changes (75). A purification factor of about 400 was achieved in a three-step fractionation: the final solution was able to split 21,000 mg. acetylcholine per mg. protein per hour; the yield was approximately 10%.

A further separation of inactive protein from the enzyme protein was obtained by high speed centrifugation. When a solution previously purified by ammonium sulphate fractionation was spun at 48,000 r.p.m. in the ultracentrifuge the enzyme was found in the pellet. The solution of the pellet had a specific activity of 75,000 mg. acetylcholine split per hour per mg. protein and shown only one component in an analytical ultracentrifuge run.

Although the method described afforded highly purified samples of the specific acetylcholinesterase it is undoubtedly laborious and requires a large amount of starting material. For this reason a simplified procedure was sought by other authors.

In 1959 Lawler devised such a method for the preparation of the enzyme with high specific activity (76). This ammonium sulphate fractionation procedure gives a 200 fold purification with a 20 per cent yield.

It differs from Nachmansohn's method (75) by using as low a pH as 4.2. Inactivation of the enzyme is avoided by keeping the temperature at 2° at low pH values and completing operations in less than thirty minutes at pH 4.2.

A relatively simple method requiring less starting material has been introduced by Hargreaves and co-workers (77,78) for the preparation and purification of acetylcholinesterase from the electric organ of the Electrophorus electricus. The enzyme was precipitated at its isoelectric point (pH 5.1), adsorbed on tricalcium phosphate gel and eluted in a 25 per cent saturated magnesium sulphate solution at pH 6.8 -7.0. Further precipitation with 75 per cent saturated magnesium sulphate, and chromatography on N N' -diethylaminoethyl-cellulose were carried out (79). The enzyme fraction was displaced from the column by 0.5 M sodium chloride solution in 0.05 M Tris buffer. The factor of purification was 200 and the yield 10% (77).

Similarly encouraging results cannot be found in the literature of work done with the mammalian brain; only a small fraction of the enzyme activity can be extracted from the brain in a soluble form by ordinary buffer extraction (80-85). Low ionic strength (83,84) and non ionic detergents such as Lubrol W (cetylalcohol-polyoxyethylene condensate) and Tween 20 have been used to solubilize the enzyme (83,85-87).

The use of butanol at low temperature for the breaking of protein-lipid bonds has been introduced by R.K.Morton (80,81) who obtained a soluble enzyme preparation from dry brain powder in this manner.

A rapid, convenient and inexpensive method for the preparation of a soluble fraction is described by Tauber (87). Washed, frozen pig brain is ground into a paste with sand, stirred with acetate buffer at pH 5.4 containing Tween 21 and toluene. The dialysed filtrate is subsequently treated with bentonite, calcium phosphate and Hyflo suspensions. A clear yellow solution obtained after filtration or centrifugation contains specific, but no unspecific, acetylcholinesterase, however, only a 3-4 fold purification of the original homogenate is achieved.

Ord and Thompson obtained a tenfold purification of the enzyme from rat brain(83). The supernatant of a homogenate, centrifuged at 4000 g was cleared with 1% Lubrol W. The pH was adjusted to 4.5; after removal of the precipitate the supernatant contained 30% of the original cholinesterase activity. Further impurities were removed by adjusting the pH to 8.0 and bringing the solution to 24% saturation with ammonium sulphate. The preparation was filtered, the precipitate discarded, and the filtrate brought to 50% ammonium sulphate saturation. The sediment was soluble in water, and contained 25% of the activity of the homogenate. The factor of purification was 10.

Purification of cholinesterases to the same extent was achieved from soluble microsomal fractions of other kind of mammalian brain (85,86), which were then processed by column electrophoresis (86) or gradient elution (89).

CHAPTER 2: EXPERIMENTAL

A. MATERIALS.

(1) Biological Materials:

Sheep brains were obtained at the slaughter house from freshly killed animals, and were kept at + 1° until used. No attempt has been made to standardize sex or age of the animals.

(2) Chemical Materials:

The non-ionic detergent Lubrol W was purchased from Imperial Chemical Industries.

Analytical grade organic solvents, n-butyl-alcohol and acetone (May & Baker) were employed.

Substrates for true and pseudocholinesterases were acetylcholine bromide and butyrylcholine chloride respectively (Light & Co.).

Salt fractionation procedures were carried out by Analytical Grade ammonium sulphate.(May & Baker).

Apart from common buffers, Tris hydrochloric acid buffer, made up from tris-(hydroxymethyl) - aminomethane (Light & Co.) was used.

Folin & Ciocalteou reagent for protein determination was purchased from B.D.H.

Cellulose derivatives used in gradient elution experiments were an anion exchanger, diethylaminoethylcellulose DE 50 and a cation exchanger, carboxymethylcellulose CM 70 (Whatman).

B. INSTRUMENTS.

Brain tissue was homogenised in a Potter-Elvehjem type homogenizer, (all-round clearance 0.004") manufactured by B. Braun, Melsungen.

The homogenate was dehydrated in an Edwards Freeze-Dryer, Model 10 P.

Centrifugation was carried out generally in a refrigerated centrifuge (MSE 'Superspeed 25'). An angle head rotor was used (35° angle, 107.65 mm, maximum radius).

For ultracentrifugal studies a Spinco Model E ultracentrifuge operated on a schlieren system was employed. Analysis was carried out in a Spinco fixed angle analytical rotor An-D. For preparative purpose a Spinco fixed angle preparative rotor was used (angle of inclination from vertical: 20°, centrifugal force at maximum speed at the middle of the tube: 197,040, maximum speed: 56,100 rpm).

Manometric measurements were carried out in a Warburg apparatus (B.Braun, Melsungen) supplied with a magnetic thermometer to keep the temperature constant within ± 0.01 degree inside the Pyrex waterbath.

In the gradient elution experiments a Towers fraction collector was used.

Spectrophotometric measurements were made with a Hilger & Watts H700 spectrophotometer; complete absorption spectra were taken with the aid of a Perkin-Elmer 4000A Spectracord. A Philips portable pH meter was employed.

C. ANALYTICAL METHODS.

(1) Enzyme Activity was measured by the Warburg manometric method in most cases. The incubation mixture contained 0.1 M NaCl.0.001 M $MgCl_2$ and 0.03 M $NaHCO_3$ in final concentration (85). The final concentration of acetylcholine bromide substrate was 0.01 M. The measurements were carried out at 37°C in a gas atmosphere containing 95% N_2 and 5% CO_2.

Activities of samples at varying pH values were determined either by Hestrin's colorimetric method (91) or by Malmstrom's combined electrometric and colorimetric method (66).

Specific acetylcholinesterase activities are expressed in this Thesis in units of μl CO_2 evolved per mg. protein per hour, unless otherwise stated in the text.

(2) Protein Concentration was measured according to Lowry et al. (92) after calibration with crystalline bovine serum albumin. Insoluble proteins were solubilized according to Aldridge and Johnson (90).

Relative protein concentrations of fractions eluted from ion-exchange columns were determined by measuring the difference in absorption between 280mμ and 260mμ (93).

D. BIOCHEMICAL PROCEDURES

(1) Preparation of sheep-brain powder:

(a) For each experiment four sets of fresh sheep brain were placed in a thermos flask over ice. After about 30 minutes the brains were washed with 0.25M ice-cold sucrose solution, frozen hard in liquid air, then left to thaw at room temperature. When soft they were quickly cut up into small pieces with scissors and homogenized in a blender at $+2^{\circ}$ in about one-third volume of ice cold 0.001M sodium chloride. The homogenate was frozen with liquid air, and dried in the frozen state.

Freeze-drying took about 6 hours for a wet weight of 320g. starting material.

The cream, light powder was collected, weighed and stored in a vacuum desiccator over calcium chloride (dry weight: 16-17% of starting material).

(b) Fresh or deep frozen sheep brain (washed and roughly cleaned) was cut up with scissors and then homogenized in a Potter-Elvehjem type homogenizer with 2X10 volumes of A.R. acetone. The procedure was carried out in a salt-ice bath of a temperature of -15°. The homogenate was rapidly filtered by suction on a Buchner funnel through Whatman No.5 filter-paper. The powder was rinsed with a few

mls of cold acetone and dried under suction. The pink powder was stored in a vacuum desiccator over calcium chloride.

(c) The dehydrated powder (acetone-treated or freeze-dried) was extracted twice with A.R.butanol (10 mls per gram dry weight) at -6° using a blender (10 minutes each). The extracted powder was filtered to dryness by suction, and stored in a vacuum desiccator over calcium chloride. The powder was fine and had an intense reddish brown colour.

(2) Extraction of sheep brain powder with detergent:

Flakes of Lubrol W were dissolved in 0.05 M NaCl to a final concentration of 0.8% and cooled to + 2°. The extraction was carried out using a blender in the cold room. The dehydrated powder was taken up in twenty volumes of the detergent solution and transferred to the blender. The extraction took 5X2 minutes with thirty seconds intervals to avoid excess foaming. The homogenates were kept in the cold for a few hours, decanted from foam, then centrifuged at 54,000 g for 25 minutes in a refrigerated centrifuge.

(3) Ammonium sulphate fractionation of extracts from dry powder:

The Lubrol W extract of the freeze dried powder or freeze dried and butanol treated powder had a protein content in the range of 16-20 mg/ml, and the specific activity varied from 20- to 100 units. The concentration of ammonium sulphate was adjusted carefully to 12%; the

precipitation was carried out with magnetic stirring in a cold room (+ 2°). The precipitate was removed by centrifugation at 54,000 g for 30 minutes in a refrigerated centrifuge. The sediment was firmly packed, colourless and insoluble in water.

The clear, intensely orange-coloured supernatant was brought to 28% ammonium sulphate concentration by addition of the solid under the conditions described before.

The precipitate was left in the cold for 16-24 hours, then collected by centrifugation at 13,000 g for 10 minutes. The supernatant was clear and reddish brown.

The sediment was dissolved in cold distilled water sufficient to make a solution containing 16 mg. protein per ml. The solutions thus obtained were clear and bright yellow. After dialysis against a 0.01M phosphate buffer, pH 7.0 (93a) for 24 hours a small amount of colourless precipitate was formed and removed by centrifugation after dilution of the dialysate approximately five times with distilled water with the buffer described before.

To 200 mls of the clear, yellow supernatant saturated ammonium sulphate solution was added to raise the concentration to 7%. The sample was placed into an ice-bath and the pH was adjusted to 4.1 with 1N sulphuric acid, while magnetic stirring was applied. The precipitate was removed by centrifugation at 54,000 g for 5 minutes and discarded. Care was taken not to keep the preparation at pH 4.1 for more than 30 minutes. During this period the temperature did not rise above + 2°.

The pH of the clear, yellow supernatant solution was adjusted to 6.1 with the dropwise addition of 1N ammonia, while magnetic stirring was applied. If the supernatant remained cloudy after centrifugation the whole procedure was repeated. The solution was brought to 25% concentration of ammonium sulphate by the addition of an estimated volume of the saturated solution of the salt. The precipitate formed was collected after centrifugation at 54,000g for 1 hour and dissolved in the minimum amount of cold water. To this solution saturated ammonium sulphate solution was added to raise the concentration to 14%. The pH was adjusted to 4.2 with 0.3 N sulphuric acid under the same circumstances as mentioned before. The precipitate was removed rapidly by centrifugation at 54,000 g and discarded.

The clear, yellow supernatant was adjusted to pH 7.0 with the addition of 0.3 N ammonia, and saturated ammonium sulphate solution was added to raise the concentration to 21 per cent. The precipitate formed was collected after one hour by centrifugation at 54,000 g , and dissolved in the minimum amount of cold water. The solution was then brought to a 5% ammonium sulphate concentration, the pH was adjusted to 7.0 with phosphate buffer (93/a) (final concentration 0.01M) and stored in the cold (+ 2°).

(4) <u>DEAE cellulose column - chromatography</u>:

56 grams of diethylaminoethylcellulose powder (Whatman Powder DE 50) was washed with KH_2PO_4 on a Buchner funnel until the pH of the washing solution reached 7.0. Washing was continued with about 500 ml. of 0.005M phosphate buffer pH 6.98 (93/a). Finally a slurry of the powder was made up

in 540 ml. of the same buffer.

280 ml. of this slurry was filled into a glass column, that had a diameter of 2.5 cm, and it was 47 cm. long. The column had a B24 Quickfit top with a stopper, that was suitable for gradient elution attachment. Into the outlet of the column an airtight polyethene capillary tubing was fitted. (See Figure A).

The column was filled with the slurry; care was taken to avoid air bubbles. The slurry was left to settle under gravity and was washed with about two column volumes of buffer. Finally a water suction pump was applied to pack the column tight. This assured that the column would not run dry under atmospheric pressure. When the column was packed tight and even it was mounted above a Towers automatic fraction collector in a cold room at 3°. The column was equilibrated in the cold with several column volumes of the above described buffer, during one night. It was ready for loading, unless a different buffer or ionic strength was needed for the experiment. In those cases the column was equilibrated with the buffer in question.

The material to be fractionated on the column was prepared as follows:-

(a) Soluble sheep-brain extract:

10g. freeze-dried powder was extracted with 20 volumes of 0.8% Lubrol W, dissolved in 0.05M sodium chloride in a blender at 3°. The homogenate was centrifuged for 30 minutes at 54,000 g in a refrigerated centrifuge. The pink, turbid supernatant was then dialysed against 5 litre of 0.005M phosphate buffer, pH 6.98 (93/a) for 16 hours. The dialysed supernatant

was freeze-dried. The powder was extracted with A.R.butanol (30 ml. per gram dry weight) repeatedly in a Potter-Elvehjem type homogenizer; the temperature was kept at -15°. The defatted powder was dried in vacuum over calcium chloride and dissolved in a 0.005M phosphate buffer pH 7.0 (93/a). The solution was dialysed against 5 litre of the same buffer for 48 hours in the cold and then centrifuged for 1 hour at 54,000 g. The yellow, crystal clear, supernatant containing 225 mg protein (15mg/ml.) was loaded on the column. After most of the solution had entered the column it was washed in with several mls of the above mentioned buffer.

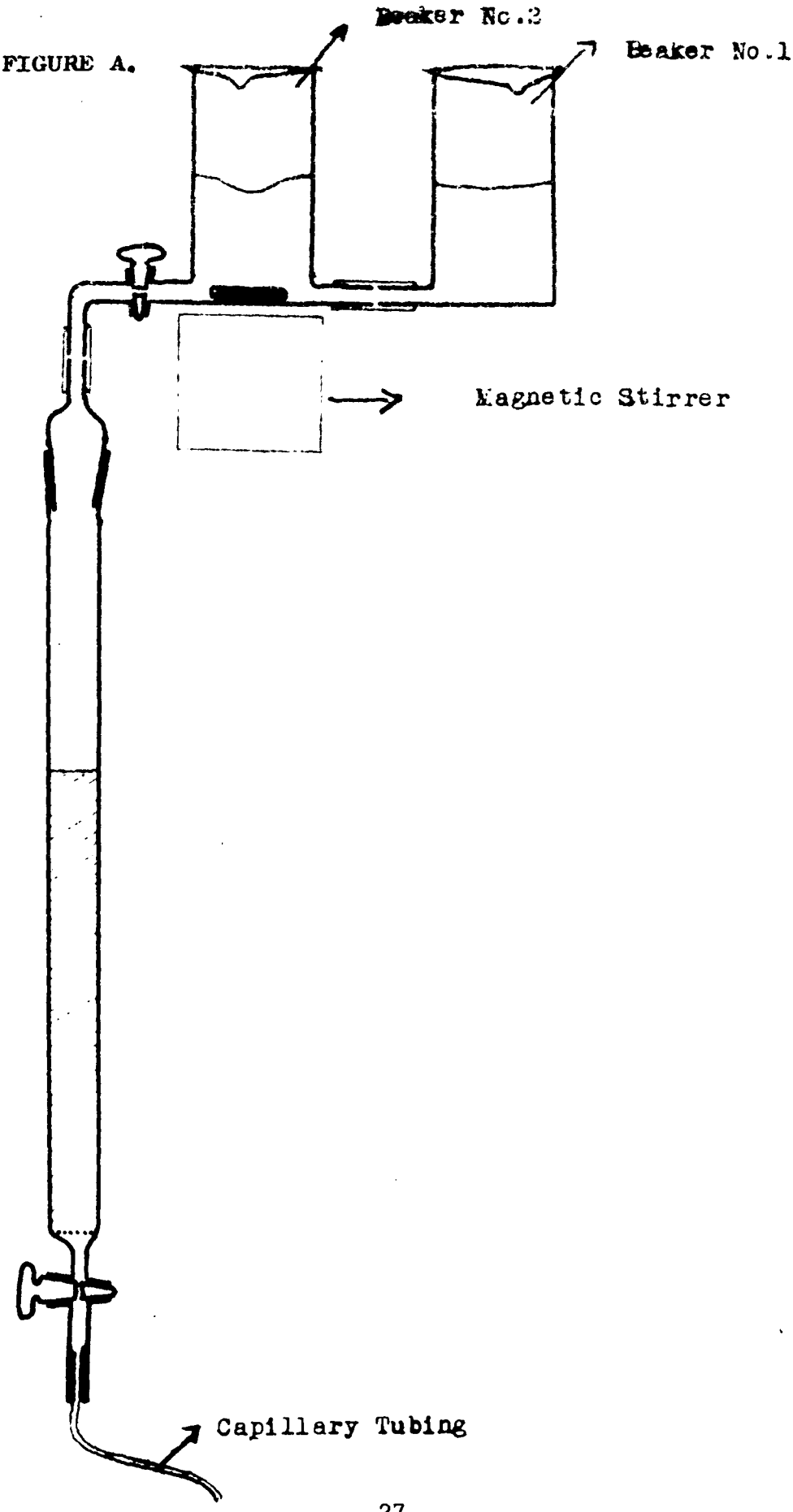
Gradient elution then began. The eluants were placed into two beakers joined together, the solution in the second beaker was stirred constantly with a magnetic stirrer. The next eluant was always put into beaker No.1, when the curve indicated that no more protein is eluted under the actual pH and ionic strength (Figure A).

(b) Purified enzyme samples:

Purified enzyme preparations, resulting from an ammonium sulphate fractionation (specific activity ranging from 100-1000 units) were concentrated with freeze drying. The powder was dissolved in the loading buffer to gain a protein concentration sufficient to carry out a gradient elution experiment. The solution was then dialysed against the same buffer for 24 hours in the cold.

(5) Elution of soluble sheep-brain extract from carboxy-methylcellulose was carried out batchwise in a few orienting tests only.

FIGURE A.



CHAPTER 3: RESULTS AND DISCUSSION

The apparent concentration of acetylcholinesterase (mostly specific) is much lower in brain than in electric tissue. Furthermore the isolation of the enzyme is made more difficult by the presence of lipids which are associated with it (8).

The powder (Chapter 2. Section D.1a) is a stable source of the enzyme, and can be stored indefinitely in vacuum. It can be seen (Figure 1) that the freeze-thaw treatment of the organs, preceding dehydration, resulted in a 40% increase of the total activity of the freeze-dried powder.

The fraction of the total activity that can be extracted with buffer is still very low. Preparation of a dry powder with acetone led to a similar result, and the freeze-drying method is more economical in the case of large scale preparations.

Removal of lipids with butanol may be accomplished by extraction of the aqueous homogenate (80), or the dry powder(81), with the solvent. The second method has been employed in this work. (Chapter 2. Section D.1c).

Most of the lipid is removed from the powder by repeated butanol extraction. This does not affect the total activity of the buffer homogenate of the dry powder, but repeated extraction increases the solubility of the enzyme (Figure 2). It is obvious from the same experiment that the removal of butanol soluble material from the dry brain powder does not satisfactorily increase the solubility of the enzyme in buffer.

As stated in the literature, acetylcholinesterase is bound not only to lipids (8) but is also associated with membrane structures (65,66). Detergent was used to release the enzyme from this association. Ionic detergents have a powerful effect on the membrane structures, but inactivate the enzyme (83). The non-ionic detergent Lubrol W has a solubilizing effect without destroying the enzyme activity (83,85): 80% of the enzyme was found in the supernatant of the buffer extract after centrifugation at 54,000 g. However, other proteins are also mobilised by this method; thus the specific activity of such extracts, with one exception, was low (Table I).

Ammonium sulphate fractionation of the soluble extract gave encouraging results in the case of electric tissue (36, 75, 76), but Toschi had difficulty in purifying brain-microsome acetylcholinesterase in the presence of Lubrol W(85). Thus preliminary experiments with albumin had to be carried out to investigate the effect of non-ionic detergent on the precipitation of protein. No disturbing effect was found except that in the presence of Lubrol W the quantitative determination of protein was impossible by the Folin method. The ammonium sulphate fractionation in almost all cases was carried out according to Lawler's method (76) with slight modifications of the concentration of ammonium sulphate and pH (Chapter 2, Section D 3). The fractionation was successful (Table I). The factor of purification was in the range of 10-50: in one experiment (No.5) it reached 1000, but in this case the yield was very low. (The average specific activity of Lubrol W

homogenates was 30 units). These values are higher than results noted in the literature concerning acetylcholinesterase from brain tissue.

The yield of specific protein was low (Table II). The first ammonium sulphate precipitation step results in a 40% loss of enzymatic activity carried away by the precipitate without purification of the soluble enzyme. Yet this step could not be avoided if one wished to gain a clear solution practically free of detergent.

Purification was most effective in the second and fourth steps, but the adjustment to a low pH had to be made very carefully and still caused a great loss in the total activity (Table II).

The highly active enzyme preparation (35,000 units) lost its activity during storage in the cold, while the activity was retained by similarly stored preparations of lesser purity. (500-1500 units).

