STUDIES ON NEURONAL PROTEINS

Abstract of a thesis submitted for the degree of D. Phil.

by

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ABSTRACT

This thesis is concerned with studies on acetylcholinesterase. The problem of the role and fate of the protein has been studied using a variety of techniques.

1. Biochemical experiments

When homogenates, in isotonic media (0.3 M sucrose), of bovine splanchnic axons and adrenal medullae were subfractionated in a centrifuge, it was found that the acetylcholinesterase did not give the expected distribution. Much of the early work in this field had resulted in the conclusion that acetylcholinesterase was a uniquely membrane-bound enzyme (see e.g. Boell and Nachmansohn, 1940: Toschi, 1959) but my results suggested that a considerable proportion of the activity in both tissues was soluble. Thus, 47% of the acetylcholinesterase activity present in the splanchnic axons and 31% of the enzyme in the adrenal medullae could not be sedimented by prolonged high-speed centrifugation. The possibility that the preparation of the tissue for the centrifugation experiments might have "solubilized" the acetylcholinesterase from its normal, membrane-bound localization was investigated in several ways. It was found: that different strengths of homogenization, while having the expected effect of breaking up the tissue into smaller pieces, had no effect on the proportion of the enzyme which was not sedimentable; that suspension and "homogenization" of the membranes themselves did not result in a solubilization of their acetylcholinesterase; that incubation, at 25°, of a washed fraction containing only sedimentable acetylcholinesterase activity did not result in any solubilization of

the enzyme. It was concluded that the soluble enzyme was not artifactually derived from the membrane-bound form during the preparation of the tissues for the earlier experiments.

Because acetylcholinesterase is an enzyme which will hydrolyze a variety of substrates, it can be localized by histochemical methods. In addition to using this technique on tissues (see below) the method has also been used to locate the enzyme on polyacrylamide gels after different tissue extracts had been subjected to electrophoresis. The results of this type of analysis showed that the acetylcholinesterase of the two tissues was separable into several isoenzymes (see Webb, 1964 for definition of term isoenzyme). The simplest case was found in splanchnic nerve axons, where there was only one membrane-bound form of the enzyme (revealed by an extraction of the enzyme activity with Triton X-100 prior to electrophoresis) and another, different, form in the high-speed supernatant. The membrane-bound isoenzyme was always unique but occasionally there was more than one soluble isoenzyme. However, even in these rare cases, it was qualitatively estimated that more than 95% of the total activity on the gel was due to the one normal isoenzyme. The adrenal medullae presented a more complex picture. Again, there was only one membrane-bound isoenzyme, which had the same electrophoretic mobility as the axonal form, but there was a greater number of soluble forms of the acetylcholinesterase. One of these appeared to be the same form as was soluble in the axons, but in addition there were usually four others unique to the medulla. These latter isoenzymes had higher electrophoretic mobilities than either of the two isoenzymes of the splanchnic nerve. The different isoenzymes were numbered from 1, the fastest migrating, to 6, the slowest. Thus, $AChE_6$ and $AChE_5$ represent, respectively, the membrane-bound and the slowest migrating of the soluble isoenzymes. These are the two forms which are common to both axons and medullae.

The relationship between $AChE_5$ and $AChE_6$ was investigated in two ways. Electrophoresis over a range of polyacrylamide gel concentrations showed that they each changed their mobility in an identical fashion, i.e., a plot of $\log_{10}(R_m \times 100)$ v. polyacrylamide concentration yielded two essentially parallel lines. This, according to Hedrick and Smith (1968), indicates that the two isoenzymes have very similar molecular sizes but different electrical charges. Supporting evidence for this suggestion was obtained by centrifugation of the acetylcholinesterase on stabilizing sucrose gradients; both the soluble and, after extraction with Triton X-100, the membrane-bound acetylcholinesterase sedimented to the same area of the gradient. Thus, when it was found that $AChE_5$ behaves as though it has a molecular weight of 240,000, this also indicated a similar size for $AChE_6$. Experiments of this type were also used to analyze the relationship between the soluble isoenzymes of the adrenal medulla. These results showed that whereas medullary $AChE_5$ was identical to the soluble isoenzyme in the axons, the faster migrating forms all differed from it in size at least, and possibly also in charge.

Because of the ease with which $AChE_5$ and $AChE_6$ can be separated, a few properties of each were compared without prior and extensive purification of the proteins. It was found that they had an identical apparent K_m (7.5×10^{-5}M, acetylthiocholine as substrate), identical Q_{10}'s of 1.2 and identical heat denaturation properties. Thus, the only differences between them were in their relative solubilities and their electrophoretic mobilities.

To try ~~and~~ to distinguish whether the soluble isoenzyme in the axons was contained within the cytoplasm, or whether it was within a labile particle which homogenization broke to liberate the soluble internal constituents, we investigated the rate at which acetylcholinesterase accumulated in constricted splanchnic axons and analyzed the types of isoenzyme involved in the movement. Both $AChE_5$ and $AChE_6$ were found to accumulate at an identical, relatively rapid rate in these nerves. It was concluded that the $AChE_5$, and at least some of the $AChE_6$, was contained within a fragile particle and that the soluble enzyme was liberated from this particle during the homogenization procedure.

2. Studies on the release of acetylcholinesterase from the isolated adrenal gland.

A rapid flow rate of the acetylcholinesterase is a strong indication that there is an equally rapid removal of the enzyme from, presumably, the nerve endings. One way in which such a rapid removal could take place is by a release of the protein from the nerve. To investigate this possibility, we stimulated the isolated, perfused bovine adrenal gland with a variety of secretagogues to try and induce release of the acetylcholinesterase. It was found that the enzyme could be released by administering either a high concentration of K^+, Dimethylphenylpiperazinium Iodide or Carbachol to the gland. The release was dependent upon the presence of Ca^{2+} ions in the perfusing fluid. Electrophoretic analysis of the perfusate showed that only $AChE_5$ was released from the glands. The amount of enzyme appearing in the perfusate was related to the amount of catecholamines, probably reflecting the efficacy of the stimulation, but not to the amount of acetylcholinesterase in the adrenal gland.

3. Cytochemical studies.

Because only one of several soluble isoenzymes of acetylcholinesterase was released, and since we were unable to analyze the storage characteristics of the protein by the normal methods (centrifugation) we resorted to a cytochemical analysis of the bovine adrenal medullae and splanchnic axons in an attempt to find how, and in what structure, the acetylcholinesterase was stored. These results showed that the only intracellular acetylcholinesterase activity which was demonstrable with this technique was within elements of the endoplasmic reticulum (agranular in the axons and granular in the chromaffin cells). Apart from the finding that most chromaffin cells contained appreciable amounts of the enzyme, three other notable findings were made using this technique. First, acetylcholinesterase was never found within nerve terminals. Secondly, there were occasions when the acetylcholinesterase-rich endoplasmic reticulum appeared to be in the process of fusing with either the axonal or chromaffin cell's plasma membrane. On such occasions, the reaction product within the tubules seemed to be continuous with that outside the cell. Finally, there was demonstrable activity outside adjacent cells only when at least one of them contained intracellular activity.

4. Extracellular fluid levels of acetylcholinesterase.

(i) Serum: The levels of the enzyme were first examined in the sera of calves and sheep of widely differing ages. It was found that the level in bovine serum was very high some 20 days before birth (the earliest time studied) and that from this peak there proceded a seemingly exponential decline in the serum level up to about 15 years of age (the oldest animal studied). In between these extremes it was found that the levels of the enzyme varied according to the health of the animal, the gestation time at parturition, and whether the birth was normal or induced. Sheep samples also showed a downward trend in the later stages of gestation, but they differed from the calf in that the acetylcholinesterase level of the newborn lamb was already the same as the concentration in the serum taken from adults. These results are discussed in relation to the development of the nervous system rather than to neonatal physiology.

(ii) Cerebrospinal fluid: It was found that the concentration of acetylcholinesterase in rabbit cerebrospinal fluid could be altered by stimulating various peripheral nerves (stimulation of these nerves was known to release acetylcholine from some structures within the brain). An electrophoretic analysis of the cerebrospinal fluid revealed the presence of only one iso-enzyme of acetylcholinesterase. It was found that the relationship between the brain and the cerebrospinal fluid was very similar to that which existed between the adrenal and its perfusate; there were several soluble isoenzymes in the brain but only one of these was released into the cerebrospinal fluid.

5. Turnover of acetylcholinesterase.

This was studied from a combined biochemical and cytochemical point of view. The recovery of enzyme activity in the superior cervical ganglion of the rat after poisoning with DFP was found to be complex, consisting of at least two parts. However, in the initial stages, when the recovery was most rapid, all of the enzyme activity appeared to be restricted to the postganglionic cells. Thus, postganglionic adrenergic cells are capable of synthesizing acetylcholinesterase very rapidly. There was no selective reappearance of any of the isoenzymes, nor was there found to be any selective disappearance induced by denervation. Cytochemistry revealed that, in common with splanchnic axons, there was never demonstrable enzyme activity within the nerve terminals of the normal preganglionic fibres. These results are used to discuss the importance of the contribution of preganglionic fibres to the total acetylcholinesterase activity of ganglia.

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CONTENTS

PREFACE

The work described in this thesis is almost totally confined to only one protein of the nervous system : acetylcholinesterase (AChE). My interest in this enzyme developed during the course of some work on its biological substrate, acetylcholine (ACh), when it became apparent that to answer some questions raised by this work it was necessary to study AChE in some detail.

It soon became obvious that to study AChE in any detail at all was a full-time occupation. The sheer volume of literature devoted to the family of enzymes to which AChE belongs, and to various aspects of their localization, inhibition, activation and sundry other properties, is vast. After the first paper appeared in 1914 there were few publications until 1934. Thereafter, a steady increase in the annual number occurred until 1955, the peak year, when some 600 papers were published. By 1960 some 2000 papers were available and looking at random samples of the years between then and now suggests that, with an annual rate of some 200-250 papers, there are more than 5000 papers devoted to these enzymes.

To survey all of the work in this field is not impossible but it would be a mammoth task. Quite apart from the volume, it must be said that not all workers have applied the available criteria for distinguishing the various esterases sufficiently stringently (see later). Thus, the interpretation is difficult of a paper purporting to be a study of AChE, the sole criterion for which is an ability to hydrolyze ACh.

Part of this trouble is undoubtedly due to the way in which the nomenclature of the cholinesterases has evolved. As shown in Table 1, the number of different names for these enzymes is surprisingly high, principally because it was not until 1965, when over half of the

TABLE 1

NAMES IN GENERAL USE TO DESIGNATE VARIOUS TYPES OF CHOLINESTERASE [a]

Authors	Designation	
Stedman et al. (1932)	not studied	choline-esterase
Most of the authors 1933-42	cholinesterase	cholinesterase
Mendel and Rudney (1943) and subsequent publications including those by Myers and the British investigators of Sir Henry Dale's school	true cholinesterase (specific cholinesterase)	pseudo-cholinesterase
Zeller and Bissegger (1943) and subsequent publications	e-type cholinesterase	s-type cholinesterase
Nachmansohn and Rothenberg (1945) and subsequent publications until 1949	cholinesterase	unspecified esterase
Glick (1945)	specific cholinesterase	non-specific cholinesterase
Augustinsson (1948), Burgen Hobbiger (1951)	cholinesterase I	cholinesterase II
Augustinsson and Nachmansohn (1949b) and subsequent publications by both authors and those by most American investigators	acetylcholinesterase	cholinesterase
Koelle and Gilman (1949) and subsequent publications by Koelle until 1955	specific cholinesterase	non-specific cholinesterase
Richter, cited by Sturge and Whittaker (1950), Whittaker (1951)	acetocholinesterase	butyrocholinesterase
Karczmar et al. (1953)	acetylcholinesterase	pseudocholinesterase (cholinesterase)
Jacob (1954)	acetylcholinesterase	XChE

[a] Adapted from Augustinsson (1963).

available papers had already been published, that the I.U.B. began to define the esterases. Of the 40 different classes of esterases defined by the I.U.B., use of differential substrate and inhibitor specificities has established that six of them are present in vertebrate tissues. Of these six only two are of real interest for the work described in this thesis - AChE (E.C.3.1.1.7.) and non-specific cholinesterase (ChE; E.C.3.1.1.8.). Both of these enzymes hydrolyze esters of choline at greater rates than they do other esters, but they do hydrolyze other esters. For example, they will both hydrolyze aliphatic esters of naphthylene - a property which has helped enormously in histochemical studies of their distribution. But it is this lack of absolute specificity which has led to much confusion and difficulty in interpretation of a proportion of the literature. Thus, use of an acetate ester, of choline or any other base, without the use of specific inhibitors and an analysis of the enzyme's kinetics can obviously lead to an erroneous and confusing nomenclature being applied.

In this thesis, use of the term AChE will be confined to an enzyme (enzymes) which hydrolyzes acetylthiocholine (ATCh), is inhibited by low concentrations of eserine (2.0×10^{-5}M) and BW 284C51 (1.5×10^{-6}M) and by higher concentrations (> 2 mM) of the substrate. ChE will be the term used to denote an enzyme which hydrolyzes ATCh but, while being inhibited by eserine at 2.0×10^{-5}M, is unaffected by BW 284C51 at low concentrations, and whose activity increases with an increasing substrate concentration.

The literature covered for this thesis will be confined where possible to those papers directly related to the work - by virtue of the organs and types of nerves studied. No attempt has been made, for example, to review systematically the publications on neuromuscular or plasma cholinesterases, although reference to them in a work of this type cannot, and will not, be avoided.

Finally, I would like to acknowledge the contributions made by various people to this work. First to my supervisor, Dr. A.D. Smith, I extend my sincere thanks for the help, kindness and interest that he has expressed in many ways over the past three years. I am very grateful to Professor Paton, not only for allowing me to undertake this project in his department, but also for the many helpful and friendly discussions we have had during the course of the work. I also acknowledge with considerable pleasure the collaboration of Mr. P. Somogyi from Budapest, who worked with us during the year 1973-1974. Apart from the many discussions we had, the results of our technical collaboration are presented in Chapters 5 and 7. This work was financed by a grant from the Wellcome Foundation, to whom I am indebted for their support.

At various times during this work I was grateful for the technical assistance of Mrs. P. Booth and Mrs. M. Burnett, the latter being responsible for many of the drawings.

I am extremely pleased to be able to acknowledge the collaboration of Drs. R. Comline and M. Silver from the Physiological Laboratory, Cambridge, from whom various bovine tissue and plasma samples were obtained. Without their cooperation, some of this work would not have been possible.

TABLES

FIGURES

facing page

CHAPTER 1

GENERAL INTRODUCTION.

INTRODUCTION

Dale (1914) found that the injection of acetylcholine (ACh) into an anaesthetized cat produced severe depression of both heart-rate and blood pressure. He noted, however, "when once the normal has been regained, which, even with doses of the order of 1 mg always occurs after a few minutes, the whole effect can be reproduced in all its features by giving another, similar injection". To explain this "evanescence" of the effect of administered ACh, Dale suggested that it was due to the "readiness with which the ester is hydrolyzed into its relatively inert constituents, choline and acetic acid..... In the blood at body temperature it seems not improbable that an esterase contributes to the removal of the active ester from the circulation, and the restoration of the original condition of sensitiveness".

Thus was made the first suggestion that an enzyme might be responsible for the rapid reversal of the effects of ACh which arose when it was administered to an animal. Indeed, most of the initial observations on these enzymes (called cholinesterases by Stedman, Stedman and Easson, 1932) were made as a result of pharmacologists studying the actions of various extracts on tissues. For example, Fühner (1918) studied the action of an extract from the Calabar bean on the ACh-induced contraction of the isolated leech muscle. He showed that the extract - now known as eserine or physostigmine - increased by about one million-fold the sensitivity of the tissue to ACh, but that it had no effect on a pilocarpine-induced contraction. Following Dale, Fühner suggested that the effect of eserine was to inhibit the hydrolytic destruction of the ACh. This result obtained by Fühner (1918) is an important adjunct to Dale's observations since it showed for the first time that tissues were also capable of hydrolyzing ACh - an ability that Dale had attributed to the blood. In 1926, Loewi and Navratil used eserine to try and show that the "Vagusstoff" observed by Loewi in 1921 was really ACh. Their now classical experiments not only provided the evidence required, but they also showed that an extract of an essentially blood-free heart contained an enzyme capable of hydrolyzing ACh. These observations confirmed and extended those of Fühner (1918) in providing more evidence that tissues could hydrolyze the ester, but it was really left to Clark (1927) to show that this hydrolysis was enzymic. Clark (1927) made two other very important observations when he first showed that the destruction of ACh was not confined to those tissues on which the ester produced a specific action and then that the "ferment" was intracellular. This latter observation he made by allowing 0.2 ml of saline to remain in the heart for 2 hours. He found, when he tested the ability of this saline to destroy ACh, that it was totally unable to do so. However, when he immersed hearts in a concentrated solution of ACh (10^{-4}M) for 12 h, he found that while there was no ACh in the heart muscle there was, albeit less, ACh in the solution. Thus, Clark (1927) concluded that the destruction of ACh occurred somewhere within the cell.

The lack of tissue specificity for the destruction of ACh was suspected and finally confirmed by Plattner and Hintner (1930) when they showed that almost all animal tissues were able to destroy the ester enzymatically. Thus, the direct relevance of much of the earlier work of Loewi and Navratil (1926) and Clark (1927) concerning enzymatic destruction of ACh was thrown into some doubt since, as pointed out by Stedman et al. (1932), not only are esterases widely distributed throughout an animal, but they also hydrolyze a variety of esters; it was, therefore, not surprising that the heart could destroy ACh. The most important question in the development of cholinesterase research thus became : is there an enzyme which specifically hydrolyzes ACh and no other ester? Such a substrate specificity was given to an enzyme (called, therefore, choline esterase) isolated from horse serum by Stedman et al. (1932). However, controversy soon raged, not over this enzyme's ability to hydrolyze ACh, but over the question of whether its action was confined to choline esters rather than simply to aliphatic esters in general.

Between the years 1940 and 1943 it was quite conclusively established that there existed more than one type of cholinesterase in the animal body. This was first shown by Alles and Hawes (1940) who compared the specificity of human erythrocytes and serum. They showed that the esterases in each had different kinetic characteristics and also that only the erythrocyte enzyme could hydrolyze acetyl β-methylcholine. Mendel and Rudney (1943) called the enzyme in erythrocytes "true" and that in the serum "pseudo" cholinesterase. Later (Mendel, Mundell and Rudney, 1943) it was shown that benzoylcholine was hydrolyzed by "pseudo" and not by "true" enzyme, whereas for acetyl β-methylcholine the reverse was true. Thus, the use of these two substrates could allow distinction of the two types of cholinesterase in the blood and, presumably, in the tissues. It also became apparent that the cholinesterase isolated by Stedman et al. (1932) was of the non-specific or "pseudo" type, fairly specific for choline esters, but not for ACh. It was thus generally concluded, and is still accepted today, that those tissues which receive a cholinergic innervation contain high activities of an enzyme which, while not totally specific for ACh, has a high affinity for that ester as its substrate. It was also obvious that the converse, i.e. that high concentrations of AChE indicated a cholinergic innervation, was not true. The very tissue used to demonstrate the presence of a "true" or "specific" cholinesterase, the erythrocytes, denied this generalization. The question raised earlier, however, had been answered. There _is_ an enzyme which is relatively, but not absolutely, specific for ACh; the earlier confusion was also resolved because it could be shown that the apparently ubiquitous distribution of the esterases was due, at least in part, to the activities of different enzymes.

The problem by this time, however, had developed into whether or not the esterases were physiologically functional in the context of cholinergic transmission (cf. Loewi and Navratil, 1926; Clarke, 1927). Quite apart from the wide distribution of the esterases and their apparent lack of absolute substrate specificity (Stedman et al., 1932), there were other strong arguments against the theory of a transmitter function for ACh in all those systems where the effector cell was rapidly responding, e.g. muscle or ganglion cell (Eccles, 1937). The principal argument used by Eccles was that of the "time factor"; an argument which was crucial to the whole discussion surrounding the concept of chemical transmission. Chemical reactions connected with this type of transmission would have to occur with great rapidity. For example, if a compound were released from the nerve to induce a depolarization of the effector cell, then it must be removed from the synaptic cleft within the refractory period since, at the end of this period, the cell (e.g. ganglion cell or muscle) is again fully responsive to any stimulus. Eccles (1937) argued that there was no evidence to show that ACh could be removed at a sufficiently high speed for the above criterion to be met. Therefore, it became essential to the theory of chemical transmission to determine whether ACh could be removed from synapses and motor end-plates with sufficient rapidity to answer these arguments.

Marnay and Nachmansohn (1937 a) were the first to attempt to answer this criticism by estimating the amount, or rate, of ACh hydrolysis by ground voluntary muscle. They found that the concentration of the enzyme in muscle tissue was low. It was so low that they estimated that it would take about 300 sec in frog and 75 sec in mammalian muscles to reduce by 50% the amount of ACh likely to be liberated by a single motor nerve impulse. Since the respective refractory periods are about 5 and 2 milliseconds, the respective periods of hydrolysis were either 60,000 or 40,000 times greater than the refractory period. Marnay and Nachmansohn (1937 a) suggested that, if the enzyme were the means by which the transmitter action was terminated, it must be concentrated many thousand-fold in the region of the motor end-plate. If this could be shown to be so, it was argued, then support could be gained for the chemical transmission theory and also strong evidence for the function of tissue esterases could be obtained.

Again, the first attempts were made by Marnay and Nachmansohn (1937 b). For these experiments they used sartorius muscle of the frog, a tissue known to have nerveless (pelvic) and innervated ends (Lucas, 1907). The part of the muscle containing the nerve fibres was found to have a concentration of esterase 3-fold higher than the pelvic end. This figure was modified after Pezard and May (1937) showed that not all, in fact only one-eighth, of the pelvic end of the muscle was really nerve-free. A comparison of innervated and nerveless parts of the muscle now showed a six-fold increase in the amount of esterase in the nerve-ending-containing muscle (Marnay and Nachmansohn, 1937 c, 1938). Finally, Nachmansohn estimated, roughly, that the end-plates occupy less than 1/5000 of the total volume of the muscle. Assuming that the enzyme is evenly distributed amongst the end-plates, and all over them, it can be calculated that the nerve ending has an esterase concentration approaching 15000-30000-fold higher than the non-innervated or nerveless muscle tissue.

Marnay and Nachmansohn (1938) concluded: "... first, the chemical changes at the end-plates of voluntary muscle can occur with the flash-like suddenness necessary for a chemical transmission of nerve impulses; secondly, a concentration of a specific enzyme at the nerve-endings, many thousand times as high as that in the surrounding muscle tissue, must have functional significance, and suggests - without anticipating the function - that ACh intervenes in the transmission."

Similar reasonings, assumptions and findings were applied to and obtained for sympathetic ganglia. Glick (1938) was the first to apply the methods of Nachmansohn to sympathetic ganglia when he showed that the amount of esterase within the cat superior cervical ganglia is not sufficient to split, within the refractory period, the amount of ACh liberated by stimulation of the nerve. He too suggested the possibility of a high local concentration of the enzyme around the synapses. Some evidence for this possibility was obtained by Nachmansohn (1939) when he showed that the ganglia of the sympathetic chain contained two to four times more esterase than the nerve fibres running to them (in absolute terms). Since, he reasoned, the synapses occupy only a very small fraction of the total ganglion volume, there must be a very high concentration at the nerve terminals in order to measure a change in the content of a whole ganglion. Assuming such a concentration, Couteaux and Nachmansohn (1940) estimated that 20-30 times more ACh could be hydrolyzed by the esterase in 1 millisecond than is in fact liberated by maximal stimulation. Couteaux and Nachmansohn (1940) also showed that section of the preganglionic fibres led to a reduction of 60% in the ganglionic esterase content. Only 1-2% of this esterase, which they assumed to be associated with the preganglionic fibres, would be sufficient to hydrolyze the liberated ACh within 1 millisecond.

At around the same time as the experiments described above, Nachmansohn initiated experiments into the esterase content of the central nervous system (see Nachmansohn, 1939). Again he found high activity and a detailed analysis of the brain showed great variations in different parts of the organ. By making the same assumptions as were used for muscles and ganglia, Nachmansohn (1939) reasoned that there was good evidence that in the CNS there was a similar mechanism to that in the periphery for the removal of ACh, i.e. an enzymic destruction which, because of the vast excesses of enzyme, could occur with "flash-like suddenness". Therefore, he proposed that these results were consistent with the suggestion that ACh was a chemical transmitter of nerve impulses in the CNS as well as in the periphery.

The actual observation of the physiological function of esterase at the motor end-plate, and confirmation of Nachmansohn's hypothesis, was made by Eccles, Katz and Kuffler (1942). When sufficient muscle AChE was inactivated by eserine, Eccles et al. (1942) observed that a single presynaptic volley resulted in a marked prolongation of the end-plate potential and, possibly more importantly, a repetitive firing of muscle action potentials. Inhibit the AChE, and you increase the physiological effects of ACh - an event first noticed twenty-four years earlier by Führer (1918).

The remaining fact that was essential to ascertain was how much of a tissue's esterases was associated with the nerve supply to that tissue. The most obvious way to determine this is to denervate the tissue or organ concerned, allow sufficient time for the nerves to degenerate and then to estimate the esterase content. As has been mentioned above, Couteaux and Nachmansohn (1940) estimated by these means that approximately 60% of the esterase in the cat superior cervical ganglion (SCG) was preganglionic; Sawyer and Hollinshead (1945) showed an 80% decrease as an effect of denervation and in addition they showed that both AChE and ChE were present in the ganglion, a result which confirmed the earlier observation of Mendel and Rudney (1944). For muscle, the results of denervation experiments have not been without controversy - an increase in activity on the one hand, a decrease on the other. These problems have now been resolved and there is no longer any question that section of motor nerves leads to a decrease in the esterase content of the muscles they innervate (see Guth, 1968; Crone and Freeman, 1972).

The type of esterase in muscle was shown to be mostly "true" or AChE (Langemann, 1944; Nachmansohn and Rothenberg, 1945) with a small amount of ChE (see Guth, 1968).

At this point I will summarize the information which had accumulated during the thirty-one years between the initial postulate of the presence of esterases in 1914 and the end of 1945.

1. There are many types of esterase present, both intra- and extracellular, in many species, but there is a group designed specifically to hydrolyze esters of choline. One of this group is relatively specific for ACh.

2. The concentration of AChE at end-plates, ganglionic synapses and in the CNS is sufficiently high to be consistent with the theory of chemical transmission of nerve impulses.

3. The function of the high concentration of AChE at end-plates can be illustrated by the alteration of the action of ACh at those end-plates where the enzyme has been inhibited.

4. Generally, AChE is associated with nerves, both in axons (Boell and Nachmansohn, 1940) and terminals.

5. Degeneration of nerves results in a loss of enzyme from the affected tissue.

6. AChE is present in the extracellular fluid of several species but noticeably, and most unequivocally, in the cerebrospinal fluid (Plattner and Hintner, 1930).

POST-WAR RESEARCH:

Up until 1945 the studies on the various esterases, but most particularly the cholinesterases, were characterized by their tendency toward the relationship between enzyme specificity, activity, distribution and localization with physiological and pathological function. After 1945, i.e. after World War II, the organophosphorus compounds, many of which were stockpiled for use as nerve gases, were made available for use in scientific study. At the same time, a considerable proportion of the work secretly carried out by scientists during the war was published in freely accessible journals. The effect on cholinesterase research was spectacular; in 1946, there were twice as many publications as in 1945, with the emphasis toward the chemistry, specificity, synthesis and reversibility of the inhibition caused by these new compounds. But during this period much information about the cholinesterases was also obtained by using the inhibitors as tools. This enabled the active centre, turnover rate, rate of synthesis and other properties of the enzyme to be studied and some of these results will be discussed later.

As these massive increases in our knowledge of the actual workings of the cholinesterases were made, advances were also making it possible to begin to study neurophysiological and neuropharmacological effects at the cellular and subcellular levels. For example, Fatt and Katz (1950) observed what was at first called "biological noise". This was subsequently shown to be small changes in the end-plate potential, which were due to spontaneous changes in this "sub-threshold" activity (Fatt and Katz, 1952). These miniature end-plate potentials were finally shown to be due to the spontaneous release of small packets (or quanta) of ACh (see Katz, 1962).

Two major technical advances, the production of the electron microscope and the first high-speed centrifuges were the equivalent hopes of the morphologist and biochemist to the micro-electrode and measuring devices of the neurophysiologist. However, the microscope soon yielded morphological details of tissues which were far beyond the known physiological correlates. What was required by the morphologist was a means by which cholinergic neurons could be identified under the microscope.

There were three known and relatively specific chemical moieties in cholinergic nerves: acetylcholine, choline acetylase (Nachmansohn and Machado, 1943) and AChE. Of these, only AChE seemed to fulfill the requirements which are necessary for cytochemical localization. It is stable, it can be inhibited by the anti-ChE's, so allowing suitable controls, and it will hydrolyze a variety of substrates some of which are amenable to histological development. There also exist specific inhibitors to allow differentiation of the various types of esterase present in tissues.

The first histochemical method for the localization of cholinesterases was at the light microscope level and was published by Gomori (1948). The substrates used were choline esters of myristic, stearic, lauric and palmitic acids which, although shown by Glick (1941) to be hydrolyzed by horse serum ChE, were obviously not ideal. However, Glick (1939) had also shown that acetylthiocholine (ATCh) was hydrolyzed by ChE, a finding extended by Koelle and Friedenwald (1949) to AChE. This substrate, because of its structural similarity to ACh and because of the chemical reactivity of the enzymatically liberated thiol group, was used as a more specific and better substrate (higher rate of hydrolysis by both AChE and ChE than ACh itself) for the histochemical reaction (Koelle and Friedenwald, 1949).

Koelle (1951) first modified his original technique to eliminate diffusion artifacts, and then, between the years 1951 and 1955, studied a wide variety of tissues from many different species. One final modification was also introduced in 1951 by Couteaux, who fixed the tissues in formalin before he developed the cholinesterase-rich zones. Although, as he acknowledged, activity was lost during the fixation, he considered that it was the only way in which the structural integrity could be preserved (Koelle studied frozen sections).

As mentioned earlier, Koelle and others (notably Gerebtzoff, 1959) have extensively surveyed the tissues of many species and from the results it can be concluded that, generally, neurons established as cholinergic contain high concentrations of AChE throughout their process, including dendrites, perikarya, axons and axon terminals. Neurons which are not considered to be cholinergic, e.g. sensory neurons of dorsal root ganglia and adrenergic neurons of the superior cervical ganglion, contain lower amounts. These latter observations made during cytological studies have received some direct support from Giacobini (1957) who estimated the AChE content of single neurons using ultra-micro-techniques. A combination of both the above techniques allowed a refinement of the above generalization; it was shown that the ganglion cells of the cat fell into three classes with respect to AChE staining, a small number of heavily, an equal number of moderately, and a large number (approx. 85%, Holmstedt and Sjöqvist, 1959) of lightly stained cells. Giacobini (1957) quantitated the amount of AChE in individual autonomic ganglion cells and found that there was a wide (20-fold) range of activities but that most cells showed significant activity and only a few seemed totally devoid of enzyme.

The adrenal medulla was also used as a source of material for cytological studies by many workers. The medulla is essentially a modified sympathetic ganglion. Embryologically, both the medullary chromaffin cells and the sympathetic ganglion cells originate from the same general region of the neural crest (see e.g. Coupland, 1965). While adrenaline is secreted from the medullae of man and most other mammals directly into the circulation, sympathetic ganglion cells liberate noradrenaline from their axon terminals which are in the immediate vicinity of the effector organs. Both types of cell are innervated by preganglionic fibres which regulate their activity. It might, therefore, be expected that the distribution of AChE, as determined by light microscopic cytochemistry, would be the same in both organs. However, this expectation was only partially confirmed by the early workers in this field.

The medullae of rat, rabbit, cat, ox and human were extensively studied by Coupland (1961), Coupland and Holmes (1958), Koelle (1951) and Eränkö (1958). The chief conclusion drawn from these studies was that medullary AChE was almost exclusively confined to the nerve fibre network and that no enzyme was present within the chromaffin cells. This consistent observation differs from the equally consistent observation that ganglion cells contain AChE - indeed, it is interesting in this context that in those medullae which contained distinct ganglion cells (notably in the rat), these cells all contained quite high amounts of AChE. Eränkö, Hopsu and Raisanen (1959) showed that there was a decrease (not complete) in the AChE content of rat adrenal when the splanchnic axons were sectioned, thus adding to the evidence that most of the enzyme was associated with the nerve supply but also providing some evidence for another site of AChE activity. This was put down to the presence of the AChE-rich ganglion cells.

Meanwhile, technical advances in the manufacture of centrifuges were such that an application of the techniques first introduced by Dounce (1943) could be made to nerve tissue. One of the first to apply these techniques to the cholinesterases was Hagen (1955) who studied the distribution of AChE among fractions obtained by centrifuging homogenates of bovine adrenal medullae at different speeds. Hagen's results showed that the bulk of the AChE was sedimented in a fraction which required high speeds but that about 30% of the activity was not sedimented at all. Similar observations were made by Toschi in a series of papers in which he studied the distribution of AChE amongst brain subfractions (Toschi, 1959; Hanzon and Toschi, 1959; Holmstedt and Toschi, 1959).

It was about this time that one of the most common misconceptions regarding the subcellular localization of AChE arose. Boell and

Nachmansohn (1940) squeezed the axoplasm out of squid giant axons and estimated the AChE activity in both axoplasm and sheath. They found that "all" of the activity was associated with the sheath. However, their results clearly show that some, albeit little (6.04%), of the activity is associated with the axoplasm. Nevertheless, the inference drawn from Boell and Nachmansohn (1940) was that AChE is only a membrane-bound enzyme. When most of the activity in homogenates was found to sediment in a centrifuge (Hagen, 1955; Toschi, 1959) and when the sediment was observed morphologically and found to consist chiefly of membrane fragments (Hanzon and Toschi, 1959) it was, and has been until recently, considered the prototype membrane-bound enzyme. This conclusion was strengthened when Lawler (1964) found that the membranous AChE could only be removed or solubilized from the membrane with extreme difficulty. Despite this result, many workers still believed that any soluble AChE found in homogenates was artifactually derived from the membrane-bound form during the homogenization or centrifugation procedure (e.g. Barron and Bernsohn, 1968; Hollunger and Niklasson, 1973; McIntosh and Plummer, 1974). This point will be discussed in detail in Chapter 2.

One other piece of vital work was performed in this era by Weiss and Hiscoe (1948) - this time as a result of simple experimentation and not of a technical advance. Weiss and Hiscoe (1948) partially obstructed nerve trunks and then, at different times after the obstruction was placed on the axon, they examined the tissue on both sides of their block. They found that the diameter of the axon was markedly reduced, not only in the area of the obstruction, but also for the entire distal length which could be observed. Proximal to the obstruction they found that the fibres were dilated, with balloon-like distortions of the axons. Removal of the block led to a return to normal of the diameter of the axons, both proximal and distal to the site of the obstruction. Weiss and Hiscoe (1948) interpreted these results as showing a bulk movement of axoplasm from the cell body of the neuron, down the axons to the terminals of the nerve. Thus, they formulated the concept of axoplasmic flow which, as originally proposed, meant a slow, steady movement of material at a rate of about 1-2 mm/day.

The earliest study in which changes in AChE were examined in the axons of interrupted nerves was that of Sawyer (1946). Using the sciatic nerve of the guinea pig, Sawyer (1946) found that there was an increase in the AChE content of the proximal tip (he surgically removed a piece of nerve) and a decrease on the distal side. He did not, however, think of his results in terms of axoplasmic flow, but more of a local production of the enzyme.

The next studies to be carried out were after the Weiss and Hiscoe (1948) proposal of axoplasmic flow. Their nature, however, was purely qualitative histochemistry (Snell, 1957) and so it was not until Lubinska and her colleagues studied the transport phenomenon in a quantitative manner that interesting features of both AChE transport and the whole concept of axonal flow became apparent. In their studies Lubinska, Niemierko, Oderfeld-Nowak and Szwarc (1964) concluded that AChE flowed more rapidly than the 1-2 mm/day of bulk flow, as proposed by Weiss and Hiscoe (1948). They deduced this fact from the clear-cut accumulation of AChE activity that they found when they studied small pieces of nerve immediately proximal to the obstruction at short time intervals after the nerve was cut. Thus, 4 h after cutting the peroneal nerve of the dog, there was a 3-fold increase in the AChE content of the 2 mm segment closest to the point of transsection. The first 2 mm of nerve on the distal side, however, were also shown to have an increased AChE content, 2-fold. Sections of nerve further away from the cut had much lower levels of AChE; the proximal segments were at control levels, the distal nerve was slightly below the control activity.

Obviously, these results threw into some confusion the proponents of bulk movement of axoplasm for two reasons: first, the accumulation of AChE was much faster than 1-2 mm/day and secondly, the accumulation of AChE on the distal side of the ligation indicated the possibility that the flow could be bi-directional. Lubinska et al. (1964) made an additional important contribution at this time, when they showed that a redistribution of AChE activity occurred in an isolated segment of nerve. For these experiments they cut the peroneal nerve of the dog in two places, 50-100 mm apart. 5 h after cutting, the more peripheral end of the isolated nerve segment had a roughly 3-fold greater AChE content than the central portions. Likewise, the distal 2 mm had roughly twice the activity of nerve segments taken from mid-way between the cuts. The important additional observation made during these experiments was that the total AChE activity between the cuts was unchanged. Thus, there had been a redistribution of the enzyme in a segment of nerve axons isolated from the cell body, and not an increased synthesis of protein induced by the cutting of the fibres (cf. Sawyer, 1946). The calculated rate of transport of AChE in dog peroneal nerve was about 15 mm/day, much faster than the rate proposed by Weiss and Hiscoe (1948). AChE was not the only protein shown to accumulate soon after ligations and thus, presumably, to flow rapidly (a relative term but, in this context, it is rapid when compared with the bulk flow). Weiss (1967), as a consequence of these and similar types of experiments, modified the concept of flow by stating that "... (slow flow) in no way precludes the presence within the carrier system of separate routes or channels for express traffic." This, as we shall see later, was a prophetic remark.

Before turning to the work of the 1960's, there are two more observations made in the late 1950's which should be mentioned. The first of these is the observation of Brown (1958) who studied the distribution of AChE in rat SCG after both pre- and postganglionic axotomy. Preganglionic trunk section led to a disappearance of all activity except that present in the cell bodies. But post-ganglionic axotomy led to a decrease in activity in both the "surrounding tissues" and in the cell body. This observation, that section of postganglionic fibres led to a decrease in the AChE content of their preganglionic counterpart, was later confirmed by Härkönen (1964) and Eränkö (1967). The second observation was that of Koelle and Koelle (1959) who studied the distribution of AChE in the ganglion with the aim to determine which was "external", i.e. which was oriented so that it was readily accessible to ACh, and which was "internal" or "reserve" enzyme. They treated ganglia with the slowly reversible, potent, but membrane-impermeable anti-AChE, WIN 8077. Subsequent histochemical examination of the tissues revealed that "external" AChE was localized at the surface of cholinergic neurons, along the process and at axon terminals. The "internal" AChE was not affected. Denervated stellate and SCG were practically devoid of external AChE, an interesting observation in view of the finding of Sawyer and Hollinshead (1945) that as much as 80% of the AChE of the cat was, on the evidence of denervation experiments, preganglionic.

The work of the 1960's was notable for the introduction of electron microscopic cytochemistry into cholinesterase research. Even though the first attempts at this technique were made in 1959 (Lehner and Ornstein, 1959), the methods used were not specifically designed for the cholinesterases. The first method which was designed to detect the cholinesterases, due to the lack of use of inhibitors of the enzymes, only had such specificity as the use of ATCh as substrate could bestow (Birks and Brown, 1960). Nevertheless, these results showed fairly convincingly that there was a high concentration of esterase at the motor end-plate, on the muscle membrane. They were quickly followed by Barrnett (1962) who, by using thiolacetic acid as a substrate, tried to avoid the large crystals which were invariably formed when the original technique of Koelle (1950) was used for electron microscopy. Barrnett (1962) observed reaction product at two sites: one in the junctional folds of the muscle and, more surprisingly, one in the preterminal axoplasm. At this latter location, Barrnett could observe the end-product within what appeared to be synaptic vesicles. Miledi (1964) confirmed this result using ATCh as the substrate for the reaction. Although both workers interpreted their results with caution, there has been controversy over the presence or not of an AChE within "synaptic vesicles" ever since that time.

The introduction of this technique to the autonomic nervous system gave rise to more questions than it answered and opened up more controversies than it settled. For example, studies on the rat adrenal medulla (the only one to have been studied at the electron microscope level) led to such comments as: "... a few (i.e. adrenaline-containing chromaffin cells) showed slight staining confined to short lengths of the nuclear envelope and rough endoplasmic reticulum...", by Lewis and Shute (1969), while Palkama (1967) could not detect any intracellular activity although he did observe enzyme "... in the membrane of the noradrenaline-containing cells". This particular problem seems to be still unresolved, but it must be said that this example illustrates the problems and pitfalls in the use of histochemical techniques, whether at the light or electron microscopic level. The interpretation of results obtained with these techniques, particularly when they are negative, must always be made with care.

Another example of a freshly opened controversy is that concerning the localization of AChE in sympathetic ganglia. As has been mentioned before, the conclusion,based upon the disappearance of almost all of the functional AChE and, indeed, almost all of the total activity, following preganglionic denervation of the SCG of the cat, was that the enzyme within the ganglion was practically entirely presynaptic (Koelle and Koelle, 1959). This agreed with the conclusion of Sawyer and Hollinshead (1945) which was based on observations made using a similar technique. However, Koelle, Davis and Smyrl (1971) reported that most of the histochemically demonstrable AChE at ganglionic synapses was associated with the postsynaptic membrane. This result has subsequently been confirmed by Koelle (1971), as has the effect of denervation on the ganglion's AChE content, which falls to 20% of the control level (Koelle, Davis and Koelle, 1974). The situation is further complicated by the observation that non-specific ChE also seems to be a constituent enzyme of the neuronal processes, and it, too, decreases, albeit at a slower rate, after preganglionic axotomy (Koelle et al., 1974).