Nachmansohn states that the enzymes of electric and brain tissue are identical (8). Yet given sufficient starting material, the candidate did not achieve the same degree of purification in the case of brain acetylcholinesterase as noted in the literature for the electric organ enzyme. There appears to be a factor which places an obstacle in the way of obtaining brain preparations with a high specific activity.

Gradient elution and ultracentrifugal analysis of the soluble extract of the dry powder (Chapter 2. Section D 4a)

by the candidate revealed a complicated pattern (Figures 3,4,5). Absorbancy was measured in 5 ml. aliquots of the effluent in the gradient elution from a DEAE-cellulose column at 280mμ at 260mμ and at 408mμ (Table III). The difference between absorbancy at 280mμ and 260mμ is proportional to the protein concentration. On the other hand the excess of absorbancy at 260mμ over that of 280mμ is due to the presence of nucleic acid components. Absorbancy at 408mμ was measured to follow the distribution of haemoprotein in the effluents. The haemoprotein was present in the original solution.

The plot of difference between absorbancy at 280mu and 260mμ against effluent volume distinctly indicates the presence of at least fourteen peaks (Figure 3). Seven peaks appear with higher absorption at 260mμ than at 280mμ (Figure 4). Acetylcholinesterase activity appeared in the first 35 tubes of the effluent and the peak showing between tube 30 and 35 possessed the highest specific activity: 230 units. This corresponds to a 3.5 fold purification as the specific activity of the solution loaded on the column was 74 units.

At the beginning of the experiment a yellow band appeared on the top of the column and another brown wide band was visible somewhat lower.

Notwithstanding the use of a selective method, the resulting highest specific activity is low. This could be due to the inactivation of the enzyme during the procedure as the recovery of total activity was not satisfactory. Since great care was taken to keep the temperature below 3° during the

experiment and to avoid extreme hydrogen ion concentrations, far-going destruction of the enzyme appears unlikely.

Ultracentrifugal analysis of similar extracts revealed the presence of three components (Figure 5): one of them is recognised as a lipoprotein from flotation instead of sedimentation.

The same methods were used not only for the analysis of whole extracts, but for attempting the further purification of partially purified samples. Such a sample, previously purified by ammonium sulphate fractionation (Table I, Experiment 3) containing 108mg. protein in 13 ml. buffer, was loaded on the column. The absorbancy of the effluent read at 260mμ and at 280mμ are shown in the first two columns of Table IV. Measurements of the absolute protein concentration and specific activities appear in the last two columns. These measurements were carried out in tubes 20-29 only; the others gave values too low for estimation. The narrow yellow band and a wider brown one were again noticed; the latter moved quickly during the gradient elution.

It is apparent from Figure 6 that the enzyme is loosely adsorbed on the DEAE-cellulose at a pH of 7.0. The protein distribution curve shows three smaller peaks and a large heterogeneous one. The latter apparently consist of two components the separation of which has not been completed. The distribution of specific activity confirms this, showing two active components in this area. The less strongly adsorbed is richer in the enzyme. Yet the enzyme in this

component was purified by a mere factor of two to a specific activity of 1000 units.

A better separation of the two active components has been attempted in other experiments. 61 mg. protein with 1070 units specific activity (Table I No.4) was dissolved in 30 ml. of an 0.005 M phosphate buffer, pH 7.6(93/a) and eluted from a column.

The detailed analysis of the effluents are presented in Table V.

As shown in Figure 7, acetycholinesterase activity was eluted together with the first heterogeneous peak in the first 60 ml. of the effluent. The protein distribution shows the presence of three peaks, the better separation of which is desirable but difficult when (as in this case) the original enzyme solution was still loosely adsorbed. The specific activity was highest in the second 15 ml. of the effluent and corresponds only to a factor of purification of 1.5. The activity again resolved into two peaks but measurements of the butyrylcholinesterase activity does not show any specificity difference between the two. Both behave as specific acetylcholinesterase.

The pH of adsorption was raised to 7.85 in the next gradient elution experiment (Table VI). A soluble sheep brain extract was purified by ammonium sulphate fractionation according to Ord (83). This procedure afforded an optically clear, strongly yellow solution with a specific activity of 350 units. 9 ml. of this sample, containing 20 mg. protein, was loaded on the column.

As shown in Figure 8, the heterogeneous peak eluted in the first 15 tubes of the effluent represents a two fold purification in tubes 15 and 14 (Specific activity 640 units). The pigmentation of this function is lower than that of the loosely adsorbed one with a specific activity of 380 units.

The gradient elution technique (88) requires the use of a wide range of pH values and the enzyme is sensitive below pH 5.6 (75). DEAE cellulose chromatography, however, has not been done on acetylcholinesterase when the candidate began the above mentioned elution experiments. In the meantime Hasson eluted acetylcholinesterase of electric organ from DEAE cellulose column (79). This method of elution by variation of the ionic strength of the eluant seemed to be more convenient.

The results of gradient elution carried out by the candidate in such a manner are shown in Table VII.

260 mg. protein in 20 ml. of buffer was adsorbed on a column that was 28 cm. long and had a diameter of 1.5 cm. The specific activity of the loading material was 98 units.

The specific protein was strongly adsorbed on the anion exchanger from an 0.05M Tris-HCl buffer (,82) at a pH of 8.65 (Figure 9). The ionic strength of the eluant was raised in four steps. Seven clearly separated peaks appeared in about 260 ml. of the effluent. Only the last two had enzymatic activity. Components 6 and 7 had specific activities of 68 and 147 units respectively. In spite of the strong adsorption of the original solution and good separation of several peaks

the purification of the enzyme achieved remained disappointingly low (e.g. with a factor of 1.5 in component 7). Readings of the optical density at 408mμ did not show the presence of any eluted pigmented component noted in previous experiments.

In two orienting experiments soluble preparations of sheep brain extracts with a specific activity of 51 units and 75 units respectively were adsorbed on carboxymethylcellulose from a 0.05M phosphate buffer, pH 7.0 and eluted with a 0.02 M phosphate buffer (93/a) at the same pH. These experiments were carried out in batch procedures (Chapter 2. Section D 5). The factor of purification was 3, and the eluted samples had the intense orange colour of the solution adsorbed on the cellulose.

Purification of the specific acetylcholinesterase of the sheep brain by centrifugation at high speed has also been attempted in this work. Figure 10 shows the sedimentation of a soluble sheep brain extract in the separation cell of the ultracentrifuge (top pattern). A sample at the same stage of purification, though containing less protein, was run for reference in a conventional cell (bottom pattern). The samples contained 40 mg.(1) and 24 mg.(2) protein per ml. respectively with a specific activity of 48 units. At least three peaks show up in the sixth and seventh pictures of the top pattern; at the end of the run two of them apparently sedimented into the bottom compartment. The contents of the top and bottom compartment and that of the filter paper were analysed for activity and

protein. Measurable enzyme activity was found in the bottom compartment only but the specific activity remained practically the same as it was in the original sample.

It is clear from Figure 10 that a separation of the enzyme from all the other proteins has not been achieved. Some protein remained in the supernatant, yet no purification occurred.

The analysis was repeated under slightly altered conditions (Figure 11). This time the separation cell and the conventional cell were filled with sample (1). The pattern reveals three boundaries (bottom pattern 4 and 5) only one of which is expected to be found in the supernatant at the end of the run (top pattern5). The contents of the top and bottom compartments and that of the filter paper were analysed for activity and protein. 90% of the activity was found in the bottom compartment. Together with the activity, however, 80% of the protein also sedimented. The factor of purification was only 1.2, corresponding to a specific activity of 57 units in the bottom compartment of the separation cell.

Highly purified enzyme preparations resulted from ammonium sulphate fractionation (Table I, Experiments 5 and 6) have also been analysed in the ultracentrifuge (Figures 12,13). The sedimentation pattern of the sample with a specific activity of 15,000 units is shown on the top pattern of Figure 12 and that of the further purified sample , with a specific activity of 35,000 units on the bottom patterns.

Both samples with specific activities of 15,000 and 35,000 units respectively emerged with a broad boundary in the cell. Yet

this level of specific activity is far from those reported by authors working with electric organ (36,75-77).

The limit of the purification of the acetylcholine splitting enzyme from the brain tissue is too sharply defined to be ascribed to large amounts of lipids only.

The binding of the enzyme to membrane structures (85,86) does explain some of the difficulties of the isolation. Once the structure is destroyed by detergent, separation of the enzyme from the protein present in the membrane should be possible, employing the usual techniques of separation, unless some special protein-protein, or protein-protein-lipid bond exists and survives the detergent treatment.

Research workers dealing with tissues other than electric organ, encountered similar limitations. Zittle and co-workers(72) noted that in the purification of erythrocyte acetylcholinesterases in most cases increase in specific activity on repetition of the procedure diminished rapidly at certain level. Recently an article was published by Lawler (74) on preparation of soluble brain acetylcholinesterase. The specific activity described in her work is lower than achieved by the candidate. These limitation in purification apparently coincides with the separation of a major protein with which the cholinesterase is tenaciously associated.

Chowdhury, in the case of cholinesterase of Bungarus fasciatus, refers to certain associated proteins which make purification of the enzyme difficult (40,41).

As acetylcholinesterase is associated with membrane structures (85,86), other proteins with affinity to membranes appeared to deserve interest. In the last ten years Green and

his school carried out brilliant work on the structure of mitochondrial membrane (99-109). Acetylcholinesterase is present in both mitochondria and microsomes (85,90).

As the next step towards the elucidation of the problem that arose from the first part of this work and which had confronted other authors quoted, the attachment of acetylcholinesterase to the mitochondria and microsomes of the sheep-brain cell and the fine structure of these cell fractions should be studied.

The second part of this Thesis is concerned with proteins that may be associated with acetylcholinesterases in the cells of sheep brain.

FIGURE 1. Effect of freeze-thaw treatment on the enzyme activity of a freeze-dried powder.

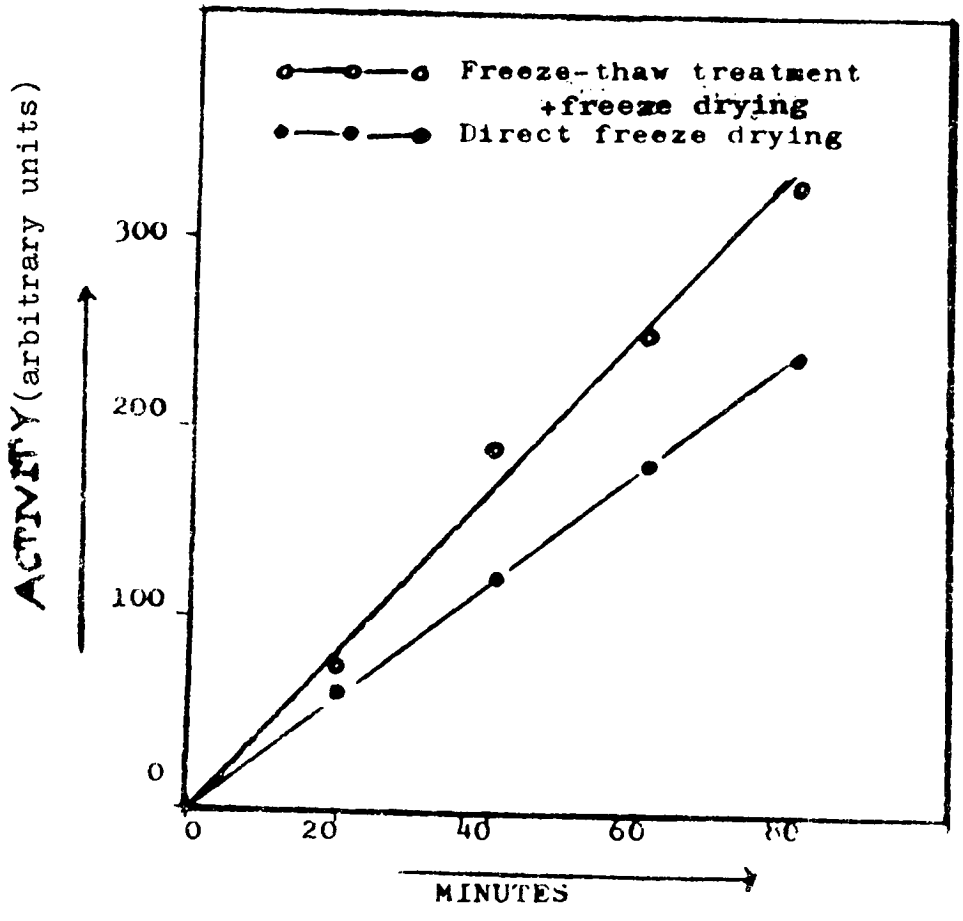
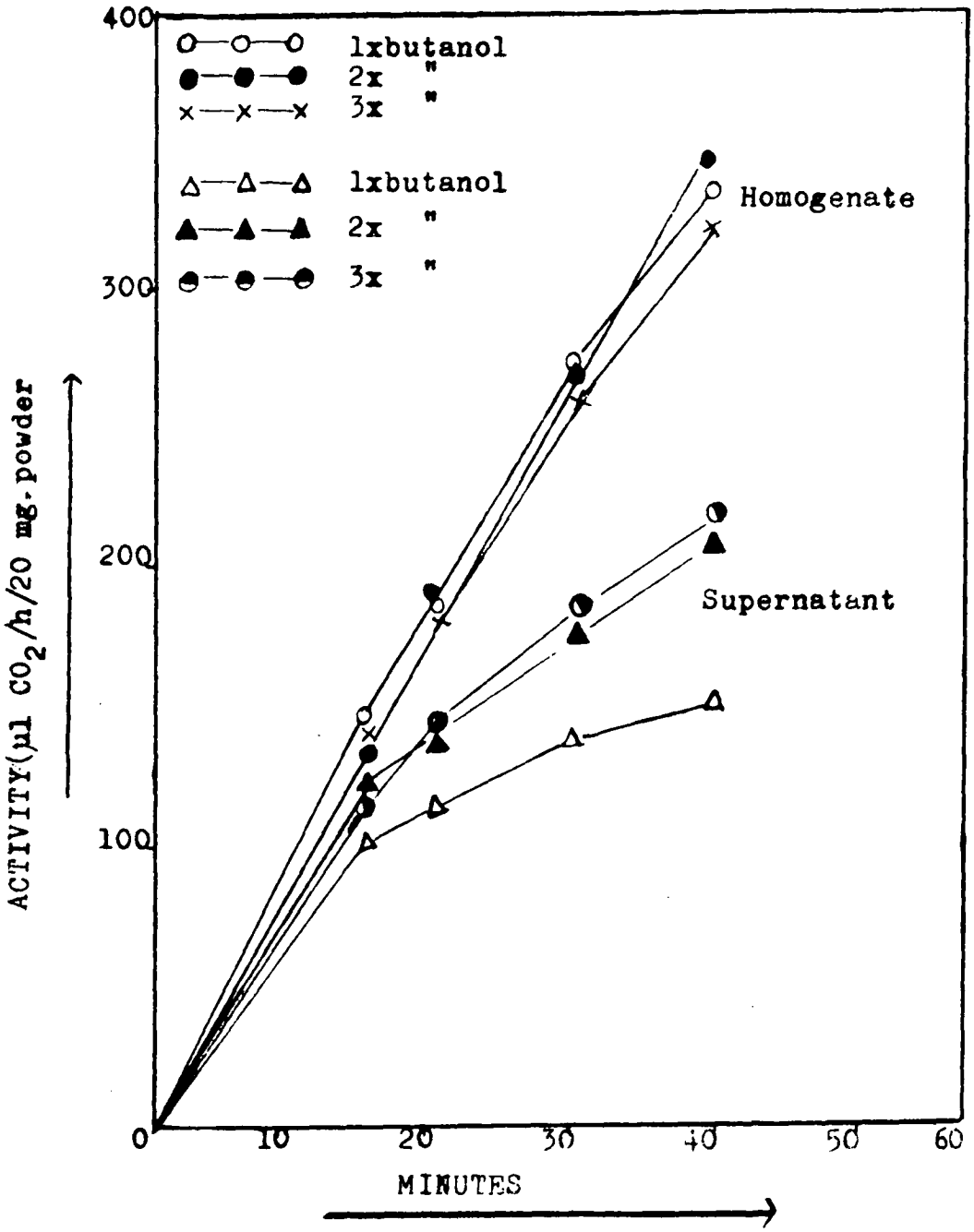


FIGURE 2.Effect of butanol extraction on a freeze-dried powder.



The homogenates were centrifuged at 54,000 g in a refrigerated centrifuge.

T A B L E I

Summary of ammonium sulphate fractionation experiments.

Specific activities in units of $\mu l CO_2$ evolved per mg. protein per hour.

No.of Experiments	Lubrol W Extract	1st ammonium sulphate supernatant	2nd ammonium sulphate sediment	3rd ammonium sulphate sediment	4th ammonium sulphate sediment
1	24	33	47	49	82
2	40	37	70	90	260
3	44	39	96	134	480
4	53	43	121	460	1070
5	53	44	136	15000	35000
6	55	50	183	-	1440
7	65	60	206	-	-
8	80	77	220	-	-
9	95	84	320	-	-
10	470	400	475	-	-

T A B L E II

Specific activity and yield in a typical ammonium sulphate fractionation experiment.

Sample	Specific activity (units)	% of original activity (total)
Lubrol W extract	48	100
First ammonium sulphate supernatant	48.5	60
Second ammonium sulphate sediment.	320	55
Third ammonium sulphate sediment	-	21
Fourth ammonium sulphate sediment	480	7

T A B L E III

Gradient elution analysis of solubilized sheep brain extract.

Tube No.	Abs. 280mμ	Abs. 260mμ	Abs. 408mμ	Abs. 280-260 mμ	Tube No	Abs. 280mμ	Abs. 260mμ	Abs. 408mμ	Abs. 280-260 mμ
1	0.027				15	0.023	0.027	0.000	0
2	0.037				16	0.012	0.008	0.000	0.004
3	0.023				17	0.026	0.110	0.085	0.016
4	0.013				18	0.267	0.271	0.310	0
5	0.005				19	0.680	0.470	0.570	0.210
6	0.004				20	0.785	0.581	0.660	0.204
7	0.000				21	0.665	0.503	0.531	0.162
8	0.000				22	0.441	0.295	0.332	0.146
9	0.000				23	0.252	0.205	0.162	0.047
10	0.000	0.003		0	24	0.167	0.140	0.087	0.027
11	.220	0.825		0	25	0.195	0.000		0.195
12	0.095	0.143		0	26	0.086	0.000	0.000	0.195
13	0.095	0.020		0.015	27	0.020	0.000		0.020
14	0.064	0.087		0	28	0.090	0.000		0.090

T A B L E III (Continued)

Tube No.	Abs. 280mμ	Abs. 260mμ	Abs. 408mμ	Abs. 280-260 mμ	Tube No.	Abs. 280mμ	Abs. 260mμ	Abs. 408mμ	Abs. 280-260 mμ
29	0.000				43	0.000			
30	0.027				44	0.000			
31	0.035	0.010		0.020	45	0.005			
32	0.047	0.030		0.010	46	0.080			
33	0.052	0.021		0.030	47	0.020			
34	0.020	0.043	0	0	48	0.010			
35	0.022	0.076	0	0	49	0.070			
36	0.200	0.300			50	0.090			
37	0.153	0.175			52	0.027			
38	0.071	0.080			54	0.000	0.040		
39	0.076	0.063		0.010	56				
40	0.073	0.092	0.000		58	0.110	0.023		0.087
41	0.032	0.080	0.000		60	0.087	0.037		0.050
42	0.032	0.072	0.000		62	0.000	0.033		0

T A B L E III (Continued)

Tube No.	Abs. 280mμ	Abs. 260mμ	Abs. 280-260 mμ	Tube No.	Abs. 280mμ	Abs. 260mμ	Abs. 280-260 mμ
64	0.000	0.021		92	0.055	0.060	
66	0.003	0.020		94	0.027	0.030	
68	0.025			96	0.097	0.145	
70	0.002			98	0.140	0.113	0.027
72	0.052			100	0.167	0.143	0.021
74	0.000			102	0.306	0.232	0.074
76	0.000			104	0.144	0.130	0.014
78	0.010			106	0.211	0.195	0.116
80	0.040			108	0.223	0.201	0.022
82	0.055			110	0.145	0.113	0.032
84	0.077			112	0.210	0.130	0.080
86	0.113	0.099	0.033	114	0.251	0.198	0.053
88	0.165	0.127	0.038	116	0.055	0.092	
90	0.047	0.085		118			

T A B L E III (Continued)

Tube No.	Abs. 280mμ	Abs. 260mμ	Abs. 280-260 mμ	Tube No.	Abs. 280mμ	Abs. 260mμ	Abs. 280-260 mμ
120	0.065	0.087	0	148	0.214	0.138	0.076
122	0.137	0.110	0.027	150	0.231	0.142	0.089
124	0.132	0.097	0.035	152	0.245	0.150	0.095
126	0.125	0.095	0.030	154	0.251	0.170	0.081
128	0.080		0.036	156	0.295	0.155	0.140
130	0.067	0.100	0.040	158	0.265	0.180	0.085
132	0.103	0.100	0.003	160	0.267	0.200	0.067
134	0.010			162	0.320	0.145	0.175
136	0.000			164	0.285	0.160	0.125
138	0.117	0.095	0.022	166	0.250	0.135	0.115
140	0.137	0.107	0.030	168	-	-	-
142	0.175	0.120	0.055	170	-	-	-
144	0.227	0.150	0.077	172	0.197	0.150	0.147
146	0.266	0.175	0.091	174	0.220	0.150	0.070
				176	0.235	0.146	0.089

FIGURE 3. DISTRIBUTION OF PROTEIN IN THE EFFLUENTS OF THE GRADIENT ELUTION, PRESENTED IN TABLE III.

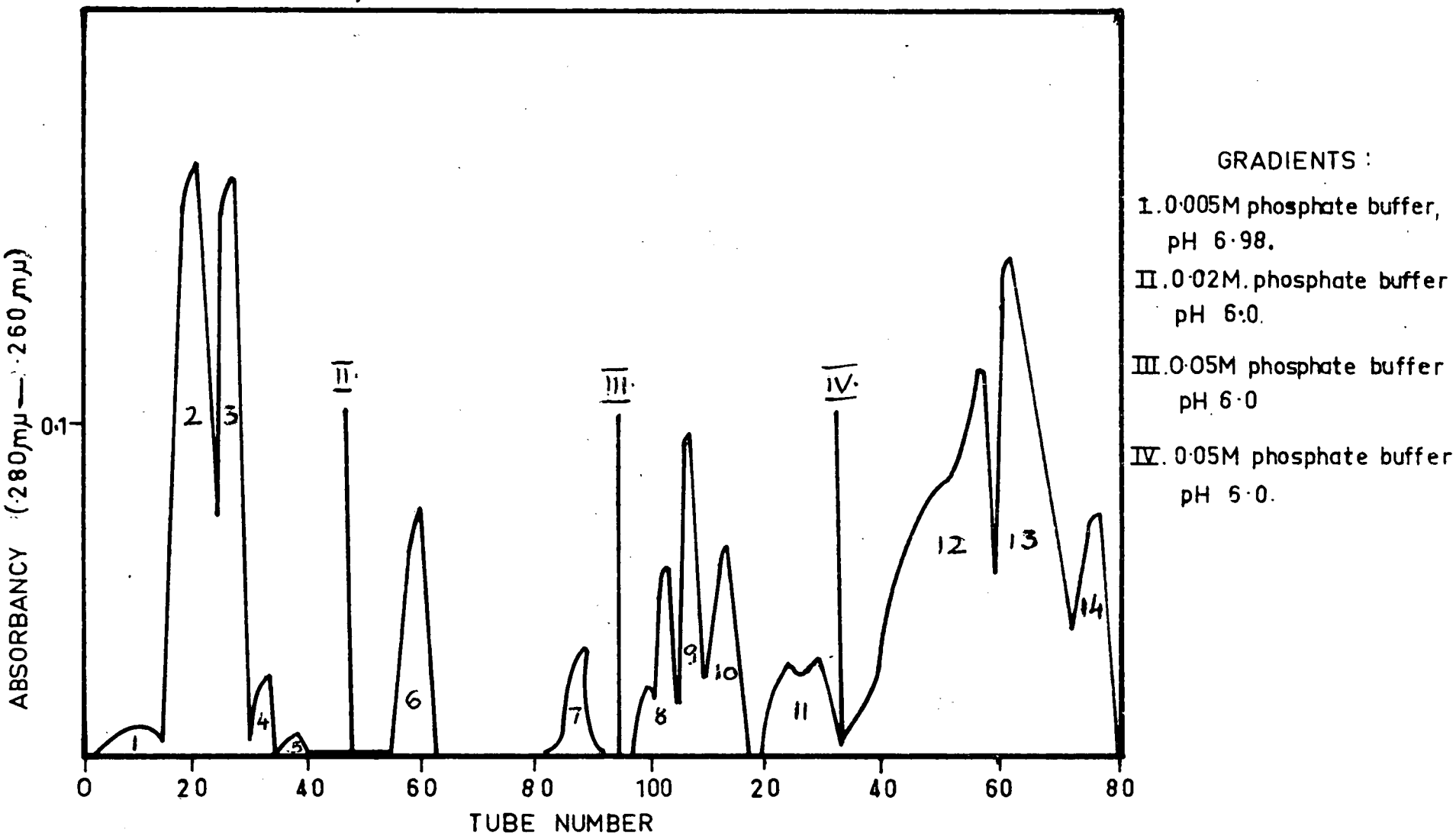


FIGURE 4. Distribution of nucleic acid in the effluents of the gradient elution; presented in TABLE III.

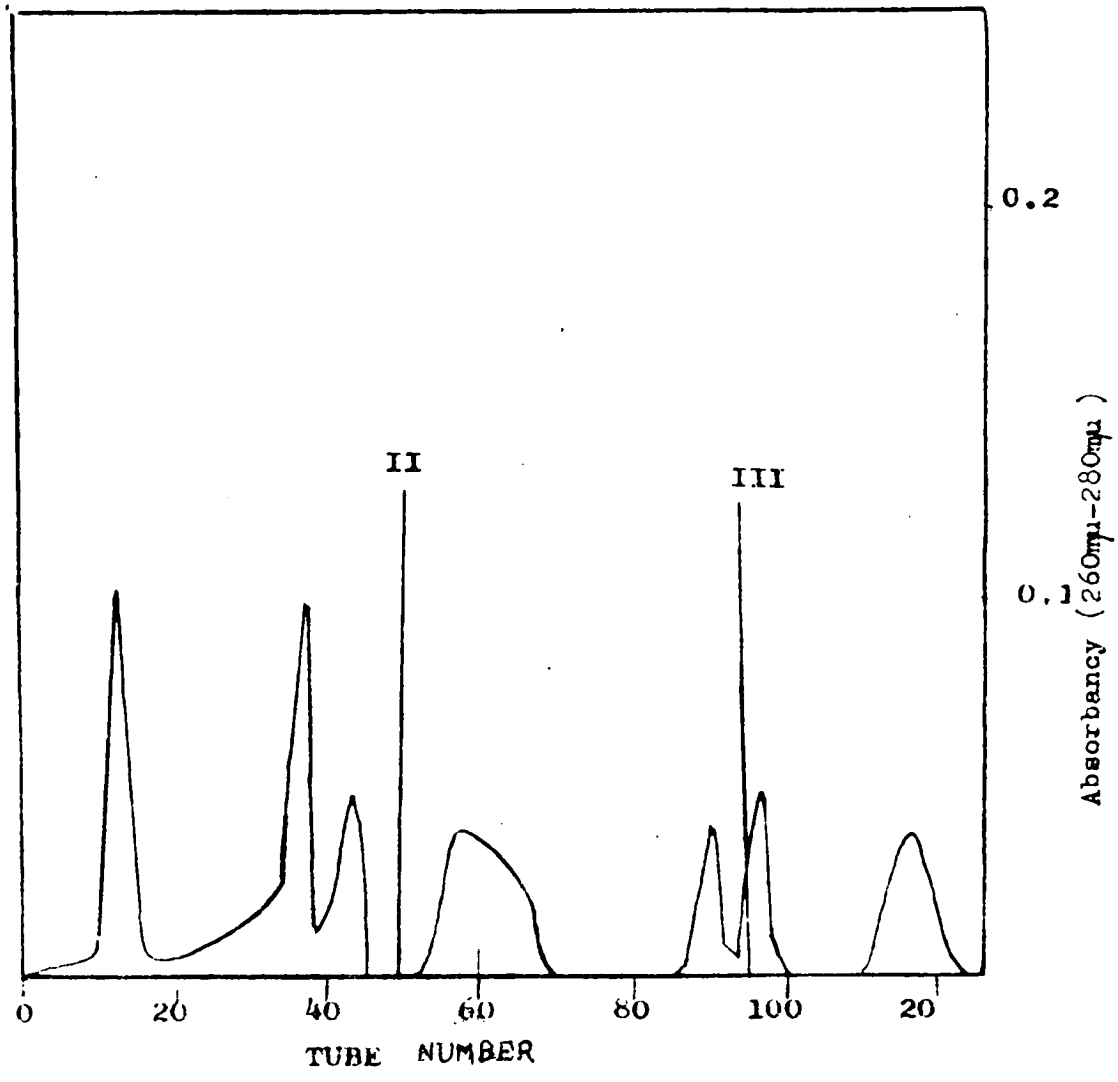
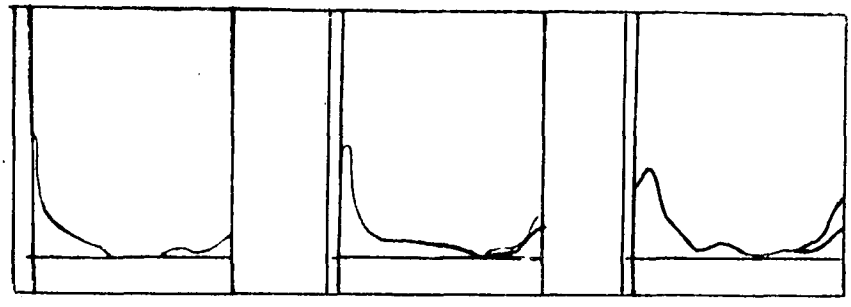
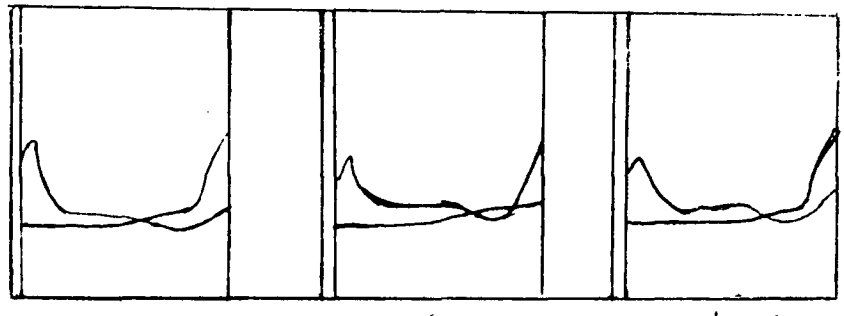


FIGURE 5.Typical ultracentrifugal pattern of a soluble sheep-brain extract.(see Table III)



24 min 32 min 40 min



48 min 56 min 64 min

The ultracentrifugal speed was 39,500 r.p.m. and a double sector cell was employed.

Photographs were taken in 8 minute intervals after the two third of the speed was reached.

Diaphragm angle:60°

T A B L E IV

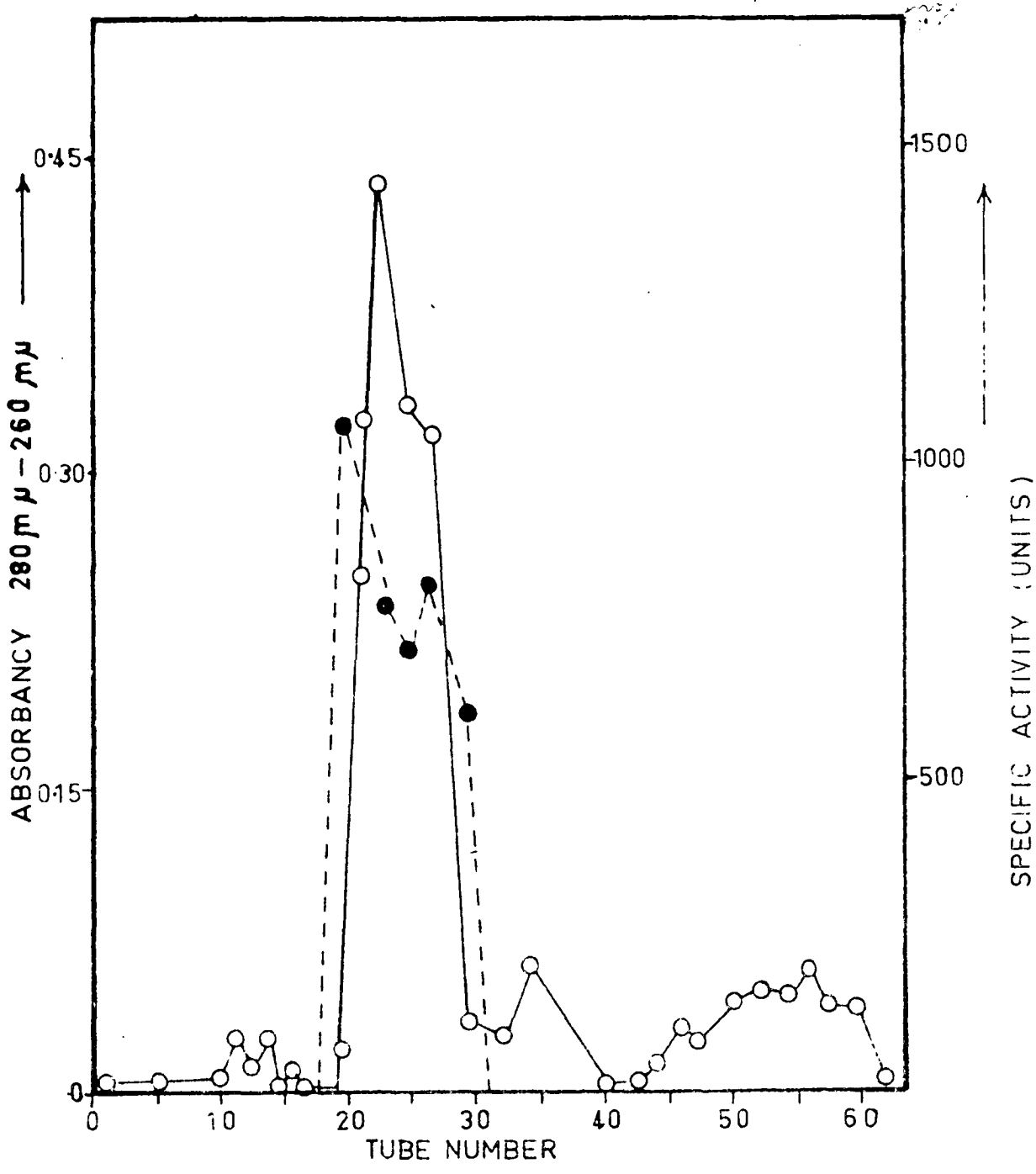
Gradient elution analysis of a partially purified sheep brain extract (Chapter 1, Section D 4.b)

Tube No.	Volume	Absorbancy 260mμ	Absorbancy 280mμ	Protein mg/ml	Specific activity
1-4	16ml.	0.000	0.000		
4-8	6ml.	0.000	0.000		
8-10	"	0.000	0.000		
11	"	0.027	0.058		
12	3.6ml.	0.000	0.015		
13	3.6ml.	0.065	0.094		
14	4.5ml.	0.070	0.000		
15	3.7ml.	0.077	0.090		
16	3.7ml.	0.000	0.000		
17	2.3ml.	0.015	0.000		
18	4ml.	0.052	0.028		
19	2.7ml.	0.250	0.272		
20	3.4ml.	2.620	2.860	0.175	1080
21	2.5ml.	"	"	0.060	"
22	2.9ml.			0.195	770
23	"			0.075	"
24	3.1ml.	3.86	4.40	0.155	700
25	2.9ml.	2.86	3.28	0.290	700
26	3.1ml.	1.45	1.85	0.105	800
27	3ml.	"	-	0.065	800
28	2.9ml.	0.790	0.84	0.200	600
29	3ml.	"	"	0.160	600

T A B L E IV (Continued)

Tube No.	Volume	Absorbancy 260mμ	Absorbancy 280mμ
30	3ml.	-	-
31	"	0.360	0.386
32	"	"	"
33	2.8ml.	0.140	0.207
34	1.0 "	"	"
35	2.9 "	0.146	0.220
36	3.0 "	"	"
37	2.8 "	0.247	0.280
38	3.0 "	"	"
39	5.5 "	0.243	0.296
40	"	"	"
41	5.7 "	0.254	0.20
42	5.7 "	0.254	0.200
43	5.5 "	0.042	0.057
44	"	"	"
46	5.5 "	0.016	0.045
48	6.0 "	0.023	0.048
50	5.80ml.	0.157	0.202
52	6.0 "	0.177	0.224
54	5.80 "	0.170	0.220
56	5.90 "	0.144	0.204
58	5.5 "	0.150	0.197
60	2.6 "	0.305	0.347

FIGURE 6. DISTRIBUTION OF PROTEIN AND ENZYME ACTIVITY IN THE EFFLUENTS OF THE GRADIENT ELUTION PRESENTED IN TABLE IV.



GRADIENT ELUTION I. 250 mls OF 0·005 M PO_4 BUFFER pH 7·08

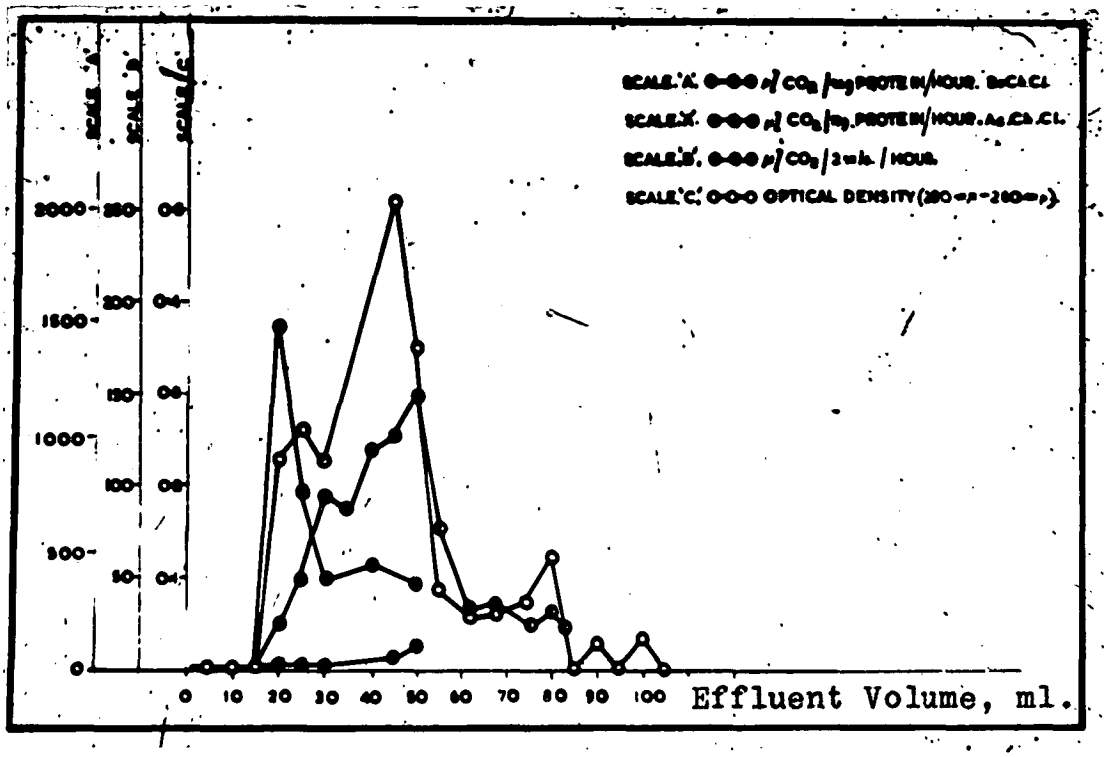
II 500 " " " " " pH 3·00

T A B L E V

Analysis of the effluent from a gradient elution of purified acetylcholinesterase.

Effluent Volume	Absorbancy 260mμ	Absorbancy 280mμ	Acetylcholin-esterase activity $\mu l CO_2$/2ml. hour	Butycylchol-inesterase activity $\mu l CO_2$/2ml. hour
5ml.	0.032	0.020		
"	0.000	0.030		
"	0.004	0.020		
"	0.292	0.350	900	0
"	0.510	0.615	1050	0
"	0.609	0.800	1000	0
"	0.716	0.900		0
3ml.	0.746	0.985		0
"	1.620	1.860	2000	60
"	0.950	1.250	1400	100
6ml.	0.124	0.256	400	0
"	0.120	0.177	280	0
"	0.112	0.177	300	
"	0.055	0.131	200	
"	0.030	0.157	260	
"	0.358	0.325	200	
"	0.150	0.143		
"	0.075	0.110		
"	0.000	0.000		
"	0.148	0.185		
"	0.000	0.000		
"	-	-		

FIGURE 7. Distribution of protein and of acetylcholinesterase and butyrylcholinesterase activity in the effluent of gradient elution of Table V.



Gradient elution: I. 0.005 M phosphate buffer, pH 7.6

II. 0.005 M phosphate buffer, pH 7.1

T A B L E VI

Absorbancy measurements in the effluent of a gradient elution of a purified sheep brain acetylcholinesterase from DEAE cellulose column.