The observations made when the ganglia are the tissue under investigation are clearly very dependent on the species studied. The best illustration of this is the work of Klingman and Klingman (1969) who showed that preganglionic denervation of rat SCG results in only a 45% fall in the AChE content; a result interpreted to mean that 55% of the activity is postganglionic. Clearly, postganglionic cells do contain AChE, but the question is, are they cholinergic? The answer to this question is almost certainly no. Hebb and Waites (1956) showed that preganglionic denervation of sympathetic ganglia resulted in the loss of 98-100% of the choline acetylase. More recently, Häggendahl, Dahlström and Saunders (1973) have drawn a similar conclusion from observations made using ACh disappearance as an estimate of the degree and rate of denervation. In the former experiments the tissues used were from sheep, a species about which little is known in respect of AChE responses to denervation, but in the latter they were from rats. A decrease of almost 100% in choline acetylase and ACh levels and a concomitant decrease in AChE of only 45% offers strong evidence that all of the choline acetylase, ACh, and hence all of the cholinergic elements are preganglionic, while only part of the AChE is associated with the cholinergic nerves. Thus, it must be concluded that adrenergic nerve cells contain AChE, a suggestion for which Robinson (1971) has provided some cytochemical support. Stronger support was provided by the work of Klingman (1970) who showed that 7-19% of all ganglion cells remain after immunosympathectomy. Klingman (1970),using cytochemical techniques, observed that in rat SCG most of the cells have marked to moderate AChE levels (cf. the cat); she also found that the cells which remained after the immunosympathectomy represented a random population with regard to their AChE content, cells with marked AChE levels did not show any unique survival properties. In other words, it was most unlikely that they were cholinergic neurons.

The results obtained with the use of the electron microscopic cytochemical technique can be summarized fairly briefly. The most thorough and detailed studies of the distribution of AChE within neurons has been carried out on the cell bodies and axons of motor nerves, and there seem to be many similarities between these and all other nerves. Generally, all nerves exhibiting AChE activity seem to be characterized by a dual localization of the enzyme: within endoplasmic reticulum, rough in the cell body and smooth in the axons, and along the surface of both the cell body and the axons (Novikoff, Quintana, Villaverde and Forschirm, 1966; Schlaepfer and Torack, 1966; Brzin, Tenysson and Duffy, 1966; Kasa, 1968; Tenysson, Brzin and Duffy, 1968; Kokko, Mautner and Barrnett, 1969; De Lorenzo, Dettbarn and Brzin, 1969; Kasa, Mann, Karcsu, Toth and Jordan, 1973).

Rapid advances were also being made in the investigation of the AChE content of the CNS since, as mentioned earlier, Nachmansohn (1940) first examined the distribution of the enzyme within the brain of several species. Most of his observations were later confirmed and extended by Burgen and Chipman (1951, 1952), the principal one being that the caudate nucleus contains by far the highest concentration of AChE; the other structures contained less, but nevertheless appreciable amounts, of the enzyme. These observations have also been confirmed by many workers, using many species (see Silver, 1967).

These biochemical results also confirm one of the earliest histochemical surveys of the CNS (Koelle, 1954). Cytochemically, there were very few neurons or synaptic regions which were totally devoid of AChE; the highest activity was associated with those neurons giving rise to known cholinergic peripheral fibres, all of which exhibited intense staining. The caudate nucleus was also very heavily stained.

The first application of electron microscopic histochemistry to the CNS appears to be that made by Lewis, Pine and Shute (1965). They used the thiocholine technique and applied it to rat tissues. Known cholinergic cells (e.g. ventral horn cells) showed intense staining within the endoplasmic reticulum and the nuclear envelope. Basically, these observations have stood the test of time and the presence of AChE at these sites is not disputed.

A considerable proportion of the rest of the work devoted to cholinesterases in the CNS is oriented toward studies of development. These studies will not be discussed in detail, but will be briefly mentioned later.

There are two more salient points which have arisen out of the studies on the CNS, however. Because of the work that will be presented in Chapter 6, it is necessary to have some idea of the gross distribution of AChE within the various structures from which ACh is known to be released - basically, the caudate nucleus and the cortex. The evidence concerning ACh release will be dealt with in the appropriate Chapter.

As shown above, it was well known from the earliest studies that the AChE content of the caudate nucleus was the highest of any structure in the brain - it was also apparent that the cortex had a fairly low content (Burgen and Chipman, 1952). The reasons for this difference have become apparent through the use of histochemistry. The caudate nucleus is known to contain a high concentration of choline acetylase in addition to the AChE and thus almost certainly has a genuine cholinergic component (see Hebb, 1963), the source of which is unknown. In an attempt to determine the origin of the cholinergic nerves, McGeer, McGeer, Fibiger and Wickson (1971) lesioned several anatomically established caudate afferent systems but were not able to detect any change in either AChE or choline acetylase levels in the caudate. This result was confirmed by Lynch, Lucas and Deadwyler (1972) who concluded that failure to alter the staining of the caudate (theirs was the histochemical study) after lesion of the afferents meant that the cholinergic nerves were either caudate efferents or interneurons. To test this hypothesis, Lynch et al. (1972) used diisopropylfluorophosphate (DFP), a potent irreversible inhibitor of all cholinesterases (see Holmstedt, 1963), which they injected into the caudate with a micro-syringe. By studying the tissue at different times after DFP treatment, it was found that there were considerable numbers of neuronal cell bodies which, as soon as 4 h after DFP, stained for AChE. Lynch et al.

(1972) concluded that, since the cells rich in AChE were numerous and since only a very small percentage of striatal cells give rise to axons which leave the structure (Kemp and Powell, 1971), then the cholinergic component of the caudate is a system of interneurons which are "unique in their tendency to show extensive bifurcation". This situation is quite different from that which exists in the cortex. Krnjevic and Silver (1965) showed that there were few AChE-rich cells in the cortex of the cat, and further studies showed that most of the AChE-rich fibres in the cortex were derived from sub-cortically situated cells. This possibility was given biochemical support when it was shown that isolation (by undercutting) of the cortex resulted in a decrease in the AChE content of the tissue (Duncan, Rutledge and Domino, 1968). The evidence, therefore, suggests that, while both the cortex and the caudate have cholinergic nerve terminals in them, one has the cell bodies of these terminals within it, and the other receives the fibres from elsewhere.

Significant advances in directions other than histochemical were also being made, however. One of these was the discovery of isoenzymes, or multiple enzyme forms, by Hunter and Markert (1957), which were first observed after electrophoresis of tissue extracts on starch-gels and subsequent incubation of the gels with α-naphthylacetate and α-naphthylbutyrate as substrates. Although the authors used eserine to attempt to distinguish cholinesterases from the other esterases, there was still very considerable hydrolysis of the substrates and so only general conclusions could be drawn about the nature of the cholinesterases in these tissues (mouse liver and lung). Nevertheless, the important observation was that total esterase activity could be electrophoretically separated into many component parts, a finding which led Markert and Moller (1959) to define the isoenzymes. Originally, they used the term "isozyme" to describe "the different molecular forms in which proteins may exist, with the same enzymatic specificity". Recently, both "isozyme" and "isoenzyme" have been used in the more limited context of multiple molecular forms of an enzyme present in a single species (Webb, 1964). The term "isoenzyme" will be used throughout this thesis.

In parallel with these studies on isoenzymes ran an increasingly analytical use of high-speed centrifuges. Wherever possible in this Introduction, I have tried to keep separate the advances in our knowledge due to the use of different techniques. However, the use of the centrifuge as an analytical tool (de Duve, 1965) and its effect on the study, not only of the distribution of AChE within tissues, but also the isoenzymic make-up of the total enzyme activity means that both must be discussed together.

As mentioned earlier, Toschi was the first to apply these new techniques of centrifugation to brain tissue, with an accompanying morphological examination of the fractions obtained (see Toschi, 1959; Hanzon and Toschi, 1959). It was , therefore, probably natural that the first attempts to study the isoenzymic characteristics of the esterases were also applied to brain, both total and substructural. It was established quite early that the human basal ganglia (Bernsohn, Barron and Hess, 1962) contained esterase isoenzymes. A saline extract of the ganglia and subsequent starch-gel electrophoresis, with development of the esterase activities, using ATCh as substrate, showed the presence of 3 isoenzymes. Because of the centrifugation conditions used by these workers (i.e. low speed) considerable enzymic activity was left at the origin - only a small proportion entered the gel. One year later, Bernsohn, Barron and Hedrick (1963) repeated the earlier experiments, but with the major difference that the separated enzymes were eluted from the gels and analyzed further (pH optima, substrate optima, K_m values, pI_{50}

for eserine, mytelase and DFP). They concluded that, since they were unable to detect any differences, the enzymes are probably functionally similar, although they may be structurally different. Isoenzymes of the esterases have since been found in many tissues, either in a variety of aqueous extracts (Härkönen, 1964; Maynard, 1964, 1966; Christoff, Anderson, Slotwiner and Song, 1966; Ecobichon and Israel, 1967; Hollunger and Niklasson, 1967, 1973; Wilson, Mettler and Asmundson, 1969; Eldefrawi, Tripathi and O'Brien,1970; Bajgar and Zizkovsky, 1971; Chan, Shirachi and Trevor, 1972) or after a partial solubilization of the tissue by detergents (Davis and Agranoff, 1968; Lim, Davis and Agranoff, 1971; Skangiel-Kramska and Niemierko, 1971; Shafai and Cortner, 1971; Coates and Simpson, 1972), or in the powder remaining after the extraction of the tissue with organic solvents (Jackson and Aprison, 1966; Baldwin and Hochochka, 1970). A comparison of all these results to try and find some systematic differences or similarities in the AChE isoenzymes is difficult. Not only have different species been used, but different electrophoretic systems, gel concentrations, substrates and methods of obtaining the samples, all make the comparison virtually impossible. It is, however, possible to say that in many tissues there are soluble isoenzymes of AChE. This can be deduced from studies such as those of Bernsohn et al. (1962) where a low speed of centrifugation was used, but nevertheless some activity had entered the gel; a similar result has been obtained by Eränkö, Kokko and Söderholm (1962). The first to show that soluble AChE was qualitatively different from the membrane-bound activity was Maynard (1966) who studied the isoenzymes liberated from chick brain by aqueous and Triton X-100 extraction. A similar observation was made for the AChE isoenzymes in the retina of the rat, i.e. that Triton X-100 or other detergent treatment which renders the AChE soluble discloses additional enzymic activity (Davis and

Agranoff, 1968). This is in contrast to Barron and Bernsohn (1968) who found that Triton X-100 did not improve separations nor disclose additional activities when developing human brain was used as the tissue source and starch-gels as the electrophoretic support.

There was also the evidence, from histochemical experiments, that not all of the AChE was membrane-bound or, at least, that some of it was readily extractable into aqueous media. Eränkö, Härkönen, Kokko and Räisänen (1964) showed what they ultimately called "lyo" and "desmo" forms of the esterases. The "lyo" form was shown to be readily extractable by water or physiological saline from frozen sections; Koelle, Hossaini, Akbarzadeh and Koelle (1970) extended this observation to show that the "desmo" form was extractable by detergents. The latter authors took this difference in solubility as indicating the presence of isoenzymes; they also observed that, whereas ganglia contained substantial amounts of the "lyo" enzyme, the motor end-plate AChE was predominantly of the "desmo" type.

Quantitative estimates of the proportion of the total AChE activity which is soluble in different tissues have been made by a variety of workers. The results show a wide range of soluble activities: 8% in rabbit brain (Nathan and Aprison, 1955), 9% (Aldridge and Johnson, 1959) or 14.5% (Bajgar and Zizkovsky, 1971) in rat brain, 30% in bovine adrenal medullae (Hagen, 1955), while in calf caudate nuclei it ranges from 7% to 19% depending on the homogenization conditions (Hollunger and Niklasson, 1973). These variations in amounts and differences in extraction methods have made some of the earlier work difficult to interpret because several workers extracted AChE from unfractionated tissue and believed that they were studying the membrane-bound enzyme.

The first evidence that different esterase isoenzymes might be localized in different subcellular structures was presented by

Bernsohn, Barron, Doolin, Hess and Hedrick (1966). An extensive subfractionation of rat brain, followed by quantitation and electrophoresis of the esterases in the fractions obtained, showed marked differences in the distribution of various esterases amongst these fractions but, unfortunately, the methods used do not allow too firm a conclusion to be drawn regarding the distribution of cholinesterases within that tissue. A similar result was obtained by Hopwood (1970), using bovine adrenal gland. He found that some of the cholinesterase was membrane-bound and some was probably soluble. Hopwood's (1970) main aim was an overall study of all esterases and not specifically of the cholinesterases.

The most extensive subfraction of brain tissue has been carried out by the groups of Whittaker and De Robertis. The driving force behind this work was the observation that carefully homogenized brain tissue produced an artifact which, with careful subfractionation, enabled an enormous amount of information about neuronal processes to be gained. The artifact is a nerve-ending which has been pinched off from its preterminal axon during the homogenization procedure; the membranes fold over, giving rise to a completely sealed nerve-ending which is attached only to that part of the postsynaptic membrane which is at the cleft. This particle has been called a "synaptosome" and has been extensively characterized (see e.g. Whittaker, 1969 a).

The subcellular distribution of AChE in the brain, as shown by Whittaker (1969 b), is consistent with the histochemical observations; part of that proportion of the enzyme which is sedimentable does so in a way which is characteristic of light membranes. It is found chiefly in the "microsomal" fraction which is mainly derived from endoplasmic reticulum, a result which confirms the earlier observation of Hanzon and Toschi (1959). However, over half of the sedimentable activity is recovered in the synaptosomal fraction and further sub-fractionation reveals that most of this activity is confined to the synaptosomal plasma membrane (see Whittaker, 1969 b). Histochemical support for this finding was also presented by Whittaker (1969 b). An isolated synaptosome can be shown to have considerable AChE activity localized on the plasma membrane, particularly at the synapse, most of which appears to be on the presynaptic side (see Whittaker, 1969 b). However, the data presented by Whittaker (1969 b) show that even in his extensively washed synaptosomes -they have been through a sucrose density gradient in addition to the normal centrifugation- some AChE is liberated when they are osmotically shocked or lysed.

The observation that synaptosomes formed when the brain was gently homogenized eventually led to attempts to make and isolate similar structures from peripheral tissues. Bisby and Fillenz (1969) succeeded in doing so with an adrenergically innervated tissue, the rat vas deferens, and Hoar, Paton and Smith (1971) found AChE-containing synaptosomes in fractions from intestinal smooth muscle. However, it was not until 1972 that a concerted attempt to isolate peripheral cholinergic synaptosomes was made (Wilson and Cooper, 1972). These workers chose to use the bovine SCG as starting material, having established that nerve-endings in both bovine adrenals and rat diaphragm were too sparse and too fragile to allow their preparation as synaptosomes. Results similar to those described by Whittaker (1969 b) were obtained with regard to ACh, but AChE was not estimated by these workers.

Thus, the use of the ultra-centrifuge has been of great benefit in the progress made toward the understanding of cholinergic structures in the CNS and it has, in fact, made this the one area in which our knowledge of the CNS exceeds our knowledge of other, peripheral, systems.

There is one other piece of information which has had a big impact on much of the work to be presented later in this thesis. As was mentioned earlier, Weiss (1967) modified the original concept of axoplasmic flow so as not to exclude the possibility that some nerve constituents could flow more rapidly than the 1-2 mm/day of the bulk phase. This was largely brought about by the observation that AChE could flow as fast as 15 mm/day.

Since 1967 there have been a vast number of publications devoted to different aspects and characteristics of axoplasmic flow (see e.g. Dahlström, 1971). Consequently, there has been a gradual evolution of our understanding of the phenomenon, an understanding which has led to yet more changes in some of the original concepts. The most significant, and certainly the most unequivocal,new finding is the very large range of mobilities which have been reported for various neuronal constituents. If we restrict the discussion to AChE, then the reported ranges are from 5 mm/day in the hypoglossal nerve of the rabbit (Frizell, Hasselgren and Sjöstrand, 1970) to 430 mm/day in the sciatic nerve of the cat (Ranish and Ochs, 1972). Between these extremes lies the 260 mm/day in the peroneal nerve of the dog (Lubinska and Niemierko, 1971) and the 99 mm/day in the sciatic nerve of the frog (Partlow, Ross, Motwani and McDougal, 1972). What is even more interesting, however, is the way in which the calculations were made.

The results from the early studies on axoplasmic flow were usually calculated by dividing the amount of material which had been accumulated above the control level in a given length of nerve, by the time during which the accumulation took place. Thus, the basic assumption was that "all" of the material in nerve axons was free to accumulate at the ligation. When this was found to be untrue for AChE (see Lubinska and Niemierko, 1971), then, obviously, a falsely low result had to be corrected.

Lubinska and Niemierko (1971) showed that only 15% of the AChE was mobile, which gave a corrected flow rate of 260 mm/day in the proximo-distal direction, which accounted for 10.5% of the mobile enzyme. The remaining 4.5% moved in the reverse direction at a rate of 134 mm/day. Partlow et al. (1972) found that 25% of the AChE in the frog sciatic was free to move, while Ranish and Ochs (1972) found that, for the cat sciatic, the amount was 15%.

This apparently "dual" mobility of AChE has some supporting evidence from the cytochemist. Kasa (1968) demonstrated that tubular AChE-containing structures accumulated at ligations placed on the sciatic nerve of the rat, with no apparent accompanying accumulation of the activity on the axolemmal membrane. It seems, therefore, that the rapidly accumulating AChE is in the intra-axonal endoplasmic reticulum, whereas the stationary fraction is probably the enzyme associated with the axonal membrane.

To summarize, an enormous amount of work has been done on the cholinesterases, both as a family of enzymes and as physiologically functional molecules, since their presence was first postulated by Dale in 1914. Consequently, much is known about such physical parameters as turnover numbers, active centres, mechanism of action, inhibition and activation and most of this information is beyond dispute. From the strictly physiological point of view much is also known but there remain many controversies to clear up and many questions still to answer.

It is hoped that the work described in this thesis will help toward answering some of the questions and, at the same time, that it will raise some more. The answers have been sought by making use of several different techniques and approaches. Thus, Chapter 2 contains the results of essentially biochemical experiments in which ultracentrifugation and electrophoresis were the main techniques used; in Chapter 3 use is made of the concept of axoplasmic flow to try and analyze the localization of AChE in axons; perfusion experiments and biochemical analyses are combined in Chapter 4 to enable a study of the release of AChE to be made; Chapter 5 contains the results of an electron microscopic cytochemical study in which an ultrastructural basis for the stored and released AChE was sought; the AChE content of extracellular fluids and variations in the level induced by various stimuli *in vivo* are described in Chapter 6; the SCG of the rat was used to study the relationship between pre- and postsynaptic AChE and the results of these biochemical and cytochemical experiments are given in Chapter 7; finally, the implications of the work are discussed, briefly, in Chapter 8.

CHAPTER 2

STUDIES ON THE TYPES OF ACETYLCHOLINESTERASES IN BOVINE ADRENAL MEDULLAE AND SPLANCHNIC NERVE AXONS

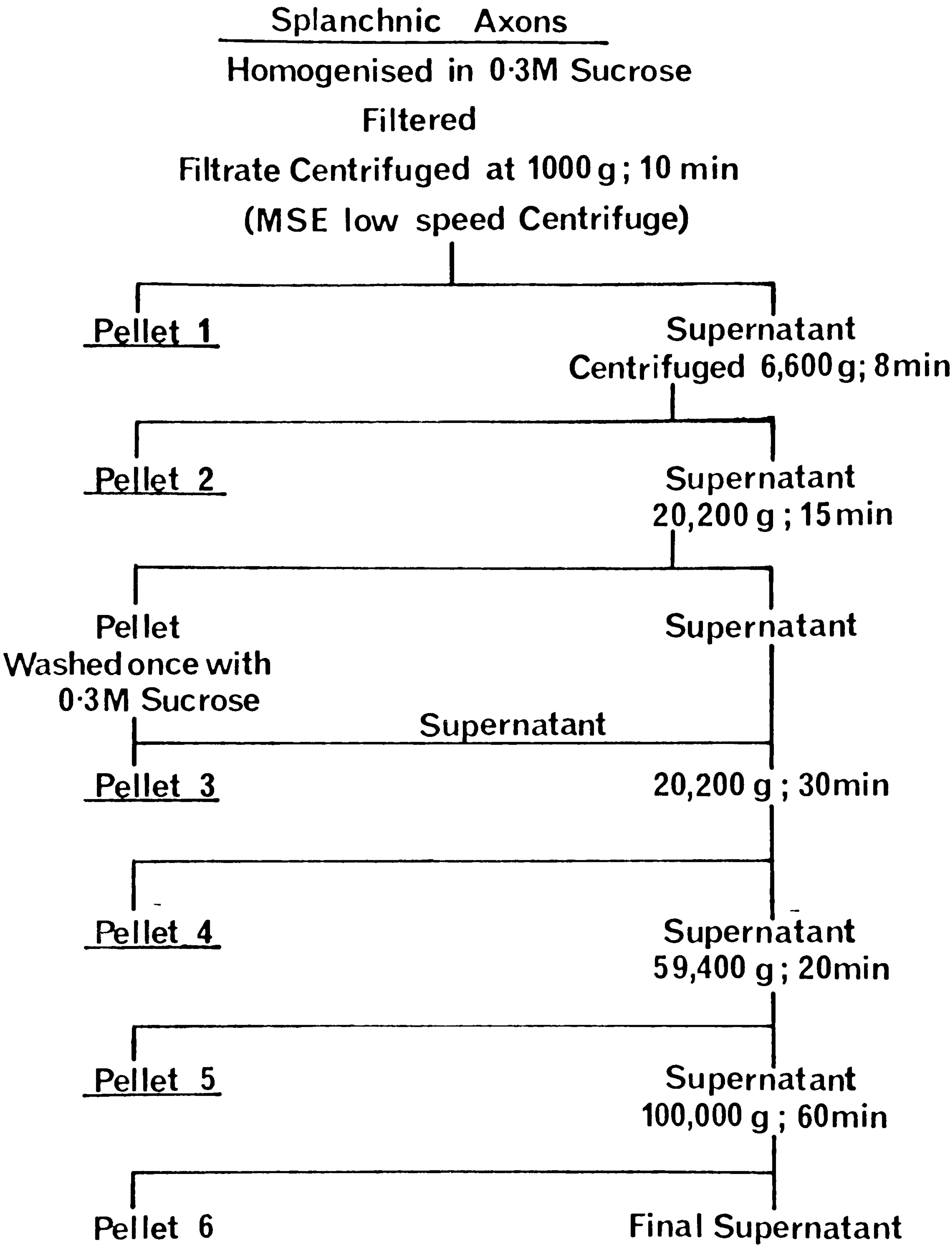


Fig. 1

The method used for the differential centrifugation of homogenates of bovine splanchnic axons. The "g" values quoted here and throughout the rest of this thesis are all g_{av}.

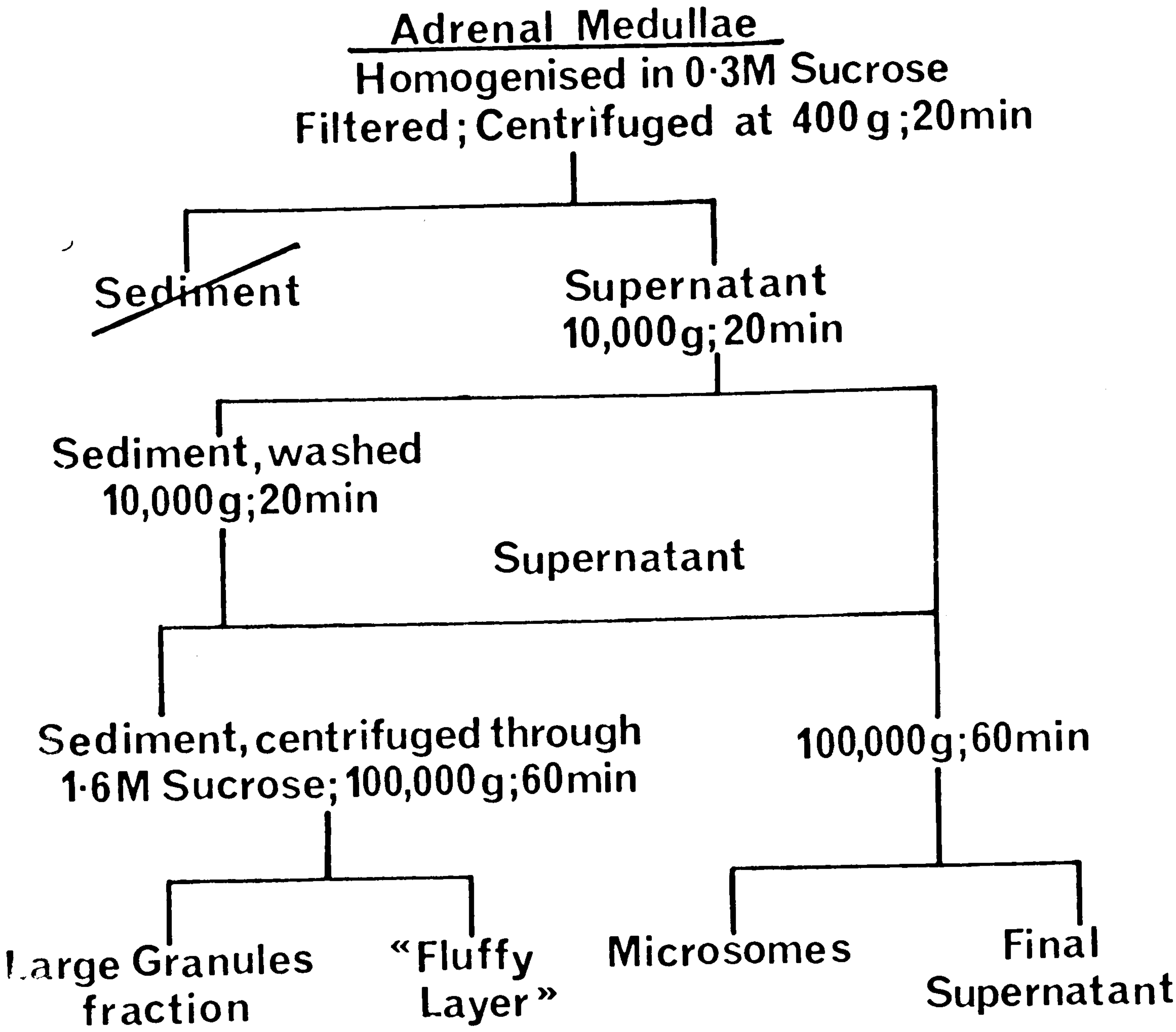


Fig. 2

The method used for the differential centrifugation of homogenates of bovine adrenal medullae.

METHODS

Bovine adrenal glands and splanchnic nerves were obtained from a local slaughterhouse within 15 min of the death of the animal. They were cleaned of fat and then transported to the laboratory in an ice-cold solution of 0.3 M sucrose. Having dissected the medullae from the adrenals and having removed as much as possible of the fat from the axons, both tissues were weighed, chopped to a fine mince with a large knife and finally homogenized in 0.3M sucrose using a glass homogenizer fitted with a teflon pestle of the Potter-Elvejhem type (clearance 0.08 mm). The extracts were then filtered through a double layer of surgical gauze and the volume of the filtrate adjusted so that 1 ml of suspension contained the equivalent of 200 mg of tissue.

The centrifugation procedure, the first step of which was carried out in a MSE refrigerated low-speed instrument, while all subsequent spins were done in the A40 rotor of a Spinco Model L2-65 B ultra-centrifuge at 2^{o}, is shown as flow diagrams in Figs. 1 and 2. All pellets obtained by these procedures were resuspended in appropriate volumes of 0.3M sucrose and kept frozen until used.

Extraction of insoluble AChE:

This was achieved with Triton X-100, a non-ionic detergent, as follows: the samples, either homogenates or membrane suspensions, were dialyzed overnight against 5 mM phosphate buffer, pH 7.0. Triton X-100 solution was then added to each sample, so that the final concentration of detergent was 0.5% (V/V). After stirring overnight at 4^{o}, which followed an initial 30 min period at room-temperature, the extracts were spun at 150,000 g for 90 min to remove any remaining membranous or other insoluble material. The efficiency of Triton extraction, which preliminary experiments showed to be independent of tissue concentration, is shown in Table 4.

Polyacrylamide gel electrophoresis:

The standard technique used was to electrophorese the dialyzed and/or extracted samples on 6.0% polyacrylamide gels, using the simplified system of Clarke (1964). The gels were cast in tubes so that their dimensions were 0.5 x 6.0 cm and were pre-run for approximately 60 min, or until the voltage stabilized, at 1 mA/tube. The samples were applied to the gel, run into it at 0.5 mA/tube for 5 min and finally run at 1 mA/tube until the marker dye - bromophenol blue - had reached the bottom. The only variations on this basic procedure were when gels of different polyacrylamide concentrations were used.

Immediately prior to electrophoresis, those samples which contained Triton X-100 were diluted so that the detergent concentration did not exceed 0.05% (v/v), and all samples had sucrose added to them so that the final concentration was about 10%. Since this was only to facilitate layering of solutions it was not done accurately.

Treatment of gels after electrophoresis:

1. Development for AChE

The gels were removed from the running tubes and immersed in a solution which was composed of 24% sodium sulphate and 0.07M sodium maleate, pH 6.5. After shaking for 20 min in this solution, the gels were transferred to another tube which contained 10 ml of the following solution (final concentrations are given): Na_2SO_4 (24%), sodium maleate (70 mM, pH 6.5), $CuSO_4$ (4 mM) in a solution of glycine (20 mM), $MgCl_2$ (3 mM) and acetylthiocholine (4 mM). This solution is basically that of Koelle (1951), who used it for the histochemical examination of cholinesterases in tissues.

A fairly standard procedure was used for the incubation of these gels in which AChE was to be visualized. They were incubated at room temperature with shaking, so that the product of time and activity on the gel was approximately 50. Thus, a gel containing 1 mU of activity would be incubated for 50 min, one with 2 mU for 25 min and one with 0.5 mU for 100 min. These conditions were a little variable simply because they were chosen to obtain considerable sensitivity with maximal resolution, the latter being dependent on the number of isoenzymes present in the extract. Generally, however, to allow comparison of the results and patterns obtained at different periods, the actual amount of enzyme was kept to between 0.5 and 1.5 mU/gel.

After incubation as described, the gels were transferred back to the original wash solution (24% Na_2SO_4, 0.07 M sodium maleate, pH 6.5) for a further 30 min period, followed by extensive washing with water. At this stage the gels contained bands of a fairly dense white precipitate which, for ease of visualization and photography, were converted into green complexes with the copper sensitive reagent dithiooxamide. This was done by shaking the gels in a saturated aqueous solution of dithiooxamide for approximately 30 min, followed by washing with water. The gels were stored, in water, in stoppered tubes.

When necessary, the specificity of the reaction was checked by the use of inhibitors. Thus, to inhibit AChE, BW 284C51 (10^{-4}M) and to inhibit all cholinesterases, eserine (2.0×10^{-4}M) were used. In each case the inhibitors were incorporated into the pre-incubation, the incubation and the first of the post-incubation solutions. In one experiment butyrylthiocholine (10 mM final concentration) was used instead of ATCh.

To calculate the mobilities of the various isoenzymes, the following procedure was adopted: first, the length of the gel and the distance that the dye had moved were measured. Then, after incubation, the length of the gel and the mobilities of the enzyme bands were measured. This allowed a corrected dye mobility to be calculated, from which the relative mobilities of the isoenzymes could be calculated. This value was then multiplied by 100 (simply for ease of handling), the logarithm taken, and this figure was plotted versus the concentration of the gel.

2. Protein

The distribution of proteins on the gels was visualized by immersing the gels in a 0.1% solution of amido black in 7.5% acetic acid for 60 min. The gels were then washed by shaking in 7.5% acetic acid until all background dye had been removed - a procedure which usually took about 24 h. Gels were stored in 7.5% acetic acid if necessary, but were usually photographed and discarded.

Sucrose density gradient centrifugation:

This technique was used to study the sedimentation characteristics of a Triton X-100 and soluble cytoplasmic extract of splanchnic axons and adrenal medulla. The sedimentation of the AChE in these extracts was compared with that of cytochrome C and Catalase. The gradients were made by pipetting 1.5 ml volumes, on to a 1.0 ml cushion of 1.0M sucrose, of 0.8M, 0.7M, 0.6M, 0.5M, 0.4M, 0.3M and 0.25M sucrose into a tube designed for the SW 36 swinging

TABLE 2

The assay method used to determine AChE activity.

Tube	Buffer	BW 284C51	Eserine	DTNB	ATCh	Enzyme
1.	2.6 ml	0	0	0.1 ml	0.1 ml	0.1 ml
2.	2.5 ml	0.1 ml	0	0.1 ml	0.1 ml	0.1 ml
3.	2.5 ml	0	0.1 ml	0.1 ml	0.1 ml	0.1 ml

The buffer used was sodium phosphate (50 mM) at pH 7.0; the concentration of BW 284C51 was 1.5×10^{-6}M and of eserine 2×10^{-5}M. The ATCh concentration was 10^{-3}M (all concentrations are final). DTNB is the chromophore used by Ellman et al. (1961). AChE results throughout this thesis will be expressed in mU which are defined as being the appearance of 1 nMole of product/min (i.e. 1 nMole of thiocholine).

To calculate the results, the difference in E_{412} between tubes 1 and 2 was taken as an estimate of the AChE activity, while that between tubes 2 and 3 was considered as an indication of the amount of ChE.

bucket rotor of the L2-65B ultra-centrifuge. After standing for 6 h at 4^{o}, the gradients were loaded with 1.0 ml of the appropriate sample (to which 1,000 units of Catalase and 1 mg of cytochrome C had been added) and centrifuged at 105,000 g for 16.5 h. The gradients were then sampled at 0.5 cm intervals using a Shuster cutter and, latterly, by collecting drops through a needle inserted in the bottom of the centrifuge tube.

Methods used for the estimation of tissue constituents

The most important single method used has been the estimation of AChE activity. This was done by the procedure of Ellman, Courtney, Andres and Featherstone (1961), which was modified to make it more amenable to the handling of large numbers of samples. The modification was simply to do the assay in a test-tube rather than a spectrophotometer cuvette, and to stop the reaction with eserine after a known time had elapsed rather than to estimate continuously a rate of hydrolysis as proposed in the original method. The composition of the incubation mixture used is shown in Table 2. Enzymic specificity was verified by the use of the inhibitors BW 284C51, at a final concentration of 1.5×10^{-6}M and eserine (2×10^{-5}M).

Protein was estimated by the method of Lowry, Rosebrough, Farr and Randall (1951) using bovine serum albumin as the standard. Succinic dehydrogenase was estimated according to the method of Porteus and Clark (1965); Lactate dehydrogenase (Wroblewski and La Due, 1955); β-glycerophosphatase (Berthet and de Duve, 1951); Acid ribonuclease (Smith and Winkler, 1966); Inorganic phosphate (Chen, Toribara and Warner, 1956).

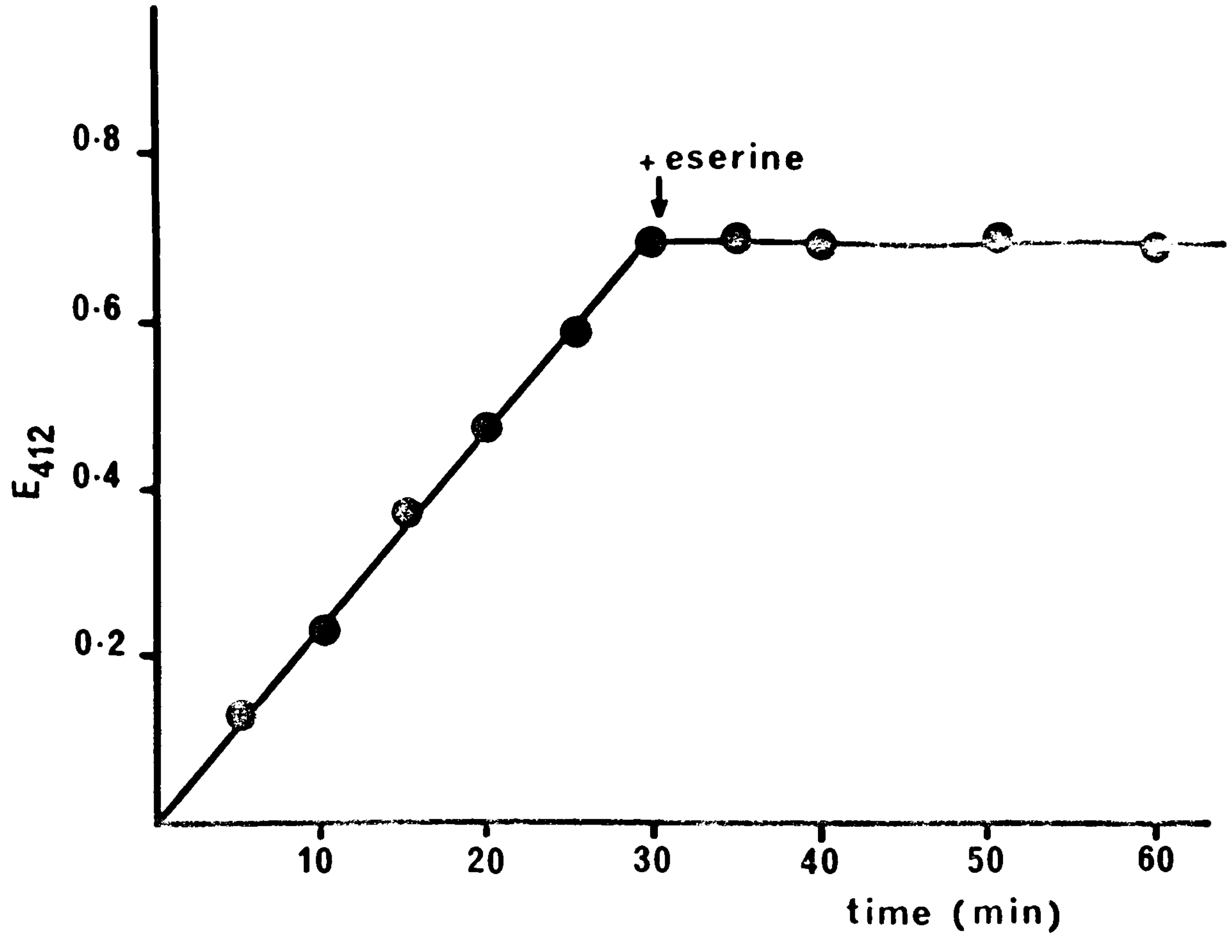


Fig. 3

Acetylcholinesterase assay: the activity is proportional to the time of incubation and eserine, added where indicated, at a final concentration of 2.0 x 10^{-5}M immediately stops the reaction.

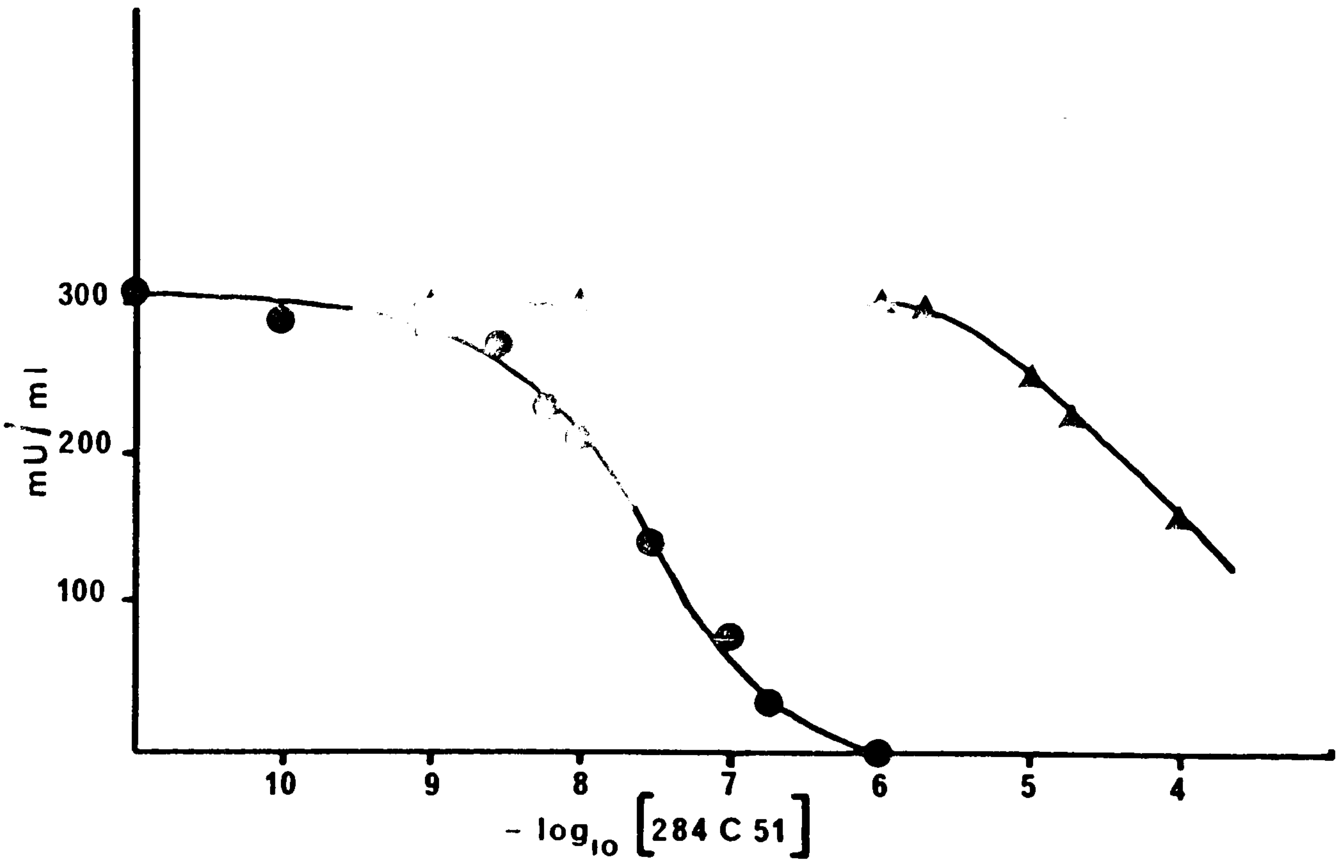


Fig. 4

Inhibition of AChE (●) and ChE (▲) by BW 284C51.

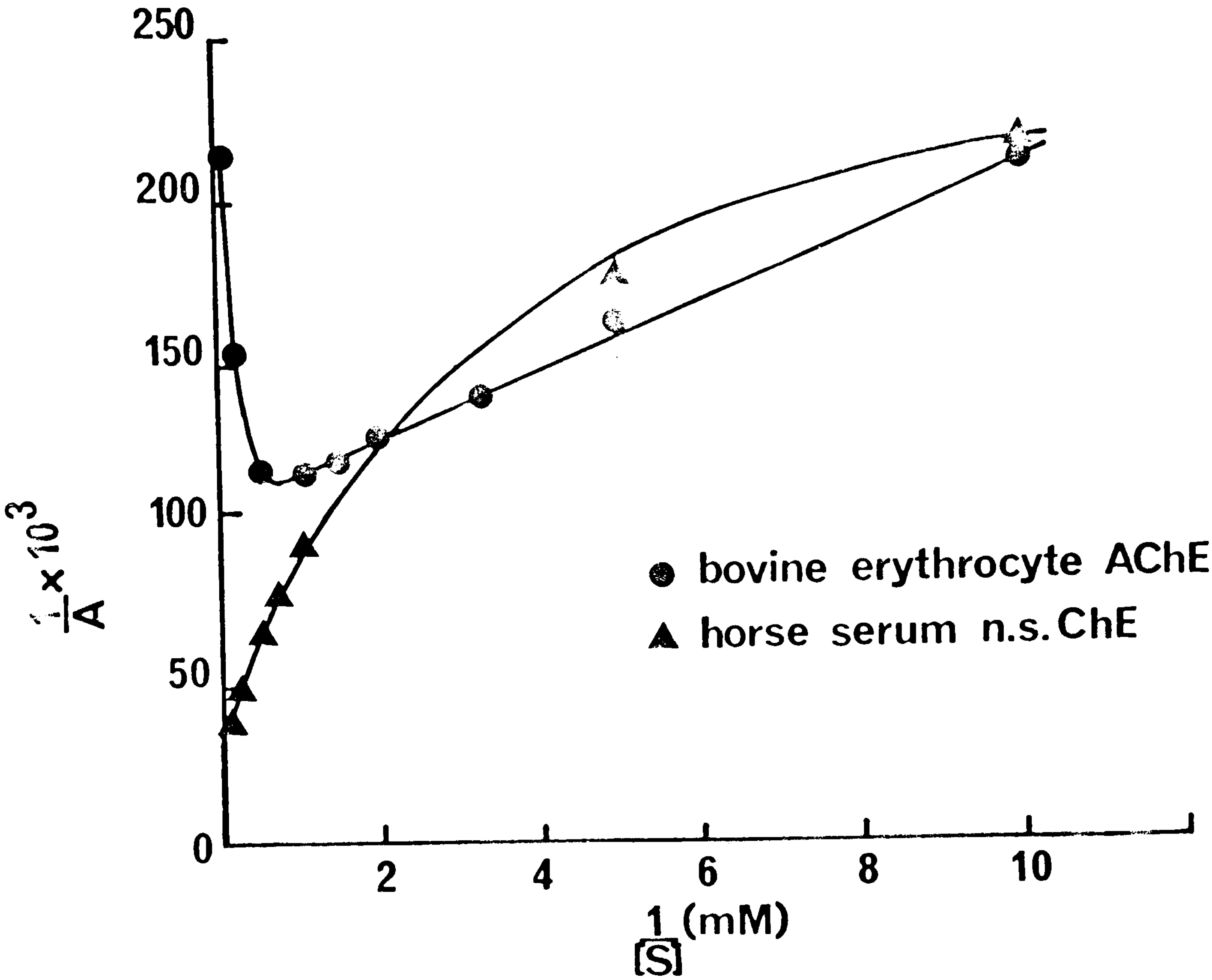


Fig. 5

The double reciprocal plots of the activity of AChE and ChE when measured over a range of substrate concentrations.

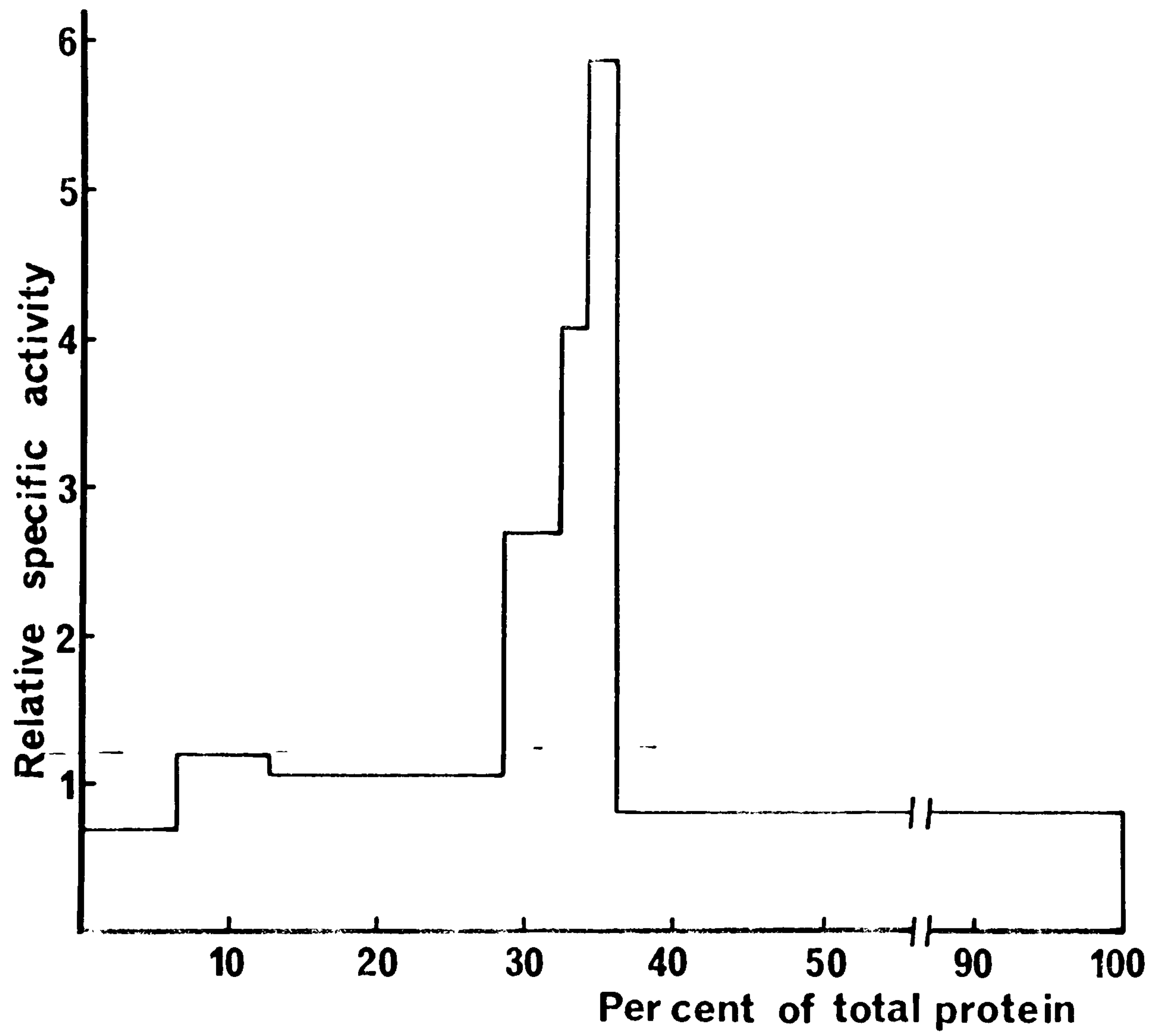


Fig. 6

The distribution of AChE amongst subfractions obtained by differential centrifugation of splanchnic nerve homogenates. Relative specific activity of the AChE in any fraction is defined as the percentage enzyme activity divided by the percentage of the total protein in the fraction.

TABLE 3

Distribution of some constituents of bovine splanchnic nerve axons and adrenal medulla among fractions obtained by differential centrifugation of homogenates.

Constituent	n	Absolute amount	1	2	Fraction 3	4	5	6	Sol.	Rec.
A. Splanchnic nerve axons:										
Protein	6	18.2	6.7	5.7	16.3	3.4	2.0	1.3	64.7	100.5
Acetylcholinesterase	12	1270.0	4.7	6.8	17.2	8.9	8.0	7.4	47.4	93.0
Succinic dehydrogenase	2	1.7	8.5	58.0					24.0	76.5
Lactate dehydrogenase	2	4.1	0.6	1.4					96.0	90.0
Acid ribonuclease	3	7.0	0	19.0					81.0	94.0
Acid β-glycerophosphatase	2	0.6	4.9	42.0					53.0	87.0
B. Adrenal medulla:										
Acetylcholinesterase	6	1200	-	70.0					30.7	93.0

The concentration of each constituent in the filtered homogenate is expressed per g wet weight of the original tissue. Succinic dehydrogenase, acid ribonuclease and acid β-glycerophosphatase results are expressed in units which refer to the decomposition of 1 μMole of substrate per h; Acetylcholinesterase units are as defined in the text and lactate dehydrogenase units refer to the decomposition of 1 μMole of substrate per min. Protein is in mg. The proportion of each constituent found in a fraction is given as a percentage of the amount recovered. The actual recoveries are given in the last column.

RESULTS

The first results which should be discussed are those concerning the assay of AChE. This is of fundamental relevance to the whole of the work presented in this thesis and it is thus of some importance to define the criteria which have been used to establish the specificity of my assay. First, as shown in Fig. 3, the actual method used is satisfactory. The amount of product formed is proportional to the time of incubation, and the addition of eserine brings the enzyme activity to an abrupt end. The colour produced is stable. Preliminary experiments also showed that the above conditions were met over a wide range of enzyme activities and times of incubation, and hence amounts of substrate hydrolyzed. As shown in Fig. 4, BW 284C51 completely inhibits activity known to be due to AChE, from erythrocytes, at a concentration of between 1.0 and 2.0×10^{-6}M. As also shown in Fig. 4, ChE is not inhibited at this concentration range, but its activity is affected at only slightly higher concentrations of the BW 284C51. Thus, the concentration of BW 284C51 which was routinely used was 1.5×10^{-6}M. These inhibition curves were always obtained whatever the source of enzyme (nerve, electric organ, erythrocytes, human, horse and ox sera have all been tested). Finally, the kinetics of the enzyme reaction were always established whenever a new source of enzyme was being used. The classic type of kinetics are shown in Fig.5, where it can be seen that AChE is inhibited by excess substrate, but that the activity of ChE increases with higher concentrations of ATCh.

The distribution of AChE amongst the pellets obtained by differential centrifugation of splanchnic nerve homogenized in isotonic 0.3M sucrose is shown in Fig. 6; the data from which this Fig. was calculated are given in Table 3. It can be seen

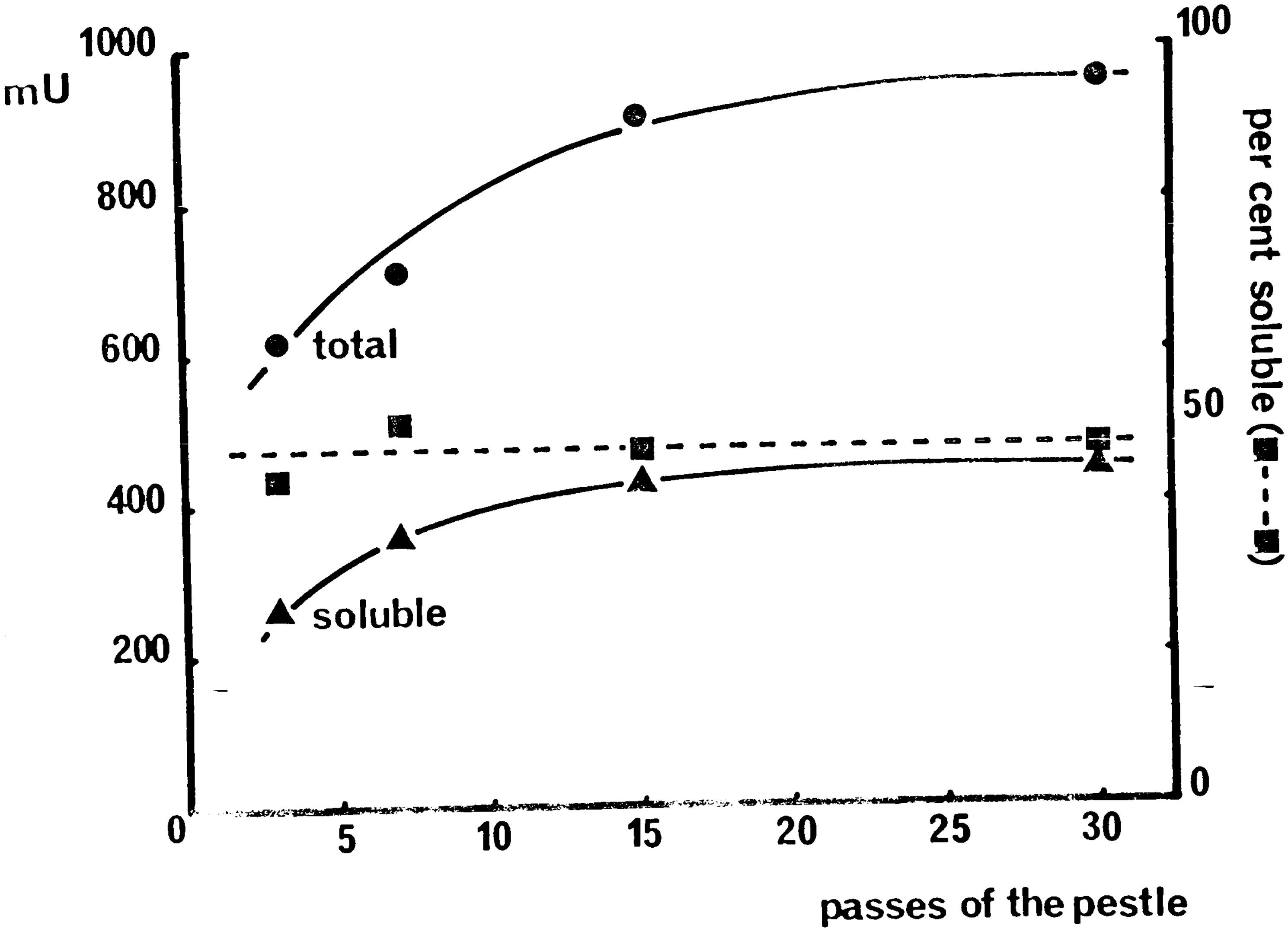


Fig. 7

The effect of different strengths of homogenization on the proportion of the AChE in splanchnic axons which is recovered in the supernatant fraction.

in Fig. 6 that the distribution of AChE in the splanchnic nerve is fairly complex. While it is obvious that there is an enrichment in a "small" membrane fraction (N^o6), it is also apparent that there is a considerable proportion of the activity in the high-speed supernatant fraction; Table 3 shows that 47% of the activity is in this fraction. Henceforth, this AChE will be called soluble AChE since it was established that it could not be sedimented by centrifugation at 200,000 g for 90 min, and was thus most unlikely to be due to small particles rich in enzyme, but which were not sedimented under the conditions used to obtain fraction 6. As also shown in Table 3, there is a high concentration of AChE in the adrenal medulla and a proportion of this activity is also soluble. In each tissue there is, when measured with 10 mM butyrylthiocholine, a very low ChE activity.

To homogenize splanchnic axons is a difficult task. To ensure that the homogenization itself was not in some way detaching AChE from its normal site, i.e. solubilizing membrane-bound AChE, the influence of the degree of homogenization on both the absolute amount and on the proportion soluble of the enzyme was studied. The axons were first homogenized for between 3 and 30 passes of the pestle, filtered through gauze to remove large lumps of tissue, sampled for total activity and then centrifuged at 100,000 g for 90 min to separate the membranous from the soluble AChE. The results are shown in Fig. 7, from which it can be seen that the absolute amount of AChE in the filtrate increases with increasing homogenization. But it can also be seen that the amount of soluble AChE also increases with further homogenization, thus keeping constant the proportion of the enzyme which is soluble.

The aim of the next experiment was to determine whether mere suspension of the tissue in sucrose was washing AChE off the membranes. For these experiments the axons were homogenized in

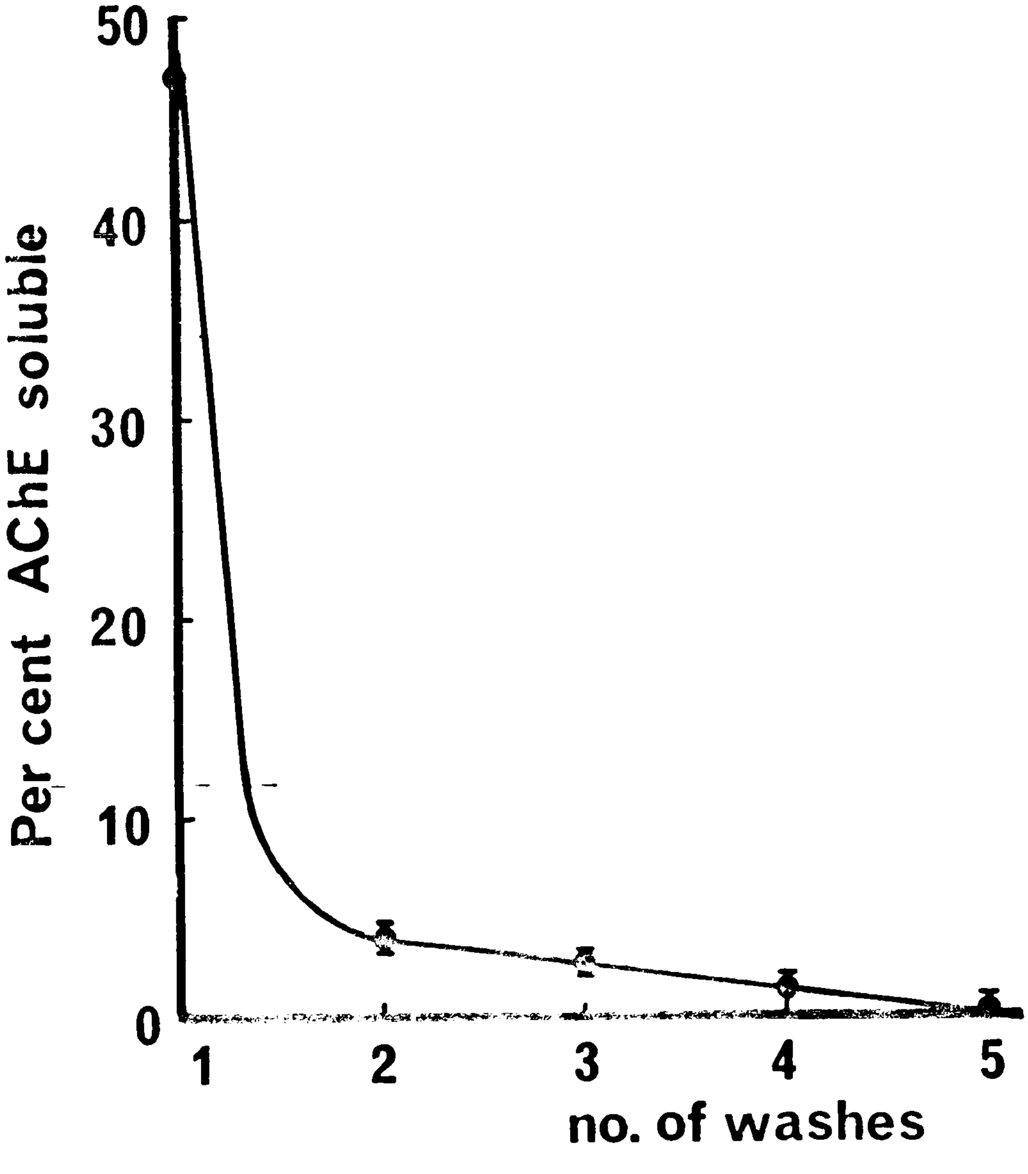


Fig. 8

The washout of AChE from an homogenate of splanchnic axons.

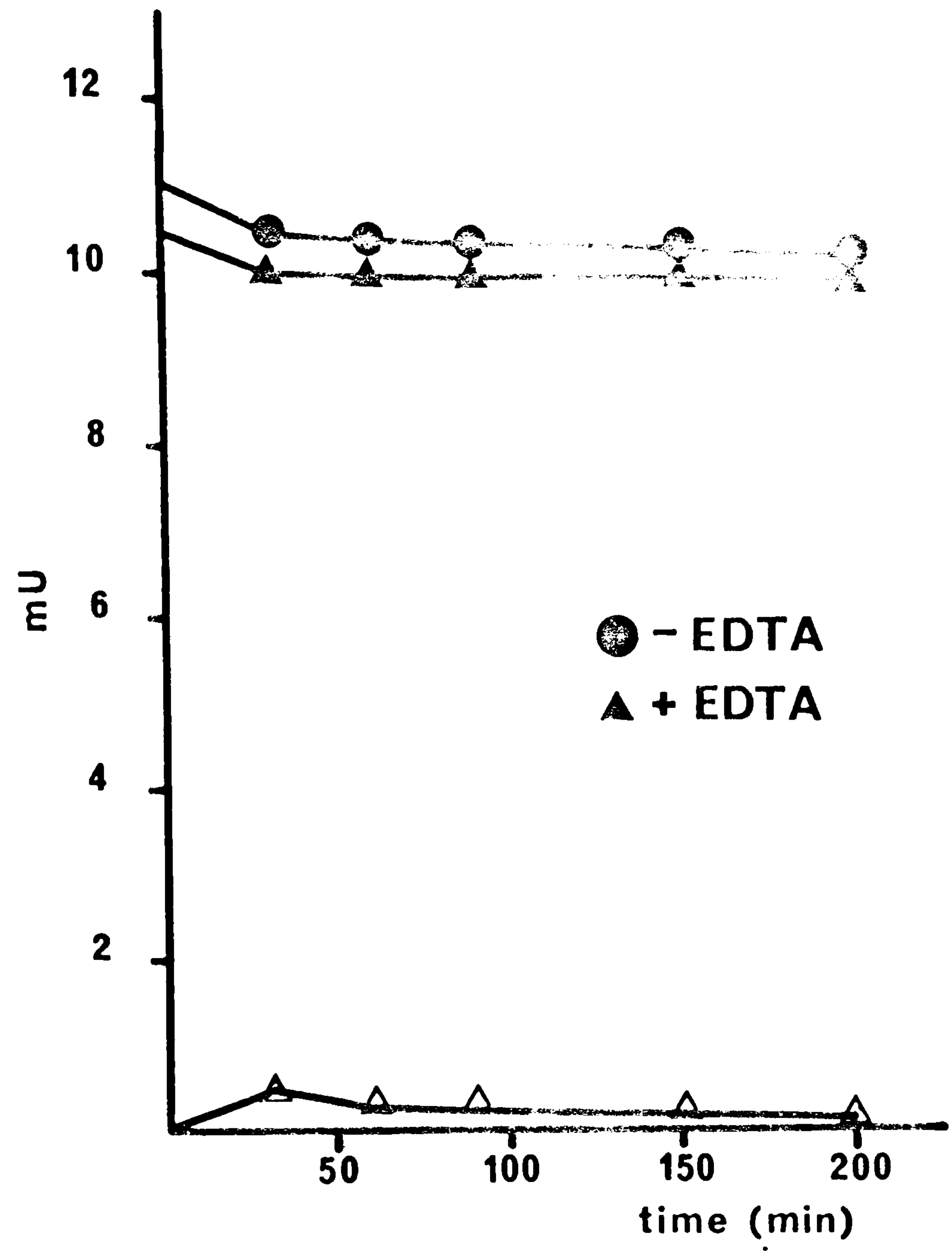


Fig. 9

The effect of incubation at 25°, with (▲) and without (●) EDTA, on the solubilization of AChE from a washed membrane pellet from the splanchnic axons.

either 0.3M or 0.03M sucrose so that the effect of osmolarity could be studied at the same time. After homogenization and filtration, the axons were centrifuged to separate membranous and soluble AChE. The membranes were then resuspended in the same volume of sucrose, "homogenized" in the same way and resedimented. This was repeated to give 4 supernatants, as well as the first, which were essentially washes of the membrane pellet. Fig. 8 shows that the soluble AChE is rapidly washed out of the pellet and that insignificant amounts of membranous AChE are subsequently solubilized - at least by sucrose alone. Homogenization of the axons in 0.3M sucrose results in good recoveries of enzyme activity after either sub-fractionation or, as in this case, washing (92%). In 0.03M sucrose, however, the recovery was only 50%, and activity was lost from both the membranous and the soluble fractions, although the overall result was similar to that obtained with 0.3M sucrose.

Finally, washed membranes were incubated at 25° with and without 1 mM EDTA pH 7.4 in the 0.3M sucrose suspending medium. This was done because of reports in the literature which suggest that incubation in EDTA solutions can solubilize AChE from brain homogenates (e.g. Hollunger and Niklasson, 1973). These experiments were repeated with the washed membrane fraction to see whether incubation could solubilize the AChE and thus offer some explanation for the soluble enzyme. As shown in Fig. 9, incubation of the membranes at 25° does not result in the solubilization of the enzyme - nor does it result in any significant change in the measured enzyme activity. These experiments have not been repeated with whole homogenates of the axons because, when it was attempted, a marked loss of activity resulted. This will be discussed later.

The centrifugation data and the other experiments described

TABLE 4

The extraction of AChE by Triton X-100.

	mU	% of original
1. Total activity	1080	
2. Total activity + 0.5% Triton X-100	1080	100
3. Supernatant after centrifugation of N°2	920	85

The enzyme activity was determined before and after centrifugation at 100,000 g for 60 min.

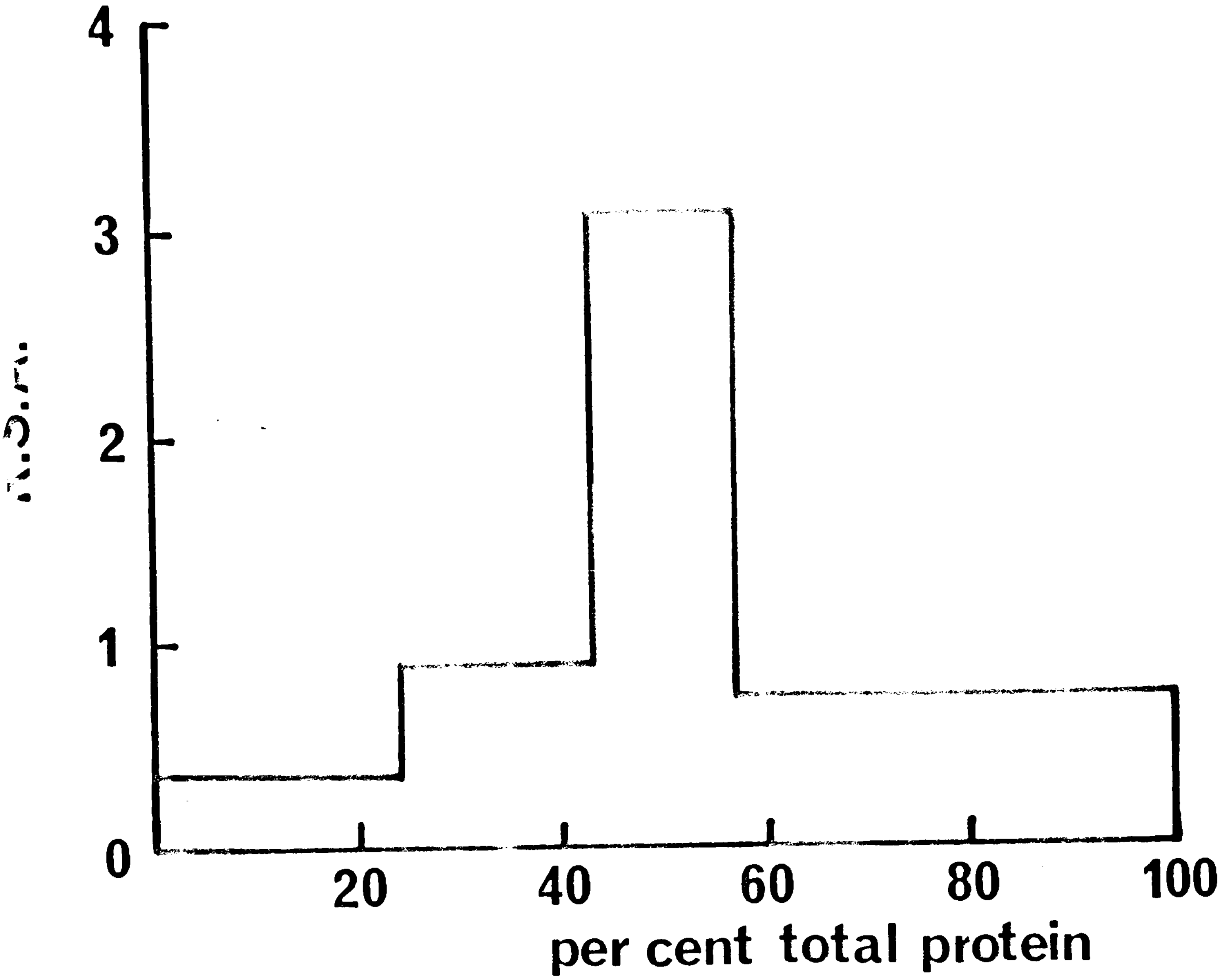


Fig. 10

The distribution of AChE amongst subfractions obtained by centrifugation of bovine adrenal medulla homogenates.

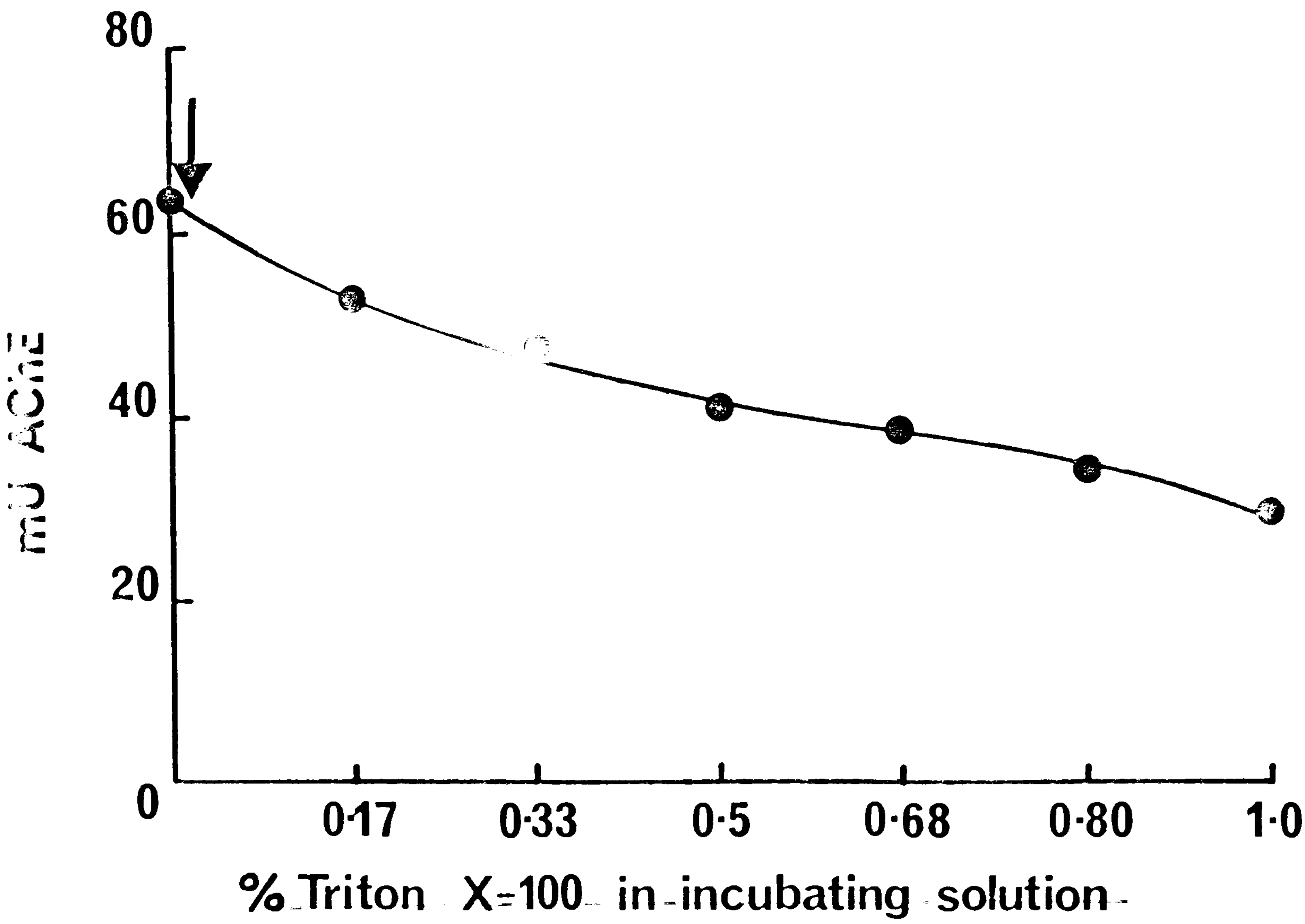


Fig. 11

The effect of different concentrations of Triton X-100 on the activity of AChE. The concentration in a normal assay (i.e. 0.1 ml of 0.5% Triton X-100 in a 3.0 ml incubation volume) is shown by the arrow.

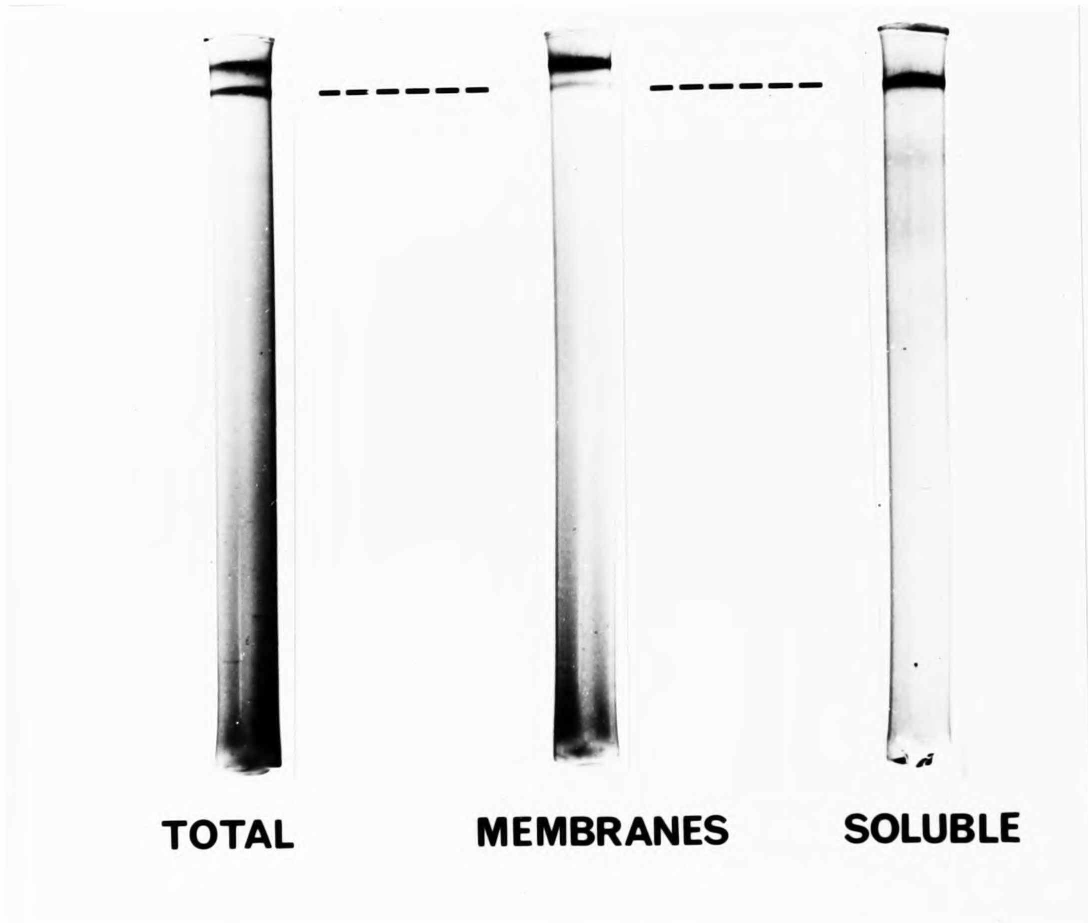


Figure 12.

AChE isoenzymes in bovine splanchnic axons. The "total" and "membranes" were exposed by first solubilizing the AChE with Triton X-100 before electrophoresis. The "soluble" is the AChE in the high-speed supernatant fraction. In these and in all subsequent gels, the mobility is from the top to the bottom, the anode.

above provide strong evidence that there normally exists both membranous and soluble AChE in splanchnic nerve axons. As shown in Fig. 10, a considerable proportion of the AChE in the bovine adrenal medulla is also recovered in the high-speed supernatant fraction. Similar criteria to those applied to the axons indicate that this AChE is also truly soluble and not an artifact of preparation.

Because of the data presented above, and because of the reported occurrence of isoenzymes of AChE, it was decided to determine the isoenzymic composition of the enzyme in the axons and the medullae. Since this would require electrophoretic analysis of the homogenates and since, with polyacrylamide gels, only proteins which are in solution enter the gels, the first necessity was to show that the membrane-bound AChE could be extracted by detergent. As shown in Table 4, 85% of the AChE activity can be solubilized by the procedure described in the Methods section. The next requirement was to show that the extracted enzyme was not inhibited by the detergent and, as shown in Fig. 11, it is only at much higher final concentrations than those used in these experiments that the detergent becomes inhibitory.

The results of electrophoresis of samples from the axons, a Triton X-100 extract of a total homogenate, an extract of a washed membrane fraction and a high-speed supernatant are shown in Fig. 12. There are two major isoenzymes present in the total homogenate and, as shown in Fig. 12, these can be resolved into one membranous component, while the other is soluble. Since all the activity on the gels could be inhibited by either eserine or BW 284C51, it can thus be attributed to AChE activity. There is always a possibility that Triton affects the mobility of the AChE on the gels, and so

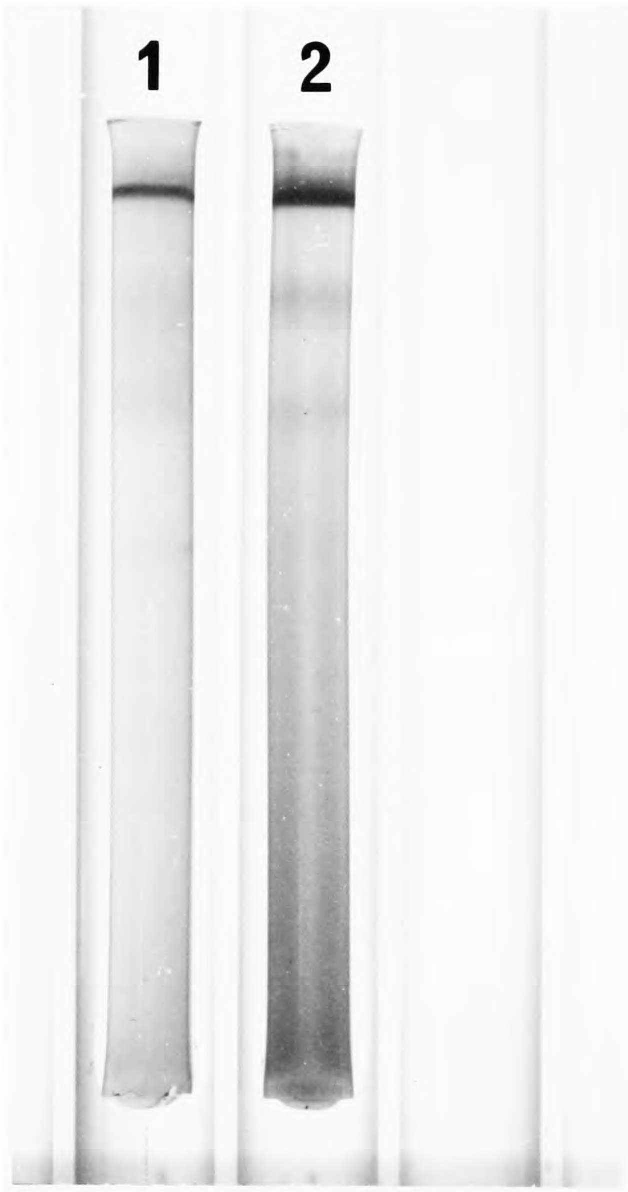


Fig. 13

The effect of Triton X-100 on the electrophoretic mobility of the soluble AChE in splanchnic axons. The detergent was added to gel No 2 before electrophoresis. Note the ill-defined zone of activity with a faster mobility than the major isoenzyme.