Tube No.	Absorbancy 280mμ	Absorbancy 260mμ	Absorbancy 280mμ-260mμ	Absorbancy 408mμ
2	0.012	0.030		
4	0.006	0.023		
6	0.000	0.020		
8	0.017	0.038		0
10	0.215	0.211	0.014	0.019
2	0.360	0.330	0.033	0.043
3	0.153	0.097	0.056	0.030
4	0.137	0.075	0.062	0.032
5	0.070	0.066	0.004	0.000
6	0.030	0.075		
7	0.019	0.019		
8	0.000	0		
9	0.043	0.043		
20	0.051	0.051		
1	0.060	0.060		
2	0.037	0.037		
3	0.050	0.050		
4	0.030	0.030		
5	0.047	0.047		
6	0.023	0.023		
7	0.010	0.010		
8	0.030	0.030		
9	0.067	0.066		
30	0.087	0.066		

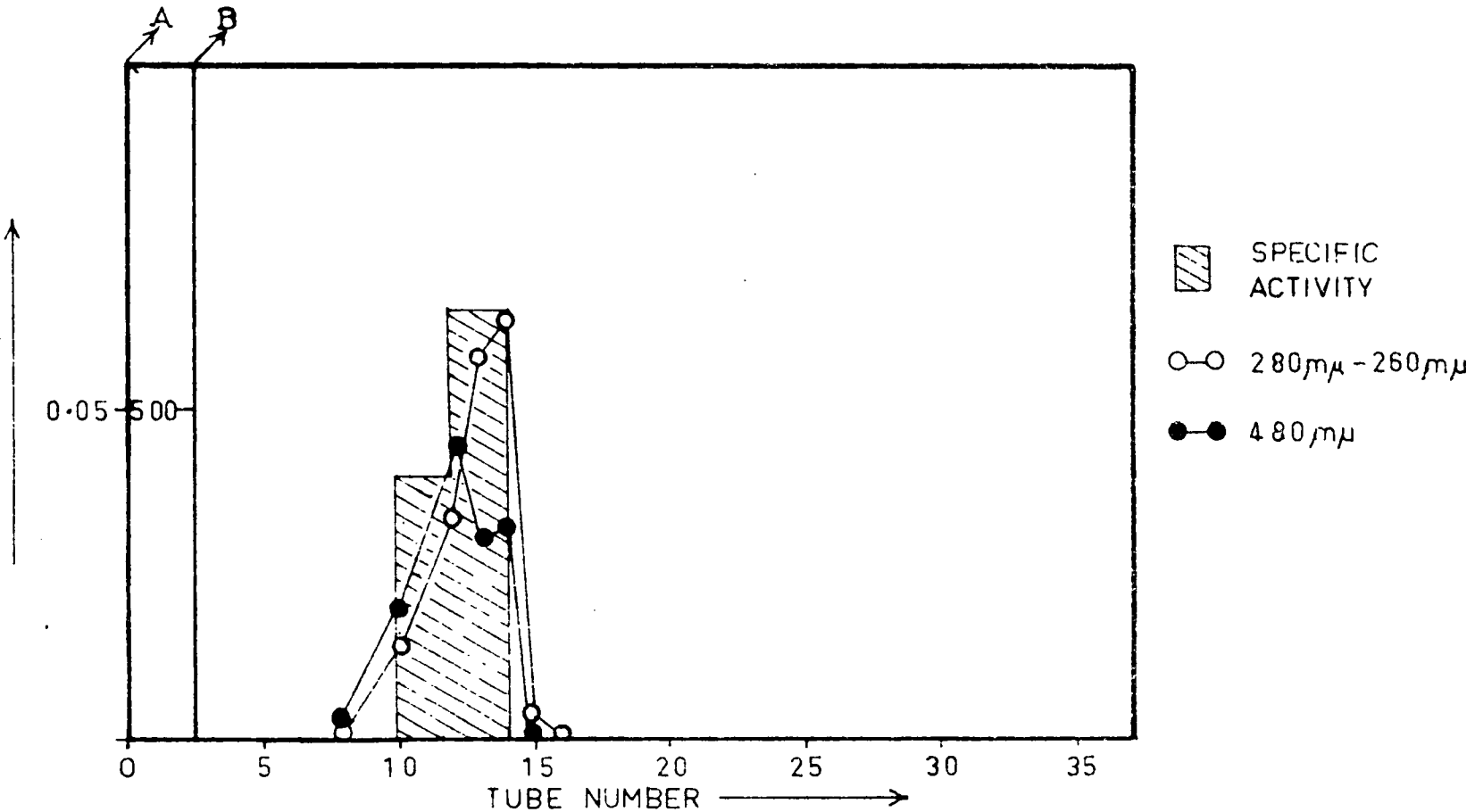
T A B L E VI (Continued)

Tube No	Absorbancy 280mμ	Absorbancy 260mμ	Absorbancy 280mμ-260mμ	Absorbancy 408mμ
4	0.010	0.033		
6	0.010	0.033		
8	0.025	0.061		
40	0.000	0.026		
2	0.013	0.057		
4	0.040	0.045		
6	0.000	0.010		
8	0.000	0.017		
50	-	-		
2	0.020	0.026		
4	0.027	0.033		
6	0.015	0.040		
8	0.080	0.066	0.014	
60	0.141	0.127	0.014	
2	0.152	0.145	0.007	
4	0.015	0.003	0.012	
6	0.022	0.010	0.012	
8	0.025	0.015	0.010	
70	-	-	-	
2	0.021	0.012	0.009	
6	0.022	0.013	0.011	
80	0.022	0.005	0.017	

T A B L E VI (Continued)

Tube No.	Absorbancy 280mμ	Absorbancy 260mμ	Absorbancy 280mμ-260mμ
84	0.016	0.011	0.005
86	-	-	-
88	0.026	0.017	0.009
92	0.020	0.015	0.005
96	0.012	0.004	0.008
100	0.017	0.008	0.009

FIGURE 8. DISTRIBUTION OF PROTEIN, PIGMENT & SPECIFIC ACTIVITY OF GRADIENT ELUTION OF TABLE VI.



GRADIENT ELUTION: I 0·005 M PO_4 pH 7·85
II 0·005 " " pH 6·98.
III 0·02 " " pH 6·00

SCALE A: ABSORBANCY

SCALE B: μlCO_2/mg. PROTEIN PER HOUR

T A B L E VII

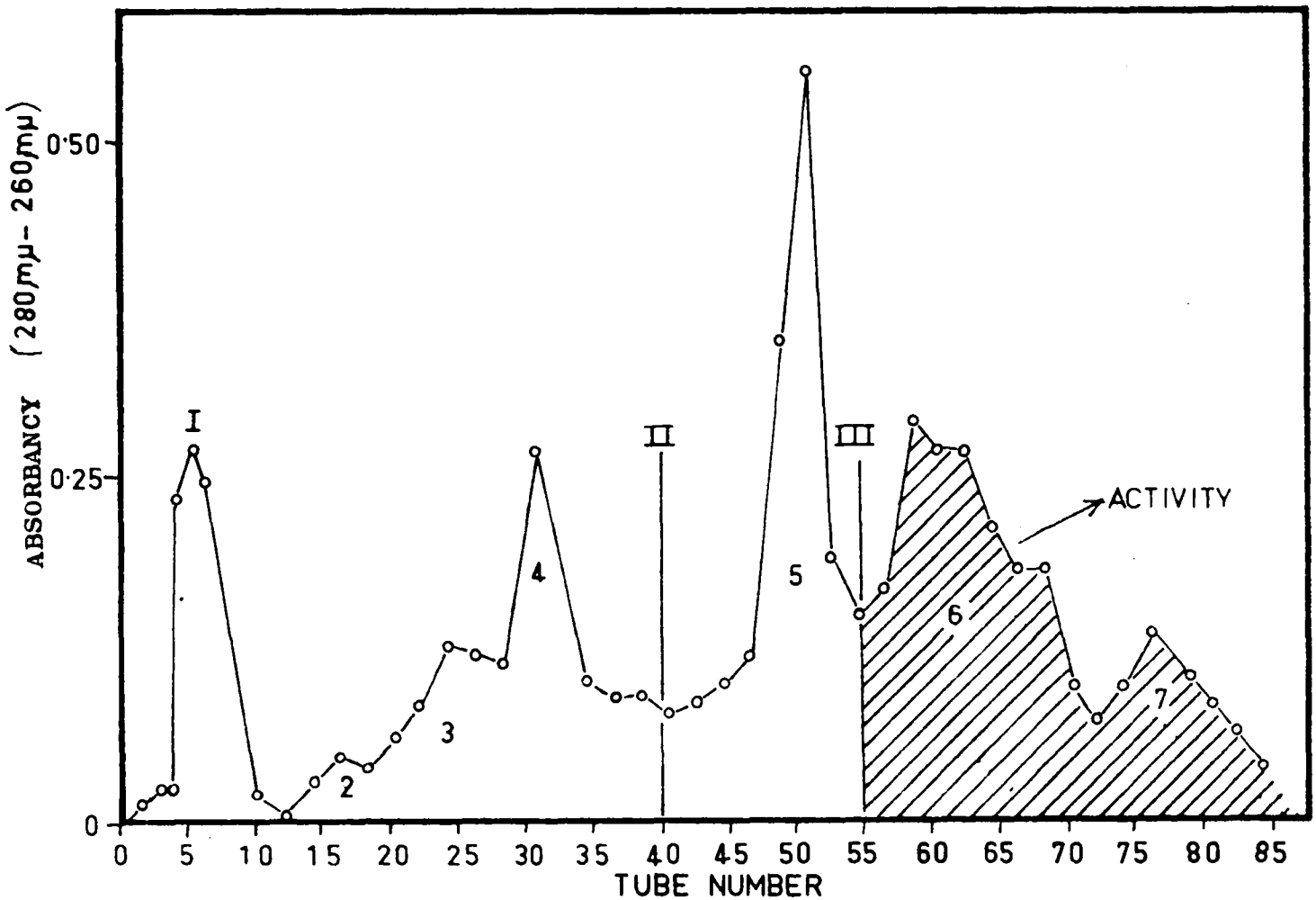
Absorbancy measurements in aliquots of the effluent of gradient elution of a soluble sheep brain extract from DEAE cellulose column.

Tube No.	Absorbancy 280mμ	Absorbancy 260mμ	Absorbancy 408mμ
12	0.160	0.152	0.000
14	0.230	0.205	0.005
16	0.280	0.235	0.027
18	0.336	0.292	0.005
20	0.445	0.386	0.014
22	0.455	0.370	0.010
24	0.495	0.365	0.014
26	0.425	0.300	0.012
28	0.375	0.262	0.014
30	0.530	0.255	0.014
32	0.260	0.295	0.014
34	0.335	0.232	0.014
36	0.285	0.195	0.014
38	0.270	0.180	0.014
40	0.266	0.185	0.023
42	0.240	0.155	0.015
44	0.275	0.172	0.015
46	0.275	0.150	0.010
48	0.630	0.275	0.022
50	0.900	0.340	0.026
52	0.565	0.370	0.030
54	0.630	0.480	0.047
56	0.590	0.420	0.030

T A B L E VII (Continued)

Tube No.	Absorbancy 280mμ	Absorbancy 260mμ	Absorbancy 408mμ
58	0.635	0.335	0.026
60	0.655	0.380	0.030
62	0.652	0.380	0.030
64	0.530	0.310	0.027
66	0.495	0.300	0.020
68	0.485	0.295	0.013
70	0.362	0.263	0.017
72	0.326	0.250	0.013
74	0.355	0.250	0.010
76	0.322	0.182	0.030
78	0.295	0.185	0.010
80	0.272	0.190	0.015
82	0.210	0.140	0.025
84	0.225	0.182	0.037

FIGURE 9. DISTRIBUTION OF PROTEIN & ACETYLCHOLINESTERASE ACTIVITY OF THE GRADIENT ELUTION EXPERIMENT OF TABLE VII.

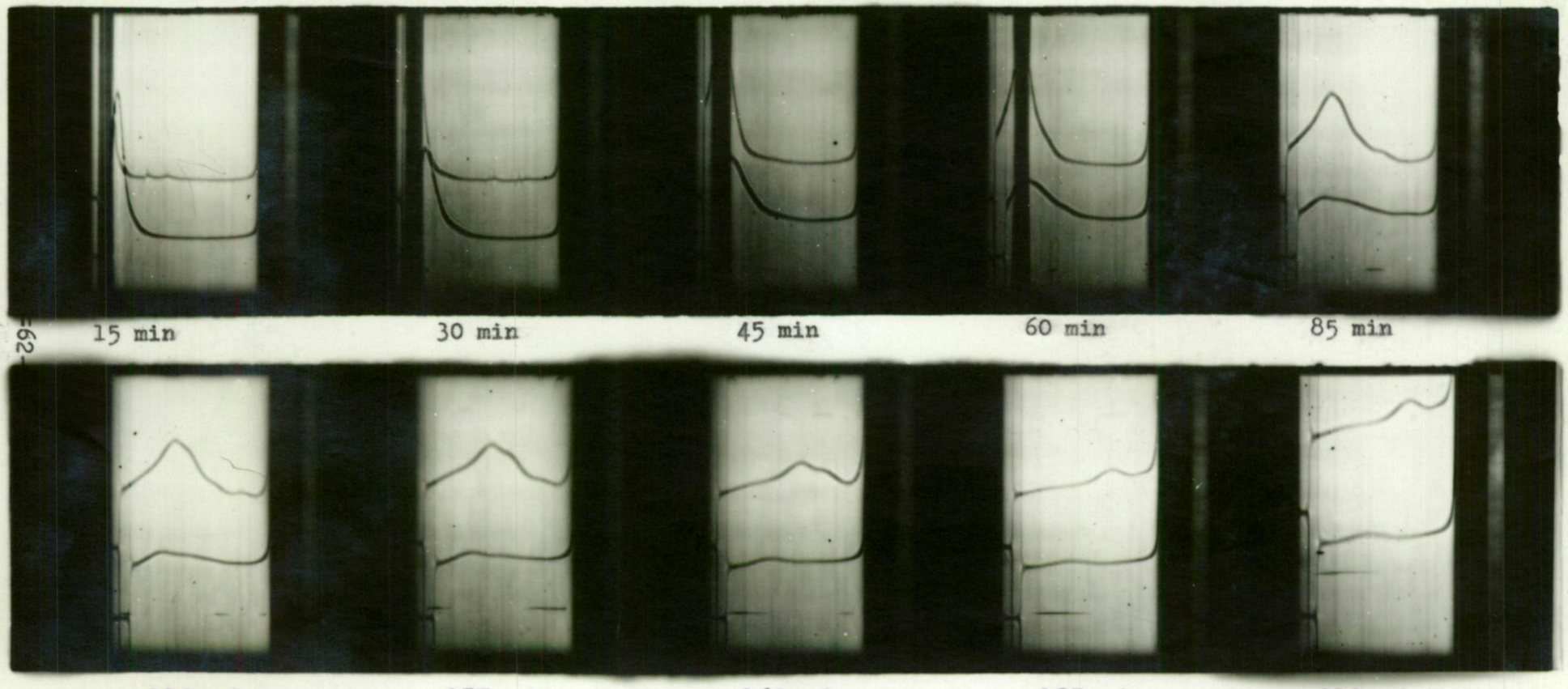


GRADIENT ELUTION: I. TRIS-HCl BUFFER, 0·05 M, pH=8·65, · · 0·1 N. SODIUM CHLORIDE

II " " " " " " 0·2 N " "

III " " " " " " 0·4 N " "

FIGURE 10. Ultracentrifugal analysis of a soluble sheep brain extract in a separation cell. I.

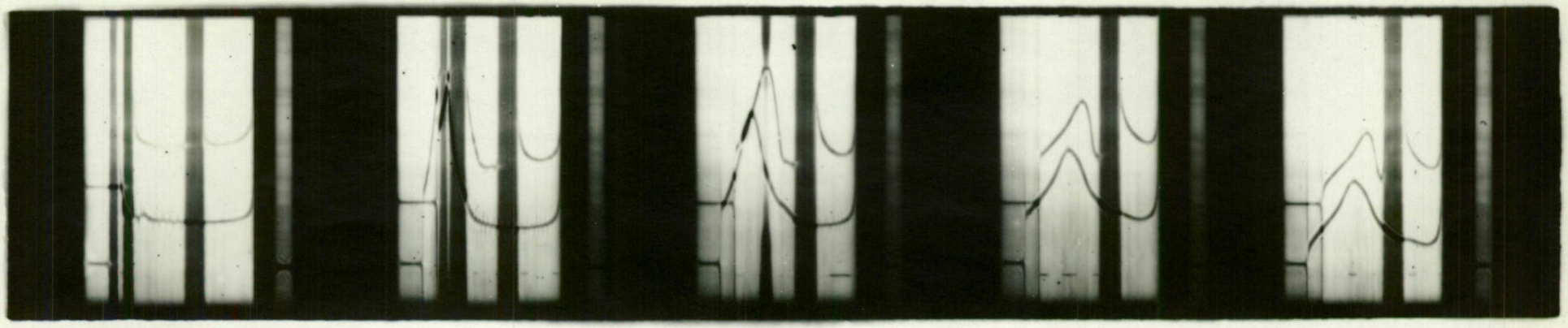


Centrifugal speed: 44,770 r.p.m.
Top pattern: separation cell
Bottom pattern: conventional cell

Diaphragm angle: 65°

Time intervals are counted from the time when two-thirds of the speed was reached.

FIGURE 11. Ultracentrifugal analysis of a soluble sheep brain extract in a separation cell. II.



16 min 32 min 48 min 64 min 80 min

The centrifugal speed was 59,780 r.p.m.

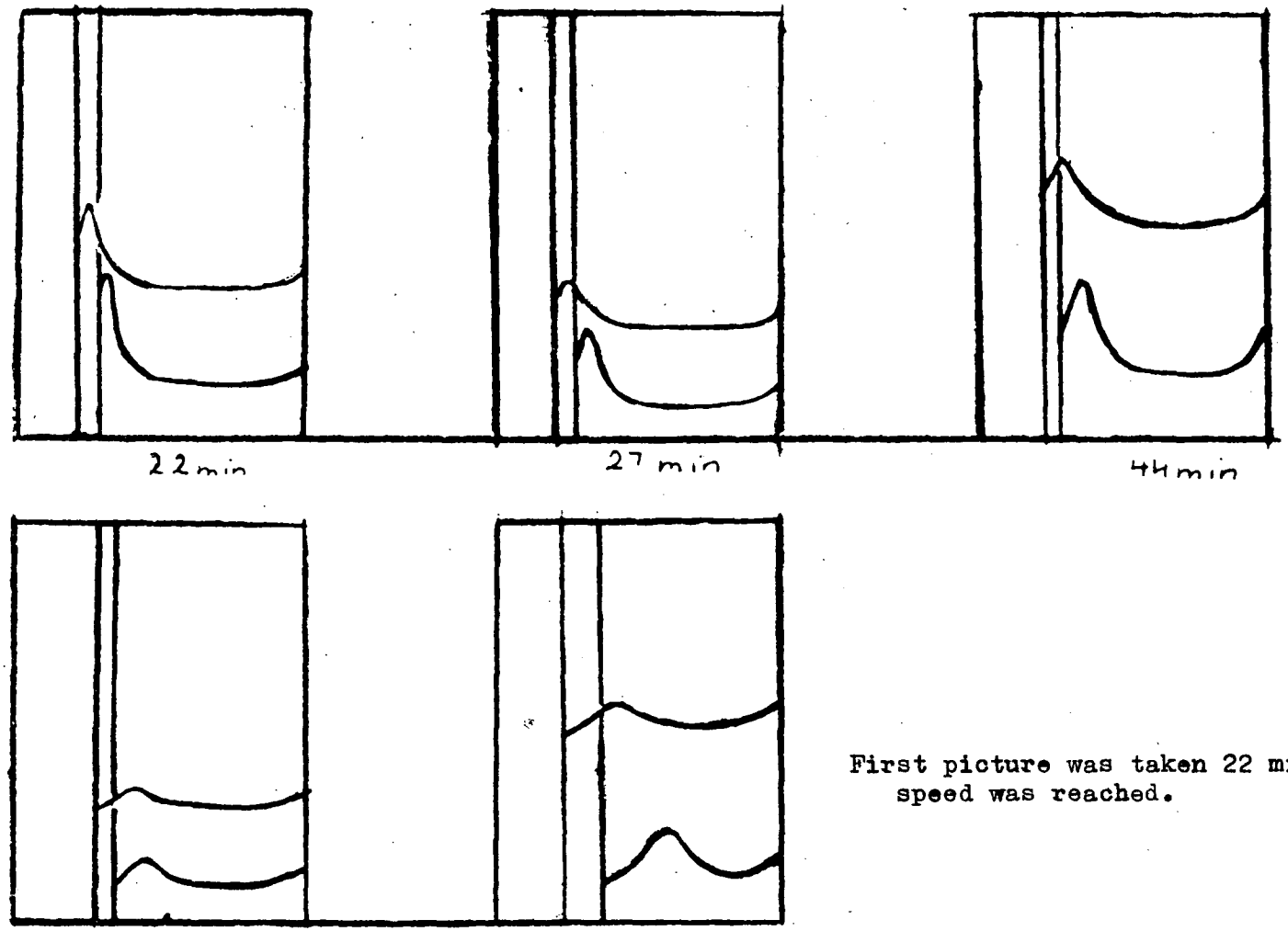
Top pattern: separation cell

Bottom pattern: conventional cell

Diaphragm angle: 65°

Pictures taken in 16 min. intervals after two-third of the speed was reached.

FIGURE 12. Ultracentrifugal pattern of highly purified enzyme sample I.

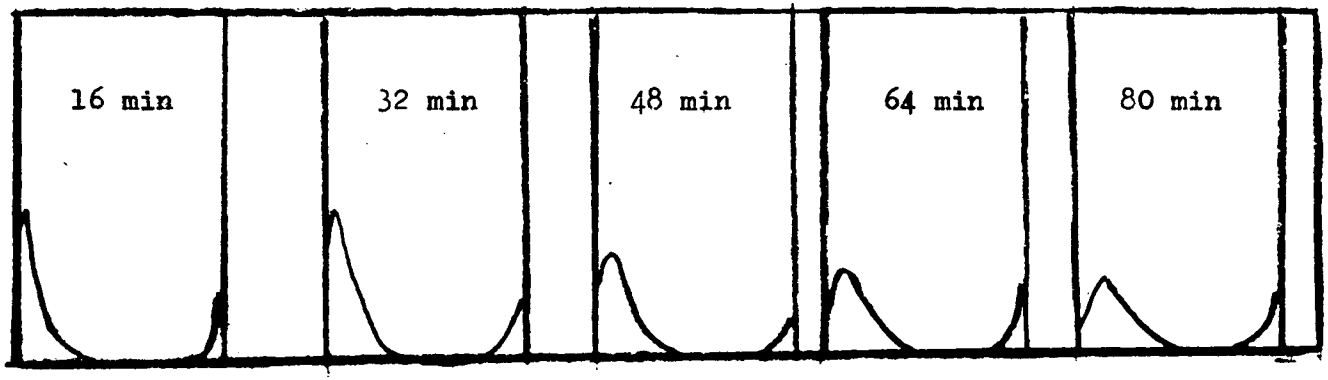


First picture was taken 22 min. after speed was reached.

Centrifugal speed: 56,100 r.p.m.
Conventional cells were employed.

Diaphragm angle: 70° in the first 4 pictures, 55° in the last one.

FIGURE.13. Ultracentrifugal pattern of highly purified enzyme
Sample II



Centrifugal Speed: 39,500 r.p.m.

Double sector cell.

Diaphragm angle: 65° in the first picture, 55° in the others.

Pictures were taken in 16 min. intervals after two-thirds of the speed was reached.

PART II

ACETYLCHOLINESTERASE IN RELATION TO CARRIER PROTEINS IN THE SHEEP-BRAIN

CHAPTER 1. INTRODUCTION

A. THE DISTRIBUTION OF ACETYLCHOLINESTERASE IN BRAIN CELL FRACTIONS.

The distribution of acetylcholinesterase within the cell of the mammalian brain has been examined by Aldridge and Johnson(90). They based their experiments on the knowledge gained from studies on liver homogenates (94-96) and used succinic dehydrogenase and deoxyribonucleic acid to assess the contamination of fractions by mitochondria and nuclei, respectively. Homogenates of rat brain have been separated by centrifuging into a nuclear fraction, two mitochondrial fractions, a microsomal and a supernatant fraction; 38% of the true cholinesterase was found in the microsomal fraction with a specific activity 2.6 times that of the homogenate. As the mitochondrial fractions contained 40% of the true cholinesterase the enzyme appeared evenly distributed between the mitochondrial and microsomal fractions. The nuclear fraction contained 36% of the pseudocholinesterase, the microsomal fraction 24% and the supernatant 27%.

Toschi and Hanzon have shown that microsomal fractions from rat brain contain both cholinesterase and RNA (85,97). The cholinesterase may be partially separated from RNA by centrifuging and by treatment with deoxycholate, some detergents or ribonuclease. Toschi has correlated these findings with electron micrographs of the fractions and considered that cholinesterase is concentrated in a "heavy" fraction whereas RNA is associated with electron-dense granules which are concentrated in a "light" fraction.

Frontali and Toschi (98) have reported a similar association of cholinesterase with membrane structures present in subcellular fractions from electric tissue of Torpedo.

B. STRUCTURAL PROTEIN OF MITOCHONDRIA.

Data on the relationship of structure and functions in different cell fractions are becoming more readily available than before. The complexity of the relationship, the difficulty of working with particulates, and various technical factors presented barriers to the study of such systems. These difficulties explain why biochemists have not studied the structure-function relationship until recently.

In 1955 Green and Beinert reviewed biological oxidation and discussed the highly organised and regular internal structure of mitochondria (99). Structure-bound enzymes are seated in the lamellae, while soluble enzymes are present in the remainder of the mitochondrion.

The meaning of terms such as "solubilization" of particulate enzymes is ambiguous. Deoxycholate or cholate "solutions" of succinic dehydrogenase and cytochrome oxidase can be transparent and without evidence of any Tyndall effect. They, however, do not satisfy the criteria of a true solution. Deoxycholate interacts with lipid rich material and the interaction is reversible (99/a). The solubilization of the particles can take place stepwise depending on the concentration of the detergent. These " solutions" become turbid as soon as deoxycholate or cholate is dialysed out and the original particulate character of the "solubilised" enzyme is restored(99).

A fundamental question in connection with mitochondria is the state of the enzymes that catalyse oxidative phosphorylation. Green discussed this problem in a symposium on enzymes (100) where he stated that oxidative phosphorylation is specifically related to the molecular structure of this system and proposed that electron transfer occurs through single structure in which all the oxidation-reduction elements are firmly bound one to the other. Pursuing this problem further, Green and his co-workers explored the molecular organization of the mitochondrial transducing system.

A minimum of 20 and perhaps as many as 40 different enzymes are involved in the basic mitochondrial transformation(101). There is electronmicroscopic evidence that each mitochondrion is surrounded by a double-layer membrane. The interior contains similar double-layer structures called cristae, which are continuous with the structures of the outer membrane. These observations suggest that the normal mitochondrion is a polymer or aggregate of many units. Green et al. (104) found that certain reagents effective in fragmenting mitochondria are soluble both in organic solvents and water; they include bile salts (e.g. cholate and deoxycholate), short chain alcohols (e.g. butyl, isobutyl, t-amyl alcohols), and detergents (e.g. sodium lauryl sulphate). The action of these reagents is exerted primarily on the lipid structure of the particle, most likely on the interface between the aqueous and lipid phases.

In the last few years Green and his school succeeded in explaining the mechanism of the transducing system of the mitochondria; some excellent reviews were published on this subject (102-109).

An important step in the elucidation of transducing systems was the isolation of a mitochondrial protein which contains no oxidation-reduction components, yet is intimately associated with both the oxidation-reduction protein of the electron transport chain and lipid (102). Iron (haem or non haem), copper, lipids and flavin are not found in this structural protein which is completely insoluble in water but readily soluble in aqueous media containing reagents which induce major charge repulsion between subunits or attack hydrophobic bonds. The intimate association of the structural protein with other protein molecules or lipids is due to hydrophobic bonds (101).

A detailed physicochemical investigation of the structural protein and an amino analysis were carried out (107). The protein that has been isolated in large amounts from beef heart mitochondria is a structural protein. Ultracentrifugal, electrophoretic and end-group analysis failed to show any heterogeneity of the isolated structural protein. At neutral pH it forms a water-insoluble polymeric aggregate, which can be depolymerised in the presence of anionic detergents or at pH 11. A monomeric form with particle weight in the range of $2 \times 10^4 - 3 \times 10^4$ can be demonstrated. Cytochromes a, b, and c_1 show analogous behaviour with respect to polymer-monomer transition. Moreover structural protein is able to form one-to-one water-soluble complexes with these cytochromes.

The hydrophobic bond is the predominant type responsible for the polymerization phenomenon and for complex formation between the monomeric species of the structural protein and of the cytochromes. Structural protein is capable of binding phospholipid; so are the cytochromes. The interactions between structural protein and cytochromes and between structural protein and lipid have considerable relevance to the problem of mitochondrial organization. This is discussed by Green and Fleischer in a review on the molecular organization of biological transducing systems (106). Transducing systems in the living cell transform energy from one form to another. They are contained within subcellular particles or membranes. The mitochondrion is a particle, a water-insoluble complex that is readily sedimented in a relatively low gravitational field. There is a continum between particles and soluble proteins. The isolated mitochondrion particle consists of highly reproducible molecules or complexes. It is the introduction of structure into a molecular array.

Oxidation-reduction proteins of the electron transport chain and the associated complexes constitute 50% of the total protein of mitochondria. The balance is made up of the structural protein.

C. STRUCTURE AND FUNCTION RELATIONSHIP IN THE MICROSOMES.

The organised structure of the mitochondrion visible in electronmicrographs consists of membranes. The cytoplasm, however, had previously been regarded as structureless substance.

A system of membrane-bound cavities has now been recognised in the cytoplasm of most cells.

The microsomal fraction is derived from the cytoplasmic matrix. The term 'microsomes' has only a methodological meaning (118). The microsomal pellet, obtained by classical differential centrifugation, consists of membrane-bound, rough-surfaced and smooth-surfaced vesicles; ribonucleoprotein particles attached to the outer surface of the membranes. Free ribosomes are also present in the pellet (110-112).

As in the case of mitochondrion (99-103), the search for the function of these membrane structures requires extensive biochemical studies. It should be noted that, while a great deal was known of the biochemical function of the mitochondrion before the structure had been revealed (114), the metabolic activities of the microsomal fraction do not provide an overall pattern yet (113). Although many microsomal enzymes are listed in the literature (113,115), the only case in which it is possible to relate biochemical function to cell structure is protein synthesis (113).

The complexity of the enzyme reactions is in agreement with the findings of the morphologists (110-117).

CHAPTER 2: EXPERIMENTAL

A. MATERIALS

(1) Biological Materials:

Structural protein was prepared from sheep-brain cell fractions (Section C).

Multiprotein systems used for interaction experiments were prepared from sheep-brain (see Part I, preparation of soluble sheep-brain extracts), or extracted with Tris-HCl buffer (82) from the freeze-dried powder of the electric organ of *Narcine tasmaniensis*. Other multiprotein systems interacted with structural protein were normal and pathological human sera (see also legend of Figure 19).

Haemoproteins used in interaction experiments were electrophoretically pure cytochrome c (Fluka) and crystalline bovine haemoglobin (Light & Co.).

Purified β-lactoglobulin was kindly supplied by Dr. H. A. McKenzie (A.N.U. Canberra).

The venom of *Crotalus adamanteus* was purchased from Fluka.

(2) Chemical Materials:

The water insoluble proteins of mitochondria and microsomes of sheep-brain were solubilized with sodium cholate, sodium deoxycholate, and sodium dodecyl sulphate (SDS) (Fluka).

B. DETERMINATION OF PHYSICO-CHEMICAL CONSTANTS, ASSAY OF ENZYMATIC ACTIVITY.

Sedimentation coefficients were evaluated from the slope of the line relating the logarithm of the boundary position to the time (moving boundary method) from the equation:

$$S = \frac{1}{\omega^2} \frac{d\ln\bar{x}}{dt}$$

Particle weight was determined according to Ehrenberg (119) or from measurements of sedimentation and diffusion coefficients.

Diffusion coefficients were determined by the porous disc method (120) or from the spreading of the synthetic boundary (119).

Partial specific volume was determined by pyknometry (121). Viscosities were measured in Ostwald pipettes at or corrected to the temperature of centrifugation.

Succinic dehydrogenase activity was estimated by Quastel's method (124) as modified by Aldridge and Johnson (90).

C. BIOCHEMICAL PROCEDURES.

(1) Cell Fractionation.

The brain was removed at the slaughter-house immediately after killing the sheep. It was kept on ice for about half an hour, then rinsed repeatedly with 0.3 M ice cold sucrose solution, quickly dried between sheets of filter-paper and weighed (wet weight: 80 ± 4 gm. per set of brain). After mincing the brain with a surgical blade, during which operation it was kept over ice, it was homoegenized in a Potter-Elvehjem type homogenizer in a final volume of 30 ml. 0.3 M sucrose solution per each 10 gm. of wet weight. During this procedure the temperature was kept at 2°. The combined homogenates were diluted with one volume of ice cold sucrose solution.

Differential centrifugation of the homogenate was carried out in the refrigerated centrifuge at 1°, and in the preparative rotor of the ultracentrifuge (see page 19 for particulars of the centrifuge and rotors).

The first sediment was collected after centrifugation of the homogenate at 1000g for ten minutes. This sediment was washed with a small volume of ice cold sucrose solution and the large amount of lipid that covered the sediment was combined with the supernatant together with the washing.

The sediment was taken up in ice cold 0.3 M sucrose solution and kept in the cold. This fraction is denoted as the nuclear fraction, 1 N10 (90).

The supernatant was centrifuged at 7000g for 15 minutes and the sediment collected, representing the heavy mitochondrial fraction, 7 M_1 15.

The remaining supernatant was centrifuged at 13,000g for 15 minutes yielding the light mitochondrial fraction, 13 M_2 15. From the supernatant left after this fractionation step the microsomal fraction was collected in the ultracentrifuge at 145,000g for 60 minutes. This sediment is denoted 145 P 60.

The final clear, pink supernatant was denoted "S". All the sedimented fractions were rehomogenized in sucrose at 1°, and used for analysis or as a source for preparation of acetylcholinesterase and structural protein.

"S" was left in the cold room for a fortnight. After this period a lipid-like precipitate appeared in the solution.

This may have been due to the disorganisation of the micellar state of lipids at low temperature. The precipitate has been collected by centrifugation and the remaining clear, pink supernatant was brought to 90% saturation with ammonium sulphate to yield partially purified haemoglobin.

Cell fractionation for the routine preparation of the structural protein was carried out in a slightly different way. The organ was homogenized in 0.25 M sucrose and differential centrifugation was carried out without dilution of the homogenate. Lipids sedimenting loosely at low speeds (1000g, 7000g) were kept with the firmly packed sediment as much as possible.

(2) Preparation of Structural Protein:

Structural protein was prepared from combined mitochondrial fractions, and from microsomal pellets.

The sucrose homogenates of these sediments, containing 10-20 mg. protein per ml., were treated with sodium deoxycholate (4 mg. per mg. protein), sodium cholate (2mg. per mg. protein), and sodium dodecylsulphate (1.5mg. per mg. protein) in the cold with magnetic stirring. The detergents were slowly added to the homogenate; the mixture was stirred for half an hour, then clarified by centrifugation at 40,000 g. The mitochondrial fraction threw down the protein-free brown-green residue observed before (107), the microsomal fraction did not afford this material but deposited lipids and protein during centrifugation.

The pH of the clear, brown solution, light yellow in the case of microsomes, was then adjusted to 7.0 with phosphate

buffer (final concentration 0.05 M). Sufficient sodium dithionate was added to reduce the cytochromes. Solid ammonium sulphate was added in the cold, with magnetic stirring to bring the concentration to 12% saturation; the precipitate was collected by centrifugation and was treated as follows to gain soluble structural protein.

The sediment was suspended in a small volume of sucrose solution (0.25M), the suspension brought to 20% saturation by the addition of an estimated volume of saturated ammonium sulphate solution. Analytical grade butanol was added to the mixture with magnetic stirring at 1° to make the concentration of the organic solvent 20% (v/v). The extraction was carried out for 25 minutes, the firmly packed sediment collected by centrifugation and suspended in sucrose solution, after being washed several times with sucrose solution.

Deoxycholate treatment, although recommended by Green (107), was avoided at this stage because the following extraction of the detergent with warm (50°) methanol denatured the protein.

1 N sodium hydroxide was slowly added to the mixture to bring the pH to 10.5, and then sodium dodecylsulphate was added, in a final concentration of 0.1%. This mixture was left overnight in the cold, then dialyzed for 24 hours against 0.1N sodium chloride, pH 10.5. The dialysate was cleared by centrifugation and stored in a cold room until used for analysis

or interaction experiments. Alternatively, samples were dissolved in 0.3% or 0.2% sodium dodecylsulphate (pH 10.5) when a higher concentration of the detergent was needed for depolymerisation (Table IX). In such cases the suspension was left at 25° for 24 hours, then centrifuged. This was necessary to avoid precipitation of the detergent, which is only slightly soluble at low temperature.

Before ultracentrifugal analysis the solutions were dialyzed for 24 hours against 0.025 M Tris-HCl buffer, pH 8.6 (8.2) containing 0.1 M sodium chloride, unless otherwise stated in the experiment.

(3) Ammonium Sulphate Fractionation of Mitonchondria

Mitochondria obtained from the cell fractionation of sheep-brain homogenate were suspended in sucrose (15mg. protein per ml.) and freeze-dried. The dry powder was extracted with 15 volumes of 0.8% Lubrol W solution in the cold. The homogenate was centrifuged at 54,000g for 25 minutes and the turbid, pink supernatant was dialysed against a 0.05M phosphate buffer, pH 7.0(93/a) for 24 hours in the cold. The small amount of white precipitate that appeared during dialysis was removed by centrifugation at 75,000g for 40 minutes and the supernatant was brought to 12% saturation with solid ammonium sulphate. After the sediment was collected, two more steps of ammonium sulphate precipitation were carried out, in the same way as described in the first part of this Thesis (Part I, Chapter 2, Section D3).

The water insoluble sediments were solubilized, fractionated and prepared for ultracentrifugal experiments in the same way as described in this section for the preparation of structural protein.

CHAPTER 3: RESULTS AND DISCUSSION

A. DISTRIBUTION AND NATURE OF THE STRUCTURAL PROTEIN OF THE SHEEP-BRAIN CELL.

The limitation of obtaining cholinesterases with high specific activity was assumed to be due to the presence of carrier protein or proteins to which the enzyme is strongly attached.

The distribution of the enzyme activity in the different fractions of the sheep-brain cell was investigated in order to test whether this attachment is specific toward proteins present only in certain cell-fractions.

The results of such an experiment (Table VIII) are in good agreement with those published by Aldridge and Johnson(90). The cholinesterase activity is almost uniformly distributed between the mitochondrial and microsomal fractions. However, the microsomal fraction is clearly the site of the highest specific activity. The latter was also found by Toschi et al. (85,86) working with other mammalian brain tissues.

The ratio of true to pseudo-cholinesterase is the same in all fractions; 76% of the succinic dehydrogenase activity is linked with the mitochondrial fraction. Contamination of the other cell fractions with mitochondria is within the limits of differential centrifugation techniques.

Results of a typical experiment are presented instead of statistical data, because no attempt has been made to multiplicate such determinations in statistically significant

numbers. The total protein content of sheep brains and the distribution of protein between cell fractions are variable, hence averages of a few experiments would present a more misleading picture than single records.

It is concluded that if carrier proteins exist, they would be present both in the mitochondria and in the microsomes. Knowledge gained during the purification experiments, pointed towards the possibility of the presence of a protein, similar to that found by Green and co-workers (99-109) in beef heart mitochondria, both in the mitochondrial and in the microsomal fractions of sheep-brain. Figure 14 shows the ultracentrifugal analysis of such proteins prepared from both mitochondrial and microsomal fractions (Chapter 2, Section C 2).

The time curve of sedimentation coefficients furnishes evidence for the similarity of the structural proteins prepared from mitochondria (SP 3.b) and from microsomes (SP 2.a,SP 4.a) of the sheep-brain. Similarity of both to those prepared from heart mitochondria by Green and co-workers (107) can also be postulated. The S values appearing in the figure are in the range of 2.5-3.0. The sedimentation analysis of a microsomal structural protein (SP 4.b) dialysed for a longer period (48 hours) against a pH 8.6 Tris buffer (82), gives a significantly higher sedimentation coefficient (top line).

All preparations presented in Figure 14 have a time-independent sedimentation coefficient, which suggests mono-dispersity.

Other preparations have sedimentation coefficients varying with time (Figure 15): SP 2.b, a microsomal structural protein has a constantly decreasing sedimentation coefficient indicating either the presence of different particle sizes or a system undergoing rapidly reversible equilibrium. In the cases of SP 1 and SP 3.a, a marked change in slope is noted; the system probably contains two or more distinct molecular species. After the first thirty minutes the S values reached a level in the same range as the ones in Figure 14. The $S_{20,w}$ value of SP 1 had been determined as a function of concentration (Figure 16) and the value extrapolated to infinite dilution was 3.8.

In order to determine the particle weight of the structural protein of sheep-brain mitochondria and microsomes, a number of preparations have been carried out. Physico-chemical characteristics such as sedimentation coefficients, particle weight, and diffusion coefficients of these preparations have been determined and the data are summarized in Table IX (see also Chapter 2, Section B, for methods).

It is clear that both sedimentation coefficients and particle weights of structural proteins isolated either from mitochondria or from microsomes vary through a wide range. Cell fractions were prepared under standardized conditions and the distribution of succinic dehydrogenase was checked each time. The intracellular distribution of lipids in the sheep-brain, however, was not determined.

Data are available in the literature on the distribution of lipid in the different fractions of rat-liver cell. According to Collins (125) and Getz et al. (126,127) the microsomes contain twice as much lipid as the mitochondria Phospholipids are responsible for a great amount of the lipid in both fractions. Lecithin and kephalin are present in the mitochondria in almost equal amounts, whereas there is twice as much lecithin as kephalin in the microsomes. Green and Fleischer have shown that the structural protein of mitochondria readily reacts with phospholipids (128).

In experiments shown in Table IX the two samples corresponding to a particle weight in the range of 27,000-29,000 are SP 3.b and SP 1.c. The former is a mitochondrial preparation, the latter is a microsomal one incubated in the presence of snake venom. The particle weights of preparations obtained from different cell fractions are in good agreement though determined by different methods. These findings confirm the similarity of the mitochondrial and microsomal structural protein as it was postulated before on the basis of sedimentation coefficient measurements (see also page 80).

The microsomal preparation appeared in a high polymeric form before treatment with snake venom (Table IX, SP 1.b). The sedimentation coefficient shows only a slight change upon the effect of phospholipase but the diffusion coefficient increases about sixfold. The significant change in the frictional ratio indicates a change in the shape of the molecule.

The species with low particle weight (SP 1.c) approaches a spherical shape, whereas the polymer is a "cigar-shaped" molecule.

The significance of the amount of phospholipid in the polymerisation and depolymerisation of structural protein may explain the wide range of particle weights demonstrated in Table IX. It should be remembered that with extremely high phospholipid concentration of the brain tissue the presence of some of this substance in the final preparation is very hard to avoid.

Further evidence to prove that the phospholipid influences the molecular size and shape of the microsomal structural protein is furnished in Table IX (SP 4.b, SP 11.b & c.) The protein preparation SP 4 has been tested immediately after solubilization (SP 4.a) and after dialysis against Tris-HCl buffer at pH 8.6 (SP 4.b). The $S_{20,w}$ values were 3.23 and 4.15 respectively; the particle weights were 22,000 and 54,000 respectively. In both cases particle weights were calculated from sedimentation coefficients and diffusion coefficients, but the latter were determined by different methods: direct measurement (120) was possible in the case of SP 4.b while calculation from spreading boundaries was used in the case of SP 4.a (119).

Preparation SP 11 containing 9 mg. protein per ml. has shown a high polymeric form with a particle weight of 263,000. The particle weight of SP 11.b (containing 3.6 mg. protein per ml.) was 154,000. The frictional ratio in the case of

the former was 1.84 and in the case of the latter 1.56, indicating ellipsoids with axial ratios 15-20 and 10 respectively (122).