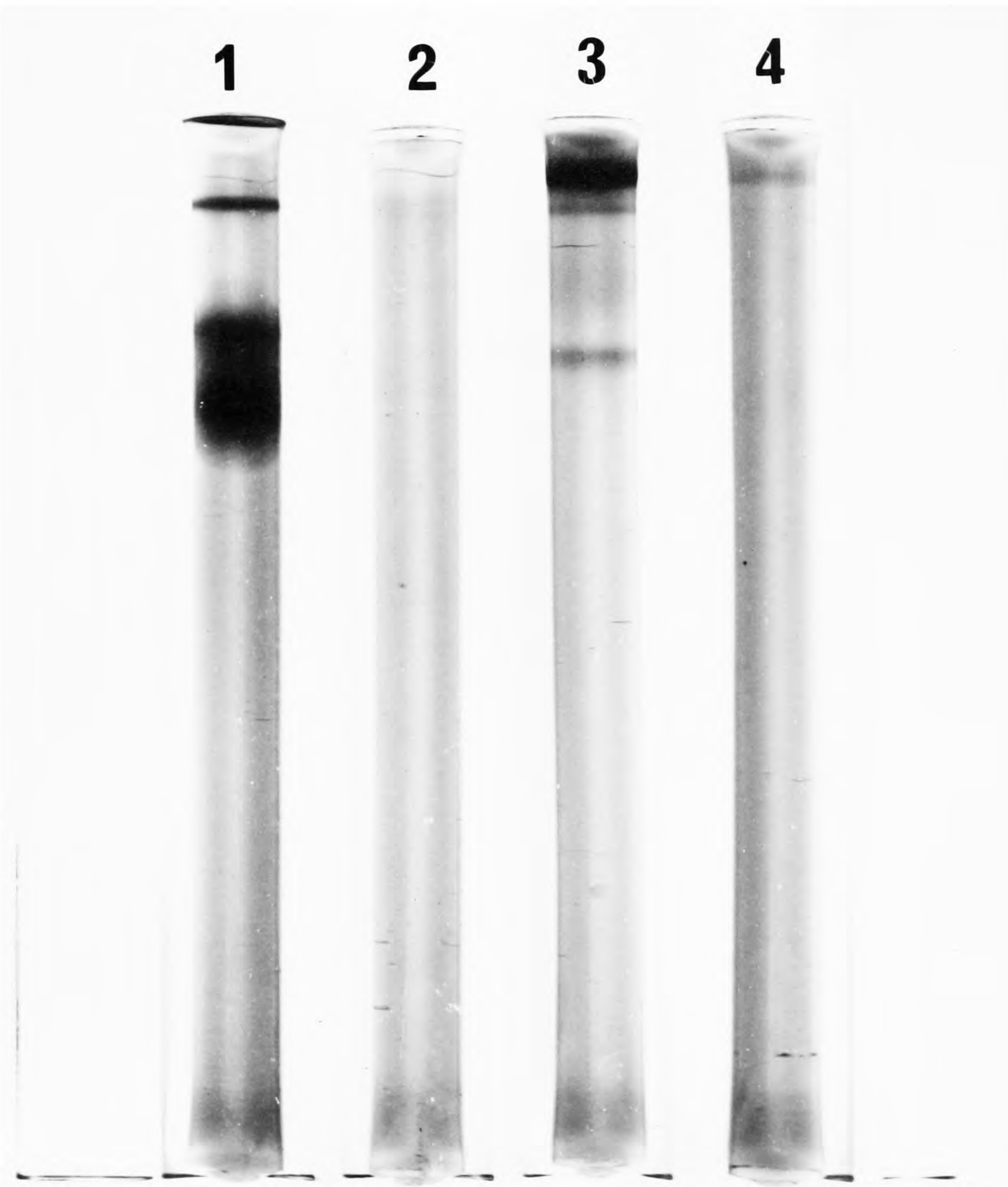


Fig. 14

The AChE isoenzymes in bovine adrenal medulla.

Gel 1: AChE in the high-speed supernatant. Gel 2: as for 1, but with 10^{-4}M BW 284C51. Gel 3: a washed and Triton X-100 solubilized extract from the total membrane fraction of the adrenal medulla. Gel 4: as for 3, but with BW 284C51.

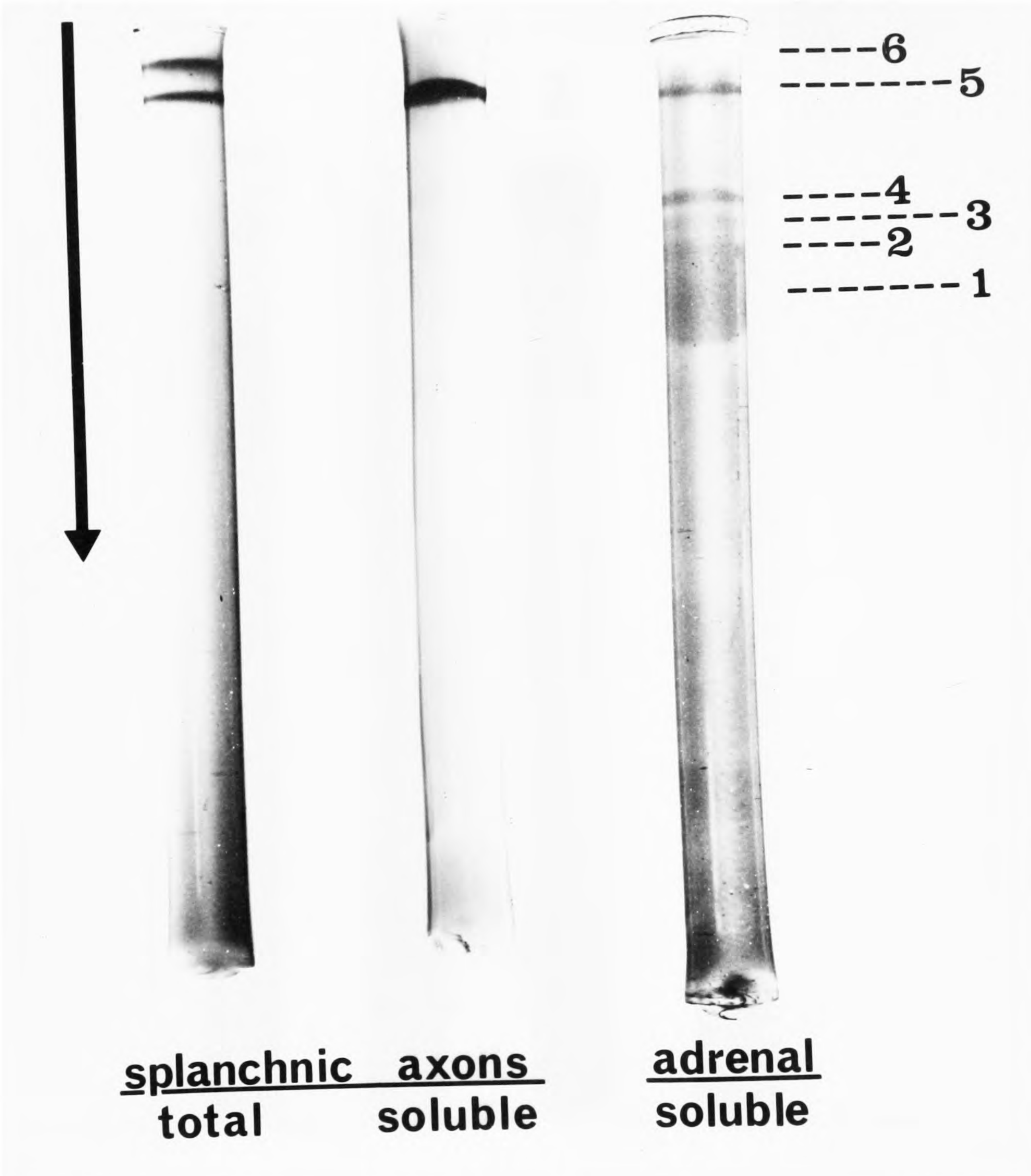


Fig. 15

The AChE isoenzymes in the splanchnic axons, both total and soluble, and the adrenal medulla from one animal. The isoenzymes are numbered, according to convention, from 1, the fastest migrating, to 6, the slowest.

artifactually produces two isoenzymes but, as shown in Fig. 13, the presence of Triton has no effect on the mobility of a sample of soluble AChE to which the detergent was added prior to electrophoresis. Fig. 13 can also be used to illustrate that there occasionally appear faster moving isoenzymes in the axons. Three times more activity was placed on the Triton X-100 containing gel, as compared with the other, and both were then incubated according to the Methods section. It can be seen on these gels that there is, in both, an ill-defined zone of AChE activity with a considerably faster mobility than the principal isoenzyme.

Fig. 14 shows that the isoenzymes of AChE in the adrenal medulla are more complex than those in the nerve axons. Whereas it can safely be said that more than 95% of soluble AChE in the majority of axon preparations is due to one isoenzyme, there are many more in the supernatant from the adrenals. The pattern obtained, particularly of the faster moving isoenzymes, is variable in the number of bands resolved, but not in their qualitatively estimated proportion of the total activity on the gel. As shown in Fig. 15, the axons and medullae have the membrane-bound isoenzyme in common.

A comparison of the splanchnic nerve total and soluble activity with the isoenzymes in the adrenal gland which those same axons innervate is shown in Fig. 15. The same amount of activity was run on the two "soluble" gels, so a direct comparison of the isoenzyme composition of the two tissues can be made. It is clear that the medulla does contain a far greater proportion of the faster moving isoenzymes than the axons. These isoenzymes are numbered from 1, the fastest moving isoenzyme in the medulla, to 6, the slowest moving isoenzyme, which is the membrane-bound form. The next set of experiments was designed to determine whether there

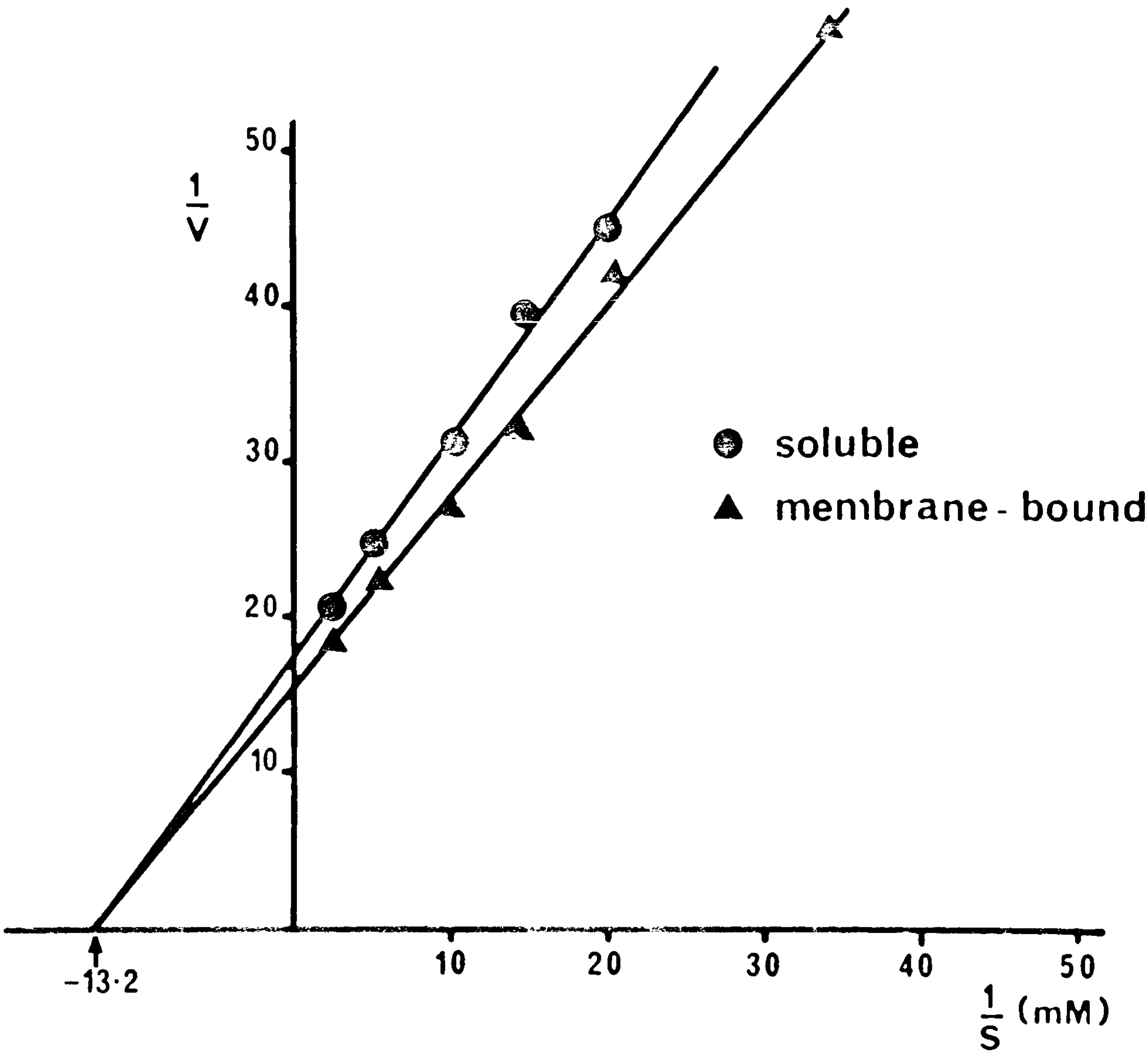


Fig. 16

The apparent K_m of the soluble (●) and membrane-bound (▲) AChE in bovine splanchnic axons.

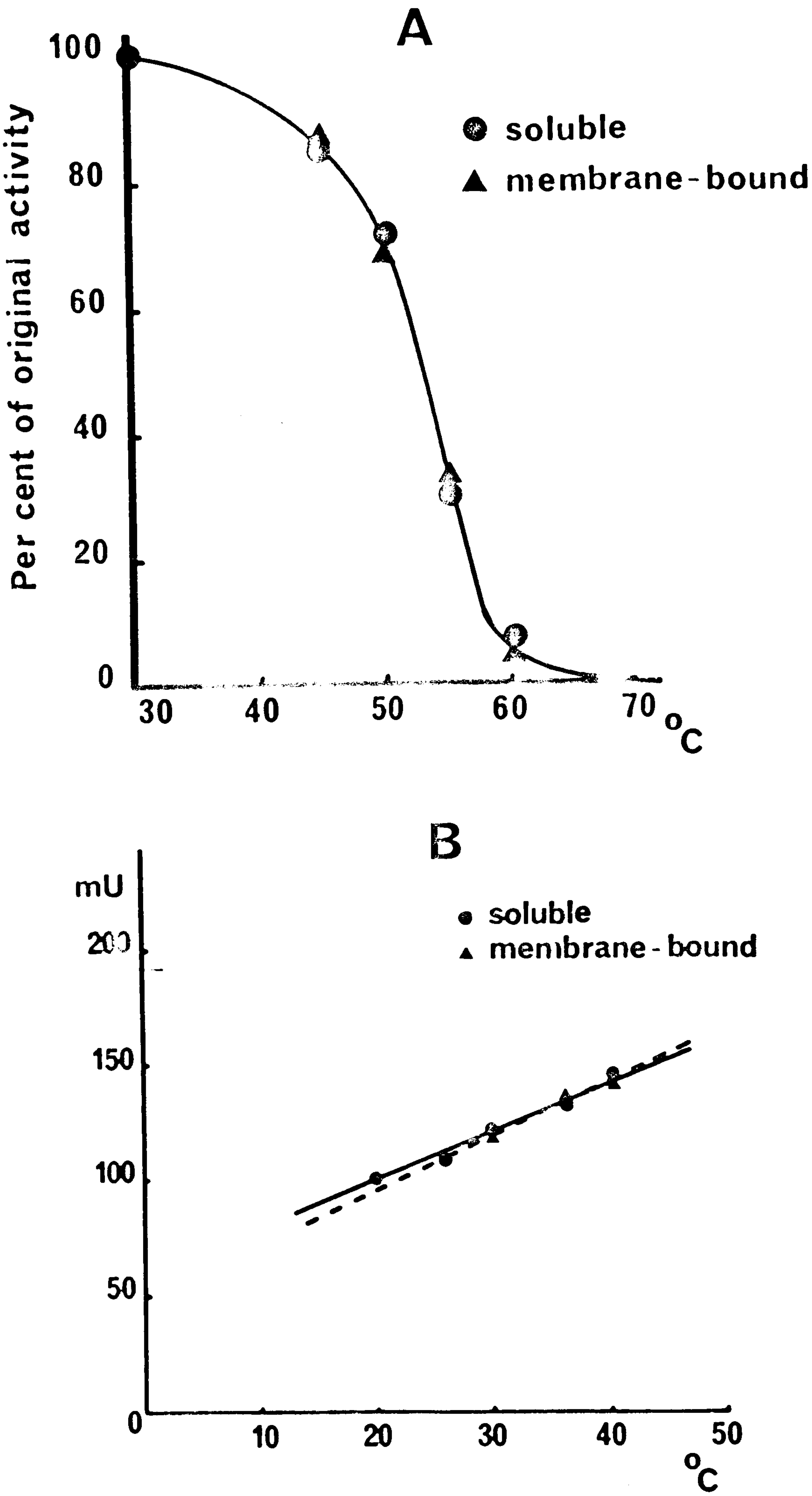


Fig. 17: The effect of excess heat (A) and different incubation temperatures (B) on the activity of the AChE from splanchnic axons.

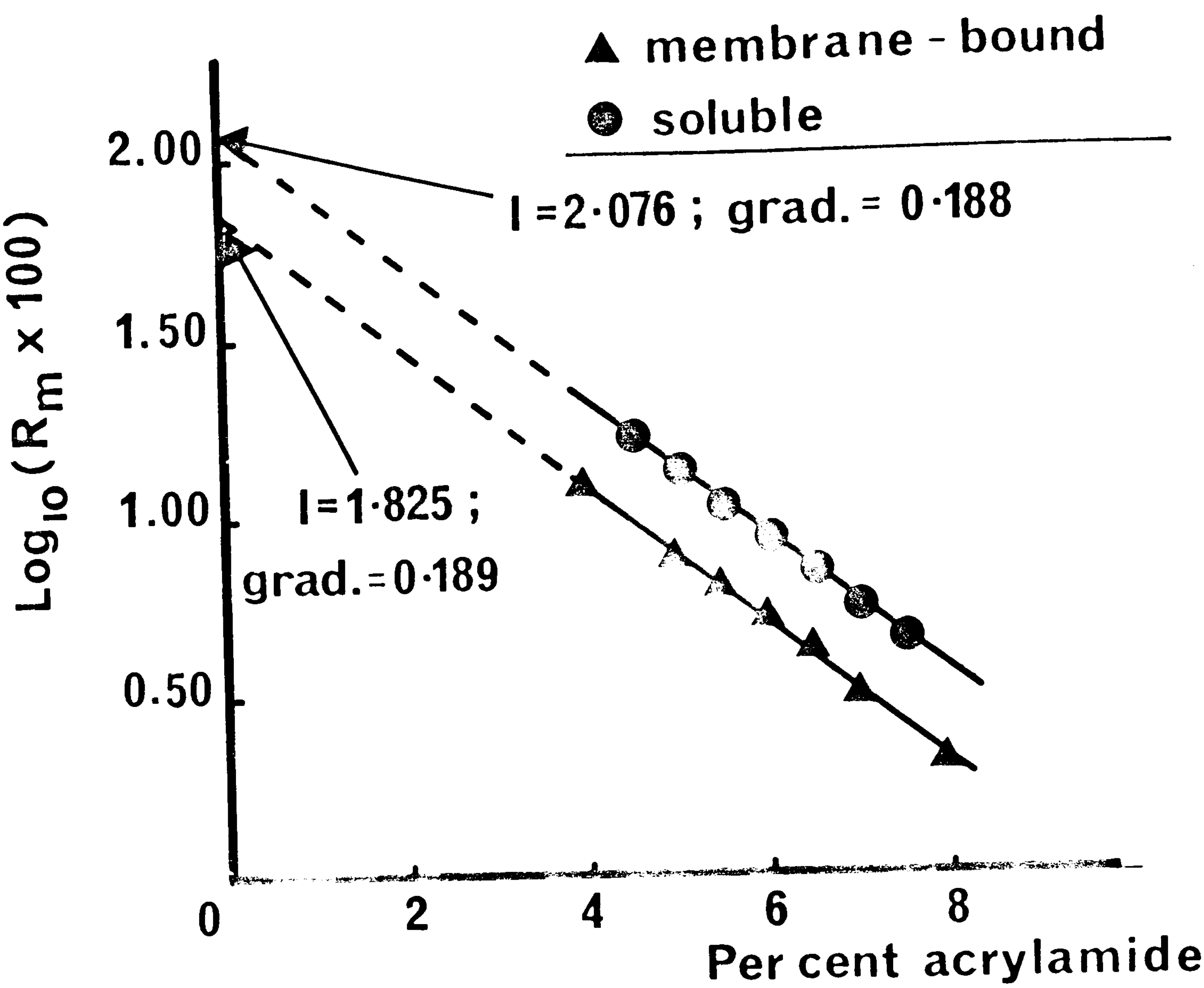


Fig. 18

The mobility of membrane-bound (▲) and soluble (●) AChE from bovine splanchnic axons over a range of polyacrylamide gel concentrations. The lines drawn through the points were calculated by the "least-squares" method.

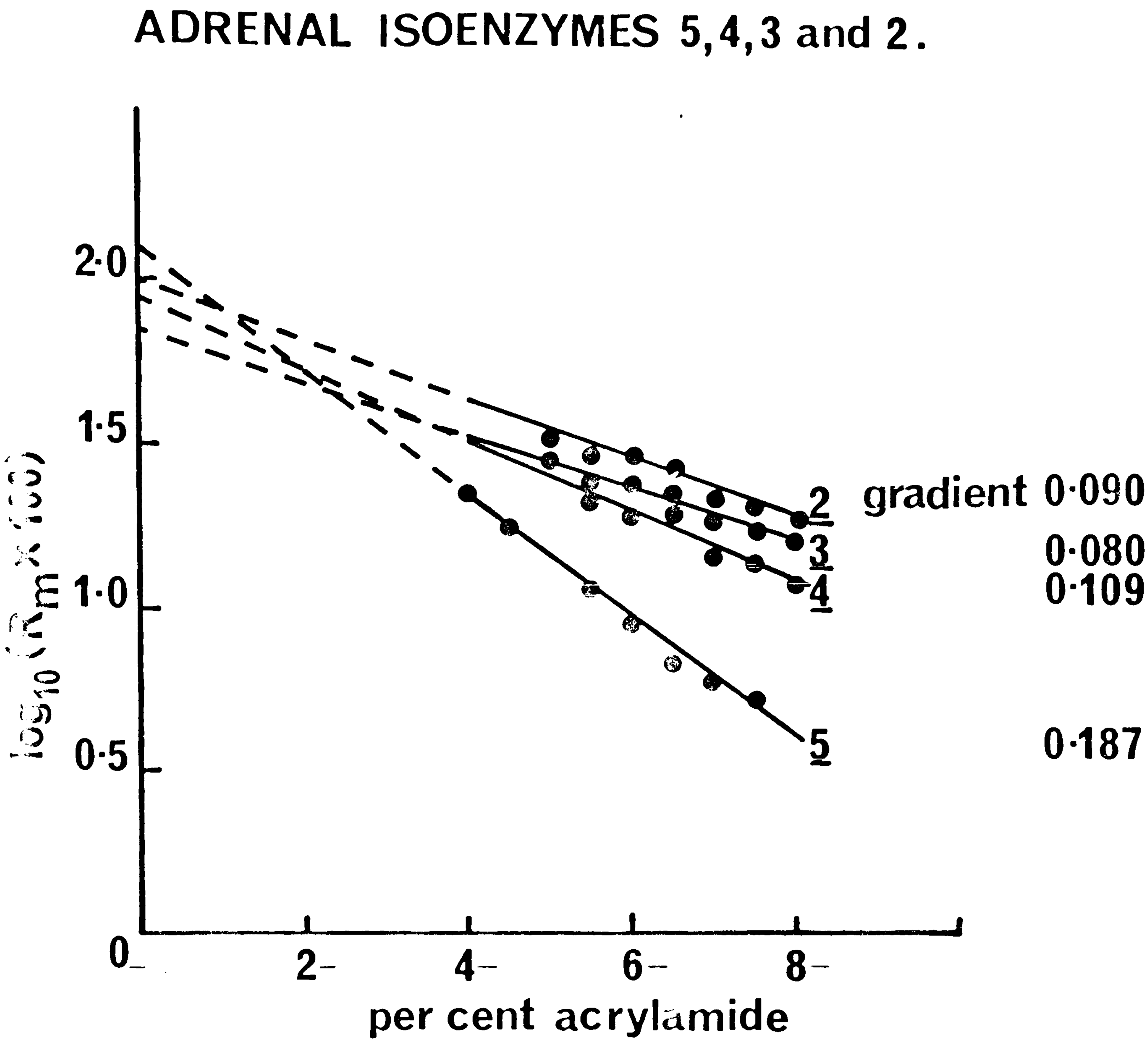


Fig. 19

The effect of different polyacrylamide concentrations on the electrophoretic mobility of the various soluble AChE isoenzymes in bovine adrenal medullae.

were similarities, or differences, between the soluble and membrane-bound forms of AChE. Initially, the experiments were carried out on extracts from the splanchnic nerve axons, where there was principally one of each isoenzyme.

As shown in Fig. 16, the bound and soluble AChE have identical apparent K_m values. For simplicity of presentation, the inhibition of the activity by higher substrate concentrations is not shown in Fig.16. Fig. 17 shows that the forms respond identically both to excess heat (Fig. 17 a) and to increasing incubation temperatures (Fig.17 b). Thus, both have identical Q_{10}'s of 1.2.

Finally, the relationship between the forms of AChE was investigated in two ways. First, the changes in their mobility when they were electrophoresed on acrylamide gels of different concentrations were measured. As shown in Fig.18, both the membrane-bound and soluble forms of the enzyme changed their mobility in an identical fashion. The parallel curves obtained by this method indicate that the two forms in the axons are charge isomers and are of very similar molecular weights (Hedrick and Smith, 1968). Similar experiments using the adrenal medulla enzymes are difficult to carry out successfully because of the large number of isoenzymes present. They become increasingly difficult to resolved because, for example, size isomers change their relative positions on the gel. Nevertheless, the results obtained are shown in Fig.19. They show that the medullary $AChE_5$ is identical to the major axonal soluble AChE - also termed $AChE_5$; it has an identical intercept at an extrapolated 0% gel and an identical change in mobility or slope. The other isoenzymes differ from $AChE_5$ and from each other principally in size (Fig.19). Similarly, the membrane-bound isoenzymes in the tissues behave identically under conditions similar to those described above. They are, therefore, both called $AChE_6$.

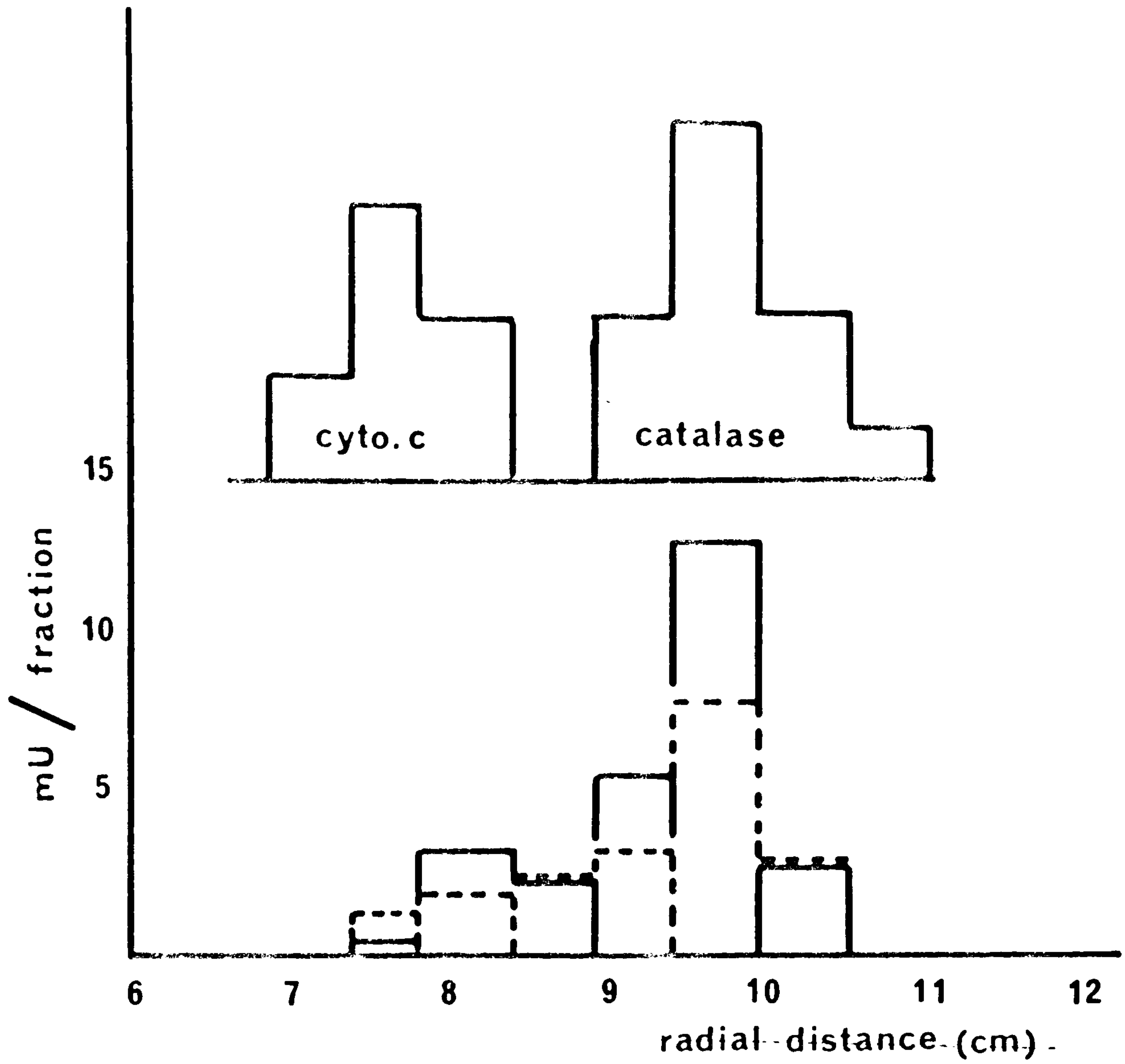


Fig. 20

The sedimentation on sucrose density gradients of total (——) and soluble (- - -) extracts from bovine splanchnic nerve axons.

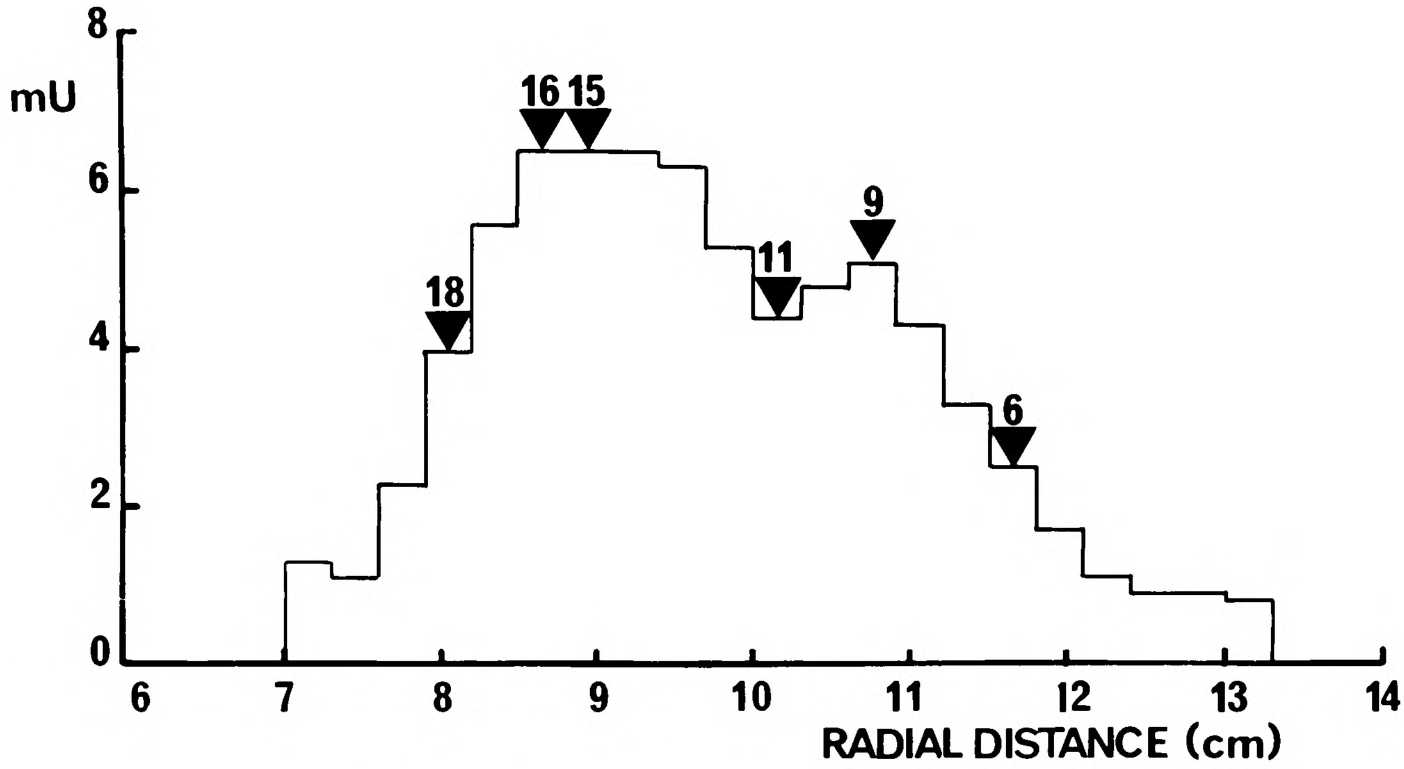


Fig. 21

The sedimentation on sucrose density gradients of the soluble AChE from bovine adrenal medulla.

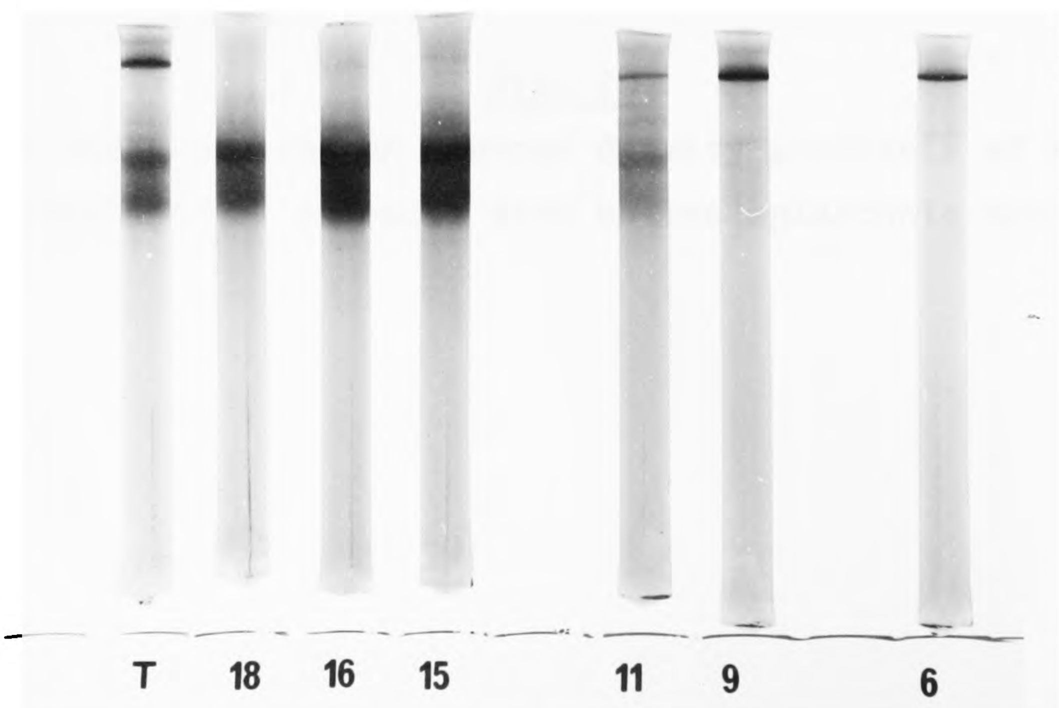


Fig. 22

An electrophoretic analysis of the fractions marked in Fig.21. T is an analysis of the total isoenzymes in the extract applied to the gradient.

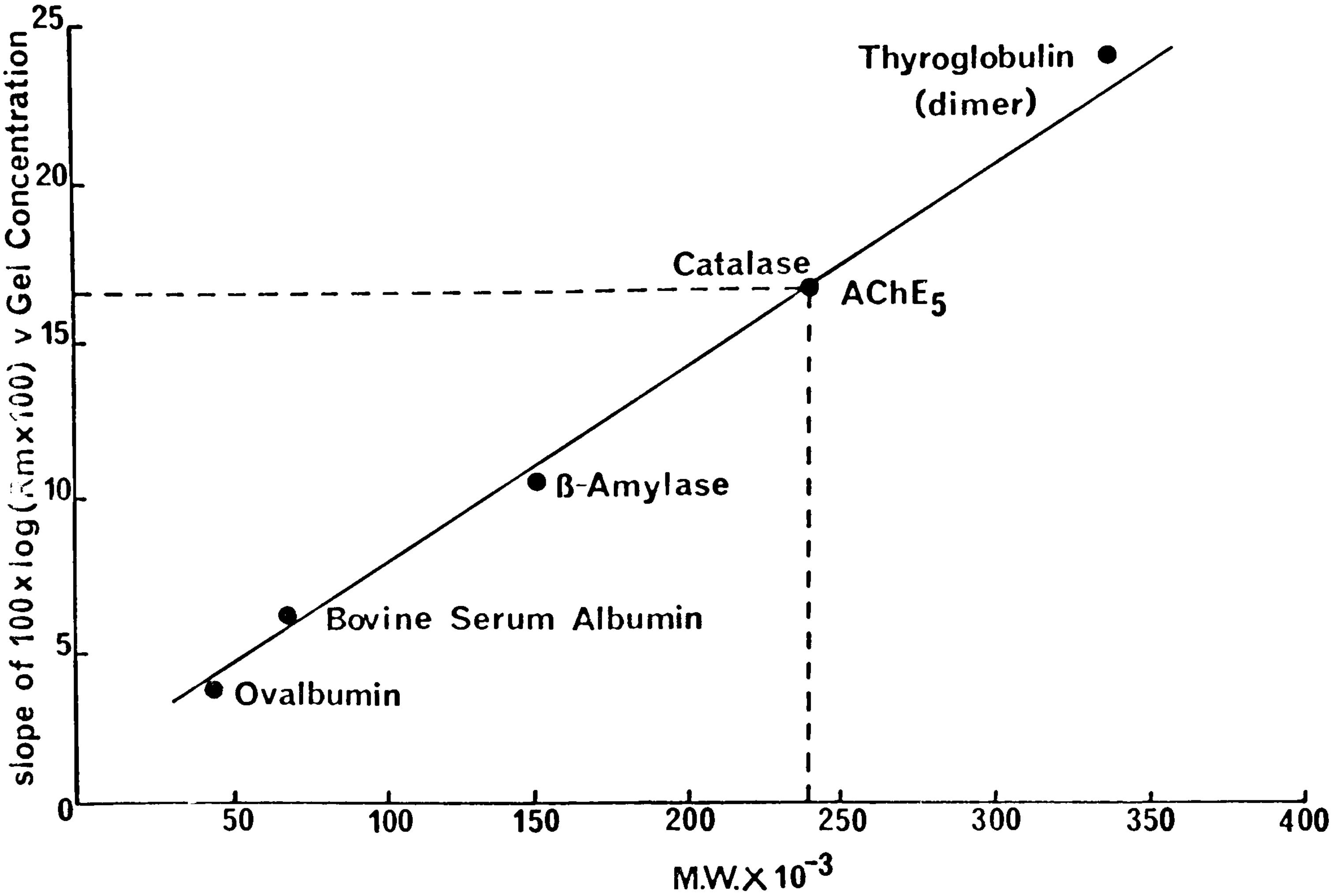


Fig. 23

Molecular weight calibration graph plotted according to Hedrick and Smith (1968).

Sucrose density gradients have also been used to try and establish the relationship between $AChE_6$ and $AChE_5$ on the one hand, and the various medullary isoenzymes on the other. Fig.20 shows that the Triton X-100 extract of the axons sediments as a single component on the density gradient. Electrophoresis of the peak fraction reveals a typical pattern for a total extract (see e.g. Fig.12). A soluble extract's AChE also sediments to the same place on the gradient (Fig.20) as does Catalase, a protein of molecular weight 240,000.

The adrenal medulla, as might be expected, gave a more complex distribution of AChE on the gradients than did the axons. As shown in Fig.21, the enzyme activity was distributed, very broadly, over the gradient's entire range. Fractions from this gradient were sampled through a needle so that small (approximately 0.7 ml) fractions could be collected. When those fractions marked with arrows in Fig.21 were electrophoresed on 6% gels, the patterns shown in Fig.22 were obtained. $AChE_5$ sediments furthest into the gradient and, as would be expected from its similarities with axonal $AChE_5$, it sediments very like Catalase. The other isoenzymes sediment fairly similarly to each other, with the possible exception of $AChE_4$, which seems to be intermediate between $AChE_5$ and the others. From the gradient it can be calculated that $AChE_5$ comprises about 25% of the activity of the soluble AChE in the medulla.