On treatment of the latter with snake venom (100γ/mg. protein) the particle weight decreased to 47,000-56,000 and the frictional ratio to 1.17, in good agreement with the data obtained on SP 4.b, though in this case two different methods of molecular weights determinations were used. An axial ratio of 3-4 corresponds to the frictional ratios of SP 4.b and SP 11.c.

The polymerizing effect of phospholipid is proved further by addition of lecithin to SP 5,a microsomal structural protein. A polymer has been formed with a particle weight of 525,000, the sedimentation coefficient being 6.8 svedberg (SP 5.c). It is interesting to compare these data with the ones afforded by SP 5.a which has a sedimentation coefficient of 7.7 svedberg corresponding to a particle weight of 287,000. Once again the presence of phospholipid appears to have a strong influence on the diffusion coefficient.

The data presented so far suggest that structural proteins from sheep-brain mitochondria and microsomes have comparable particle weights in the range of 20,000-30,000 as observed by Criddle, Bock, Green and Tisdale (107) for similar preparations from beef heart mitochondria.

Taking this molecular weight as that of the monomeric

structural protein from both mitochondria and microsomes, SP 4.b would represent a dimer, SP 5.a, and SP 11.a aggregates of ten molecules. On the other hand the particle weight of preparations SP 1.b, SP 6 and SP 11.b can be regarded as 5-6 times that of the monomer.

When frictional ratios are plotted against particle weights, Figure 16/a, a straight line can be fitted to the data with regression analysis which suggests linear aggregation of the globular protein molecules.

This postulation is in good agreement with the hypothesis made by Green and Fleischer on the structural protein-lipid network of the mesolayer of the mitochondrion (128). Then SP 4.b and SP 11.c can represent a trimer (see also page 84) and indeed many of the polymers would satisfy the criteria of a particle weight of nx18,000 (Table IX). The lowest particle weight obtained at pH 8.6 is higher than this value, but the affinity of structural protein for phospholipids is obvious from the results, and one may apply a partial correction for lipid binding. Another possible explanation is that the monomer contains some dimeric or trimeric form, or it is a monomer-dimer system. Low sedimentation coefficients obtaibed at high pH values (SP 1.a, SP 2.a, SP 3.a) favour this explanation. Whether the particle weight obtained for SP 2.a is the molecular weight of the monomer or the splitting of the molecule occurred at this high pH can not be concluded from these experiments.

B. INTERACTIONS OF THE STRUCTURAL PROTEIN.

The possible relation of structural protein to other proteins within the sheep-brain cells was investigated in interaction experiments. The next three figures present the results of complicated interactions. Figure 17 illustrates the ultracentrifugal analysis of the interaction (B) of structural protein preparation, SP 4b(A) with enzymatically active, soluble sheep-brain extract (C). The stream of heavy proteins and light lipoproteins moving in opposite directions are clearly visible in the sequence (C): interaction with structural protein virtually suppresses this effect. A much more complicated pattern of counterflow is presented in Figure 18 by sequence (C) of an extfact of the electric organ of Narcine tasmaniensis. The same structural protein preparation as in Figure 17 is given as comparison (A). Interaction (B) is readily seen but some of the pattern of (C) remains.

The sedimentation coefficients of the peaks seen in Figure 17 and 18 have been calculated and are shown in Table X. The enzymatically active sheep-brain extract shows two main peaks, interaction with structural protein leaves one component. The electric organ extract from Narcine tasmaniensis also have two major peaks before interaction and two peaks can be observed after interaction with structural protein both having lower sedimentation coefficients than originally.

The interaction of SP 4.b (structural protein of microsomes having a molecular weight of 54,000) with sheep-brain extract or the preparation from electric organ of Narcine tasmaniensis is accompanied by depolymerisation. The experiment illustrated in Figure 17 is interpreted as an interaction of the slow component of the brain extract with structural protein which probably has been depolymerised. The experiment shown in Figure 18 results again in the disappearance of the "trimer" on interaction. The electric organ proteins are also undergone some depolymerisation. It is noted in the literature (78,129) that the purified acetylcholinesterase of electric organ undergoes polymerisation and depolymerisation. It may be postulated from the experiment described above that interaction with structural protein favours dissociation of the macromolecule.

The experiment shown in Figure 19 compares two human sera, one normal (lower pattern) and one showing anomalies, e.g. slight agammaglobulinemia (upper pattern)(A). Sequence(B) shows the same after interaction with SP 4.a. Two features of the interaction are noted: bands of heavier proteins disappear in both cases in the region of lighter proteins, the normal serum alone preserves some of its "fine structure".

This experiment does not allow discussion in the present context apart from the suggestion it offers: structural proteins of cellular fractions can act as "sweepers". Removal of interacting proteins can enhance recognition; possibly also the isolation of minor or anomalous proteins.

The interactions of the structural protein with different purified proteins were also invedtigated.

SP 4.a (see also Table IX) and cytochrome c, or haemoglobin were mixed in a ratio of 1:1 or 2:1 (Figure 20). The mixtures were dialysed against 0.025 M Tris-HCl buffer, pH 8.6 (82) for 24 hours in the cold and then analyzed in the ultracentrifuge. The ultracentrifugal pattern revealed a single peak in all of this experiments. The sedimentation coefficients decrease in time which indicates polydispersity (Figure 20). In the case of cytochrome c the single peak sedimented together with the red colour and the sedimentation coefficient extrapolated to zero time is approximately three times as high as that of the structural protein itself.

Based on these experiments an interaction of microsomal structural protein with cytochrome c is suggested resulting in the formation of a complex. Particle weights were not calculated because of the polydispersity of the system.

The sedimentation coefficient of the mixture of structural protein and haemoglobin, as a function of time, is also shown in Figure 20. The sedimentation coefficient of the single sedimenting component is in the range of those noted in experiments with pure microsomal structural protein. This has been interpreted as a lack of interaction between haemoglobin and structural protein. The explanation of the presence of the single peak could be that the sedimentation coefficient of

haemoglobin and the present form of structural protein are very close to each other and the two peaks are not resolved in the ultracentrifuge.

The supernatant left after the collection of the microsomal pellets from the sheep-brain homogenate has been fractionated (Chapter 2, Section C 1) and the purified supernatant "S" was incubated with SP 4.a in the manner described in the case of haemoproteins. Two peaks have clearly shown in the ultracentrifugal analysis (Figure 21). The slow component had an $S_{20,w}$ value of 3.3 independent cf time and that of the fast one was in the range of 14-28 rapidly decreasing with time.

As the purified supernatant has shown the characteristics of haemoglobin (molecular weight,visible spectra), the appearance of the two peaks can be only explained by a change in the size or shape of the structural protein molecule during the incubation period.

The absorption spectra of the interacted samples are shown in Figure 22. The Soret bands in the oxidized state appear at the same wavelength, 412 mμ, in presence of bound cytochrome c and unbound haemoglobin as well.

Purified β -lactoglobulin of cow's milk was also used for interaction experiments with the structural protein of sheep-brain microsomes.

When β -lactoglobulin was dialysed against Tris-HCl buffer (82) at a pH of 8.6 a much lower molecular weight was

obtained than noted in the literature (130-132). The particle weight, determined by the Ehrenberg method was 21,000 and the $S^{o}_{20,w}$ value was 2.0. An unfolding or splitting of the molecule at a pH of 8.6 was postulated. An article that became accessible during the writing of this Thesis deals with the dissociation of β-lactoglobulin B into two subunits in the pH range of 5.5 to 8.8 (134): the authors determined the sedimentation coefficient in the pH range of 7.8-8.8 and found the $S^{o}_{20,w}$ value being 1.9, in good agreement with the value found by the candidate.

For interaction experiments structural protein preparation SP 1.b (Table IX) was mixed with β-lactoglobulin, both were previously dialysed against Tris-HCl buffer (82) pH 8.6, at a ratio of 1.1 (W/W); the mixtures were left at room temperature, and the sedimentation coefficient of the mixture was determined at two different total protein concentrations, in different time intervals. The sedimentation coefficient plotted against time are shown in Figure 23. The sedimentation coefficients were extrapolated to zero time of the ultracentrifugal analysis, and results are tabulated in Table XI. It is evident that sedimentation coefficient of the mixture decreases with time elapsed after mixing. The decrease of sedimentation coefficient becomes slower at lower total protein concentration. The findings suggest interaction between β -lactoglobulin and the structural protein of sheep-brain microsomes.

To confirm this suggestion further samples of microsomal and mitochondrial structural protein were interacted with β-lactoglobulin and the particle weights of the complexes obtained were determined according to Ehrenberg (119). The values obtained were in the range of 60,000 - 70,000; sedimentation coefficients (3.1-3.3 svedbergs) and diffusion coefficients ($5.3\text{-}5.8 \times 10^{-7}$) were determined.

These interaction experiments confirm the assumption that microsomal structural protein of the sheep-brain behaves similarly to the mitochondrial one. Concurrently with the work described in this Thesis, Richardson and co-workers(128/a) carried out experiments indicating the existence of a general class of protein responsible for the maintenance of structural integrity of the membrane, which is in good agreement with the conclusions derived by the candidate.

C. AMMONIUM SULPHATE FRACTIONATION OF SHEEP-BRAIN MITOCHONDRIA.

It became obvious during the experiments which resulted in the partial purification of sheep-brain acetylcholinesterase that a large percentage of the activity is lost at a certain level of ammonium sulphate saturation (Table II).

In order to show that this is due to the presence of structural protein, an ammonium sulphate fractionation of the freeze-dried mitochondria of sheep-brain was carried out (Part II, Chapter 2, Section C 3). The insoluble fractions were solubilized and analyzed in a similar manner as purified structural protein preparations (Chapter 2, Section C.2).

The results of two such experiments are shown in Table XII, 12% saturation of the salt threw down a white, water insoluble sediment which carried 10-20% of the total protein of the extract together with about 10% of the enzyme activity. The solubilized fraction has a slightly time-dependent sedimentation coefficient in the range of 1.4-2.1 svedbergs, suggesting the presence of a monomer-dimer system. The white, water insoluble sediment obtained at 30% ammonium sulphate saturation had different characteristics in the two experiments. The second one carried twice as much enzyme activity as the first one together with about the same amount of protein. This was therefore a typical case when enzyme activity was lost in an insoluble form (see Table II). The ultracentrifugal data obtained after the solubilization of the two sediments were also different. The first one had a high sedimentation coefficient in the range of 7.2-10.3 svedbergs, while that of the second one was much lower; 4.2 svedbergs corresponding to a particle weight in the range of 90,000-120,000 when determined by the Ehrenberg method.

It is clear from Table XII that colourless, water insoluble proteins of varying particle size can be prepared from the ammonium sulphate fractions of the Lubrol W extract of the sheep-brain mitochondria. These proteins were previously associated with acetylcholinesterase activity. The sedimentation coefficient covers a wide range, 2.5-8.7 svedbergs, but such a range has been measured when purified structural protein preparations were analyzed. Fraction 2 could be the aggregate of three structural protein

molecule and fraction 3 an aggregate of six of those, if the molecular weight of the monomer is 18,000. The protein from fraction 2, however, is a highly elongated molecule with a frictional rate of 2.5 and only fraction 3 has its place among the linear aggregates shown in Figure 16/a.

The sediment obtained at 60% saturation resulted in a water soluble protein with an intense orange colour. Although specific acetylcholinesterase activities were different in the two experiments, the sedimentation coefficients of the preparations are close to each other and correspond to a particle weight of 70,000-78,000. The spectral evidence shows the presence of a protohaem so taking the particle weight into account this protein could be haemoglobin with more or less acetylcholinesterase as impurity. The extinction coefficient of the alkaline pyridine haemochromogen at 556 mμ, however, is very low and would not account for more than 10^{-4}M haemoglobin being present. Yet the ultracentrifugal pattern reveals a single peak (Figure 24).

The splitting of this apparently complex protein was attempted in two different ways. The solution was incubated with snake venon (100γ/mg.protein) then examined in the ultracentrifuge. No quantitative or qualitative change was observed. Another attempt was made by adjusting the pH of the protein solution to 5.6 and bringing the ammonium sulphate saturation to 30%. At this salt concentration a white water insoluble precipitate was collected which upon solubilization

had a sedimentation coefficient of 3.1 svedbergs, much lower than before. The supernatant lost its enzyme activity and thus the fate of the enzyme could not be detected.

T A B L E VIII

Cholinesterase and succinic dehydrogenase in sub-cellular fractions from sheep brain.

Fraction	Protein % of homogenate	Succinic dehydrogenase activity; % of homogenate	Acetylcholinesterase activity; % of homogenate	Ratio of true:pseudo cholinesterase	Specific activity; acetylcholinesterase-unit x 10^2
1N10	7	7	6	6.8	1.6
$7M_2^{-}15$) $13M_2^{-}15$)	33	76	31	7.1	1.8
145P60	20	5	47	7.2	4.5
S	18	-	12	7.2	1.3
Loss	22	12	4		

Total protein: 180 mg/g. wet weight.

Total acetylcholinesterase activity: 3.4 units/g. wet weight/minute.

Specific activities are expressed as µM acetylcholine split per mg. protein per minute (37°).

FIGURE 14: Variation of sedimentation coefficient(S) with time in structural protein preparations. SP 3 was prepared from mitochondria, SP 2 and SP 4 from microsomes of sheep brain. The measurements were made at 14°C in a medium that was 10^{-2}M in Tris (chloride) pH 8.6 and 5 x 10^{-2}M in NaCl. Sp 4-b was dialyzed for 48 hours against this medium.

The centrifugal speed was 59,780 r.p.m.

FIGURE 14.

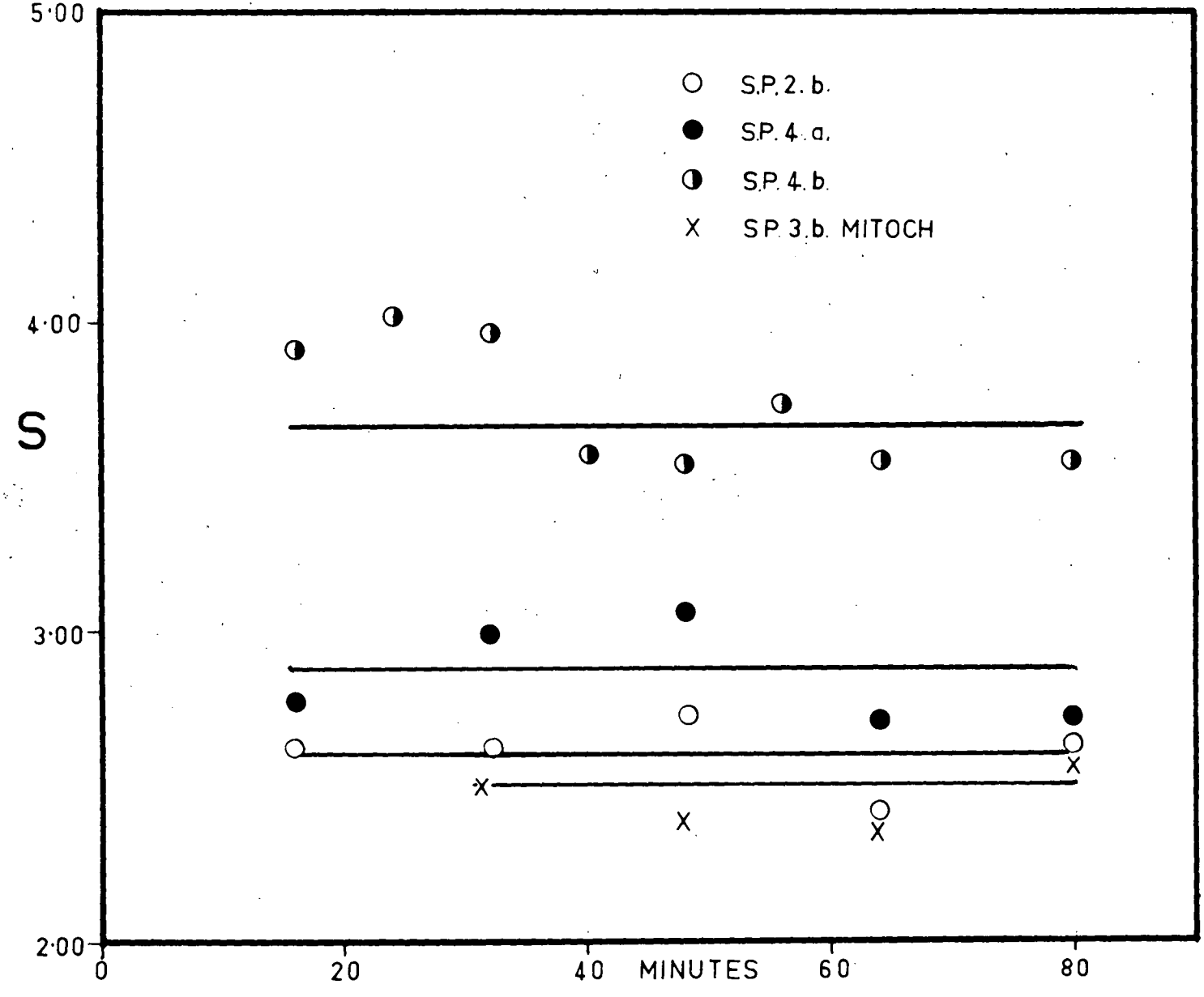

5·00
4·00
3·00
2·00
S
0
20
40
60
80
MINUTES
S.P. 2. b.
S.P. 4. a.
S.P. 4. b.
S.P. 3. b. MITOCH

FIGURE 15. Variation of sedimentation coefficient (S) with time in structural protein preparations. The measurements for SP 2.b and SP 1 were made in a medium that was 0.1M in NaCl at a pH of 10.5. Other conditions of the sedimentation analysis were the same as those described in the legend of Figure 14.

FIGURE 15

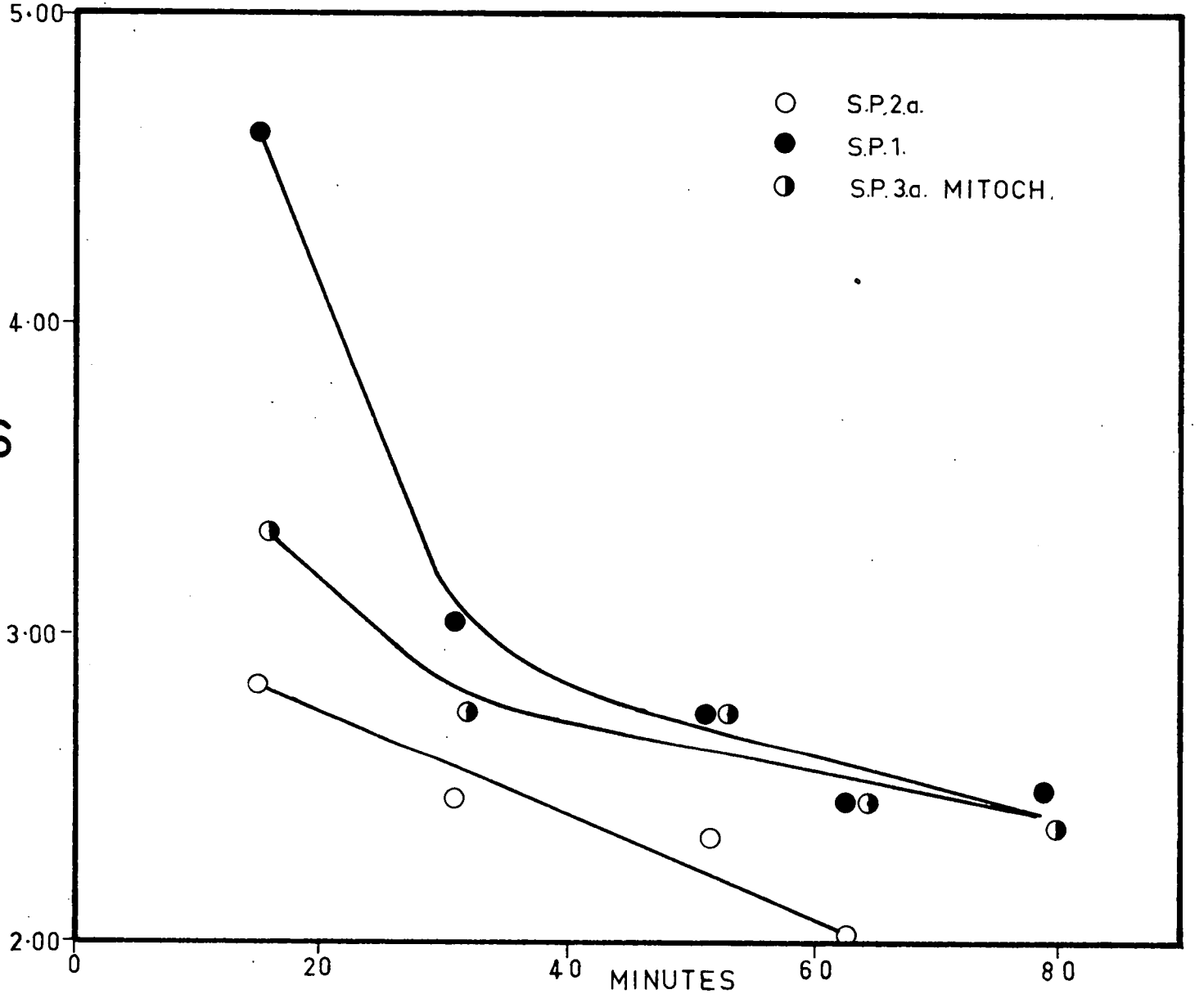


Figure 16

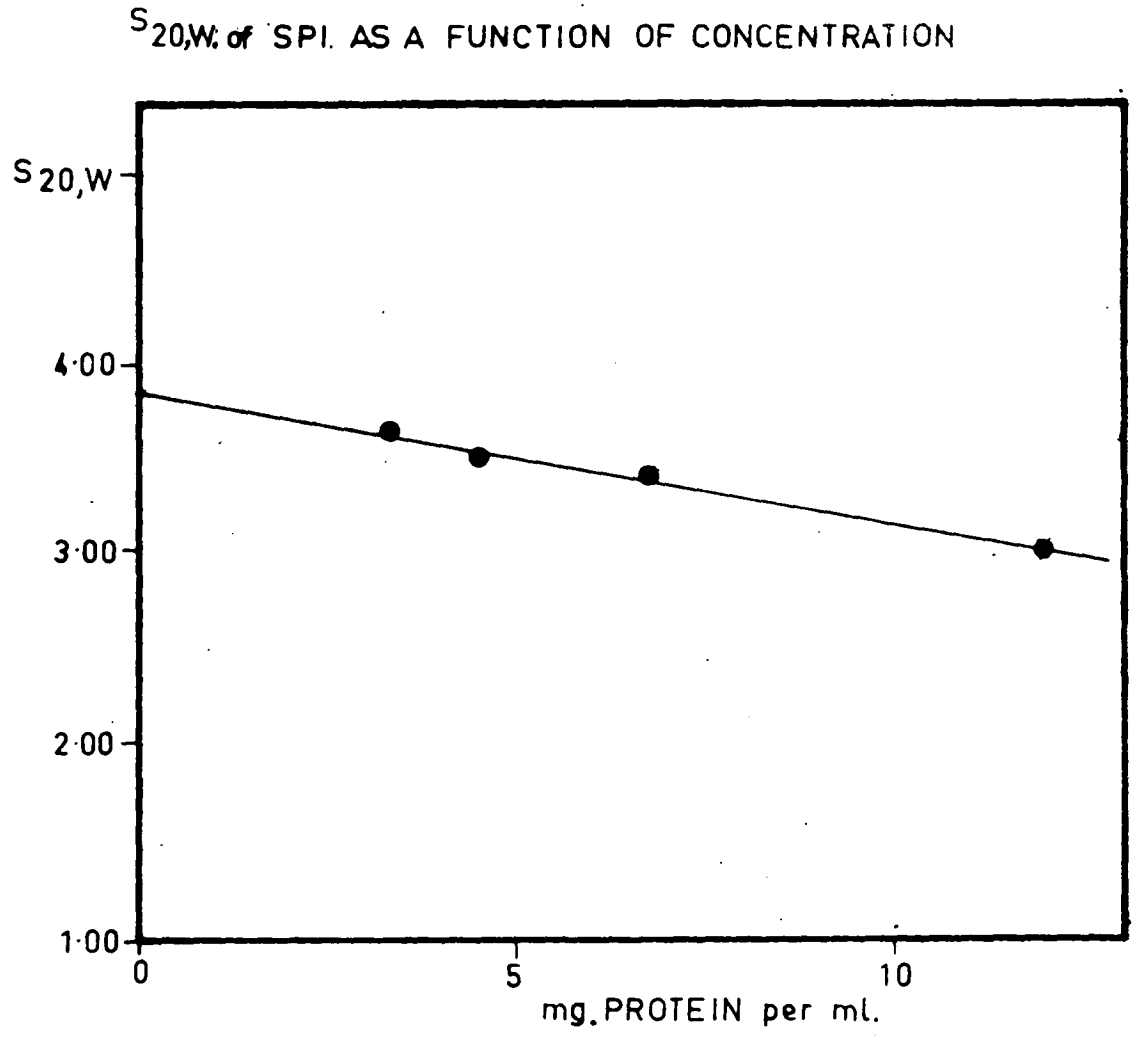

$S_{20,W}$ of SPI. AS A FUNCTION OF CONCENTRATION
$S_{20,W}$
4·00
3·00
2·00
1·00
0
5
10
mg. PROTEIN per ml.

TABLE IX

Sample	Source	Particle Weight	$S_{20,w}$	$D_{20,w}$	f/f_o	Medium
SP 1.a	Cytoplasm	-	2.8-3.5	-	-	0.1% SDS, pH=10.5
SP 1.b	Cytoplasm	160,000	3.8	2.2×10^{-7}	2.64	0.025 M Tris-HCl, pH 8.6, 0.1M NaCl
SP 1.c	Cytoplasm	29,000	3.5-5.4	1.2×10^{-6}	1.0	0.025 M Tris-HCl, pH 8.6, 0.1M NaCl + snake venom(100 γ/mg pr.)
SP 2.a	Microsomes	15,000	2.4-3.3	2.4×10^{-6}	-	0.1% SDS, pH 10.5
SP 2.b	Microsomes	30,000	3.1	1.02×10^{-6}	0.99	0.025 M Tris-HCl, pH 8.6, 0.1M NaCl
SP 3.a	Mitochondria	-	2.8-3.3	-	-	0.1% SDS, pH 10.5
SP 3.b	Mitochondria	27,000	3.4	1.24×10^{-6}	0.92	0.025 M Tris-HCl, pH 8.6, 0.1M NaCl
SP 4.a	Microsomes	22,000	3.2	1.25×10^{-6}	0.93	0.025M Tris-HCl, pH 8.6, 0.1M NaCl
SP 4.b	Microsomes	54,000	4.2	7.5×10^{-7}	1.12	0.025 M Tris-HCl, pH 8.6, 0.1M NaCl

TABLE IX (Continued)

Sample	Source	Particle Weight	$S_{20,w}$	$D_{20,w}$	f/f_o	Medium
SP 5.a	Microsomes	287,000	7.7	2.9×10^{-7}	1.62	0.025M Tris-HCl, pH 8.6,0.1M NaCl
SP 5.b	Microsomes	192,000	4.4	-	-	0.025M Tris-HCl, pH 8.6,0.1M NaCl + 0.2% SDS
SP 5.c	Microsomes	525,000	6.8	2.9×10^{-7}	1.35	0.025M Tris-HCl, pH 8.6,0.1M NaCl + lecithin
SP 5.d	Microsomes	260,000	4.6	3.6×10^{-7}	1.39	0.025M Tris-HCl, pH 8.6,0.1M NaCl + 0.3% SDS
SP 6	Mitochondria	180,000	5.1	4.3×10^{-7}	1.31	0.025m Tris-HCl pH 8.6,0.1M NaCl
SP 7.a	Microsomes	-	4.9	-	-	0.025M Tris-HCl, pH 8.6,0.1M NaCl

TABLE IX (Continued)

Sample	Source	Particle Weight	$S_{20,w}$	$D_{20,w}$	f/f_o	Medium
SP 7.b	Microsomes	-	3.2	-	-	0.025M Tris-HCl, pH 8.6, 0.1M NaCl + 0.2% SDS
SP 7.c	Microsomes	-	4.0	-	-	0.025M Tris-HCl, pH 8.6, 0.1M NaCl
SP 11.a	Microsomes	263,000	6.8-7.6	2.7×10^{-7}	1.84	0.025M Tris-HCl pH 8.6, 0.1M NaCl
SP 11.b	Microsomes	154,000	4.5-6.2	4.1×10^{-7}	1.56	0.025M Tris-HCl pH 8.6, 0.1M NaCl
SP 11.c	Microsomes	47,000-56,000	3.5-4.2	7.3×10^{-7}	1.17	0.025M Tris-HCl pH 8.6, 0.1M NaCl + snake venom (100 γ /mg.protein)

FIGURE 16a.

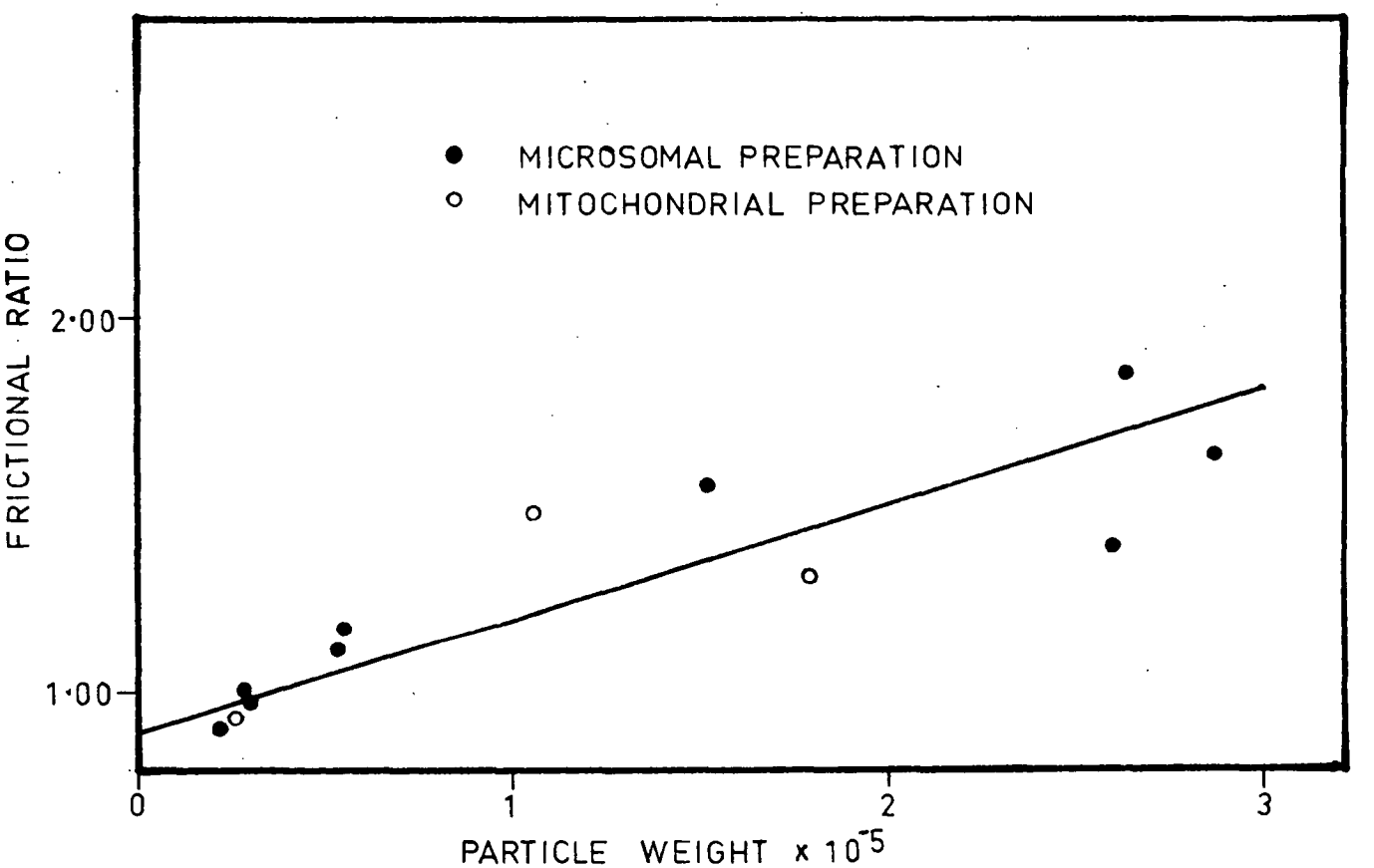

MICROSOMAL PREPARATION
MITOCHONDRIAL PREPARATION
FRICTIONAL RATIO
2·00
1·00
0
1
2
3
PARTICLE WEIGHT x 10⁻⁵

FIGURE 17: Comparison of ultracentrifugal sedimentation patterns, in

(A): structural protein from microsomes;

(B): the same preparation after interaction with sheep brain extract enriched in acetylcholinesterase;

(C): the same sheep brain extract.

The centrifugal speed was 39,500 r.p.m. Pictures were taken in 8 minute intervals after reaching speed; the first two pictures are omitted.

FIGURE 17.

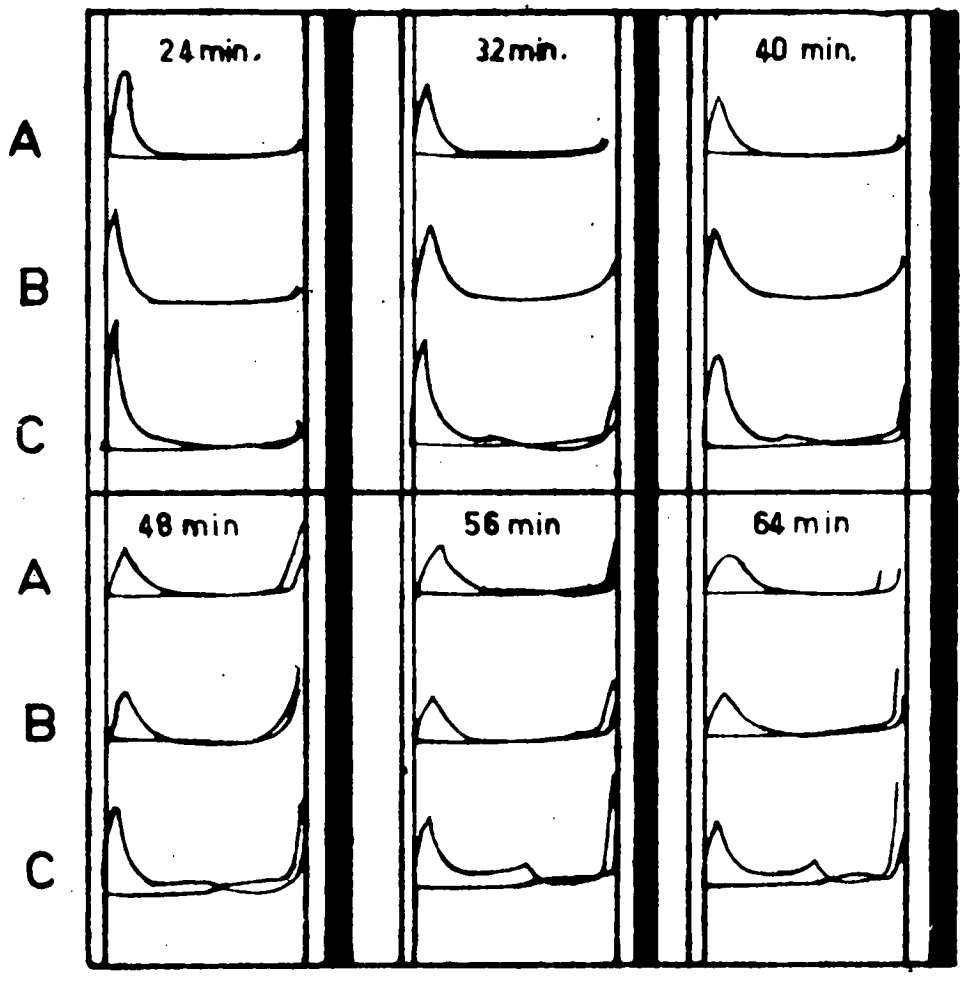


FIGURE 18: Comparison of ultracentrifugal sedimentation patterns.

(A): structural protein from microsomes;

(B): the same preparation after interaction with the extract of the electric organ of Narcine tasmaniensis;

(C); the same electric organ extract.

The centrifugal speed was 39,500 r.p.m. Pictures were taken in 8 minute intervals after reaching speed.

FIGURE 18.

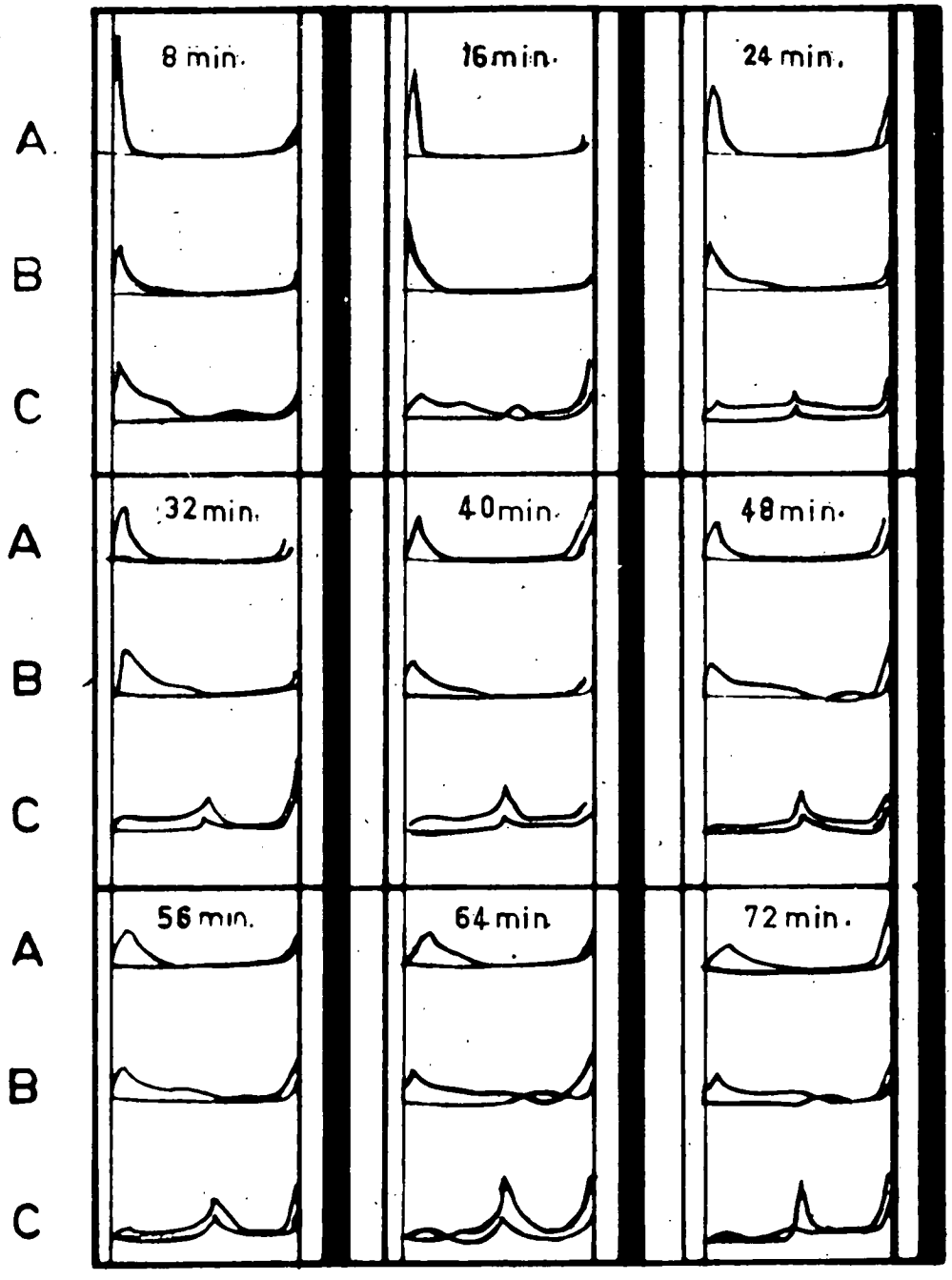

8 min.
16 min.
24 min.
A
B
C
32 min.
40 min.
48 min.
A
B
C
56 min.
64 min.
72 min.
A
B
C

TABLE X

Sedimentation coefficients of peaks present in the ultracentrifugal pattern of enzimatically active sheep-brain and electric organ extracts, before and after interaction with microsomal structural protein.

Sample	Slow Component	Fast Component
Brain extract	2.4-4.7 svedbergs	19-38 svedbergs
Brain extract + structural protein	3.7-6.2 svedbergs	-
Electric organ extract	5.5-15 svedbergs	65 svedbergs
Electric organ extract + structural protein	2.3-4.7 svedbergs	14-46 svedbergs

FIGURE 19: Comparison of ultracentrifugal sddimentation patterns.

(A): normal human serum (bottom) and a pathological human serum (top).

(B): the same preparations after interaction with structural protein from microsomes.

The centrifugal speed was 59,780 r.p.m. Pictures were taken in 16 minute intervals after reaching speed.

FIGURE 19.

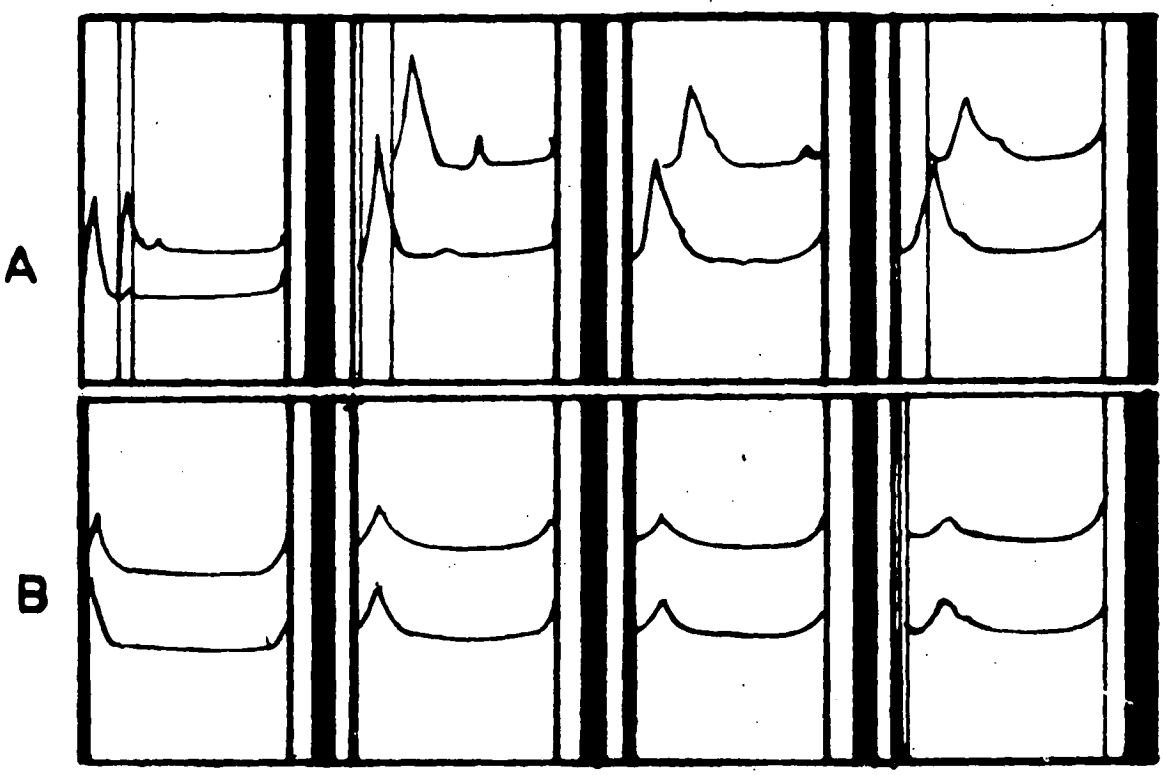


FIGURE 20.