By the use of standard proteins, measurement of their mobility on gels of different concentrations and plotting the consequent changes in mobility versus their molecular weight and comparing this curve with the mobility of AChE, it can be calculated that $AChE_5$ has a molecular weight of about 240,000 (Fig.23; Hedrick

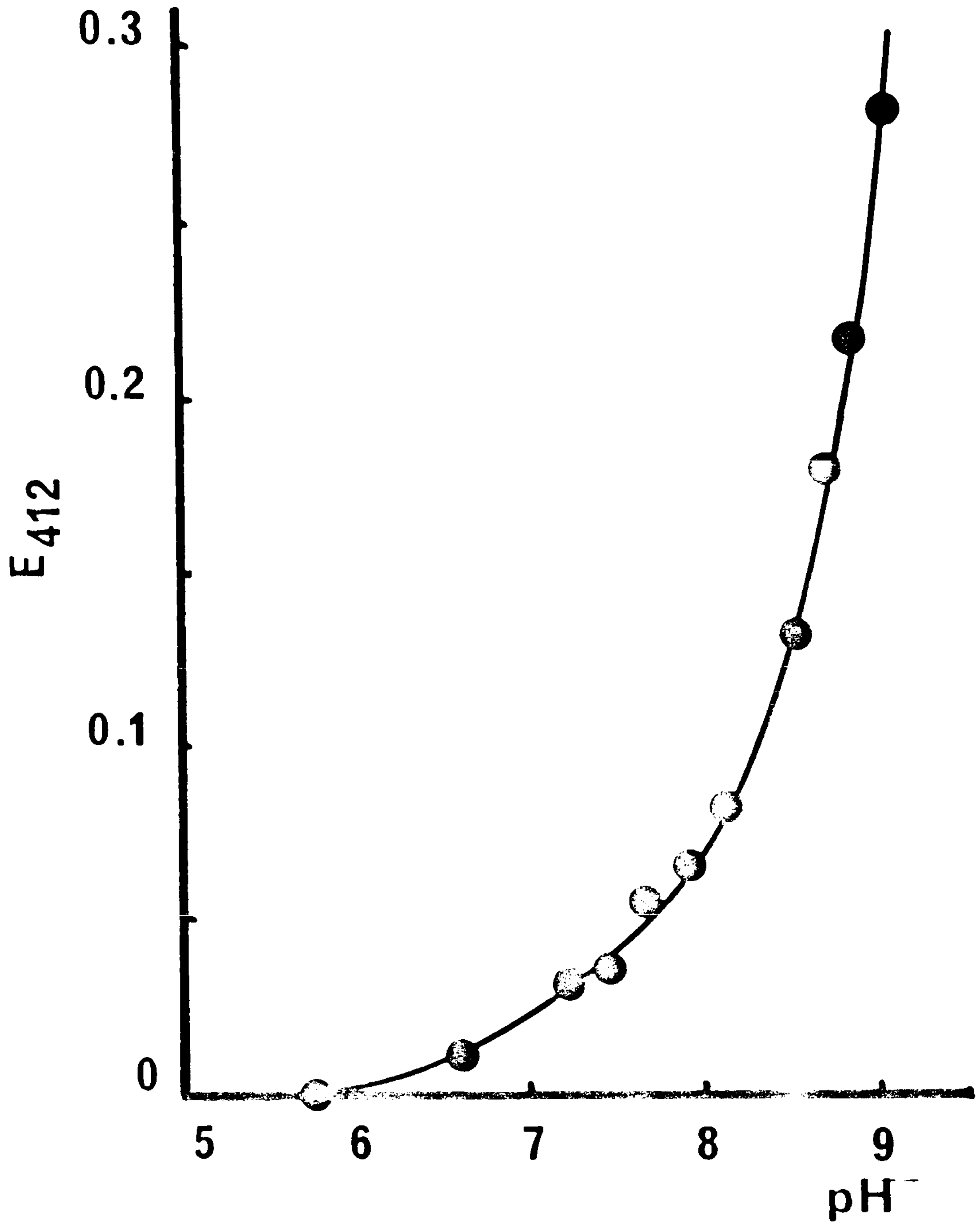


Fig. 24

The effect of increasing pH on the spontaneous hydrolysis of both ATCh and DTNB.

and Smith, 1968). From the other experiments described above it also seems reasonable to suggest that $AChE_6$ has a similar molecular weight. This result fits well with the centrifugation data, which show that both $AChE_5$ and $AChE_6$ sediment similarly to Catalase which, as mentioned earlier, has a molecular weight of 240,000.

It would have been interesting to compare one other property of the isoenzymes: their respective pH optima. It was not possible to do this with the method used for AChE assay because, as shown in Fig.24, there is an exponential increase in the blank value as the pH is increased. The increase can be shown to be due to a faster spontaneous rate of hydrolysis of the substrate, coupled with an increased, pH dependent,hydrolysis of the chromophore. Changes in E_{412} due to enzyme activity, when superimposed on a blank curve such as that shown in Fig. 24, could not be estimated accurately. Most of the methods available for measuring AChE activity all present some problem when it comes to measuring pH optima, so no other method has been tried.

DISCUSSION

First, I will discuss the method of assay of AChE activity, which has several advantages over existing, alternative procedures:

(i) it is simple and rapid;

(ii) large numbers of samples can be handled at one time;

(iii) it is sensitive - in the nMole range of product formed;

(iv) it is economical - especially when compared with other, equally sensitive, but radiochemical methods.

There are, however, some disadvantages in the method which should be kept in mind when it is used and when results from it are being interpreted:

(i) only sub-optimal activities of AChE are determined. Both ATCh and DTNB are hydrolyzed at alkaline pH (Fig.24) and the substrate becomes more unstable with increasing incubation temperature. For this reason incubations were carried out at 30° rather than 37°, and at pH 7.0 rather than the reported optimum for the enzyme of pH 8.6 (Augustinsson, 1963);

(ii) the method is only as specific for AChE as is the inhibitor used. Radiochemical and manometric methods can use acetyl β-methylcholine as a substrate quite specific for AChE. Unfortunately, acetyl β-methylthiocholine is no more specific for AChE than ATCh (Augustinsson, 1963) so it is necessary, if this current method is used, to employ an inhibitor specific for AChE or, alternatively, one for ChE. The most readily usable specific inhibitor of AChE is BW 284C51 (Austin and Berry, 1953) but, even here, care must be shown because, as can be seen in Fig.4, at concentrations only slightly higher than those which totally inhibit AChE activity, ChE activity is also inhibited;

(iii) as mentioned earlier, pH activity data for the enzyme are virtually impossible to determine accurately. This does not seem to be a difficulty confined to this particular method, indeed all of those which employ ACh as the substrate must suffer from the basic instability of the ester at higher pH values. The problem then becomes one of how the reaction is measured because, for example, the methods that measure acetate liberation from the substrate rely generally on either a subsequent liberation of CO_2 from a buffer or on a change in pH of the

solution. Obviously these methods are of only limited use in the estimation of pH optima. The investigation of the kinetics of AChE is also complicated by the spontaneous hydrolysis of the substrate. Thus, the blanks increase many-fold over the substrate range used and so errors are magnified. It is fortunate in this context that AChE has a quite high affinity for ATCh and so this latter problem is partially overcome because only low substrate concentrations are really necessary;
(iv) finally, a point which must be kept in mind whenever, and by whatever method, AChE is being estimated : a mixture of AChE and ChE assayed at one substrate concentration must give a falsely low value for one of the enzymes. This is because at substrate concentrations where the activity of AChE is maximal (i.e. about 1 mM), the activity of ChE is sub-maximal because the ChE is not saturated with its substrate (see Fig.5). At those substrate concentrations where the ChE activity is higher, the AChE is markedly inhibited. Consequently, it is impossible to quantitatively estimate both AChE and ChE in the one sample at one substrate concentration. ChE results presented in this thesis are, therefore, meant to show the presence of the enzyme and are not meant to represent absolute amounts, unless this is specifically mentioned.

The results of the centrifugation studies are interesting for two reasons. First, there was the large amount of AChE (and protein) sedimenting in Fraction 3 (Fig.6 and Table 3). The appearance of the pellet suggested that the bulk of myelin sedimented in this fraction, and an examination of the pellet with the electron microscope has revealed that this is indeed the case (Somogyi, unpublished observations). The answer to the question of whether the AChE activity is associated with the myelin, or whether it is derived from large membrane fragments which sediment in the same fraction, is not known. Attempts to remove myelin from the fraction (by flotation from 0.8M sucrose) were successful, but since they also resulted in a loss of AChE activity, the question is still unresolved. There are, however, reasons to suggest that it is not the myelin but probably membrane fragments which are responsible for the enzyme activity. These reasons will be discussed in Chapter 5.

The second interesting feature of these results, and certainly the most important so far as the rest of the work in this thesis is concerned, is the observation that a large proportion of the AChE in both splanchnic nerve axons and adrenal medullae is soluble. This observation has also been made for a number of other tissues but the proportions soluble are generally lower than those found for the tissues studied in this thesis. The lowest reported soluble AChE level is that found by Nathan and Aprison (1955), who showed that 8% of the enzyme in rabbit brain was soluble. One of the highest is that found by Hagen (1955), whose estimate of 30% soluble AChE in bovine adrenals agrees remarkably well with the figure of 31% found in this work. Between these two extremes lies a range of values obtained for different tissues from different species and using different homogenization procedures, all of which undoubtedly play some part in the breadth of the range. Nevertheless, the fact is clear that in all the tissues for which data are available there is some soluble AChE (see Chapter 1).

This observation has been largely ignored by most workers because of the feeling that AChE was specifically a membrane-bound enzyme. This was despite the evidence that a) <u>saline</u> extracts of basal ganglia contained electrophoretically separable esterases (Bernsohn et al., 1962), a result which infers that the different forms cannot be associated with membranes, since only soluble enzymes could be expected to enter the gel and b) membrane-bound activity could only be removed and solubilized with extreme difficulty (Lawler, 1964). Both of these results were obtained soon after the first extensive characterizations of AChE localization in membranes by Toschi (1959), but it was still often considered that soluble AChE was derived, artifactually, from the membranous enzyme.

Because of these considerations it is necessary to establish what we mean by the term soluble. In other words, when is a soluble enzyme cytoplasmic? (using cytoplasmic in the sense of the soluble phase of the cell). Are the biochemical criteria for solubility sufficient to allow an enzyme or other cell constituent recovered in the high-speed supernatant fractions to be designated a soluble cytoplasmic constituent? To answer this question directly is not easy, particularly when the enzyme under study is, as in this case, fairly evenly distributed between the supernatant and sedimentable fractions. When de Duve (1965) put forward his rationalisation of centrifugation experiments, he suggested that the estimation of cellular constituents in fractions obtained by centrifugation would give an indication of their localization within the cell. Obviously, this theory does not hold in cases where a dual localization of the constituent is found, and so we must turn to other, less direct methods of approaching the problem of identifying the subcellular localization of AChE.

For an enzyme to be recovered in the high-speed supernatant fraction when tissue homogenates in isotonic media are centrifuged, it must be localized in one of two sites. It can either be cytoplasmic to start with, or it can be contained within a particle or other structure which is sufficiently labile so as to be broken when the tissue is homogenized in preparation for the centrifugation procedure.

It seems most unlikely that AChE is free in the cytoplasm of the splanchnic axons, for the simple reason of transmitter economics. The available evidence strongly suggests that the biosynthetic enzyme for ACh is cytoplasmic (Fonnum, 1967; Fonnum, 1968). It is improbable that a hydrolytic enzyme with the efficiency of AChE would be in competition for this newly synthesized transmitter with the putative storage and release organelle, the synaptic vesicle. If this were so, then only a small proportion of the synthesized ACh would be expected to enter the vesicle and so one would have to postulate either a rate of synthesis far in excess of demand, or a specialized localization of choline acetylase which would give the vesicle first call on the new transmitter. Both of these suggestions are possible, but there are other reasons which tend to suggest that a fragile storage structure is the real source of the soluble AChE.

The first argument that can be used in favour of a labile AChE storage structure in the axons is one obtained from morphological studies. These will be discussed only briefly here because morphology and cytochemistry are the chief subjects of Chapter 5. However, it can be briefly said that the cytochemical observation of a dual localization of AChE in motor nerve axons was noticed from the very earliest of the studies which used the electron microscope. Barrnett (1962) and Miledi (1964) both observed intra-axonal AChE activity as well as that associated with the axonal membrane. The intra-axonal end-product was shown to be in "vesicles", but the evidence for this localization, as both authors pointed out, must be interpreted with caution. Schlaepfer and Torack (1966) also showed a dual localization of AChE in sciatic axons - membrane and axonal "vesicles". The designation of these intra-axonal structures as "vesicles" is possibly open to debate but the principal observation, that axonal AChE is found, not evenly or diffusely distributed in the cytoplasm, but within some type of membrane-limited structure, is beyond doubt.

The most convincing experiments in which the electron microscope was used for cytochemical studies on AChE distribution are those of Kasa (1968). His results showed that in longitudinal sections of sciatic nerve, the axons contained quite long tubules rich in AChE. Again, there was no evidence of cytoplasmic AChE activity. These results also offer an explanation for the observation discussed earlier of axonal, AChE-rich vesicles. If cross-sections of axons are examined, it seems reasonable that the tubules, also cut in cross-section, would have the appearance of vesicles. It is interesting in this context that, in studies in which axons were soaked in peroxidase and then examined cytochemically, the intra-axonal peroxidase was shown to be in tubules in longitudinal section, but in "vesicles" and only in vesicles in cross-section (Krishnan and Singer, 1973) . Thus, these "vesicles" might reasonably be tubules cut in cross-section, as might the "vesicles" which contain AChE. These experiments of Krishnan and Singer (1973) are of considerable interest and will be discussed later because they show that axonal tubules are, or can be, open to the extracellular space.

The fact that AChE has been shown to flow rapidly down axons is also indicative of a particle-associated localization of the enzyme. Karlsson and Sjöstrand (1971), who studied the rates of protein migration in rabbit retinal neurons, found four different rates of transport in the nerve. But most significantly, they showed that the rapid rate reflected proteins in a small (light) particulate fraction, while the slowest rate consisted predominantly of soluble proteins (see also Jeffrey and Austin, 1974). Thus, the rapidly flowing AChE is almost certainly associated with particles of some type which are, almost equally certainly, within the axoplasm (it is unlikely that the AChE is that which is associated with the axolemma because of the extreme rates of flow which have been estimated). It remains to be shown whether or not the soluble AChE is carried down at this rapid rate.

The presence and the distribution amongst the fractions of the acid hydrolases can also be used as an indication that the soluble AChE might be contained within a labile particle. In interrupted axons the acid hydrolases are known to be in elements of smooth endoplasmic reticulum (SER) (Holtzman and Novikoff, 1965; Holtzman, 1971), and it is this SER which is thought to give rise to the lysosomes, the more usual particle associated with these enzymes (de Duve, 1965). In normal axons, however, there are very few lysosomes and so it can be concluded that a considerable part of the acid hydrolase activity in normal axon homogenates is derived from the SER. A study of the distribution of these enzymes in fractions obtained by centrifugation of splenic nerve homogenates showed that varying amounts of different hydrolases were always recovered in the supernatant fraction (De Potter, Smith and

De Schaepdryver, 1970). Thus, 47% of the acid β-glycerophosphatase activity and 72% of the acid ribonuclease were "soluble", a result which is quite similar to that obtained for the splanchnic axons (Table 3).

As a working hypothesis then, it seems not unreasonable to suggest that the AChE is stored in a soluble form within elements of the SER which flow rapidly down axons and which are sufficiently labile to be unable to withstand the preparative procedures required for centrifugal analyses of tissue homogenates. Support for this hypothesis will be presented in Chapter 3.

The discussion so far has centred mainly around the AChE content of the axons, principally because all of the available evidence suggested that most of the AChE in innervated organs was neuronally derived. In other words, it was thought that the work mentioned in Chapter 1, in which denervated organs lost most, or a lot, of their AChE, meant that the medullary AChE was due to the presence of AChE-containing splanchnic nerve terminals within the tissue. It will be shown later that this is not true, but so far as a lot of the work that has been done is concerned, this is known only with hindsight. Consequently, I will first discuss this work as it was done at the time.

Most of the reasons for supposing that the adrenal medulla cells did not contain AChE were indirect, since very few cytochemical studies have been made on this tissue. Those that have (see Chapter 1) indicated that all of the AChE was associated with the nerve fibres, and that no activity was present within the chromaffin cells. The advent of the electron microscope indicated that there might be some enzyme in some chromaffin cells, but the situation was still by no means clear. However, because of the marked species differences in the response of a tissue's AChE to various treatments (e.g. the 80% decrease in cat SCG AChE versus the 45% decrease in rat SCG enzyme in response to preganglionic denervation) we concluded from the only cytochemical study that we could find which used ox medullae as tissue source (Coupland, 1961) that the chromaffin cells were devoid of activity. Thus, the working hypothesis described above was modified to include the fact that probably all of the medullary AChE was derived from the axons - the increased number of isoenzymes was taken to indicate that there was a concentration of these faster (in the electrophoretic sense) moving components in the nerve terminals.

We must now turn to the isoenzymes of AChE which are present in the two tissues studied. The first point that should be made here is the clarification of the biochemical data that the electrophoresis yields. As mentioned earlier, the dual localization of AChE complicates the situation, since the rationale of de Duve (1965) cannot be applied in these more complex cases. Electrophoresis, however, clarifies the situation when it shows, in effect, that axonal AChE does not have a dual localization, but rather that there are two AChE's, each with a unique localization as far as its distribution among fractions obtained by centrifugation is concerned. Accordingly, estimation of the total AChE activity includes contributions from the two isoenzymes and so cannot be used on its own as a means of studying the subcellular localization of the enzyme.

As mentioned in Chapter 1, there have been many reports of AChE isoenzymes obtained from many tissues and species and by using different methods of extraction. Generally, however, the total isoenzymes have been studied and no attempt has been made to study separately the membranous and the soluble enzymes.

Maynard (1966) found some qualitative differences in the distribution of AChE between different isoenzymes in aqueous and Triton X-100 extracts of chick brain and muscle; a similar observation utilizing rat tissues was made by Davis and Agranoff (1968). The work presented in this Chapter shows that in both the splanchnic nerve and the adrenal medulla there is a striking difference between the membrane-bound and the soluble isoenzymes of AChE : in both tissues there is only one membrane-bound isoenzyme, and this has a lower mobility in electrophoresis than all of the soluble isoenzymes. It has been shown recently that soluble and particulate fractions of rat cerebral cortex also contain distinct isoenzymes of AChE (Wenthold, Mahler and Moore, 1974). Thus, it is no longer justifiable to assume, as has been done in the past (Barron and Bernsohn, 1968; Hollunger and Niklasson, 1973; McIntosh and Plummer, 1973), that the pattern of isoenzymes in an aqueous extract is representative of the isoenzymes of AChE present in the membranous phase. For this reason some of the earlier work is difficult to interpret, because several workers extracted AChE from unfractionated tissue and believed that they were studying membrane-bound enzyme.

Because of the way in which the isoenzymes in splanchnic axons separate, i.e. one being uniquely membrane-bound while the other is soluble, we have been able to compare some of the properties of these two isoenzymes without the necessity of further purification or separation of the enzyme forms. The properties examined were electrophoretic behaviour, apparent molecular weight and simple kinetics of the two isoenzymes $AChE_5$ and $AChE_6$.

The greater electrophoretic mobility of $AChE_5$ could be due to a smaller size and/or greater electrical charge of the enzyme molecules. According to Hedrick and Smith (1968), three different types of protein isomers can be distinguished using acrylamide gel electrophoresis. Plots of $\log_{10}$ Rm (relative mobility) versus the gel concentration should give straight line graphs, and a comparison of different proteins would yield 3 basic families of curves. Thus :

(i) proteins which differ in both size and charge will give non-parallel lines intersecting at a gel concentration other than 0%;

(ii) proteins differing in molecular size but carrying the same net charge will give non-parallel lines, but which intersect at 0% gel concentration;

(iii) proteins differing only in charge and not in size will give parallel lines.

Fig.18 therefore shows that $AChE_5$ and $AChE_6$ most probably differ from each other in charge, but not in size, while Fig. 19 would tend to suggest that in the adrenal medulla the four fastest moving isoenzymes are size and charge isomers of $AChE_5$. This latter result is not particularly clear, however, and no great emphasis is placed upon it. On the other hand, Figs.18 and 19 provide quite convincing evidence that $AChE_5$ in both tissues is one and the same isoenzyme. In each tissue they have identical mobilities over the range of gel concentrations used, and have identical extrapolated intercepts at 0% gel.

The method of Hedrick and Smith (1968) has been criticized by Rodbard and Chrambach (1972) on the grounds that factors such as molecular shape, hydration and partial specific volume have not been taken into account. Consequently, we used an alternative method to try and separate $AChE_5$ from $AChE_6$ in the first instance, and later $AChE_5$ from the rest of the medullary isoenzymes - sedimentation on a stabilizing sucrose density gradient. The results obtained with this method essentially confirmed those obtained with the electrophoresis: $AChE_5$ and $AChE_6$ were similar in size, and $AChE_5$ was bigger than the other medullary isoenzymes.

In the literature there are reports that AChE isoenzymes can differ from each other both in terms of charge and of size. In human erythrocytes, for example, a difference in charge but not in molecular size distinguishes two of the membrane-bound isoenzymes of AChE. Similarly, the six isoenzymes of AChE in aqueous extracts of rat cerebral cortex seem to be charge isomers (Wenthold et al., 1974). However, the majority of studies on isoenzymes of AChE have shown that the isoenzymes differ in molecular weight. This is the case for the isoenzymes of AChE in the soluble fraction of embryonic chick brain and skeletal muscle (Wilson et al., 1969) and for those in aqueous extracts of bovine caudate nucleus (Chan et al., 1972; Hollunger and Niklasson, 1973), pig brain (McIntosh and Plummer, 1974) and rat brain (Wenthold et al., 1974). In a Triton X-100 extract of rabbit sciatic nerve, the two isoenzymes differed in both charge and size (Skangiel-Kramska and Niemierko, 1971). Since all possible reasons for the differences in the electrophoretic and chromatographic behaviour of isoenzymes, i.e. differences in molecular size or in charge or in both, have now been recorded for AChE, no attempts should be made to generalize about the molecular properties that give rise to the isoenzymes.

The apparent molecular weight of 240,000 for $AChE_5$ and $AChE_6$ was obtained by comparing the mobilities of the isoenzymes in gel electrophoresis with those of other proteins and, since denaturing conditions were not used, it must be considered only a rough estimate. This value is nevertheless supported by the centrifugation data, which show that the sedimentation of $AChE_5$ and $AChE_6$ is very similar to that of Catalase - a protein with a known molecular weight of 240,000. It is also very similar to the estimate of 260,000 for the AChE solubilized from membranes of the electric organ of the eel (Leuzinger, 1969). This latter enzyme preparation offers an explanation for the apparent charge differences of $AChE_6$ and $AChE_5$. Leuzinger (1969) purified his AChE sufficiently to crystallize the protein, and an examination of this protein showed the presence of two amino-sugars, glucosamine and galactosamine. If, and this must remain speculation until enzyme from both soluble and membrane-bound forms are obtained at comparable purity, the membrane-bound but not the soluble AChE contains the amino-sugars, then the molecule should have a lower net negative charge at pH 8.1, since both carbohydrates would contribute positive charges to the protein. This would result in the isoenzymes behaving as charge isomers, since the addition of carbohydrates would not alter the molecular size sufficiently for the change to be detected by the relatively crude methods used here. As has been said, this possibility must remain speculation, but one interesting observation has been made very recently which bears upon this problem; it is that $AChE_5$ binds to the plant lectin concanavalin A - a result which is preliminary, but which suggests that there is at least some type of carbohydrate on that isoenzyme. Thus, both $AChE_6$ and $AChE_5$ are probably glycoproteins, but nothing can be said about the type of carbohydrate attached to either isoenzyme, at least so far as those derived from these tissues are concerned.

With acetylthiocholine as the substrate, both $AChE_5$ and $AChE_6$ have the same apparent K_m of 7.6×10^{-5} M, which is close to the

K_m of 9×10^{-5} M obtained with ACh as the substrate and the AChE from the electric organ as the enzyme (Wilson and Quan, 1958). The finding that the soluble and membrane-bound isoenzymes in the splanchnic nerve have the same K_m can be compared with kinetic studies on other isoenzymes of AChE. The two soluble isoenzymes in human basal ganglia have the same K_m value (Bernsohn et al., 1963) and so do two of the membrane-bound isoenzymes of human erythrocytes (Shafai and Cortner, 1971). On the other hand, different K_m values were found for two soluble isoenzymes in rat brain (Bajgar and Zizkovsky, 1971), for two isoenzymes from bovine caudate nucleus (Chan et al., 1972), for four soluble isoenzymes from horsefly brain (Tripathi, Chiu and O'Brien, 1973) and for the five isoenzymes in the electroplax of the electric eel (Tripathi et al., 1973). It is not known whether different K_m values are found only for those isoenzymes of AChE that differ in molecular weight. However, it is noteworthy that $AChE_5$ and $AChE_6$ of ox splanchnic nerve have the same molecular size and the same K_m, just as do two of the isoenzymes in the erythrocyte membrane (but, see Chapter 4).

The one remaining point to be discussed, very briefly, is the observation that incubated homogenates of splanchnic axons rapidly lost AChE activity. This problem has not been studied in great detail, since it was observed only very recently, but enough has been done to be able to say that it is fairly widespread in the sense of tissue localization, i.e. incubated homogenates of splanchnic nerve axons, adrenal medullae, SCG and cerebrospinal fluid all lose AChE activity, but that it is not a complicating factor in the normal assay. If a tissue homogenate is incubated, the more dilute, the more stable the

AChE activity; thus, if tissue is incubated as in the AChE assay (Table 2) at a concentration of up to about 20 mg/ml, no activity is lost over a period of several hours. The same tissue incubated at a concentration of around 100 mg/ml loses about 70% of its AChE within 2 h. Much more work needs to be done to determine such things as whether it is an enzymic destruction of the protein or, more mundanely, the production of a compound which interferes in the subsequent assay.

Earlier in this discussion, a working hypothesis was put forward, which was later modified. As it now stands, the hypothesis is that soluble AChE is stored within the axonal SER, which flows rapidly down axons and which is a sufficiently labile structure to be broken by homogenization, thus liberating the AChE. It was later modified to include the suggestion that all of the medullary AChE was due to the presence of splanchnic nerve terminals in the tissue and that the increased concentration of fast migrating isoenzymes in the medulla reflected an accumulation of them in the nerve endings.

The only modification of the hypothesis that is now necessary is that which would give the unique localization of $AChE_5$ to the SER. This is a natural extension, given first the evidence that there is one soluble isoenzyme and, secondly, the earlier formulated hypothesis that the soluble AChE was in the SER.

It is one thing to formulate an hypothesis, it is quite another to gather evidence in its favour. Experimentally, one way to obtain evidence to support the current hypothesis is to determine whether <u>soluble</u> AChE accumulates rapidly in constricted axons. The results of experiments such as these are described in the next Chapter.

CHAPTER 3

THE AXONAL TRANSPORT OF ACETYLCHOLINESTERASE IN RAT SCIATIC AND BOVINE SPLANCHNIC AXONS.

INTRODUCTION

In the previous Chapter several arguments were used to try and show that soluble AChE in axons was not cytoplasmic but was rather stored within a fragile particle easily broken during homogenization procedures. One of the arguments used was that of axonal flow: rapid flow being equated with a particle movement, while slow flow was thought to be indicative of a slow steady movement of the axoplasm (see e.g. Ochs, Sabri and Johnson, 1969; Karlsson and Sjöstrand, 1971).

Most of the available evidence allows the conclusion that AChE flows down nerves at a rapid rate. This fact has been clearly shown by the work of Lubinska and her colleagues, Partlow and his, and Ranish and Ochs (see Chapter 1). But just as an estimate of total activity was not sufficiently informative in the centrifugation experiments described in Chapter 2, an estimation of the total AChE activity accumulating at a ligation on a nerve no longer adds much to our knowledge; to gain evidence for, or against, our working hypothesis, we need to know what type of AChE is involved in the movement. Thus, a rapid accumulation of the soluble isoenzyme would be evidence for the hypothesis, whereas a slow movement of soluble, but a rapid flow of membranous AChE, would be an argument against.

The experiments described in this Chapter were undertaken to obtain some information as to the type of AChE which is involved in axoplasmic transport.

METHODS

Preliminary experiments were carried out using rat sciatic nerve. This was done to test the viability of the method before we used the more expensive experimental animal, calves. For these latter experiments the collaboration of Drs. R. Comline and M. Silver is gratefully acknowledged.

180-220 g male Wistar rats were anaesthetized with 37 mg/Kg nembutal (i.p.) and the right sciatic nerves were exposed in the mid-thigh region. A tight ligature was placed around the nerve, the wound closed and the animals allowed to recover for different times before the nerves were removed.

The ligatured nerve, and an equivalent length of contralateral control nerve were placed on a glass plate and cut into measured segments, usually 2 mm lengths. Each segment was then homogenized using a small, all glass, hand-driven homogenizer in 1.0 ml of 0.3 M sucrose. An aliquot was taken for measurement of total activity and then the homogenates were centrifuged at high-speed (100,000 g; 60 min; A40 rotor of Spinco centrifuge) to separate the soluble from the bound AChE. This supernatant was sampled for estimation of enzyme activity and also, without other treatment, for electrophoresis.

The procedure adopted when calf nerves were used was very similar to that described above. The differences - which were only minor - were as follows: the homogenate volume was 2.0 ml; a double ligature about 2 mm apart was used so that truly proximal and distal segments could be analyzed without overlap; the samples were dialyzed overnight against 5 mM sodium phosphate, pH 7.0, before extraction with Triton X-100 and electrophoresis; the anaesthetic used was pentobarbitone sodium (13 ml of a 6%

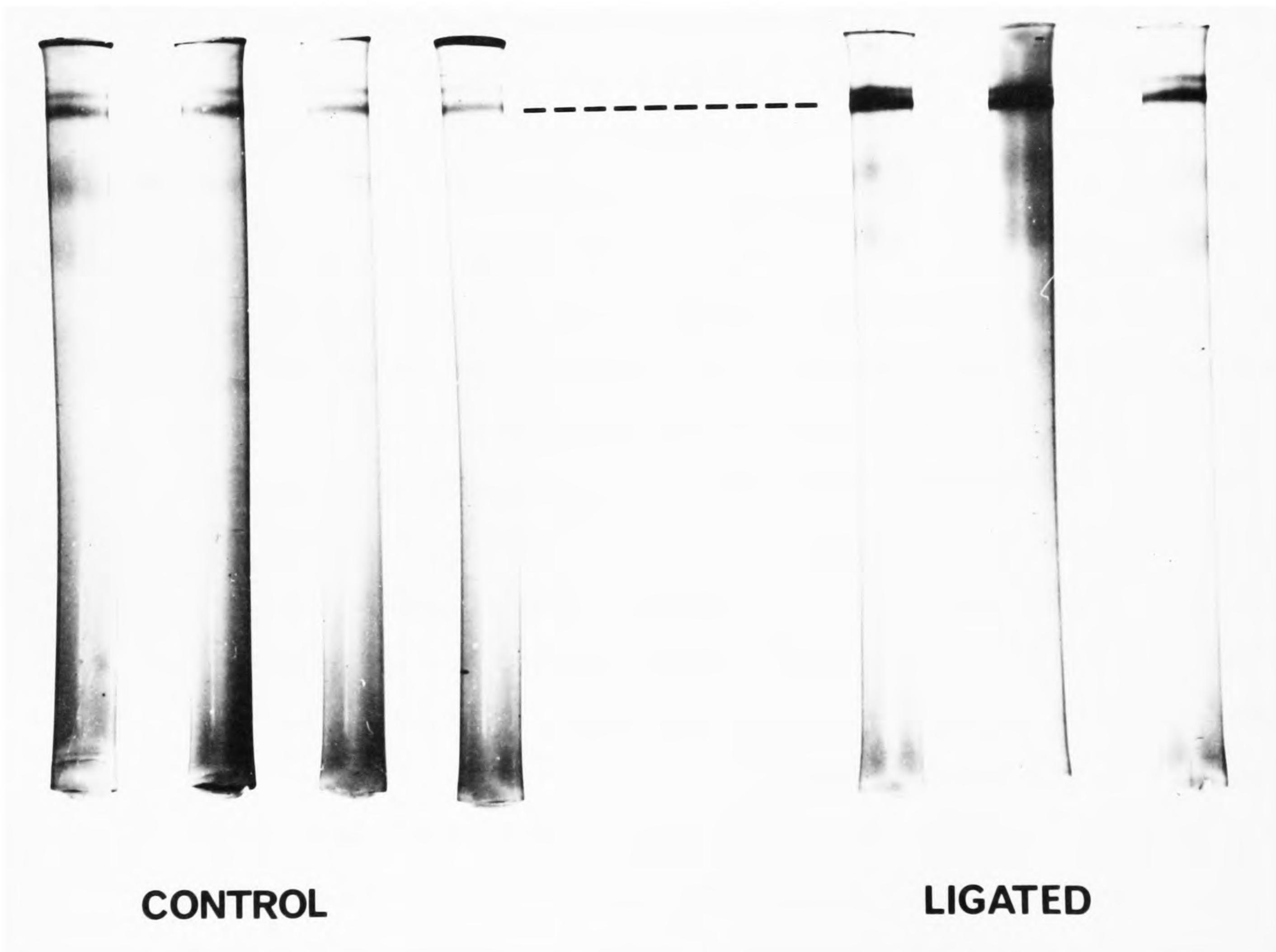


Fig. 25

The accumulation of soluble AChE in rat sciatic nerve. The same equivalent amount of nerve was run on each gel. The qualitative differences therefore reflect true quantitative changes.

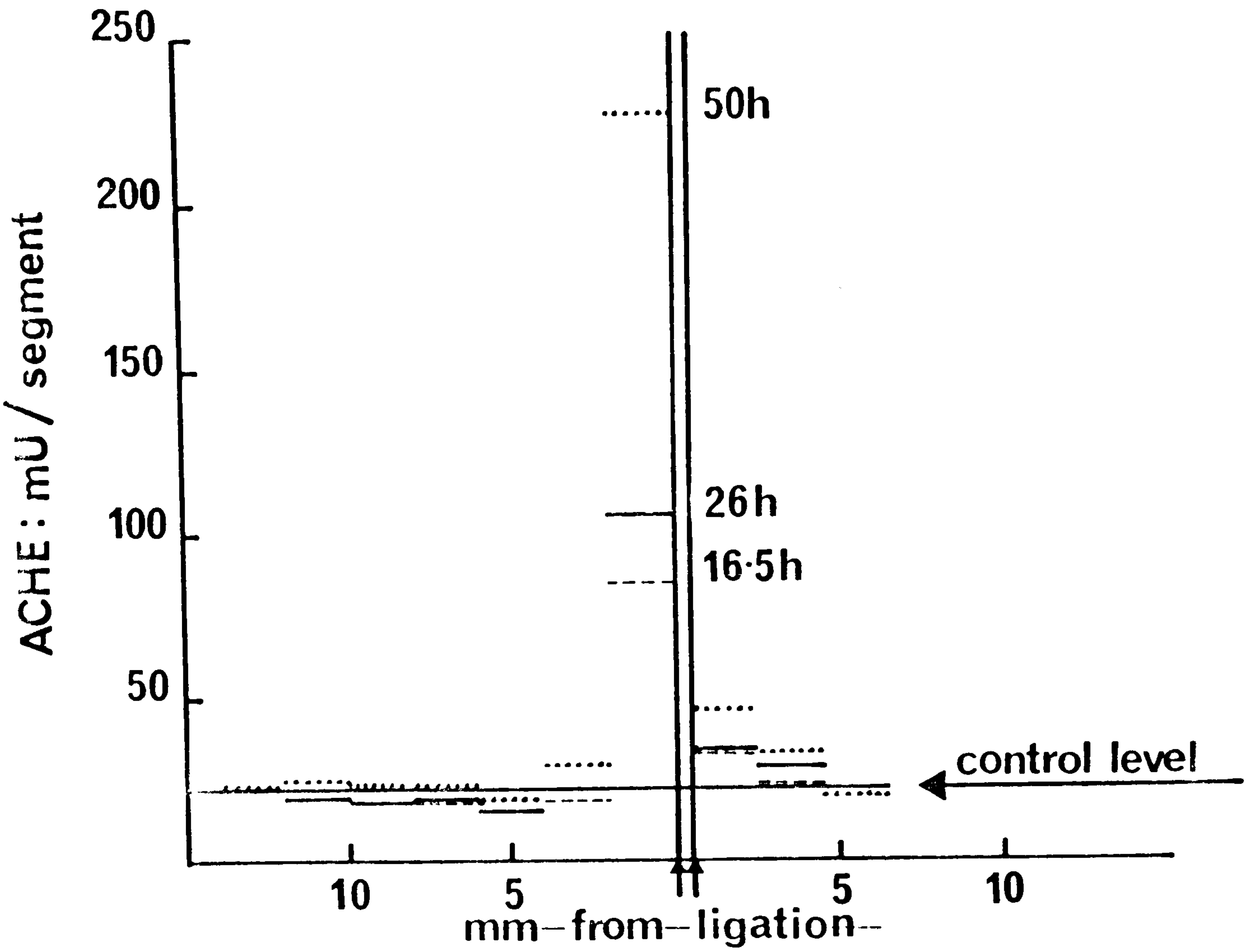


Fig. 26

The amount of AChE in different 2 mm segments of bovine splanchnic axons at different times after ligation (represented by ↑↑).

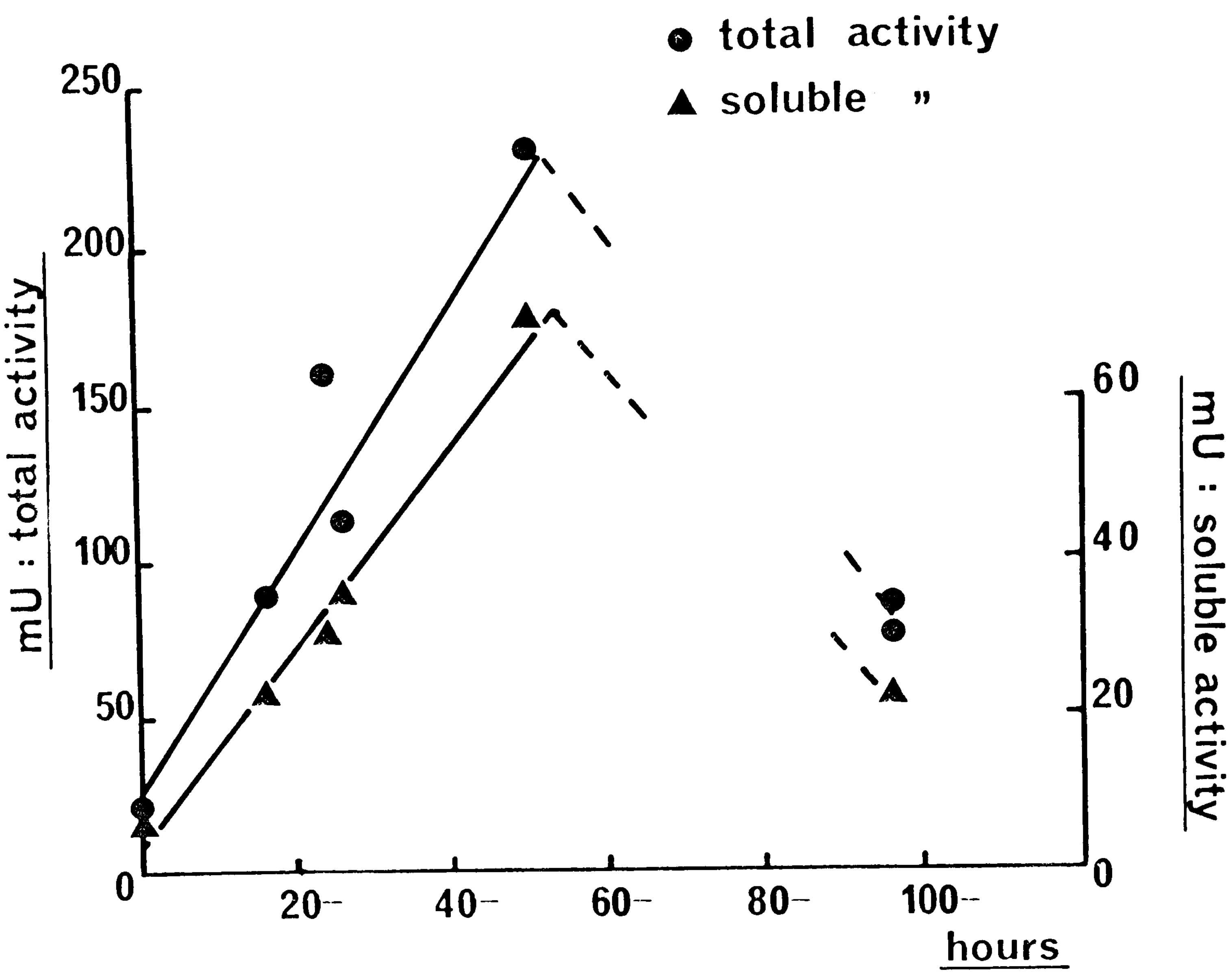


Fig. 27

The accumulation of total and soluble AChE activity in the 2 mm segment immediately proximal to a ligation placed on bovine splanchnic axons for different periods of time.

solution; i.v.) and the animals were heparinized prior to the removal of the tissues.

Electrophoresis, enzyme assays and protein determinations were made as described in the previous Chapter.

RESULTS

The isoenzyme pattern of soluble AChE in rat sciatic nerve is shown in Fig. 25. It is a more complex pattern than that obtained from the bovine splanchnic axons but it shows, nevertheless, that there are also soluble isoenzymes in this tissue. Quantitative estimates show that these isoenzymes account for about 30% of the total activity in the axons. Fig. 25 also shows that there is an increase in the amounts of all the soluble isoenzymes in a 2 mm segment of nerve immediately proximal to a ligation. These results were obtained from nerves in which the accumulation time was 18 h, and since the same equivalent length of nerve was run on each gel, the apparent qualitative differences reflect true, quantitative changes.

Much more information was obtained from calf splanchnic axons when they were analyzed at various times after ligation. First, as shown in Fig. 26, there is a clear-cut accumulation of AChE activity in the first 2 mm of nerve on both the proximal and the distal sides. This activity is rapidly reduced to control levels in subsequent segments. The rate of the down, or proximo-distal, flow is shown in Fig. 27, where both the total and soluble AChE activities in the first 2 mm are shown. The lines drawn were calculated by the least-squares method and

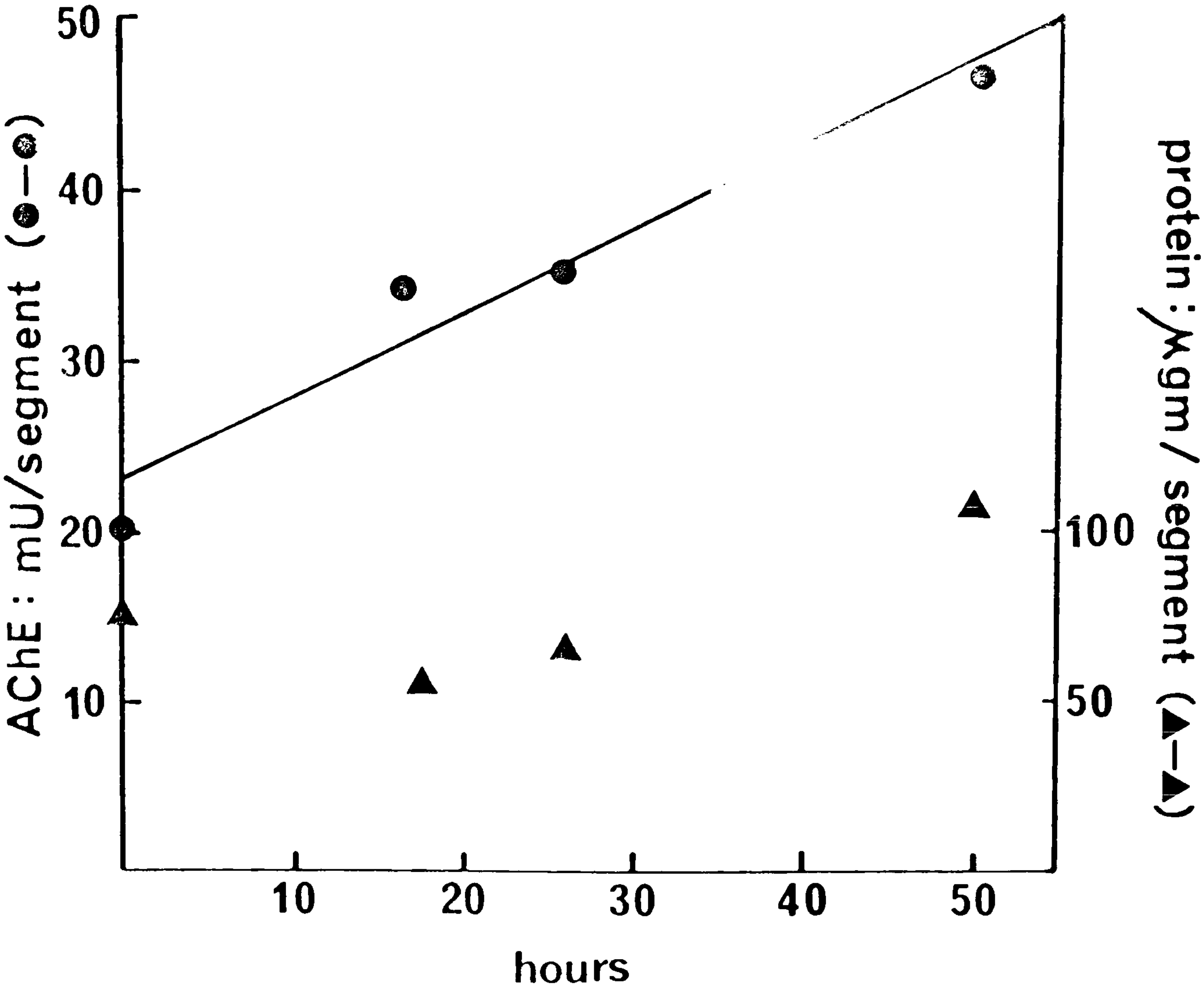


Fig. 28

The accumulation of AChE and changes in protein in the 2 mm segment immediately distal to the obstruction on bovine splanchnic axons.

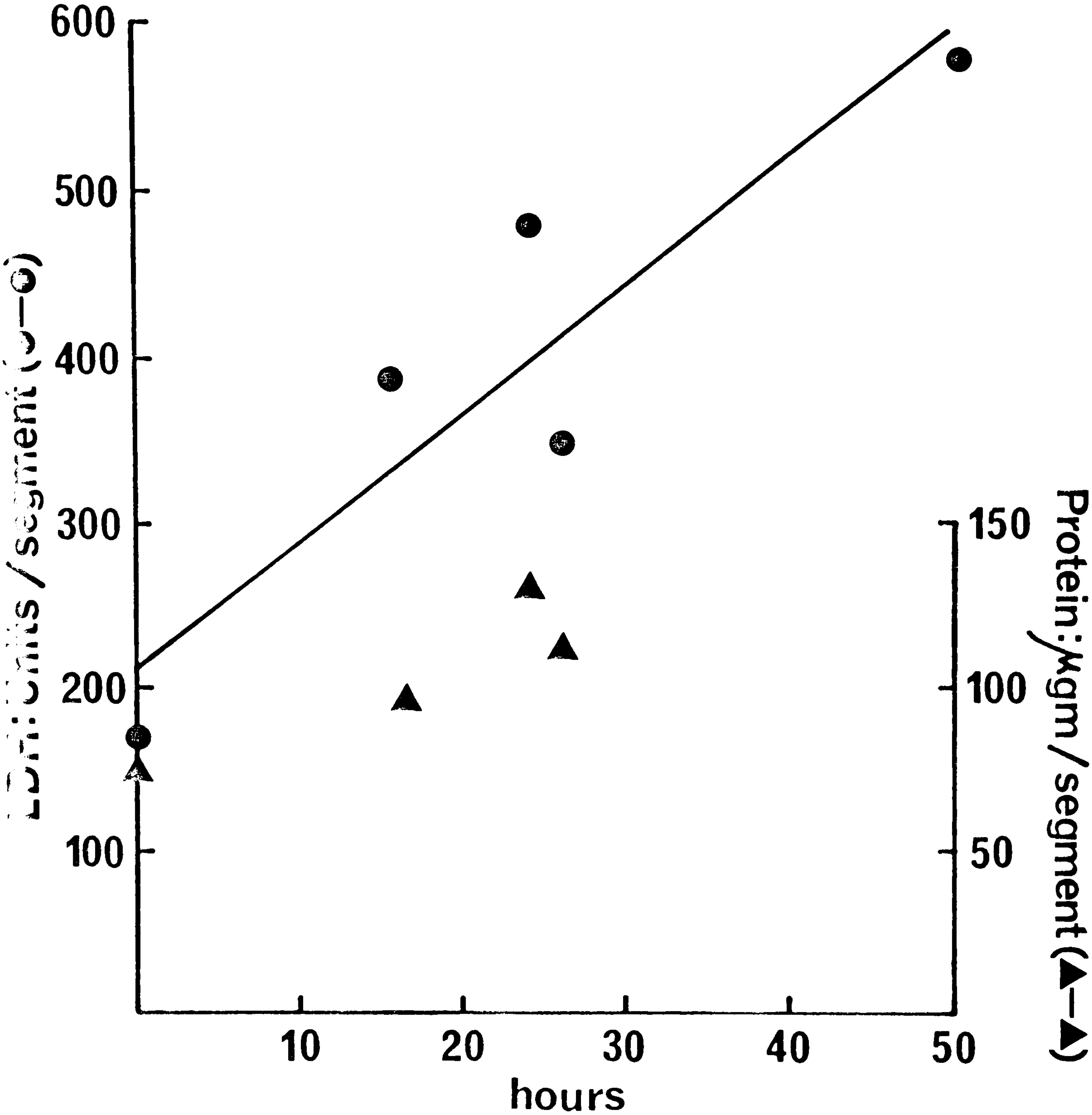


Fig. 29

The accumulation of LDH and protein in the segment immediately proximal to the ligation on bovine splanchnic axons.

are described by the equations: total, $y = 4.1x + 24.4$ and soluble, $y = 1.3x + 3.4$. The respective correlation coefficients are 0.956 and 0.990, both of which are statistically significant at the $P < 0.001$ level. The rate of accumulation of AChE activity which can be calculated from these curves is 4.1 ± 0.7 mU/h of total activity and 1.3 ± 0.1 mU/h of soluble AChE. From these figures the rate of flow of the enzyme can be calculated as follows: 4.1 mU of total activity accumulates per h and since there are about 10 mU/mm of axons (see Fig. 26), then the equivalent of 0.41 mm of AChE accumulates each hour, or 9.9 mm/day. Similarly, there are 1.3 mU of soluble activity accumulating each hour, and there are 3.2 mU of soluble activity per mm of these axons, so that the same flow rate, i.e. 9.9 mm/day, is found for the soluble AChE. Fig. 28 shows the flow of AChE in the reverse, retrograde direction. The line in this Figure is drawn according to the equation, $y = 0.48x + 23.1$; the correlation coefficient is 0.972 ($0.02 < P < 0.05$). This result, which means that about 12 mU of AChE accumulates per day, shows that the flow rate in the retrograde direction is about 1.5 mm in 24 h. Also shown in Fig. 28 is the amount of protein in the same 2 mm segments of the nerve. With the limited data, it seems that there is not a great overall change in the total amount of protein present in these segments.

Protein and LDH were also measured in the proximal 2 mm segment of nerve and, as shown in Fig. 29, both of these components accumulate at the ligation. However, analysis of the points shows that only the LDH values are significantly correlated; $y = 7.8x + 213.4$, correlation coefficient 0.915 ($0.02 < P < 0.05$). The flow rate of LDH can therefore be calculated from these results, in the same way that the flow rates of AChE were

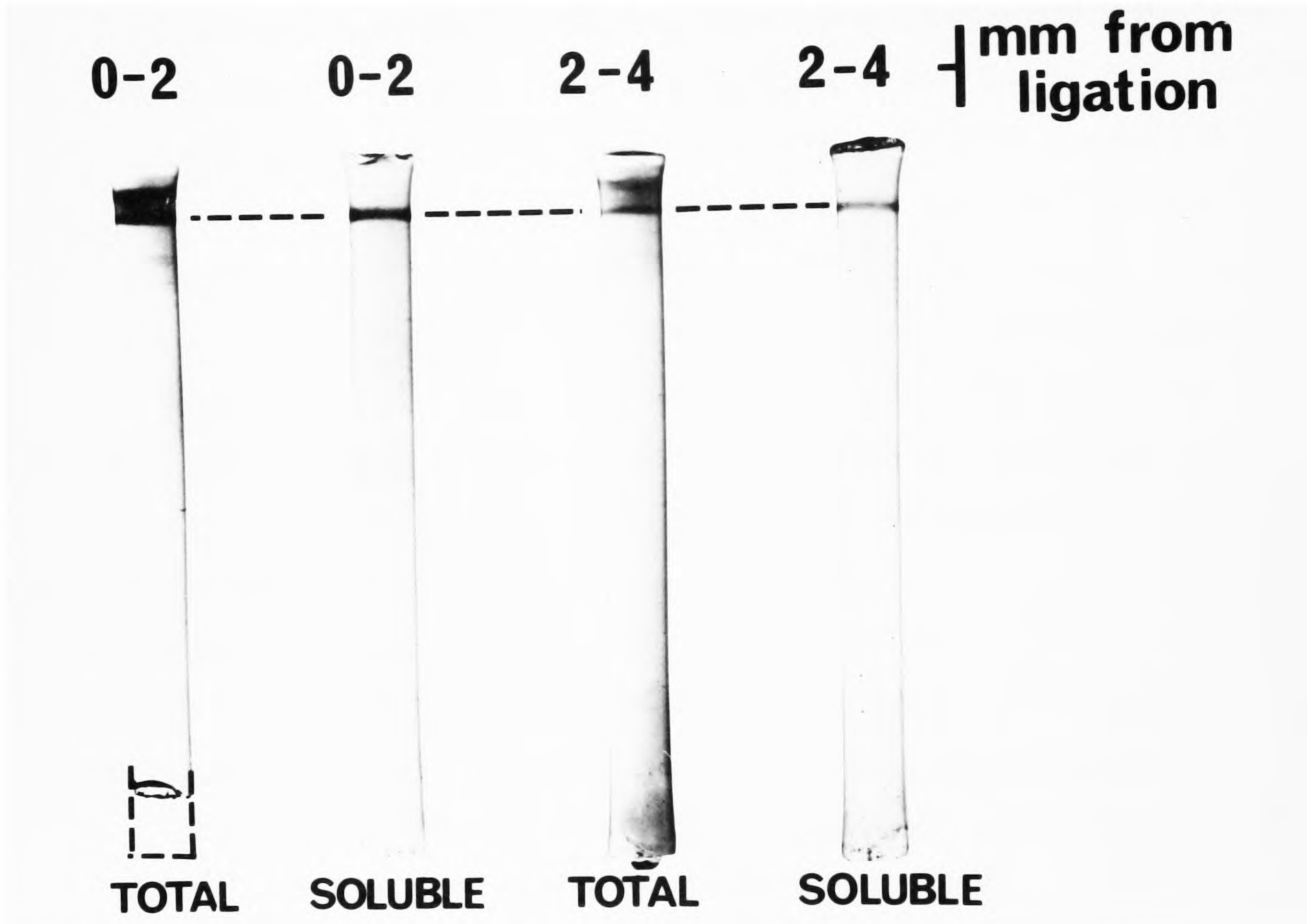


Fig. 30

The accumulation of AChE, total and soluble activity, on the central side of constrictions on splanchnic nerve axons. 0-2 mm represents the segment immediately proximal to the ligation and 2-4 is the next 2 mm segment away from the tie.

calculated, to be of the order of 1.5 mm/day.

Finally, the nature of the AChE isoenzymes in the proximal 2 mm segment was examined by electrophoresis. As shown in Fig. 30, there is an accumulation of both the membrane-bound ($AChE_6$) and soluble ($AChE_5$) isoenzymes in the first 2 mm as compared with the second (cf. Fig. 26).

DISCUSSION

These results show that soluble AChE accumulates fairly rapidly and, therefore, probably flows fairly rapidly down the axons of the splanchnic nerve of the calf. They also show that the increase in soluble activity is accompanied by a parallel increase in the membrane-bound enzyme (Fig. 27). Similar results were obtained when ligated rat sciatic nerves were used, but as has been said the results were less clear.

In some ways it might have been preferable to have used rats as the experimental animal, and this would have been done had it not been for the technical difficulties encountered in the preliminary experiments. These difficulties basically revolved around the fact that the AChE content of the sciatic nerve is very low. In addition, the nerve is heavily myelinated and this interfered with both the Triton X-100 extraction and the subsequent electrophoresis. This latter problem was not encountered when bovine nerves were used because, in contrast to the rat nerves, there is a high AChE content per cm of the axons. Thus, the detergent concentration in electrophoresis samples could be diluted (see Chapter 2) so that there was no interference with the running of the gels but so that there was still sufficient enzyme activity to develop. As a consequence of these problems the sciatic nerve was only used to qualitatively examine whether or not soluble AChE accumulated rapidly and hence to determine whether it would be worthwhile to use the calf nerves.

The main advantage of using bovine nerves, however, is that it is the same species from which most of the tissues used for this work were taken. Consequently, much more is known about the various isoenzymes, their characteristics, their distribution and some of their properties than for those from other species. But first, before we discuss the isoenzymes, we must discuss the flow rate.

There is a wide variation in the calculated rate of proximo-distal axonal transport of AChE in different nerves from different species. Thus, Frizell, Hasselgren and Sjöstrand (1970) estimated flow rates of 12.6 and 5.0 mm/day in the vagus and hypoglossal nerves of the rabbit, which are slow in comparison to the 99 mm/day in the sciatic nerve of the frog (Partlow et al., 1972), the 260 mm/day in the dog sciatic (Lubinska and Niemierko, 1971) and the 430 mm/day in cat sciatic nerve (Ranish and Ochs, 1972). Later the rates for the rabbit nerves were modified and estimated to be 430 mm/day in the vagus and 73 mm/day in the hypoglossal nerve (Fonnum, Frizell and Sjöstrand, 1973). These faster rates were calculated after it was estimated that in each of the nerves only a small proportion (5-20%) of the enzyme was free and able to move. A similar correction factor has been introduced into their calculations by all of the workers cited above and they have variously estimated the amount of mobile enzyme as comprising 5-25% of the total enzyme activity. It is, therefore, not valid to calculate flow rates for AChE by using the total amount of activity in the nerve as a basis, allowance should be made for the proportion of the enzyme which is not mobile.

In general, that proportion of the AChE which is mobile has been calculated by measuring the decrease in AChE activity midway between two widely spaced ligatures. The most clear-cut illustration of this method is that provided by Partlow et al. (1972), who showed that after sufficient time had elapsed for all of the mobile AChE to have arranged itself at the two ends of an isolated segment of sciatic nerve, there was a 25% decrease in the activity in the centre of the segment. An alternative method is that employed by Ranish and Ochs (1972), who, from the slopes of calculated regression lines, sample sizes and velocities, were able to calculate that 15% of the AChE in the cat sciatic nerve was free to move; 10% could flow in the proximo-distal direction and 5% in the reverse.

Calculations of this type are not possible with the limited data that we have for the calf nerves (technically it was possible to ligate the nerves so that the calculations could be made, but it proved impossible to obtain clean, blood-free samples of nerve from around the second ligation, principally because of its proximity to the adrenal and the consequent increase in the number of blood vessels and the increased branching of the fibres). Our figure of 10 mm/day must therefore be regarded as the minimum value for the flow rate of AChE in the splanchnic axons. If, by extrapolation from the results discussed above, only 5-25% of the AChE is free to move, then the flow rate would be between 197 and 33 mm/day. However, a note of caution should be introduced at this stage regarding the way in which estimates are made of the proportion of axoplasmic constituents which are mobile.

One of the most widely studied particles in adrenergic nerves is the synaptic or granular vesicle, a particle which is known to flow rapidly down these axons (see e.g. Banks and Mayor, 1972). It is also known that the enzyme dopamine β-hydroxylase (DBH) is a component of the vesicle, and detailed centrifugational analysis shows that as much as 80% of the activity is probably associated with the particle (De Potter et al., 1970). Wooten and Coyle (1973) have shown, however, that only 30% of the DBH is mobile within the sciatic axons of the rat. Obviously, this result implies either that only 30% of the DBH is associated with the vesicles, or that only about 30% of the vesicles are moving rapidly. A third alternative is to conclude that a way has yet to be found which will unequivocally estimate the proportion of an enzyme or other macromolecule or particle which is free to flow down, or up, axons. Because of this uncertainty, the rest of this discussion will use the actual figure for the flow rate of AChE as calculated from the experiments described in this Chapter, with the caveat that it is almost, but not entirely, certain to be an underestimate of the true rate.

The main point of these results is not to estimate with great accuracy the flow rate of AChE, but to show that the soluble AChE flows rapidly. As mentioned earlier, the estimated rate is some 6 times that of the soluble cytoplasmic enzyme LDH (see Chapter 2 for the subcellular distribution of LDH), but an addition to this observation is the one which shows that total AChE and therefore membrane-bound enzyme accumulates at the same rate as $AChE_5$. The proportion of the enzyme which is mobile therefore becomes irrelevant to this part of the discussion because, whatever the proportion may be, it is made up of both $AChE_6$ and $AChE_5$, i.e. membrane-bound and soluble enzyme. It can therefore be concluded from all these results that soluble AChE flows down the axons faster than the bulk of the axoplasm.

These results also allow some calculations to be made about the maximum turnover time for the enzymes in the adrenal medulla, just as has been done for the noradrenergic vesicles and DBH (De Potter and Chubb, 1971). For example, given that 4.1 mU of AChE flow down the axons per hour, there is a net flow of at least 100 mU of total AChE activity into the adrenal per day. The 1200 mU in the medulla ought therefore to be completely replaced in 12 days if all of the enzyme in the medulla is derived from the nerve and if, of course, the protein could be regarded as a single isoenzymic species. Interestingly, however, even after 2 days, the accumulated axonal enzymes do not show a significantly increased concentration or proportion of the four isoenzymes $AChE_1$ to $AChE_4$. If we conclude tentatively that these enzymes are not derived from the nerve, but from the adrenal medullary cells themselves, then the proportion of the medullary enzyme which could be neuronal can be reduced to 930 mU. This is calculated, considering Fig.21, by assuming that 75% of the soluble activity can be accounted for by the four isoenzymes $AChE_1$ to $AChE_4$; thus, all of the membrane-bound (840 mU) and the 25% of the soluble activity which is due to the presence of $AChE_5$ could be attributable to the nerve fibres. The turnover time for this AChE is thus about 9 days. Considering only $AChE_5$, of which 1.3 mU flows down the axons per hour, then the turnover time of this single isoenzyme could be as short as about 70 h. This latter figure is quite similar to the turnover time of DBH in the spleen (Chubb and De Potter, 1975), a protein which turns over rapidly, partly because it is released from nerve terminals at the same time as the transmitter (Smith, De Potter, Moerman and De Schaepdryver, 1970).

The results presented in this Chapter offer our working hypothesis considerable support in one respect: soluble AChE does accumulate fairly rapidly in constricted axons but, more importantly than the actual rate, it accumulates at the same rate as the membrane-bound enzyme. Our suggestion that the soluble AChE is in a membrane-limited but fragile structure is still reasonable. On the other hand, we must also suggest that the structure contains membrane-bound enzyme in addition to the soluble. It is most unlikely that the $AChE_6$ which flows rapidly is the enzyme which is bound to the axolemma, because this would necessitate an extraordinarily rapid growth of the nerve trunk. Therefore, the hypothesis can be modified further to include the proposition that the SER almost certainly contains both $AChE_5$ and $AChE_6$. Whether this is the unique localization of both enzymes is not known, but it does not seem likely - the axolemmal membrane does contain AChE activity and, given the presence of only one membrane-bound isoenzyme, it must also be $AChE_6$. The one final modification of the hypothesis which can be introduced at this stage is that not all of the medullary isoenzymes are derived from the nerve. This conclusion is based on the observation that none of the isoenzymes $AChE_1$ to $AChE_4$ were increased proximal to the ligation, even after 2 days of accumulation, so it seems highly unlikely that the tie was blocking their transport. Whether these isoenzymes are present in the chromaffin cells or satellite cells will be discussed in more detail in Chapter 5.

The working hypothesis as it is in its newly modified form is as follows: some of the axonal AChE is present within the

SER of the axons. This is the soluble ($AChE_5$) isoenzyme which differs in electrical charge from $AChE_6$, whose localization is both the SER and axolemmal membranes. It is the tubules of SER which flow down the nerve, giving rise to the relatively rapid flow of both $AChE_5$ and $AChE_6$. Finally, it is no longer thought that all of the AChE in the adrenal medulla is derived from the nerve fibres, although the origin and localization of the extra isoenzymes in the adrenal medulla is not known.

It is noteworthy that $AChE_5$ seems to have a rapid turnover in the adrenal medulla - almost as rapid as the turnover of DBH in the spleen (Chubb and De Potter, 1975). As was mentioned earlier, the rapid flow of DBH and the consequent high amounts of enzyme activity which enter the spleen per unit time are thought to be a reflection of the fact that DBH is rapidly removed from the terminals of the splenic nerve - by release of the protein by exocytosis together with the transmitter. The possibility that removal of $AChE_5$ from the splanchnic nerve terminals in the adrenal medulla occurs by a release of this isoenzyme is the subject of the next Chapter.

CHAPTER 4

STUDIES ON THE RELEASE OF ACETYLCHOLINESTERASE FROM ISOLATED, PERFUSED BOVINE ADRENAL GLANDS.

INTRODUCTION

The results presented in the previous two Chapters offer reasonable support for the idea that soluble AChE ($AChE_5$) in the splanchnic axons is an entity in its own right and not an artifact caused by the preparation of the tissue for centrifugation experiments. These observations led us to consider the role, and fate, of $AChE_5$ in the splanchnic nerve.

Chapter 3 shows that there is a relatively rapid accumulation of both $AChE_5$ and $AChE_6$ on the central side of a ligation placed on the nerve trunk, and it is this observation which raises the question of the ultimate fate of these isoenzymes when they, as they presumably must, reach the terminals of these axons embedded in the adrenal medullary tissue. Since there has never been a suggestion that the amount of AChE in any innervated organ increases throughout life, the enzyme must be removed from the terminals at about the same rate as it arrives. An attempt to calculate this rate was made in the previous Chapter and the result suggested that $AChE_5$ in the adrenal medulla could turn over in about 70 h, whereas that of $AChE_6$ is slower - to the order of 9 days. The figure of 70 h was arrived at by dividing the estimated amount of $AChE_5$ in the medulla by the amount which arrives, via the axons, per hour. A calculation such as this, but for DBH, resulted in a similar figure being suggested for the turnover of this enzyme (Chubb and De Potter, 1975).

There are two ways in which a cell might be expected to dispose of excess or unwanted material. First, it could be digested intracellularly - a method which would be difficult to examine experimentally. The second method is by an expulsion of the material from the cell, and there are three reasons why we have chosen to see whether AChE was removed from a cell by some form of secretion. The first reason is that methods are available by which cholinergically innervated tissues can be perfused and stimulated, thus enabling a relatively easy testing of the hypothesis. The second reason is that extracellular fluids of many species contain AChE (see Augustinsson, 1963) and it would be expected that, if the above supposition is correct, the level of AChE in these fluids would be brought about, and maintained, by AChE-secreting organs and/or nerves. The third reason is the one which has been mentioned earlier; there is a similarity in the suggested turnover times of DBH in the spleen and $AChE_5$ in the adrenal medulla - an observation which could, of course, be coincidental, but both are neuronal proteins and it is known that DBH can be released from stimulated nerves (Smith et al., 1970).

Thus, there are reasons for supposing that AChE might be secreted from tissues in which it is present. We examined this problem by using the isolated, perfused bovine adrenal gland as the cholinergically innervated tissue and the results are presented in this Chapter.

METHODS

Bovine adrenal glands were obtained from a local slaughterhouse and were placed in ice within 15 min of the death of the animal. The glands were perfused, in the retrograde manner with oxygenated Tyrode's solution as described by Schneider, Smith and Winkler (1967). The flow rates were variable, but were kept wherever possible to between 8 and 12 ml/min. The temperature of the perfusing fluid as it entered the gland was kept at 37°. The glands were perfused for 45-60 min prior to administration of the secretagogues, which were injected into the perfusion fluid immediately before it entered the gland. When this method was used, they were injected every 30 sec during a period of 120 sec. The volume of KCl (0.5M) which was used was related to the flow rate in such a way that it was diluted 10-fold by the perfusing medium. The concentration of KCl passing through the gland was therefore about 50 mM. In some experiments an equal amount of NaCl in the Tyrode solution was replaced with KCl so that the K^+ concentration of the medium was 50 mM and so that the tonicity of the medium was maintained. To induce secretion the two solutions were interchanged. In still other experiments dimethyl phenylpiperazinium iodide (DMPP; 5 x 0.5 mg) and carbachol (3.5 mg in 5 x 0.7 mg injections) were used to try and provoke the secretion of AChE.

AChE activity, protein determinations and electrophoresis with subsequent staining for both AChE and protein were all done as described in earlier Chapters. Catecholamines were estimated colorimetrically according to the method of von Euler and Hamberg (1949) and at pH 6.0 (McIlvaine, 1921) in order to estimate the total, i.e. adrenaline and noradrenaline, content of the perfusate. When calculations were made to exclude the chromogranins from estimates of the specific activity, use was made of the ratio 1 μmole catecholamine $\equiv$ 0.208 mg of the chromogranins (Schneider et al., 1967).

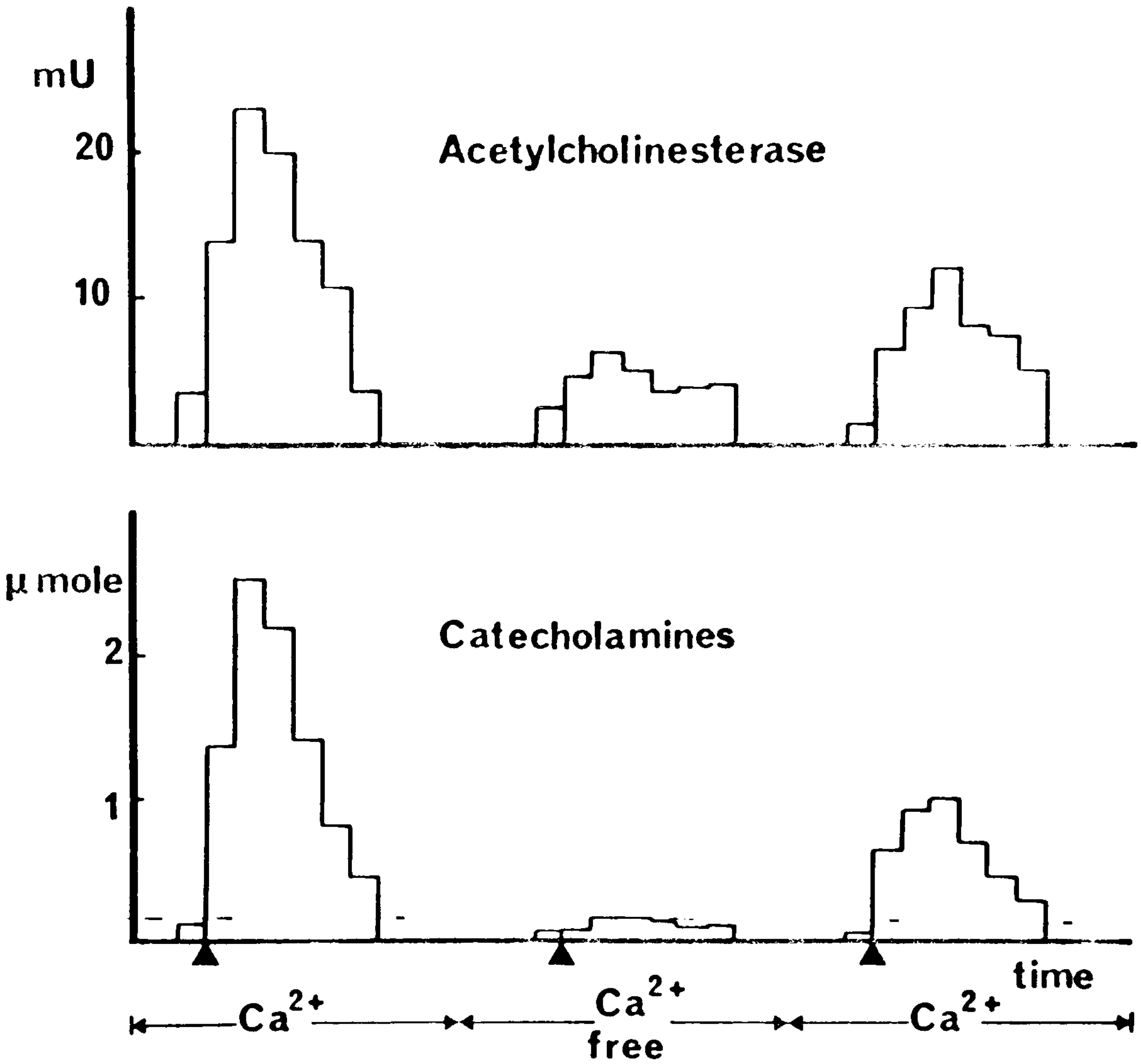


Fig. 31

The release of catecholamines and AChE from the perfused adrenal gland. At the arrow (▲) the K^+ concentration was increased to 50 mM. The enclosed rectangle immediately before the administration of excess K^+ represents the amount of each constituent in pre-stimulation control periods.

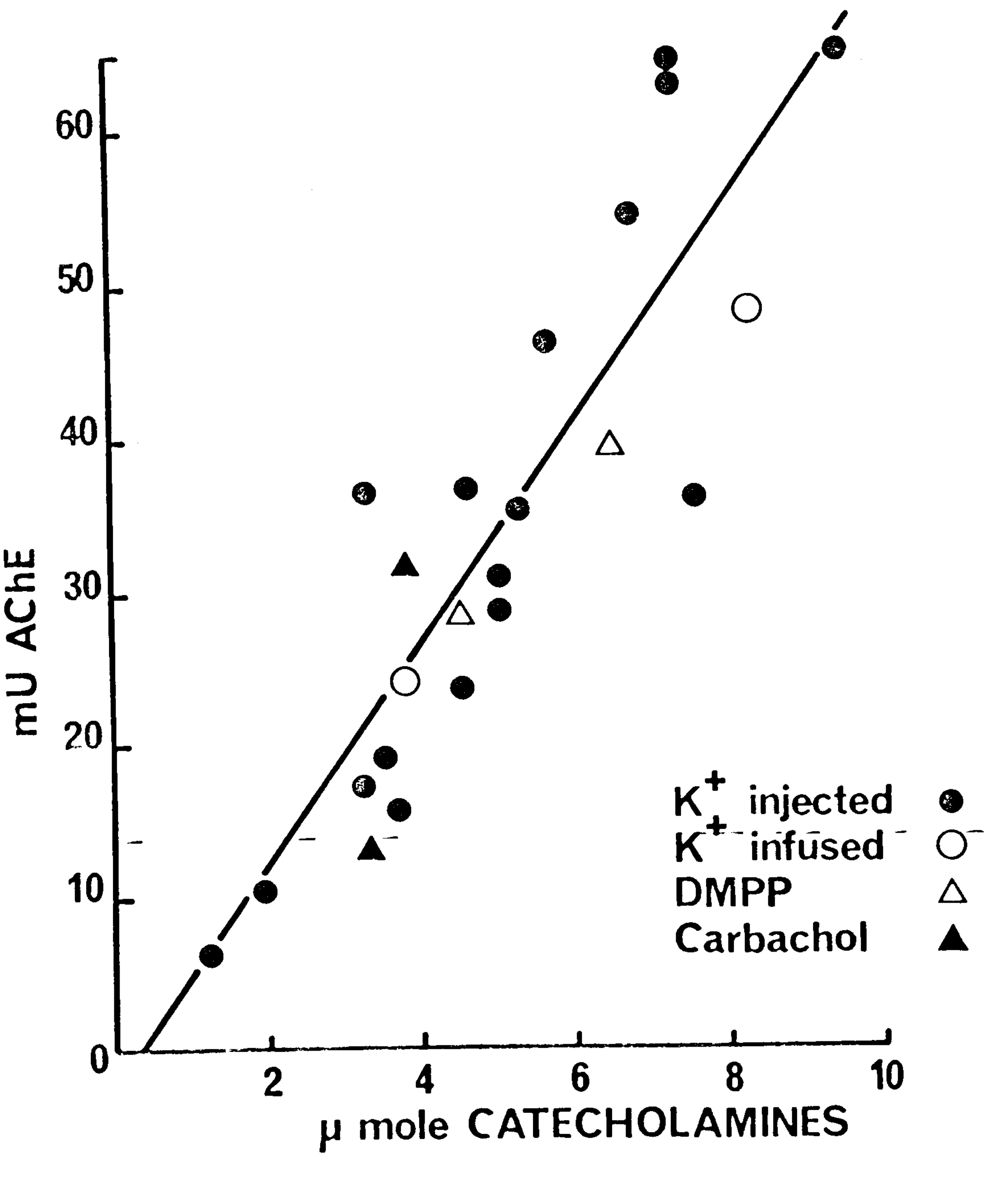


Fig. 32

The correlation between the catecholamines and the AChE released into the perfusate by different secretagogues.

RESULTS

Injection of KCl into the perfusion fluid resulted in an increase in the AChE, and catecholamine, content of the adrenal perfusate (Fig.31). The appearance of both was immediate and, as also shown in Fig.31, the increase was dependent upon the presence of calcium ions. The third set of histograms shows that replacement of the Ca^{2+} and another injection of KCl again resulted in an increased perfusate level of both the enzyme and the amines. This dependence on Ca^{2+} is characteristic of many forms of protein secretion from different tissues so, for simplicity, the terms "released" or "secreted" will be used in relation to AChE, but the arguments for why use of those terms is justified will not be presented until later.

The apparent parallelism in the appearance of both AChE and catecholamines in the perfusate, as illustrated in Fig.31, led to a more detailed analysis of the perfusate concentrations of each to determine whether there was a correlation between the amounts of the two compounds which were released from the gland. As shown in Fig.32, there is a surprisingly good correlation between the total AChE activity and the total catecholamines secreted during the stimulation and collection period. The line drawn on this Figure is described by the equation $y = 7.25\,x - 2.26$ and it has a correlation coefficient of 0.885, which is statistically significant at the $P<0.001$ level. From these data it can also be calculated that the mean catecholamine output is 5 μmole while, on average, 34.0 mU of AChE are secreted. As is also shown in Fig.32, the same catecholamine:AChE ratio was obtained whichever way the secretion was induced.

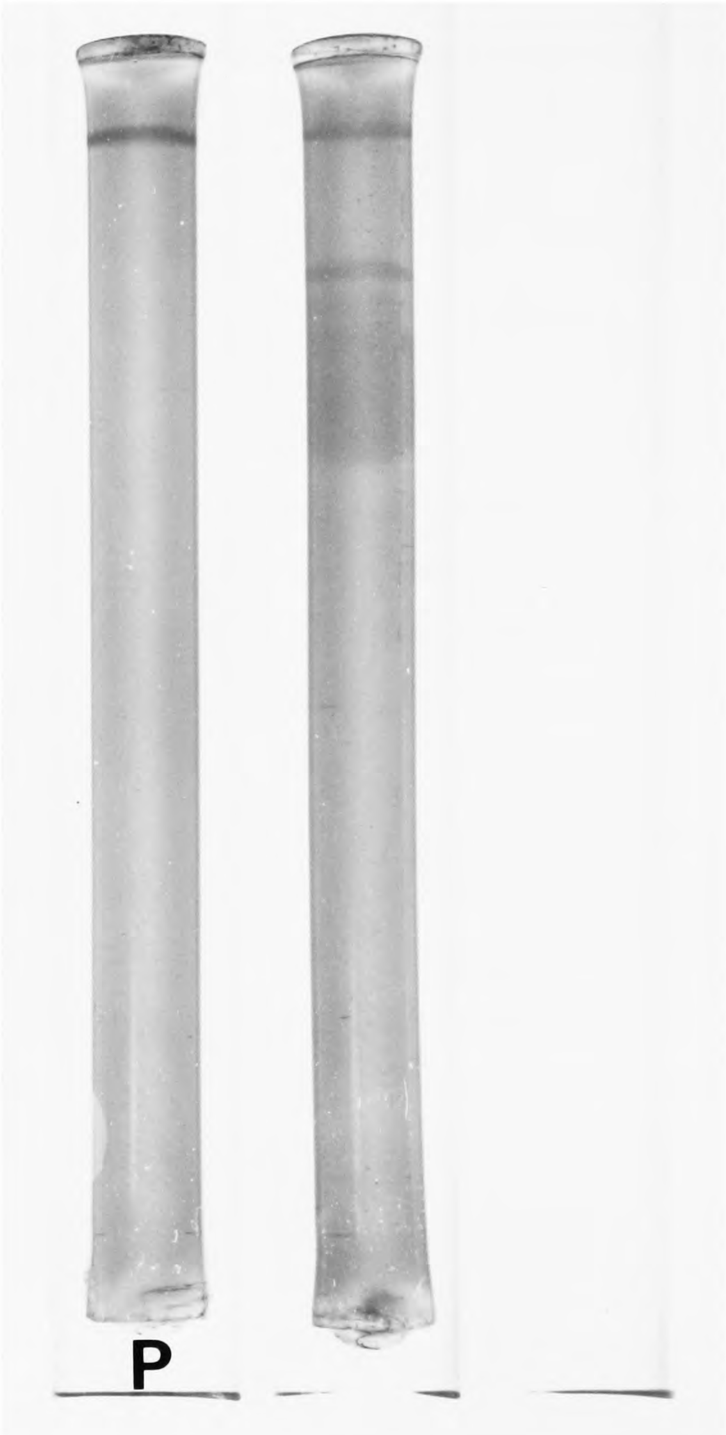


Fig. 33

The isoenzyme released into the perfusate (P) compared with the same amount of soluble AChE activity from the perfused adrenal gland.