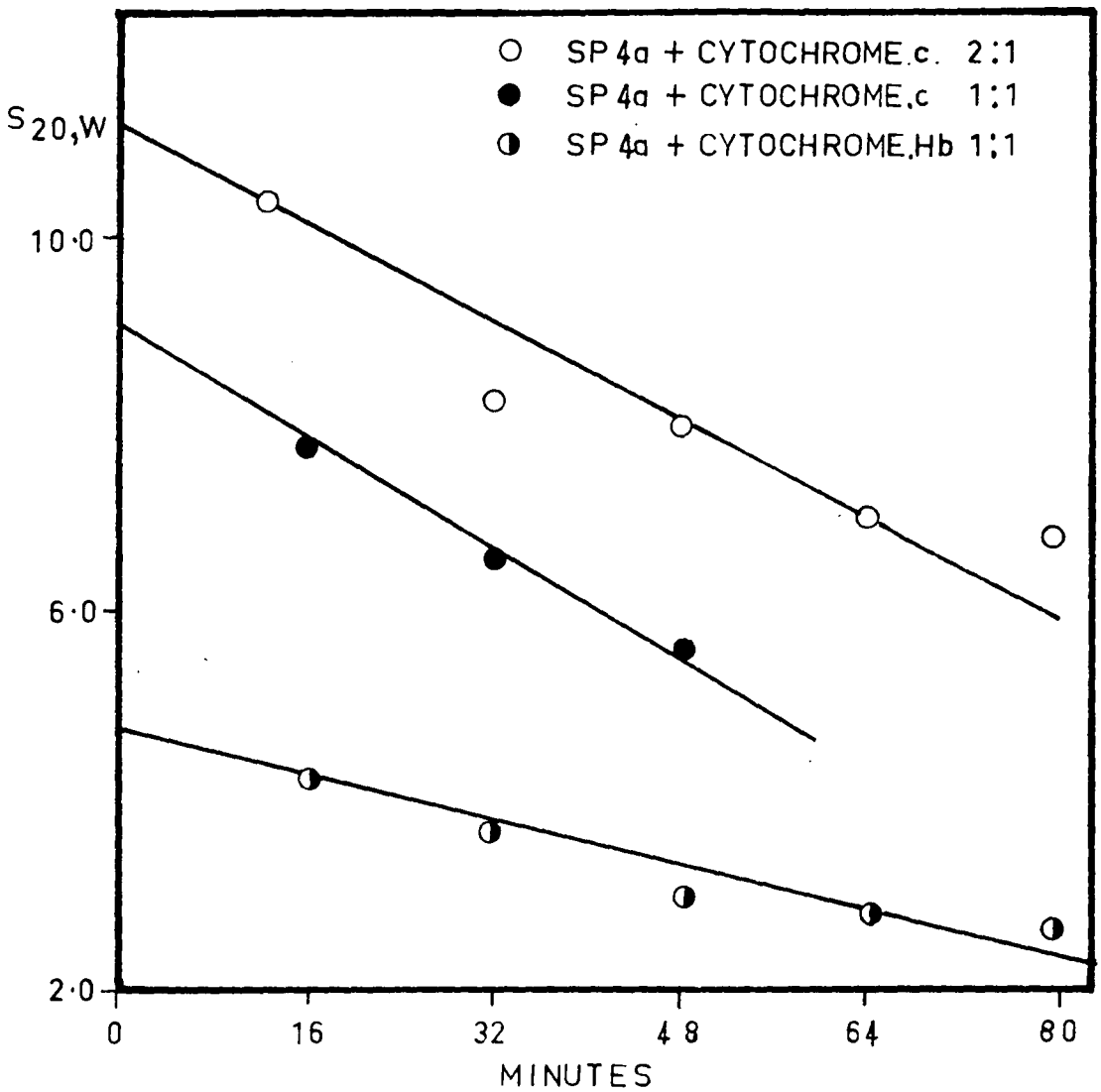
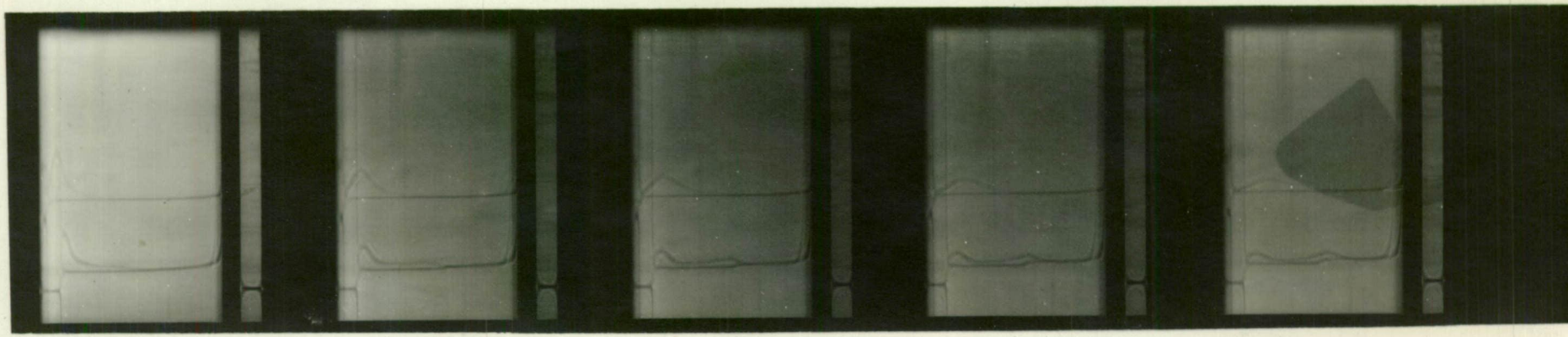

SP 4a + CYTOCHROME.c. 2:1
SP 4a + CYTOCHROME.c 1:1
SP 4a + CYTOCHROME.Hb 1:1
S20,W
10·0
6·0
2·0
0
16
32
48
64
80
MINUTES

FIGURE 21. Comparison of the sedimentation pattern of SP 4.a after interactiom with cytochrome c (upper pattern) and with purified "S" (lower pattern).



16 min 32 min 48 min 64 min 80 min

Speed: 39,500 r.p.m.

Temperature: 15°C

Double sector cells were employed

Phase angle: 60°

Photographs were taken in 16 min. intervals after the two-third of the speed was reached.

FIGURE 22.

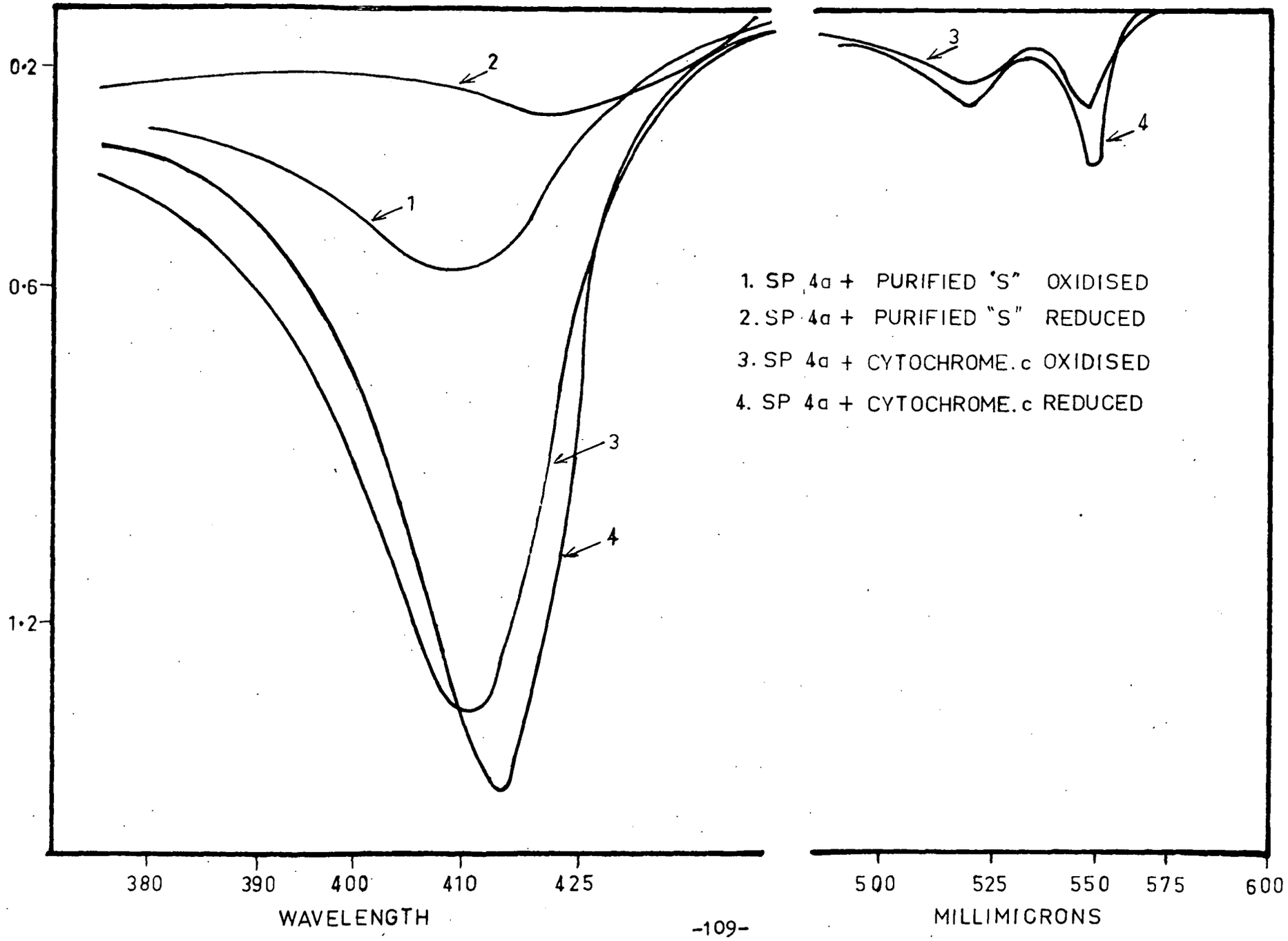
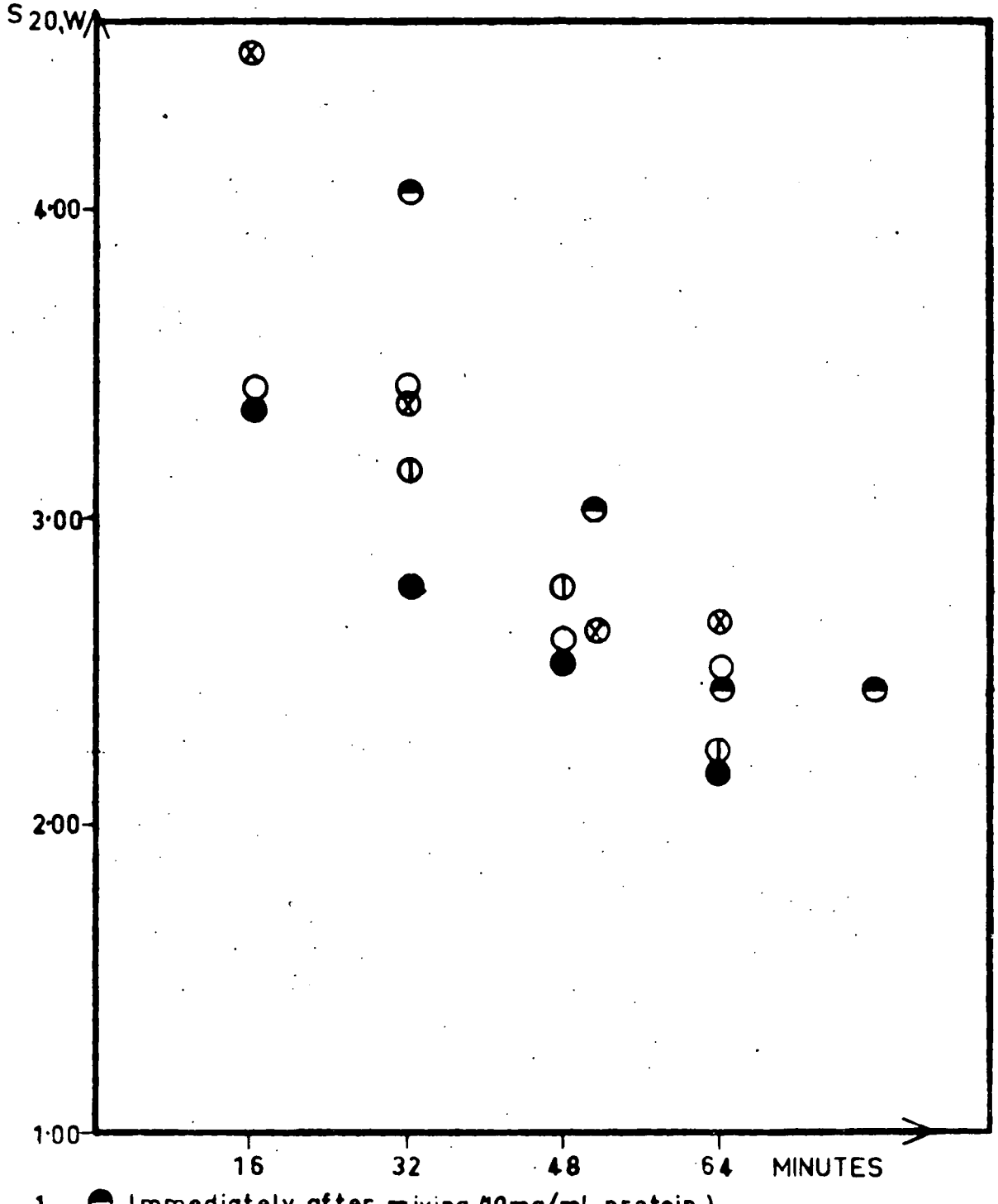

0·2
0·6
1·2
1
2
3
4
1. SP 4a + PURIFIED "S" OXIDISED
2. SP 4a + PURIFIED "S" REDUCED
3. SP 4a + CYTOCHROME. c OXIDISED
4. SP 4a + CYTOCHROME. c REDUCED
380
390
400
410
425
WAVELENGTH
500
525
550
575
600
MILLIMICRONS
ABSORBANCY

FIGURE 23.



1 ⊖ Immediately after mixing (10mg/ml. protein)
2 ● 72 hours " " "
3 ◐ 4 " " " "
2a ○ 2a & 3a the same as 2 & 3 at 5mg/ml. protein concentration.
3a ⊗ " " " " " " " " " " " "

T A B L E XI

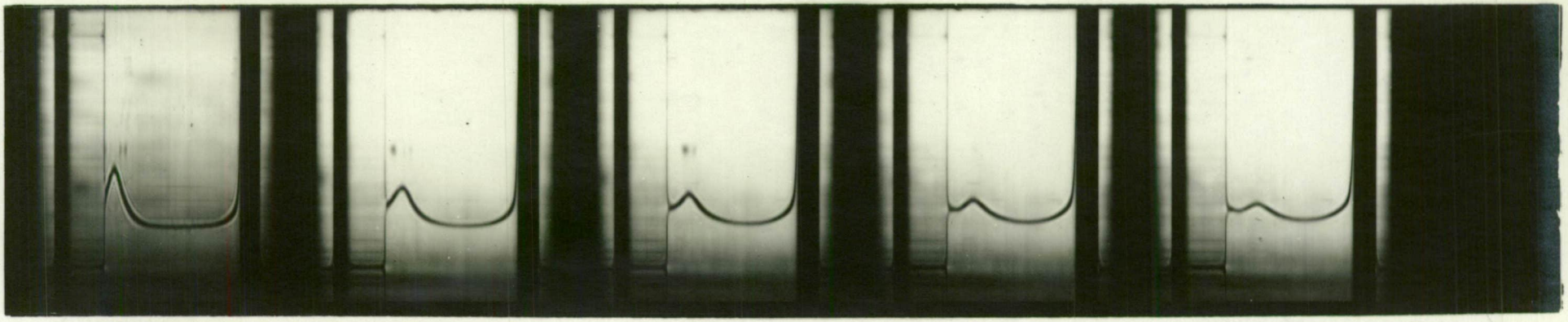
Sedimentation coefficients extrapolated to zero time. (see Figure 23)

	6 mg. protein per ml.	3 mg. protein per ml.
Immediately after mixing	5.7 svedbergs	
4 hours after mixing	4.2 svedbergs	5.3 svedbergs
72 houts after mixing	3.7 svedbergs	4.1 svedbergs

T A B L E XII

Sample	Total activity μl CO_2/hour	Total protein mg.	Specific activity (units)	$S_{20,w}$	$D_{20,w} \times 10^7$	Particle weight	f/f_o
1. LW extract	73,000	1320	55	-	-	-	-
2. 12% saturation	74,000	2400	31	-	-	-	-
	8,000	288	28	2.5	4.2	58,000	2.5
	6,000	181	33	-	-	-	-
3. 30% saturation	17,000	732	23	8.7	-	-	-
	35,000	500	72	4.2	4.6	90,000-120,000	1.5
4. 60% saturation	65,000	296	220	4.8	-	-	-
	2,500	120	21	4.1	-	70,000-78,000	-
5. pH 5.6, 60% saturation	-	-	-	3.1	-	-	-

FIGURE 24. Ultracentrifugal pattern of fraction 4 of mitochondria (see also Table XII)



16 min 32 min 48 min 64 min 80 min

Speed: 39,500 r.p.m., Conventional cell

Temperature: 20°C

Phase angle : 60°

Photographs were taken in 16 min, intervals after the two-third of the speed was reached.

GENERAL DISCUSSION.

Although the association of acetylcholinesterase activity with structural protein cannot be concluded from work reported in this Thesis alone, a structure-function relationship between the two can be postulated. The purified enzyme can hydrolyse its substrate in vitro without depending on any other macromolecule but its function within the cell is more complicated. The regulation of the enzyme-activity, co-ordination of acetylcholine synthesis and hydrolysis, may depend on the structure to which the enzyme is bound.

It is most probably the mitochondrial and the microsomal membrane structure which plays a regulating part in the function of acetylcholinesterase in analogy to the enzymes of the respiratory chain.

The mesolayer of the mitochondrial membrane contains a structural protein-lipid network. All membrane systems yet examined show similar structural pattern (128) with a mesolayer of structural protein and lipid. Purified structural protein preparations, isolated by the candidate, of sheep-brain cell mitochondria and microsomes seem to have similar characteristics. Evidence has been obtained that the preparations are linear aggregates of protein molecules the molecular weight of which has been estimated in the range of 18,000-27,000. This postulate of the candidate is in good agreement with Green's work (107), according to which the molecular weight of the monomeric structural protein is 22,500.

Different material has been used in this work and most of the experiments have been carried out on structural protein prepared from a different fraction of the cell; the microsomal fraction.

As it was mentioned before, the presence of different kind of lipids, especially phospholipids, could be expected (125-127) in different cell-fractions. Interaction between structural protein and phospholipids is shown by Green (128) and in Table IX; slight differences in molecular weight and in capacity to combine with lipids could be expected.

Interaction of the microsomal structural protein with soluble proteins, e.g. cytochrome c and β -lactoglobulin demonstrated in this Thesis supports the postulate that structural protein of mitochondria and microsomes are similar. Green proved that the mitochondrial structural protein interacts with cytochrome c only in presence of phospholipid(128). but the latter was most probably present in preparations described in this work.

In the present study of the microsomes, the highly heterogeneous fraction obtained by centrifugation of the mitochondrial supernatant was used only. The amount of detergent used for the preparation of structural protein most probably dissolved the ribosomes as well as the membrane-bound vesicles.

It can not be stated from these experiments which constituent of the microsomal fraction afforded the structural protein. Extension of these studies to more finely fractionated sheep-brain cell microsomes is intended.

SUMMARY.

1. Soluble sheep-brain acetylcholinesterase has been prepared in sufficient amount for further purification.

2. Partial purification of the enzyme has been achieved by ammonium sulphate fractionation and cellulose ion-exchange chromatography.

3. Structural protein was prepared from the mitochondria and from the microsomes of sheep-brain cell.

4. The structural proteins prepared from either cell fraction had similar characteristics.

5. The structural protein of sheep-brain mitochondria and microsomes seemed to be identical with or similar to those prepared by Green and his school (107) from beef-heart mitochondria.

6. Association of acetylcholinesterase with structural protein is postulated.

7. Interactions of structural protein from microsomes with cytochrome c, β -lactoglobulin, phospholipid and complex lipoproteins have been studied.

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   Structural protein from microsomes of sheep-brain.

NOTE: Publications 1-6 have not been used for a higher degree; 7 and 8 report preliminary studies relating to the work described in this Thesis.