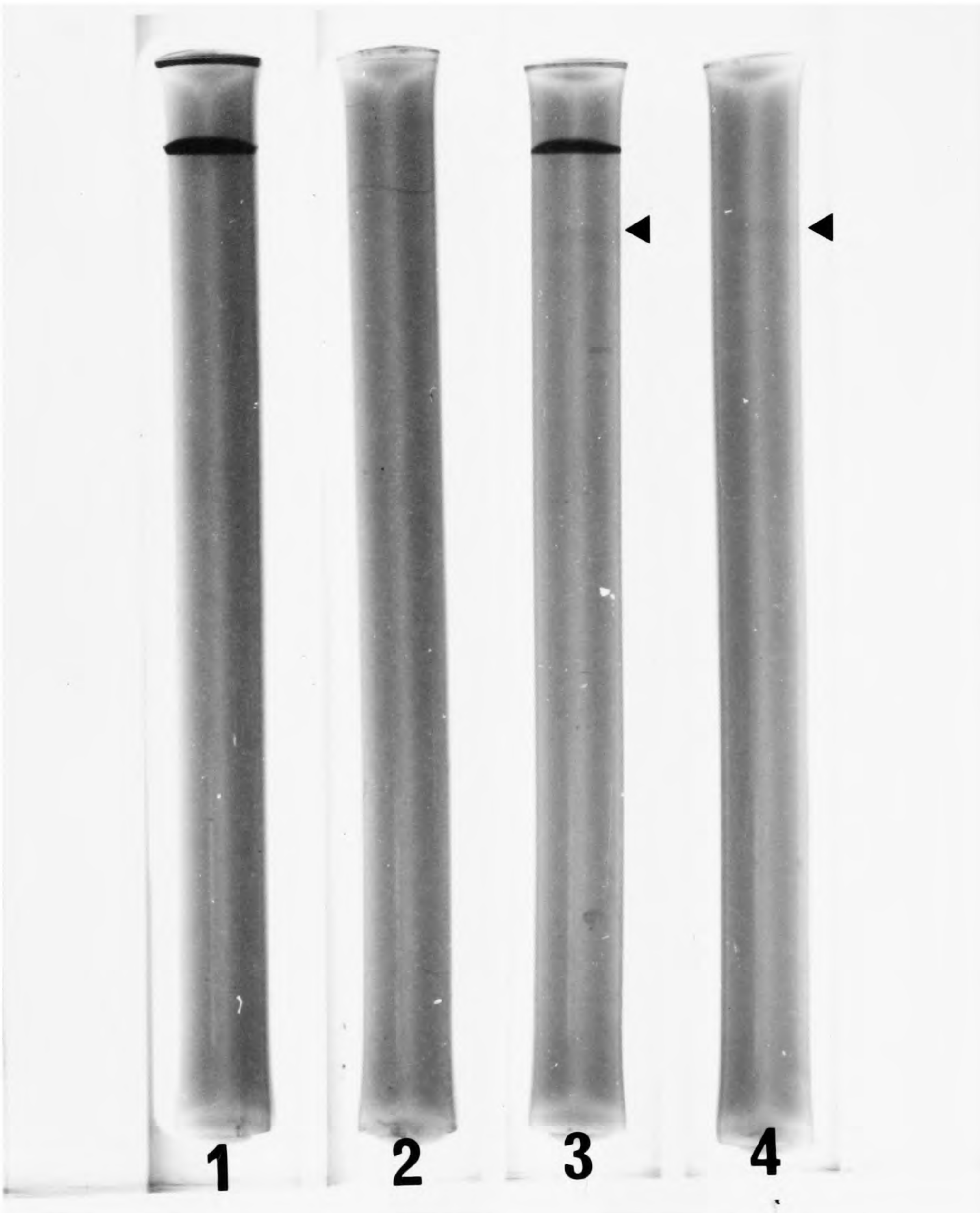


Fig. 34

The released enzyme (3) compared with the soluble AChE in the splanchnic axons which innervated the adrenal gland used for the perfusion experiment (1). The same amount of AChE activity was run on each gel. Note the very low amounts of faster migrating components (◀) visible in both gel 3 and gel 4. Gels 2 and 4 were incubated with 10^{-4}M BW284C51

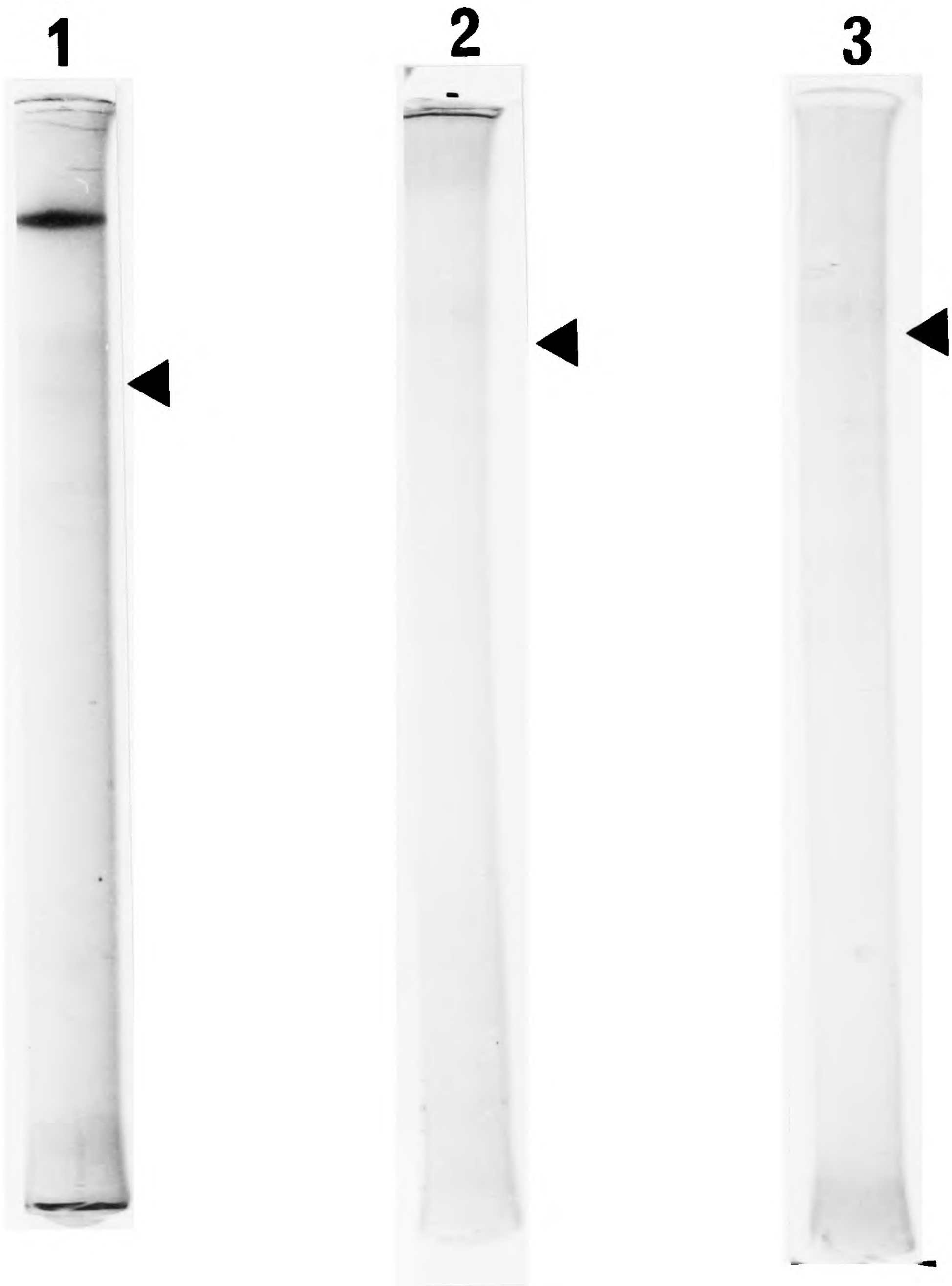


Fig. 35

The released AChE (1), with 10^{-4}M BW 284C51 (2) and with butyrylthiocholine (3). Note the very low amounts of the faster moving components in all gels (◀).

Thus, DMPP, carbachol and injected or infused KCl were all equally effective in their ability to release the amines and the enzyme.

The results of an electrophoretic analysis of the perfusate AChE are shown in Fig.33. These results clearly show that only one isoenzyme of AChE is present in the perfusate because the same amount of AChE activity was run on each gel and they were incubated for the same period. The activity on both gels could be completely inhibited by BW 284C51. Because of the apparent similarity in the electrophoretic mobility of the released isoenzyme with that of $AChE_5$, it was tentatively concluded that it is this isoenzyme which appears in the perfusate. The results in Fig.33 show, however, that the electrophoretic pattern obtained from the perfusate is more like the pattern given by the splanchnic soluble AChE than the one found in the adrenal medulla. As shown in Fig.34 there is, indeed, a very marked similarity between the two sources of enzyme. To obtain this result, a different experiment from the one in which the results shown in Fig.33 were obtained, the same amount of axonal and perfusate AChE was run on the respective gels and, as before, they were incubated for the same time. The axons used for the electrophoresis were those which innervated the adrenal gland from which the perfusate enzyme was obtained. It can also be seen in Fig.34 that the stimulated perfusate contains very low amounts of faster moving components which are also present even when the gel was incubated with BW 284C51. This latter observation infers very strongly that these faster migrating components cannot be due to other isoenzymes of AChE, so the possibility that they are due to ChE activity must be considered. However, as shown in Fig.35, incubation of a gel containing perfusate

Fig. 37

A comparison of bovine plasma AChE (2) with the soluble isoenzyme in bovine splanchnic axons (1).

with butyrylthiocholine (10 mM) does not increase the staining of the faster component as would be expected if it was due to ChE activity. Even so, the band is faintly visible in all of the three gels shown on this Figure.

The evidence presented so far suggests that $AChE_5$ is secreted probably, if our working hypothesis is correct, from the nerve (terminals?) into perfusates of the adrenal gland. Further evidence for the identity of the released isoenzyme is shown in Fig.36 from which, if this Figure is compared with Fig.18, it can be seen that axonal $AChE_5$ and the isoenzyme in the perfusate have identical electrophoretic mobilities over a wide range of gel concentrations. This leads to an identical extrapolated intercept at 0% gel and adds more convincing evidence that they are one and the same isoenzyme.

However, sources other than nerves or medullae must be considered as the origin of the "released" isoenzyme. The most likely alternative to the cells as the source of the enzyme is the blood or, since the perfusate activity is entirely soluble, the plasma. Consequently, the plasma of calves was analyzed for AChE activity and it was found to contain a significant but variable amount of the enzyme; the average for slaughterhouse animals being about 100 mU/ml (see Chapter 6). Electrophoretic analysis of the plasma enzyme shows that the principal esterase component is $AChE_5$ (Fig.37), a finding which makes it essential to discount even dilute plasma as the means by which the AChE level of the perfusate is changed. The first experiments specifically designed to show this were those in which protein levels, as well as catecholamines and AChE, were measured in the perfusate, both before and during administration of KCl.

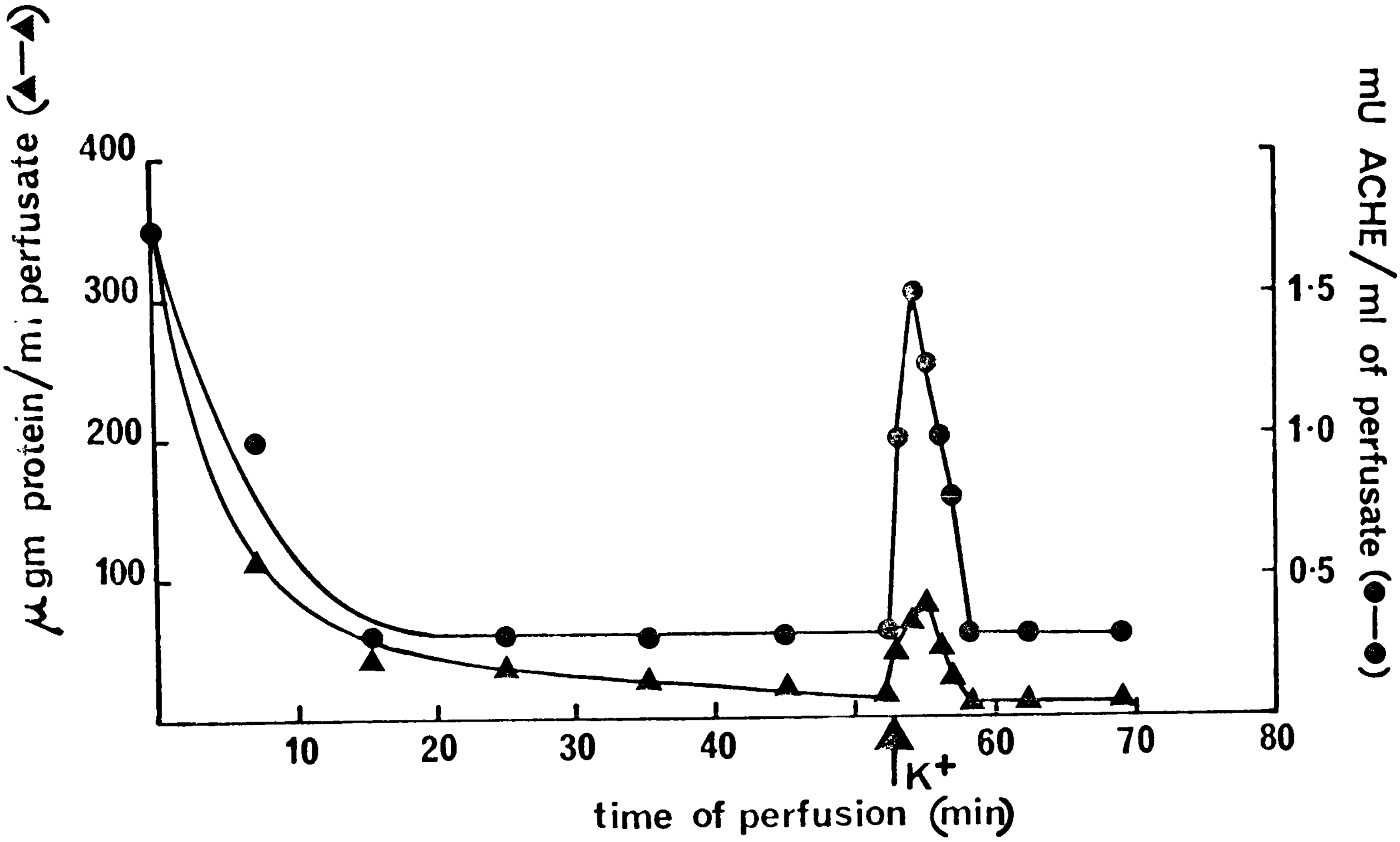


Fig. 38

The washout of protein and AChE activity from the perfused adrenal gland.

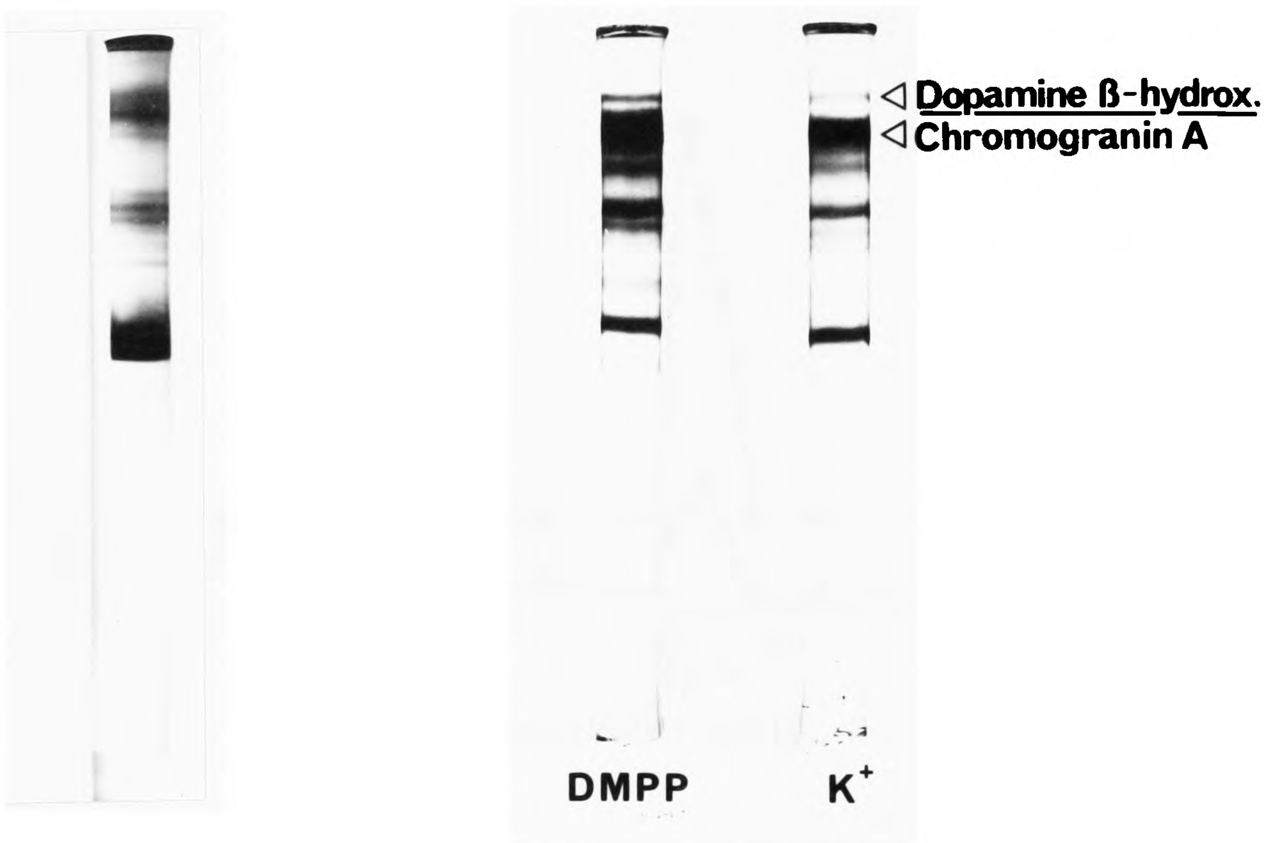


Fig. 39

A comparison of bovine plasma proteins with those released into the perfusate by both DMPP and high K^+.

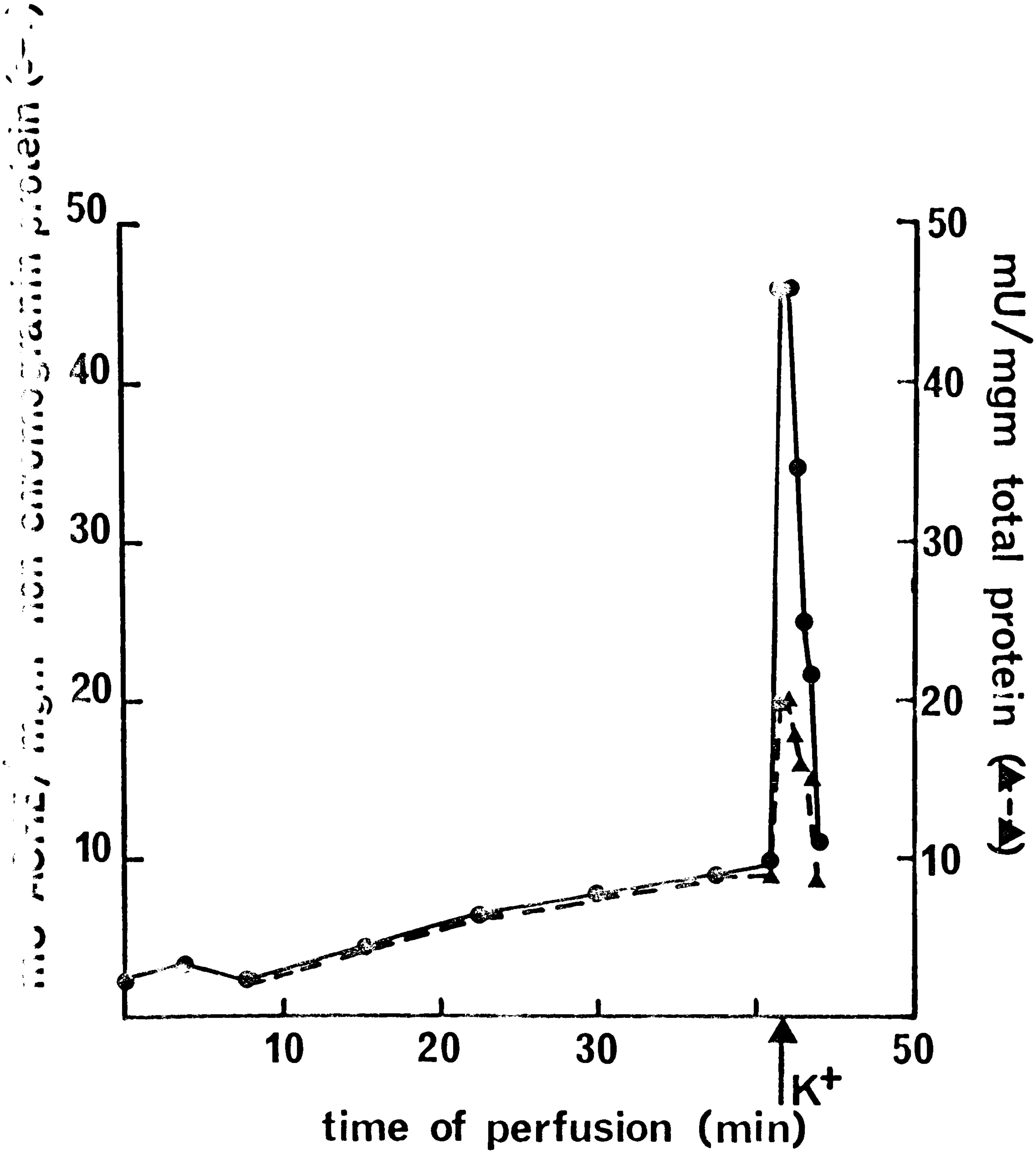


Fig. 40

The specific activity of the AChE released into adrenal perfusates by administration of high K^+.

As shown in Fig.38, there is a fairly rapid washout of total protein so that by about 20 min after the commencement of perfusion, the protein concentration of the perfusate is low and, with time, only slowly decreases further. AChE follows a similar washout curve, although there does seem to be a more constant resting level of enzyme activity (Fig.38). When KCl is injected into the gland there is, as would be expected from the previous results, an increase in the AChE content of the perfusate. Since there is also an obvious increase in the total amount of protein in the perfusate, also induced by KCl (a total of about 2.5 mg is secreted during the stimulation period), an electrophoretic analysis of the proteins in the perfusate was carried out; this yielded the results shown in Fig.39. The protein pattern is typical of the chromogranins (see Bartlett and Smith, 1974) and not, as shown, of plasma. This result was obtained whether DMPP or K^+ was used to evoke the release (Fig.39). Because of the appearance of the chromogranins (see later) in the perfusate after stimulation, estimates of the specific activity of the AChE liberated during this period would be falsely low. Fig.40 shows that,even without allowing for this fact, the specific activity of the released AChE is still 8-fold greater than the specific activity of the first sample - the sample in which the protein and the AChE can be assumed to come from the plasma. The second curve in Fig.40 shows the results obtained when an allowance is made for the presence of the chromogranins, a group of proteins which previous results have shown to be released by KCl from the adrenal medulla (Schneider et al., 1967). The specific activity of the AChE at its peak is almost 20-fold higher than that of the first sample. One final study was made in which the K_m of the released AChE was measured and compared with the K_m of the soluble AChE from splanchnic axons, all of the isoenzymes from

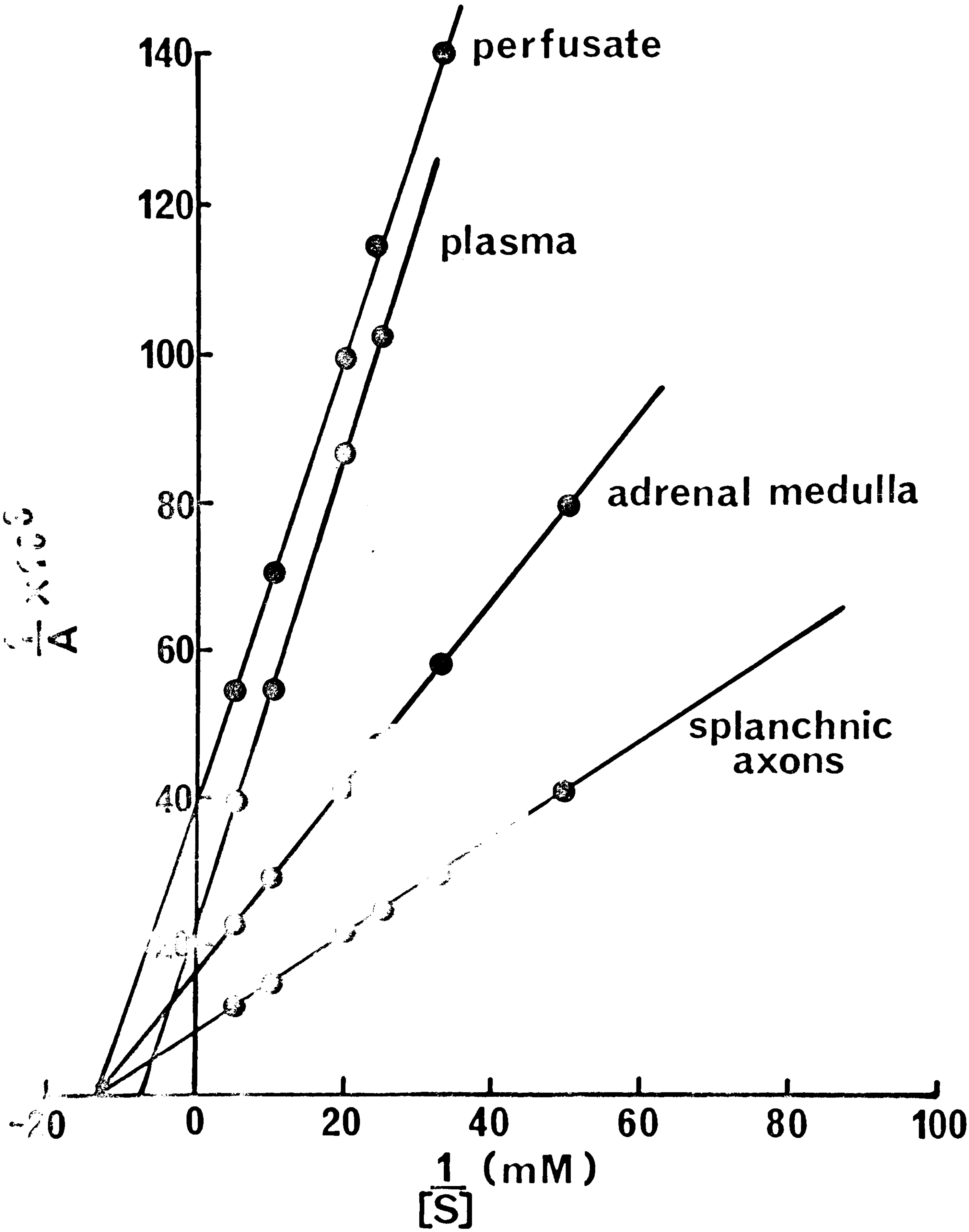


Fig. 41

Estimation of the apparent K_m of bovine AChE obtained from different sources. The value for the AChE released into the perfusate can be compared with the K_m of the soluble isoenzymes obtained from the adrenal medullae, splanchnic axons and plasma.

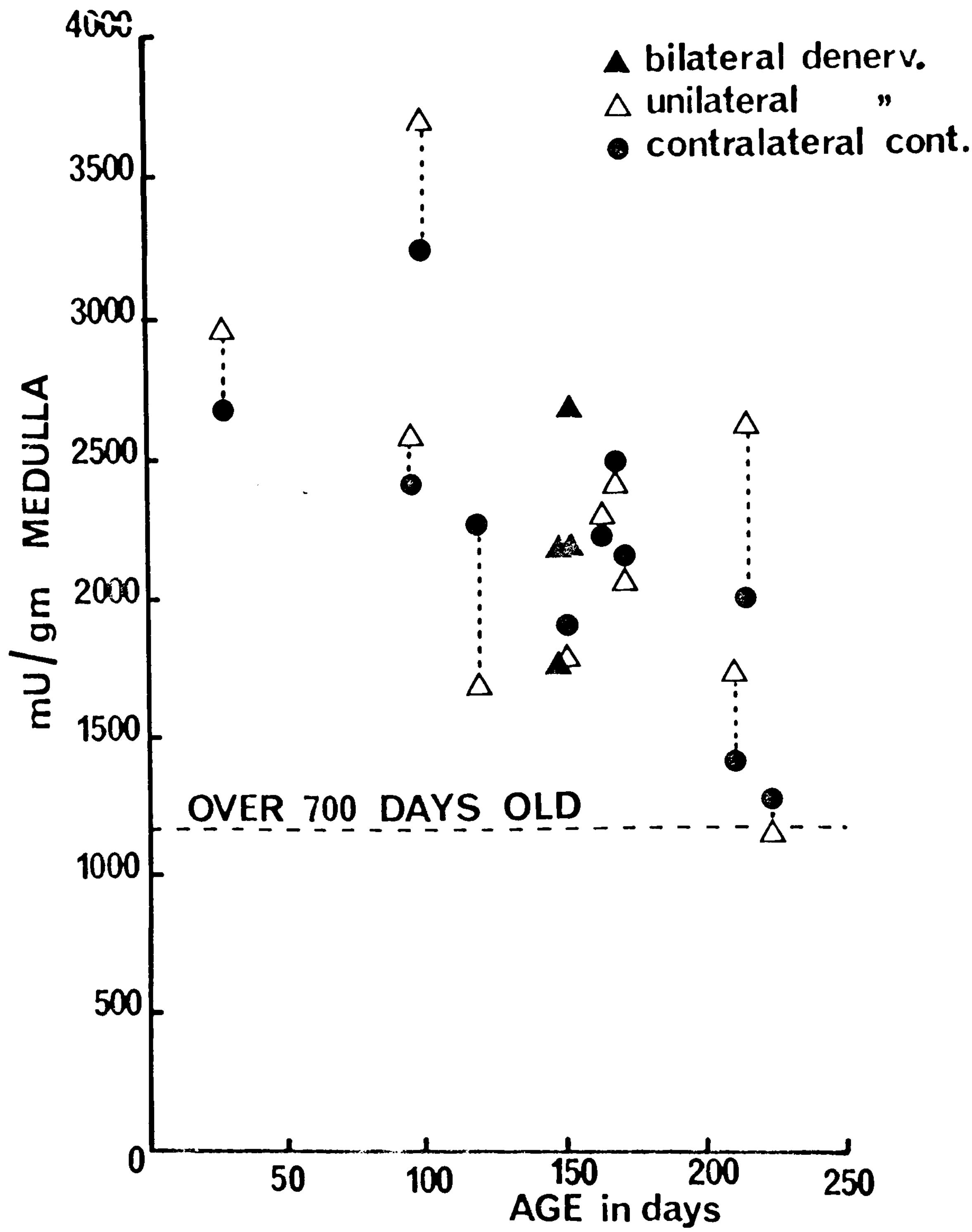


Fig. 42

The AChE content of denervated and contralateral control adrenal medullae. The level "over 700 days old" is the concentration in adrenal glands obtained from slaughterhouse animals.

the adrenal medulla (i.e. a total soluble fraction) and with that measured for bovine plasma. The results,which are given in Fig.41, show clearly that, while the soluble AChE isoenzymes originating from the axons and the medulla have K_m values which are identical with that measured for the AChE in the perfusate, the plasma enzyme is different. The lines are drawn according to the equations: axons, $y = 0.62\ x + 8.2$; adrenal medulla, $y = 1.26\ x + 16.7$; perfusate, $y = 3.07\ x + 40.7$ and plasma, $3.1\ x + 24$. All have correlation coefficients greater than 0.999 which are statistically signifcant at the $P<0.001$ level.

If it is accepted that the evidence outlined above strongly suggests that the AChE in the perfusate is not derived from the plasma, then we must design experiments to try and show that the enzyme is derived from either the nerves or the chromaffin cells (it must be one of the two because the adrenal cortex is known not to contain AChE; Antopol and Glick, 1941). We have tried, with Drs. Comline and Silver, to denervate bovine adrenal glands. Surgically, there was no doubt that the nerves were cut, but as shown in Fig.42, attempts to show a decrease in the AChE content of the medullae at denervation times ranging from 16.5 h to 6 months were, with only one exception, singularly unsuccessful. All of those animals in which the two glands contained similar AChE activities were denervated for less than 4 days before analysis - those from which the glands differed were all denervated for between 21 and 180 days. As also shown in Fig.42, the levels of AChE in the glands of all the experimental animals were higher than in those glands which were obtained from the slaughterhouse animals. The only known difference between the two sources of glands was, as shown, that the slaughterhouse animals were all considerably older than the oldest of our

Fig. 43

The soluble AChE isoenzymes in denervated (gel 1) and contra-lateral control (gel 2) bovine adrenal medullae. Gels 3 and 4 are as for 1 and 2 but with 10^{-4}M BW 284C51 in the incubation medium.

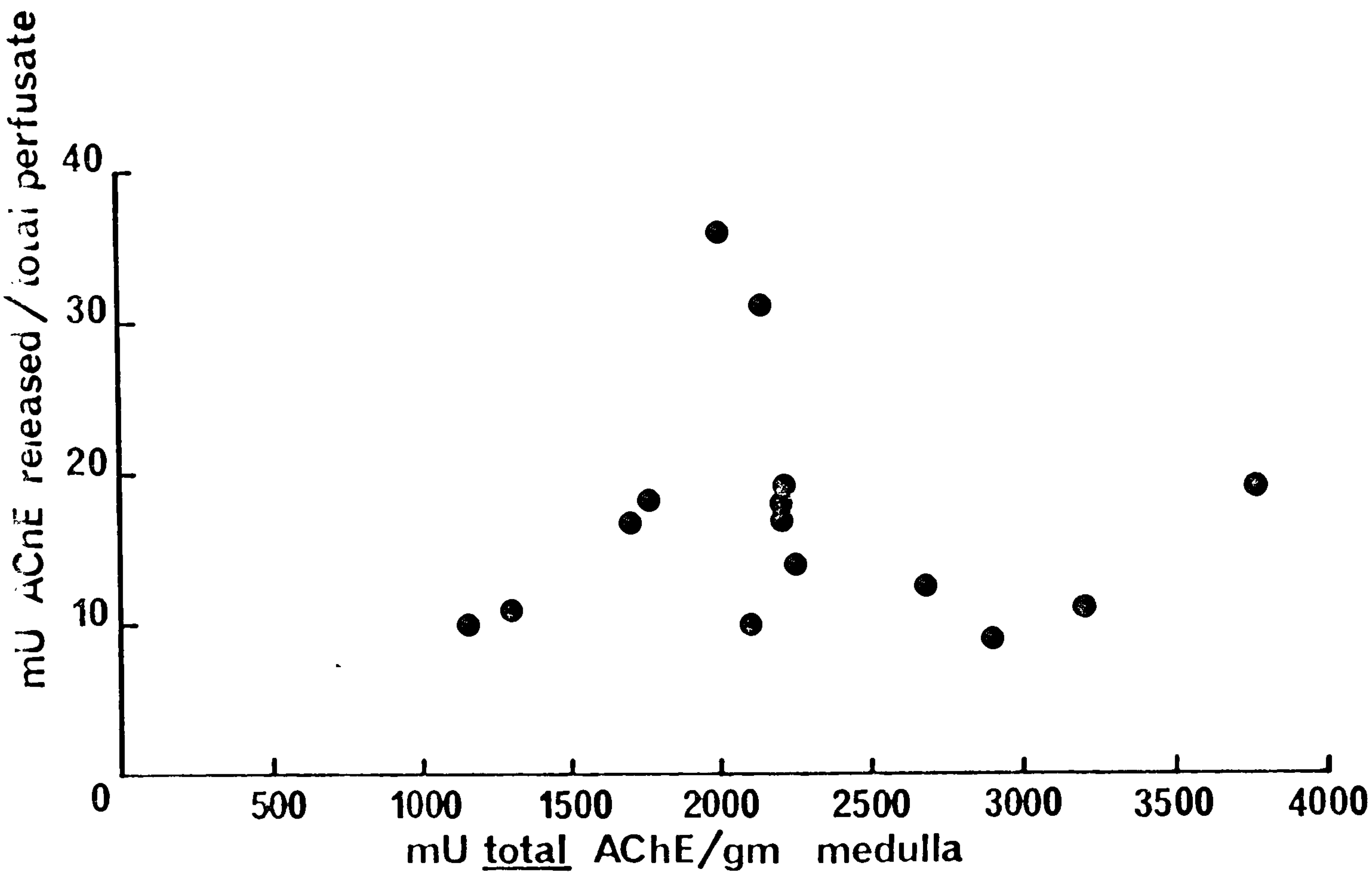


Fig. 44

A comparison of the amount of released AChE activity with the amount of enzyme per gram of adrenal medulla.

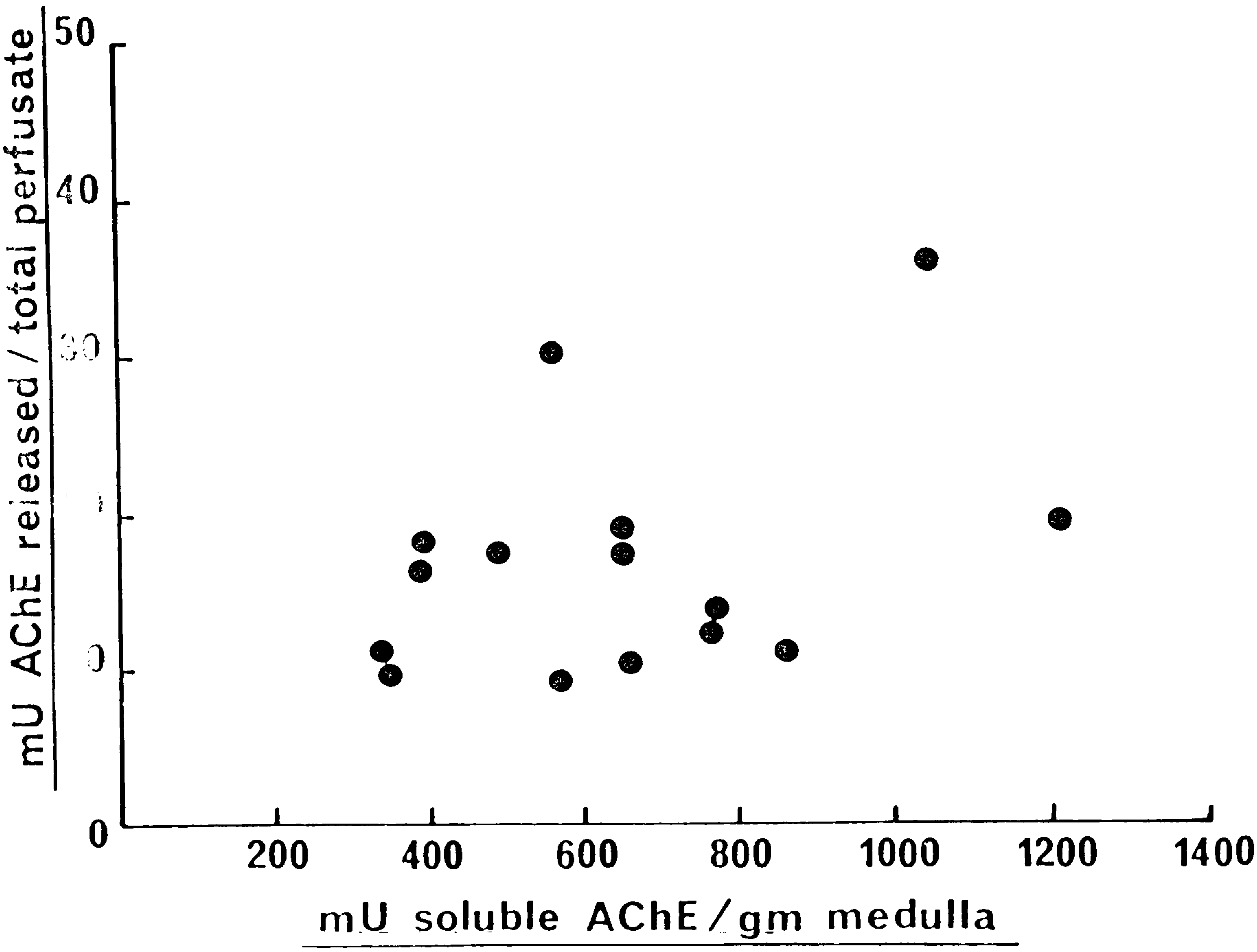


Fig. 45

A comparison of the amount of released AChE activity with the amount of soluble enzyme per gram of medulla.

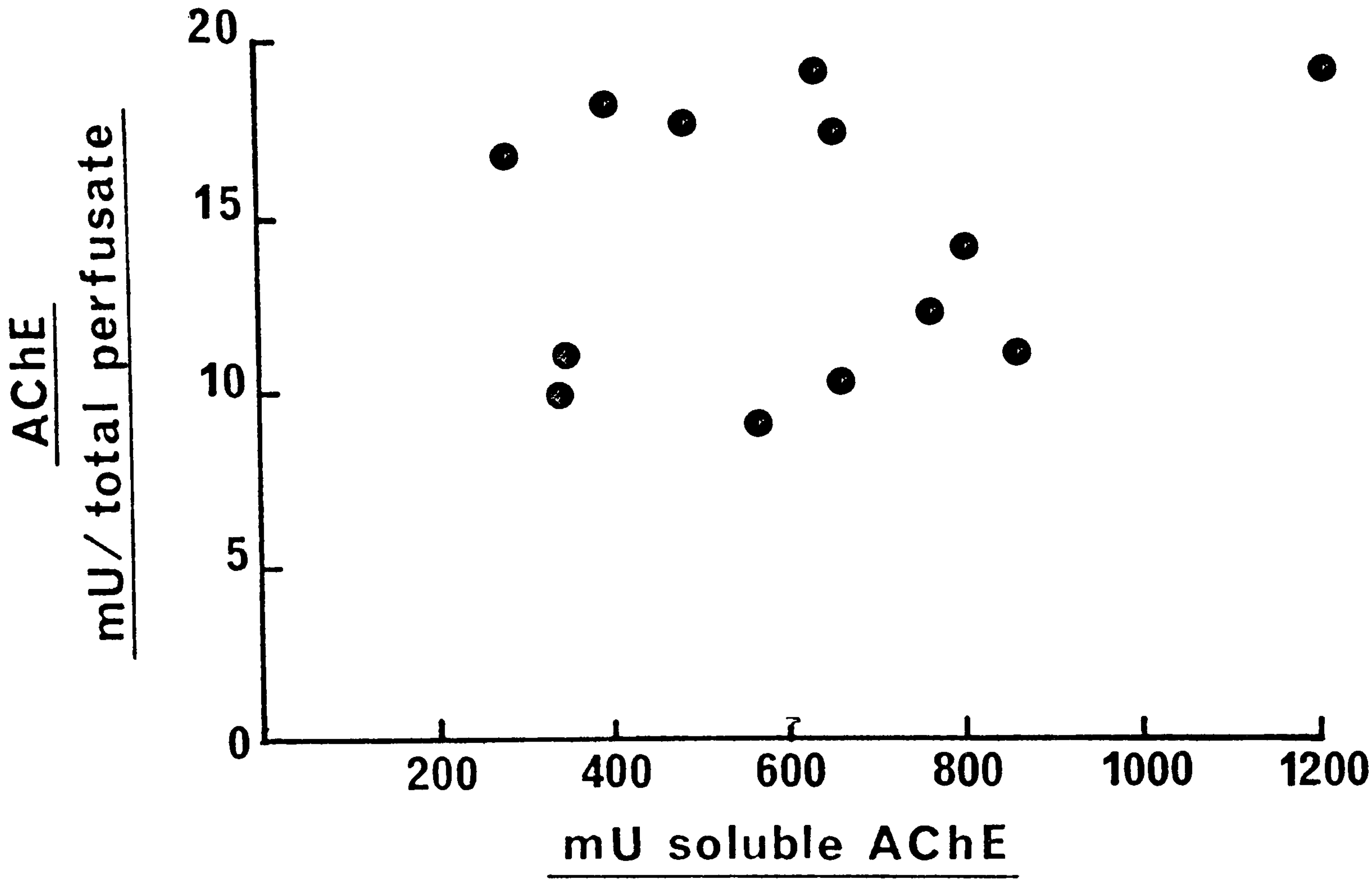


Fig. 46

A comparison of the amount of AChE released into the perfusate with the amount of soluble AChE within the same adrenal gland.

experimental animals. Perfusion of these "younger" glands, both those which had had their nerves cut and their contralateral controls, was found to release AChE and catecholamines in the same ratio to each other as the ratio found for the glands whose results are shown in Fig.32. An examination of the AChE in the glands by electrophoresis shows that there are no qualitative differences in the pattern of isoenzymes when a "denervated" gland is compared with its contralateral control (Fig.43).

Is there, therefore, a correlation between the amount of AChE released and the amount of enzyme in the medulla? Fig.44 shows that there is no correlation if the amount of AChE released is compared to the amount of enzyme per g wet weight of medulla. Nor, as shown in Fig.45, is there a correlation between the released enzyme and the amount of soluble AChE per g medulla. Finally, there is no correlation between the released and the absolute amount of the soluble medullary enzymes (Fig.46). There are too few data to compare the levels of released enzyme with the axonal enzyme levels but what data there are suggest a similar laçk of correlation.

DISCUSSION

The first question which must be answered is, how valid is the use of terms such as "released" or "secreted" when used in the context of, and applied to, the perfusate AChE? Generally, problems such as this can be approached from two angles, the first of which is to argue for the secretion concept and the second is to find an alternative to the initial hypothesis. In this particular case, the demonstration of the lack of a viable alternative to the proposition of secretion is probably the easier of the two.

Since there is no AChE in bovine adrenal cortices (Antopol and Glick, 1941) and since there is no reason to suppose that AChE is present in any cell other than the nerve or chromaffin cells (but even the latter localization is still considered doubtful, especially for $AChE_5$), the most likely alternative source of the perfusate AChE is the plasma. But there are fairly strong arguments against even this possibility. The first of these is the clear difference between protein patterns in the perfusate and the plasma as exposed by electrophoresis. Here, though, it could be argued that an excess of the chromogranins would mask the presence of a low concentration of plasma proteins in the perfusate. The argument against this proposal is one of specific activities. Apart from the 20-fold differences in specific activity shown in Fig.40, there is one additional piece of information which should be mentioned. The average output of catecholamines, from 22 glands, is 5 μmole which should, according to the ratio 1 μmole amine to 0.208 mg chromogranins, result in the release of a total of about 1.0 mg of protein during the stimulation period. During this same period, and from the same glands, an average of 34 mU of AChE is found in the perfusate. Since the plasma contains roughly 70 mg of protein per ml and has an AChE activity of about 100 mU/ml, then 34 mU of enzyme should be accompanied by around 20 mg of protein if the enzyme activity indicated the presence of plasma. Since only about 2.5 mg of protein was found in the average stimulated perfusate, if there should be an excess of one type of protein over the other, then it ought to be a 10-fold excess of plasma proteins, which is clearly not the case (Fig.39). These figures also infer that the value shown for the first sample in Fig.40 is too high. The average specific activity of the AChE in the plasma of slaughterhouse animals should be about 1.5 mU/g protein or $\frac{1}{30}$th, the highest specific activity of the perfusate enzyme. There is, it would seem, a much faster washout of plasma protein than there is of AChE. When the differences in K_m between the "released" and plasma enzymes are also considered, there seems little doubt that the former is not derived from the plasma. No matter how dilute the plasma, the K_m would remain constant and only the V_{max} would change. Thus, unless there is a specific retention or extraction of AChE from the plasma by the adrenal gland, it seems unnecessary that we continue to regard the plasma as a possible source of the perfusate enzyme.

But is the enzyme "released" or "secreted" from some site, axons or chromaffin cell, within the gland? In order to answer this question we must first discuss the evidence that <u>any</u> protein is released from the gland. In other words, we must try and establish whether it is theoretically possible for proteins to be secreted from this tissue (to clarify nomenclature, when the terms gland and tissue are used as above, they refer to the tissue as a whole and include reference to all the types of cell which are known to be present within the organ - except for the cortical cells).

There is, of course, no doubt that the adrenal medulla is capable of secreting proteins (see e.g. Kirshner and Kirshner, 1971; Smith and Winkler, 1972) amongst which are the chromogranins, the two best known of these being DBH and Chromogranin A. In addition, various acid hydrolases are also known to be released when appropriate secretagogues are administered to the isolated organ

(Schneider et al., 1967) or, as in many of the experiments described by Smith and Winkler (1972), when stimulation of the adrenal medulla is induced, reflexly, by administration of drugs to an animal. The release of the chromogranins from the chromaffin cell can be shown to parallel the release of the catecholamines, and since the proteins and the amines are stored within the one particle (Sage, Smith and Kirshner, 1967), this observation was interpreted as providing evidence for the concept that exocytosis was the mechanism by which the adrenal medulla normally released its amines and its proteins (for review, see Smith and Winkler, 1972). Exocytosis is a form of reversed pinocytosis or, more positively, it is the name given to a mechanism by which substances are released from cells when the membrane of their specific storage particle fuses with the plasma membrane, thus enabling any molecule, however large, to pass from the inside to the outside of the cell. Given the vast disparity in molecular sizes between the amines on the one hand (about 170) and some of the proteins on the other (e.g. DBH, 240,000; Friedman and Kaufman, 1965), exocytosis was proposed as the only mechanism by which the two components could be concomitantly released (see Smith, 1973). Schneider (1968) has shown that a group of proteins quite distinct from the chromogranins are also released from the perfused adrenal gland. These proteins, the acid hydrolases, are, at least for some part, known to be stored in lysosomes within the adrenal medulla (Smith and Winkler, 1966) but again, care must be taken in the interpretation of data obtained with the use of an homogenate and a centrifuge. Some of the acid hydrolases are also known to be in axonal SER (see Holtzman,1971) and certain similarities between these enzymes and the axonal $AChE_5$ have already been pointed out (Chapter 2).

Equally, a considerable proportion of various acid hydrolase activities in adrenal medullary homogenates are not sedimentable (Dubois, 1970). We cannot, therefore, be certain whether all of the soluble acid hydrolase activity is derived from broken lysosomes or from elements of SER which, by analogy with AChE, might lose their internal soluble contents when, and if, they are broken during the homogenization of the tissue. By the same reasoning, we cannot be certain from where the perfusate acid hydrolases originate. It could be medullary lysosomes, but could it be direct from the axonal SER?

The results of Schneider (1968) show that there is a significant correlation between the catecholamine and the acid hydrolase output from the isolated adrenal gland. There is no evidence to suggest that these enzymes are present in the chromaffin granules (Smith and Winkler, 1966) and so Schneider (1968) reasons that the parallelism is probably coincidental and is more an indication of the efficacy of the stimulation than it is an indication of any devious storage properties of the two sets of constituents. A similar argument can also be used to explain the parallelism between the amines and AChE observed in these experiments. Reference to Fig.10 shows that about 7% of the total AChE activity is associated with the "Large-granule fraction", the major component of which is the chromaffin granules. Since there are about 360 mU of soluble AChE in each medulla, 7% of this figure would be about 28 mU of activity. Thus, to reach the mean figure of AChE output, 34 mU, <u>all</u> of the chromaffin granules would have to release <u>all</u> of their contents if they were the origin of the perfusate enzyme. It is known that only 4-16% of the catecholamines and chromogranins are released under similar experimental conditions (Smith, 1972), and so it would seem that the fairly constant ratio between the amines and the esterase is a reflection of the efficacy of stimulation by whatever secretagogue is used, and not of a concomitant release from the one storage site. The occurrence of AChE in the chromaffin granule pellet is most likely to be due to co-sedimentation or adsorption of enzyme from another source during the centrifugation procedure.

The evidence, therefore, is quite conclusive that the adrenal medulla can secrete at least two separate sets of proteins; one type is secreted, quite without doubt, from the chromaffin cell, while the source of the other is perhaps more open to debate. But protein secretion is also a well established property of some nerves. Thus, Smith et al. (1970) have shown beyond doubt that DBH, and probably the other chromogranins, are secreted from a noradrenergic nerve, the splenic, when the spleen from either dog or calf is isolated, perfused and stimulated. We still cannot, therefore, use this evidence to help toward a conclusion as to the source of the AChE, although we can say that there cannot be any theoretical objections to the secretion of AChE by either the nerve or the chromaffin cell, simply on the grounds that it is a protein.

Up to about this stage in the course of this work, we were fairly convinced by the nature of the perfusate enzyme, i.e. only $AChE_5$, that the enzyme was released from the nerve. This was also based on the findings, mentioned in the Introduction, of many workers which suggested that if not all, then at least a high proportion of the adrenal medullary AChE was restricted to the nerve network within the gland. For example, the work of Coupland (1961) which was cytochemical in nature, and that of

Eränkö et al. (1959), who showed a decrease in the AChE content of the rat's adrenal medulla after splanchnic section, both suggested that this was a valid conclusion to draw. The first real shaking of this faith was when it was found that both DMPP and carbachol (nicotinic agonists) released AChE from the perfused gland in the proportion to catecholamines as was found with KCl. This observation could, however, be rationalized by supposing that presynaptic nicotinic receptors could be present on these axons just as they might be present on, e.g., the terminals of the fibres innervating the SCG of the cat (Katz, Salchmagherddam and Collier, 1973). This suggestion is based upon the observation that both carbachol and ACh are capable of releasing "surplus" ACh from sympathetic ganglia. (Surplus ACh is that which was shown by Birks and McIntosh (1961) to accumulate rapidly when the AChE of an organ is inhibited by a membrane-permeable anticholinesterase.) In those SCG which have been decentralized, the "surplus" ACh does not accumulate, nor does it if a membrane-impermeable inhibitor is used. Thus, Collier and Katz (1971) proposed that intracellular AChE was responsible for the lack of surplus ACh in normal, untreated ganglia, and also that, when it was present, it was in the terminals of the preganglionic fibres.

The observation that ACh and carbachol could both release this surplus ACh could therefore be interpreted as indicating the presence of a presynaptic receptor for nicotinic stimulants. The presence of a similar type of receptor has long been proposed by Koelle (see e.g. Koelle, 1962), but more recent evidence does not support this proposal (see Vizi, 1973).

One other piece of evidence that we used at this time to support the idea of the neuronal origin of the released AChE was also related to the surplus ACh. If a ganglion which is actively accumulating surplus ACh is stimulated by increasing the KCl concentration of the perfusing medium, it can be shown that some of the surplus ACh is released by a Ca^{2+} dependent mechanism (Collier and Katz, 1971). Thus, carbachol, exogenous ACh and K^+ all released surplus ACh, but nerve stimulation did not (Collier and Katz, 1970). We reasoned, therefore, that the structure which contained the surplus ACh in the treated ganglia could be the same structure which contained the AChE in normal tissues. This would explain the similarities in the requirements for release of both the protein and the ACh, and would fit with our conception of the intracellular localization of the AChE. Direct evidence does, of course, need to be supplied but, while the possibilities listed above still remain, the results of further experiments led to an alteration in our whole concept of the problem with which we were confronted. These were the results obtained with the denervation experiments.

As shown in Fig.43, the only effect of denervation for periods longer than 21 days is to increase the amount of AChE in the denervated organ as compared with its contralateral control. Shorter denervation periods appear to have little effect on the AChE level so the question must be asked: are the glands being denervated? There are probably two ways to answer this question, the most direct of which would be to estimate choline acetylase in the adrenals; a degeneration of the nerve terminals would mean a loss of choline acetylase activity. However, we have not been able to obtain consistent results with the choline acetylase assay, tried over a period of months, with a variety of enzyme sources and concentrations, substrates and concentrations, methods of separating the product, incubation times and temperatures, pH of the buffers, methods of stopping the reaction, starting the reaction, etc. Thus, we have no estimate of the choline acetylase content of the adrenals or its behaviour after splanchnic nerve section. Fortunately, there are more indirect methods by which an answer can also be obtained. First, we know that the correct axons have been tied because, if these axons are stimulated, catecholamines (Comline and Silver, 1966) and proteins (Blashko, Comline, Schneider, Silver and Smith, 1967) are released from the adrenal glands in vivo. Secondly, we know that in many of these experiments the axons have been securely ligated, because it was from these axons that the results described in the previous Chapter were obtained. Consequently, we know that the terminals were deprived of considerable quantities of AChE. Thirdly, a morphological examination of the glands,as soon as 2 days after ligation of the axons, shows an extensive degeneration of the observable axons, many of which are in advanced stages of digestion. In longer term denervations, the microscope reveals regeneration, but shows the scars of degeneration very clearly (Somogyi; unpublished observations). There are, therefore, excellent reasons for supposing that at some time between 16.5 h and 6 months, which were the shortest and longest times studied, the adrenals should have shown considerable changes in their AChE content; provided, of course, that the bulk of the enzyme in the medulla was there as a consequence of the nerve terminals within the tissue. The fact that there was no decrease in AChE activity,and experiments we have now done using rat SCG show that we chose reasonable times to examine the tissues, can only mean that a significant proportion of the adrenal medulla's AChE is present within, or associated with, the chromaffin cells. If this is so, we have to extend our working hypothesis to include all of the isoenzymes that electrophoresis of the adrenal medulla reveals. The previously postulated occurrence in the adrenal medulla of $AChE_1$ - $AChE_4$ could not account for sufficient enzyme activity for these to be the sole isoenzymes associated with the chromaffin cells.

It is tempting to speculate that the increased concentration of AChE in several of the denervated adrenals is due to a slow down in the release of the protein resulting from the loss of nervous activity, i.e. there is an accumulation of the enzyme in the tissue. Unfortunately, there is no evidence for or against this idea, but, since there is certainly no suggestion of an increased amount of AChE released from these glands when they are perfused and stimulated (either in absolute terms or relative to catecholamines), it is perhaps an unlikely speculation. But however obscure the explanation may be for why the denervated glands contain more AChE than their contralateral counterparts, it is very clear that our conception of the adrenal gland as being the meeting place of linked but mutually distinct cholinergic and adrenergic processes is too simplistic. Even if there is another unknown nerve supply to the adrenals, the gland would remain the only cholinergic tissue known in which interruption of the major innervating axons does not result in at least a partial depletion of the AChE content of the tissue. Thus, the postsynaptic adrenergic cells must have an extremely high AChE content, so high that the loss of the axonal enzyme can pass undetected. The discussion of this problem will be continued in the next Chapter.

The results presented in this Chapter make it possible to add AChE to the list of proteins which are known to be secreted from isolated perfused adrenal glands. But they also leave many questions to be answered. For example, why is only $AChE_5$ released?

Why, for that matter, is any AChE released? Do the released proteins have extracellular functions? From where is the AChE released, both at the cellular and at the ultrastructural levels? Does the presence of AChE in extracellular fluids show that release of the protein can also occur *in vivo*? Why does the AChE content per g of medullae decrease with age? Can we show that the postsynaptic isoenzymes are rapidly turning over, as they would need to do if the released enzymes originated from these cells?

The final four Chapters of this thesis will be devoted to answering and discussing some of these questions. Our first priority was to find an ultrastructural basis for the release of AChE, so we had to try and analyze the storage characteristics of the enzyme. As mentioned in Chapter 1, this problem was approached from a cytochemical point of view, and the results are presented in the next Chapter.

CHAPTER 5

A CYTOCHEMICAL STUDY OF THE DISTRIBUTION OF ACETYLCHOLINESTERASE WITHIN BOVINE ADRENAL MEDULLAE AND SPLANCHNIC NERVE AXONS

INTRODUCTION

It has already been pointed out that AChE is a widely distributed enzyme (see Chapter 1), and also that this distribution does not necessarily reflect the nature of the innervation to the AChE-rich tissue or cell. We are now able to add to these another unusual property of this enzyme, that is that it can be released into extracellular fluids. As described in Chapter 4, one of the soluble isoenzymes of the AChE present in the adrenal medulla can be released into perfusates from the gland.

It has also been mentioned that the problem of the origin of the released isoenzyme could not be resolved by biochemical experiments, probably because the structure which contains the soluble enzyme is too fragile to withstand the preparative procedures required for the biochemical analysis of tissue subfractions. Instead, therefore, we have turned to a cytochemical study of bovine splanchnic nerve axons and adrenal medullae in order to obtain some information about the distribution of AChE within these organs. It is only when the answer to this question is known that suggestions can be made about the site from which AChE is released and about the mode of release.

METHODS

Bovine adrenal glands from three oxen about two years old, and from two calves aged approximately 200 days were used for cytochemical purposes. The ox adrenal glands were obtained from the local slaughterhouse, while the calf glands were removed from animals anaesthetized with sodium pentobarbitone, in the Department of Physiology, Cambridge.

As soon as possible, and certainly within 15-25 min of the death of the animal, 1-2 mm^3 blocks of adrenal medulla from at least four different parts of the gland were cut with a razor blade, and placed into ice-cold fixative solution. Fixation was carried out for 2-4 h at 4^o, with constant agitation, by immersion of the blocks in 2% (w/v) glutaraldehyde and 2% (w/v) formaldehyde solution buffered to pH 7.3-7.4 with 0.1 M sodium cacodylate. After fixation, 40-50 μm thick sections were cut using a Sorval TC-2 Smith-Farquhar tissue chopper. Sections containing large intramedullary nerve trunks were selected and washed for 2-3 h at 4^o with 0.2 M sucrose buffered with 0.1 M cacodylate, pH 7.3-7.4. This washing solution was used throughout the procedure.

For demonstration of cholinesterases the thiocholine technique (Koelle and Friedenwald, 1949) was used as modified for electron microscopy by Kasa and Csillik (1966). Thus, the thick sections were incubated at room temperature for 30, 50 or 90 min in media containing (final concentrations): 73 mM sodium acetate pH 5.5, 13.3 mM glycine, 6.6 mM $CuSO_4$, 0.2 mM $Pb(NO_3)_2$, together with ATCh (8 mM).

To establish specificity the following incubations were carried out: (i) incubation without substrate; (ii) incubation in the presence of 10^{-4} M ethopropazine hydrochloride (an inhibitor of ChE; see Kasa and Csillik, 1966); (iii) addition of 10^{-4}M BW 284C51; (iv) both 10^{-4}M ethopropazine hydrochloride and 10^{-4}M BW 284C51; (v) with 2.0 x 10^{-5}M eserine sulphate. As is usual, samples were preincubated for 30 min in a wash solution containing the appropriate inhibitor(s).

After incubation, specimens were washed for 2 x 5 min and then transferred into 2% (v/v) $Na_2S.9H_2O$ dissolved in 0.2 M sodium acetate pH 7.0 - 7.5. After 5 min in this solution, the sections were washed again (2 x 5 min) and post-fixed for 8 min in 0.5% OsO_4, according to the method of Palade (1952).

Thin sections were stained for 30 min with saturated, aqueous uranyl acetate and then for 2 min with lead citrate. Either a Metropolitan-Vickers E.M.6 or a Philips 200 electron microscope was used to examine the sections.

To study the effect of fixation on the activity of the individual isoenzymes in the adrenal medulla, we "fixed" a gel on which a high-speed supernatant fraction from the adrenal had been run (see Chapter 2). Prior to development of the AChE activity, the gel was exposed to the cytochemical fixative for 10, 20 or 40 min. After washing, the enzyme activity was developed as described in Chapter 2.

RESULTS

By the use of inhibitors and appropriate controls, it is possible to establish the specificity of the cytochemical reaction. After incubating without substrate, or in the presence of eserine, we could not detect any reaction end-product. This result shows that, in these experiments, not only are there no detectable levels of esterases other than cholinesterases in the tissues studied, but also that there is no non-specific binding of the metal ions used in the incubation medium. It is necessary to establish this latter point because the end-product of the enzymatic reaction which is observed in these experiments is a metal sulphide.

The reaction product obtained with either of the two substrates was not formed when both BW 284C51 and ethopropazine were incorporated in the incubation medium. This was so even with extended (up to

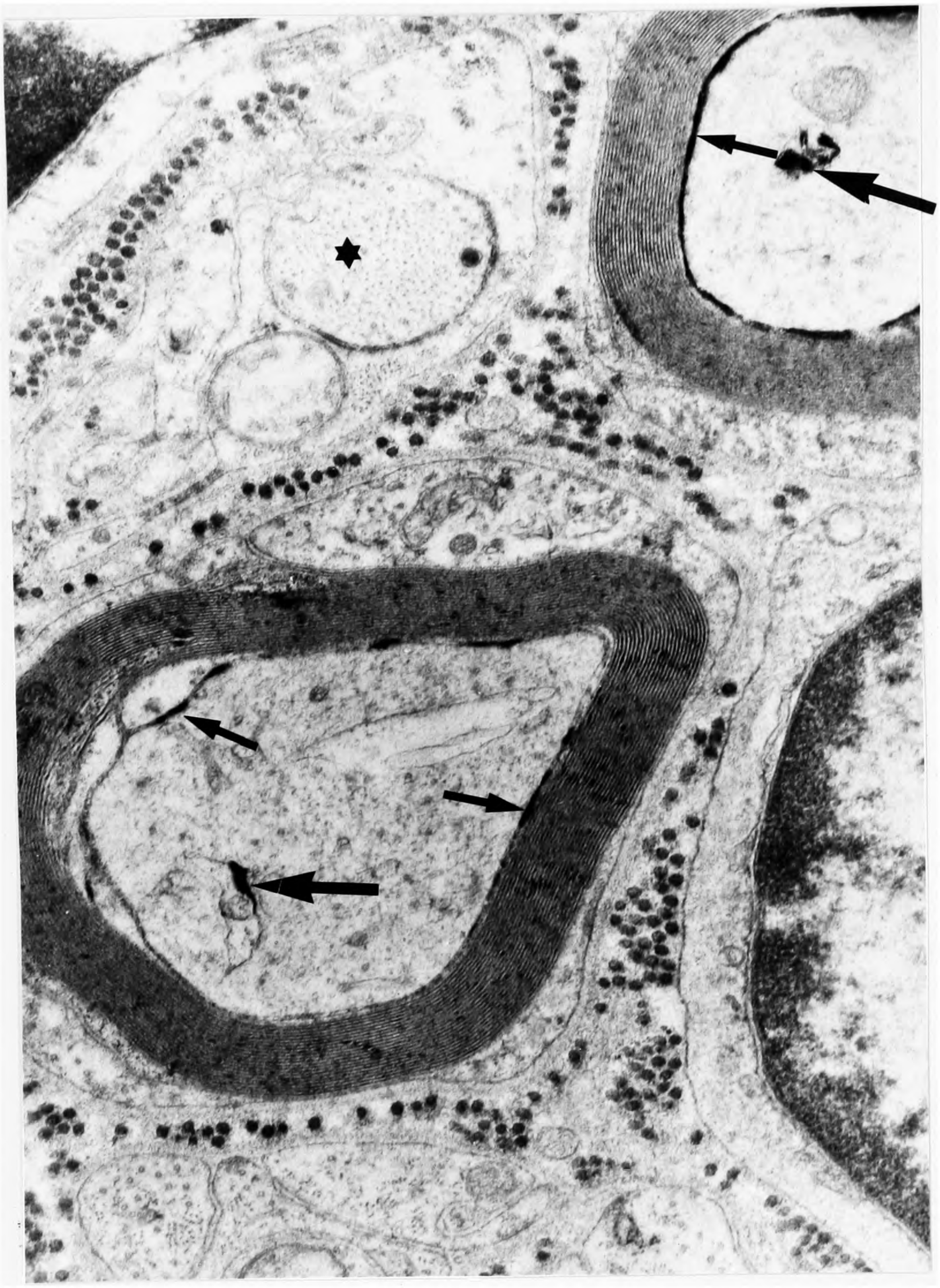


Fig. 47.

Part of an intramedullary nerve trunk with two myelinated and one unmyelinated axon (asterisk). End-product due to AChE activity can be seen around all three axons as well as associated with structures within the axoplasm (arrows). Mag. X 25,000.

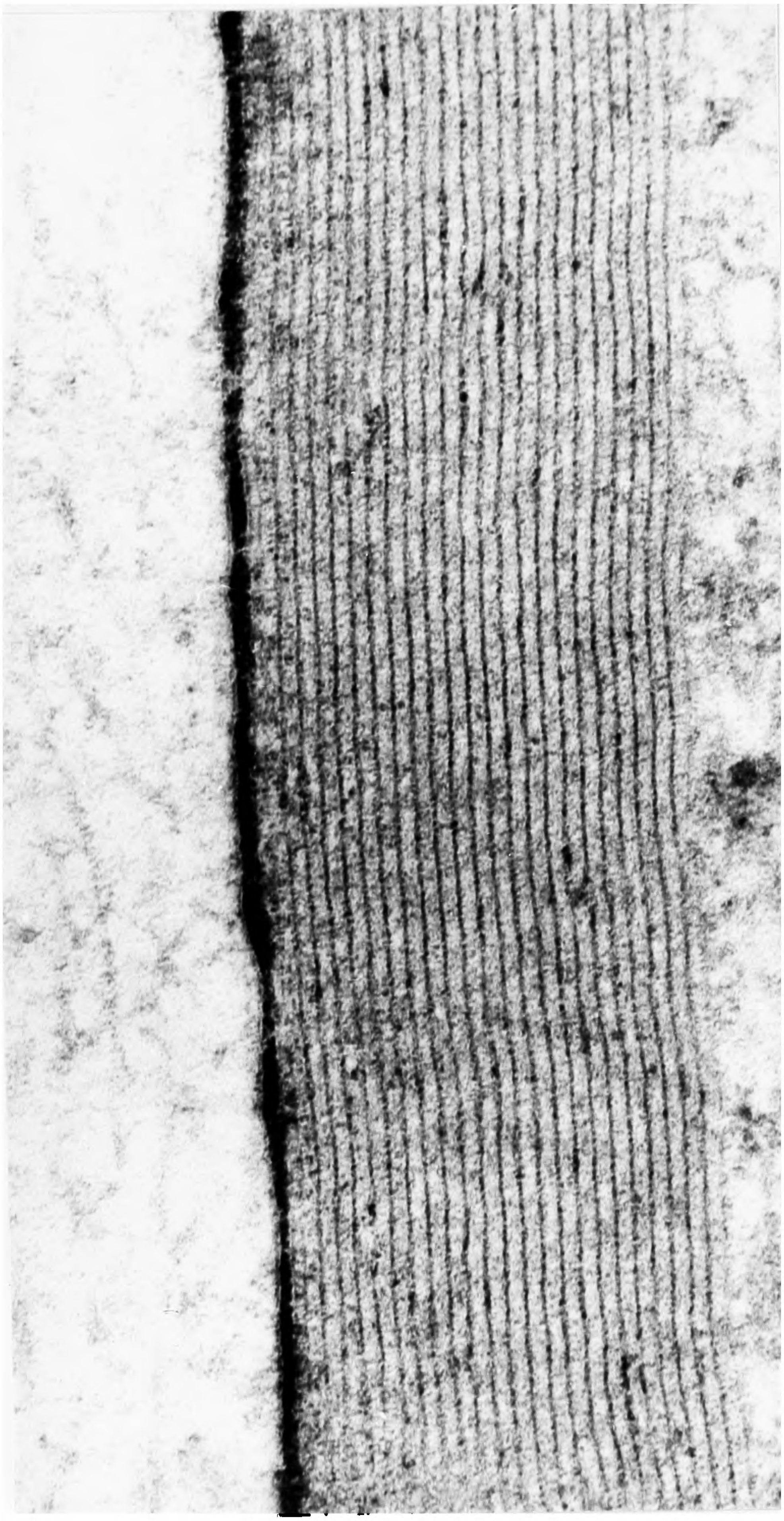
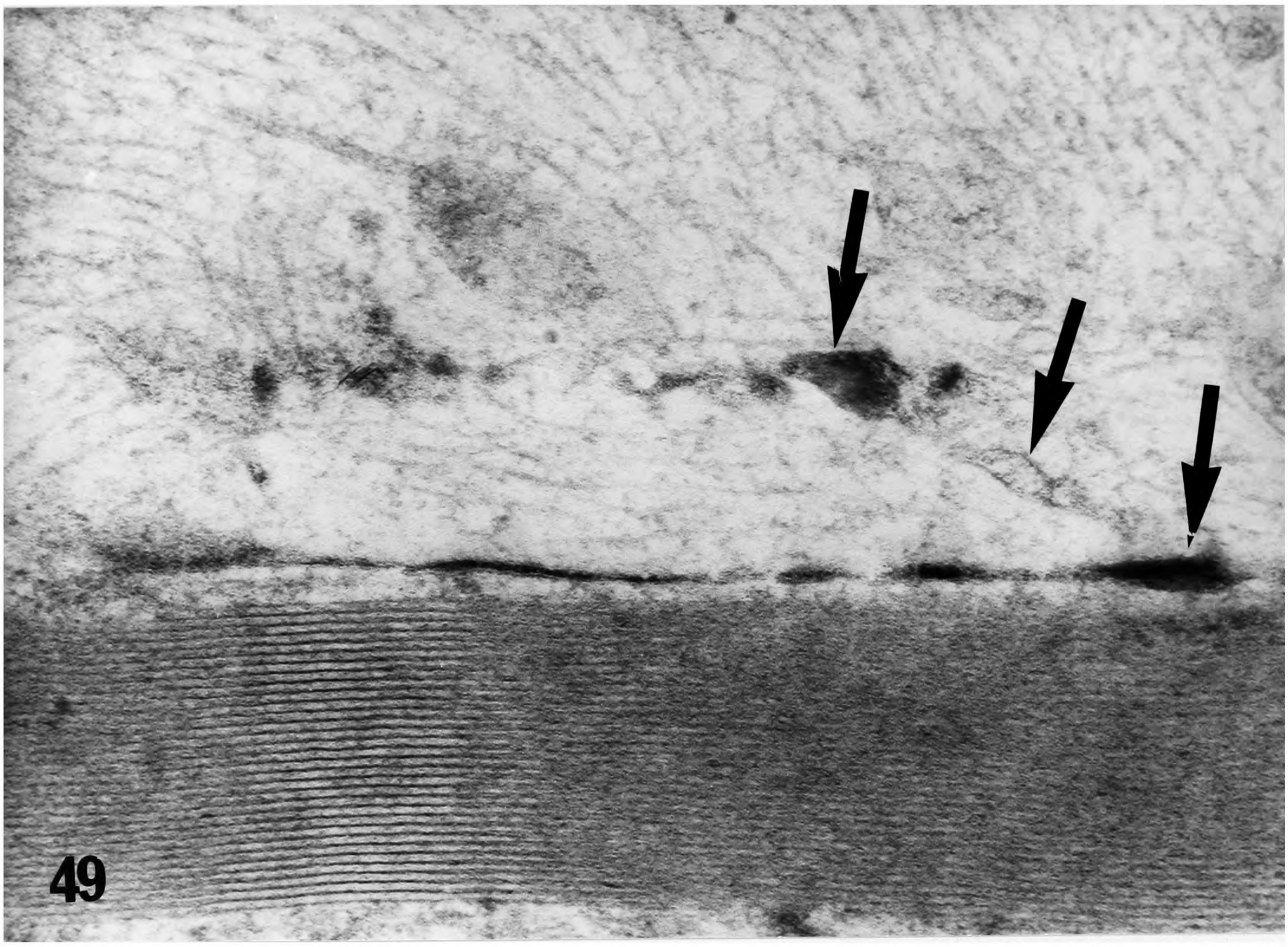
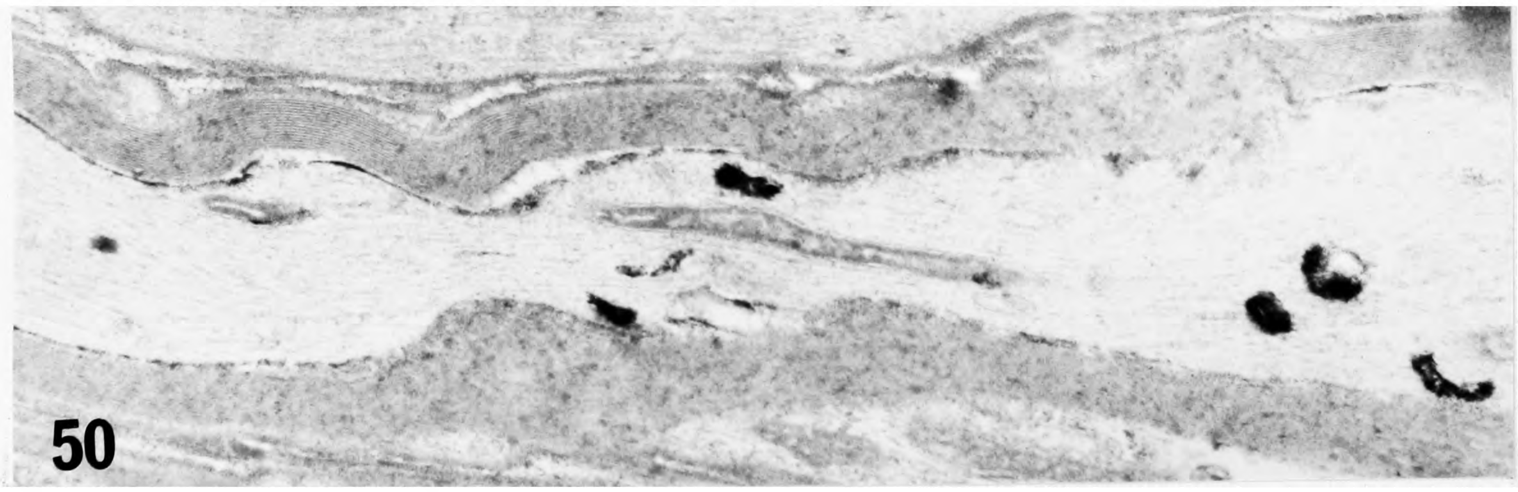


Fig. 48.

High power micrograph showing that the AChE end-product is localized between the outer leaflets of the Schwann cell and axonal plasma membranes. Mag. X 154,000.





<u>Fig. 49</u> : AChE activity in a short segment of axonal SER (arrow) near to a region of reactive axolemma. Mag. X 68,000.

<u>Fig. 50</u> : Longitudinal section of an axon containing several AChE positive SER profiles in a comparatively small area. Mag. X 18,000.

90 min) incubation times. When BW 284C51 was used alone with ATCh as substrate - and it is this substrate which was used for all of the results to be presented here - no end-product was visible after as long an incubation time as 90 min. From these controls we concluded that by using ATCh in the presence of 10^{-4}M ethopropazine we would only reveal sites of AChE activity.

Finally, we examined the possibility that the fixative might selectively inhibit some of the AChE isoenzymes, an event which could clearly lead to an erroneous or incomplete cytochemical picture of the cholinesterases. When tested by its effect on electrophoretically separated isoenzymes, it was found that the fixative caused a marked and progressive inhibition of enzyme activity, but that there was no selective inhibition of any of the isoenzymes.

To turn to the results obtained with this technique, it can first be said that no observable differences could be seen in tissues from young or adult animals. Thus, the pictures that follow are from animals of all ages.

The first tissue I will discuss will be the splanchnic nerve axons and, as shown in Fig. 47, the large nerve bundles which were selected (Methods) consist of both myelinated and unmyelinated axons. The reaction end-product, which in every case in this Chapter has been shown to be due to AChE activity, can be seen at two sites, between the plasma membranes of the Schwann cells and those of axons, and intra-axonally in ill-defined structures. Higher magnification showed first that the former activity is between the outer membrane leaflets of the two cells (Fig. 48), and secondly that the intra-axonal end-product is localized within tubules of what appears to be SER (Fig. 49). Only a minority of

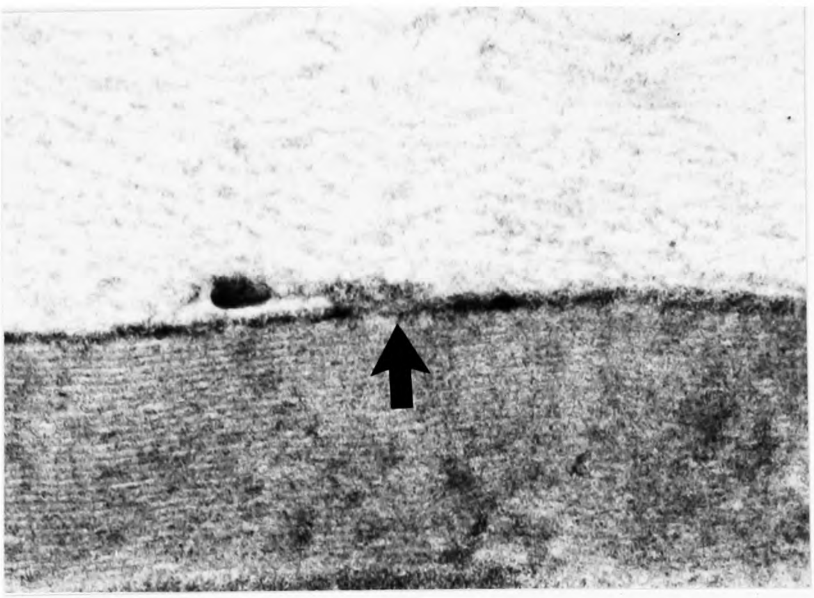


Fig. 51.

AChE activity within a tubule of SER which is continuous with the activity around the axon (arrow). Mag. X 48,000.

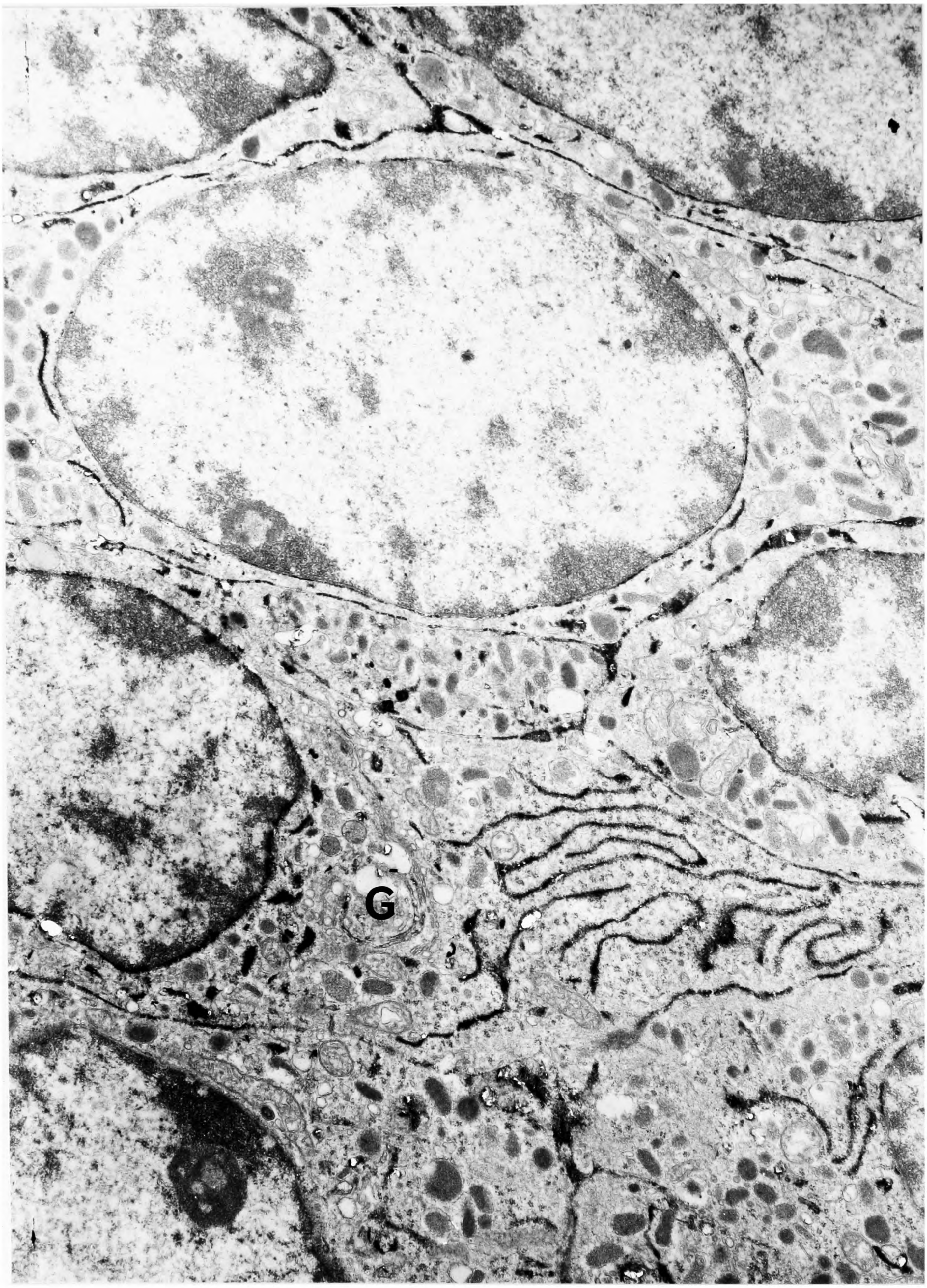
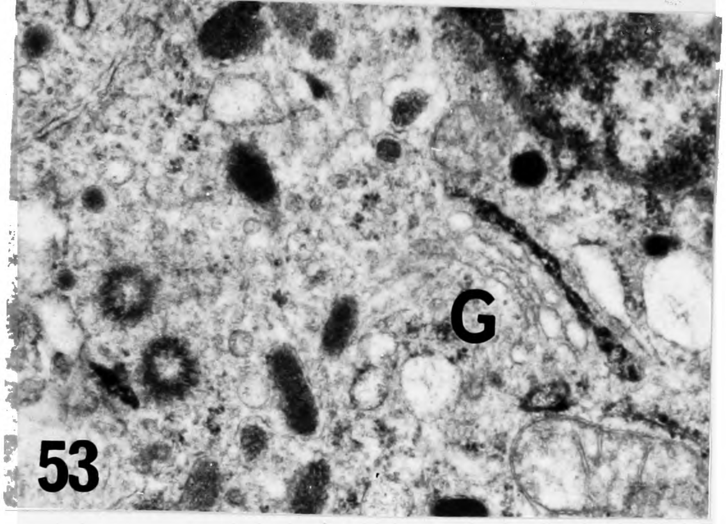
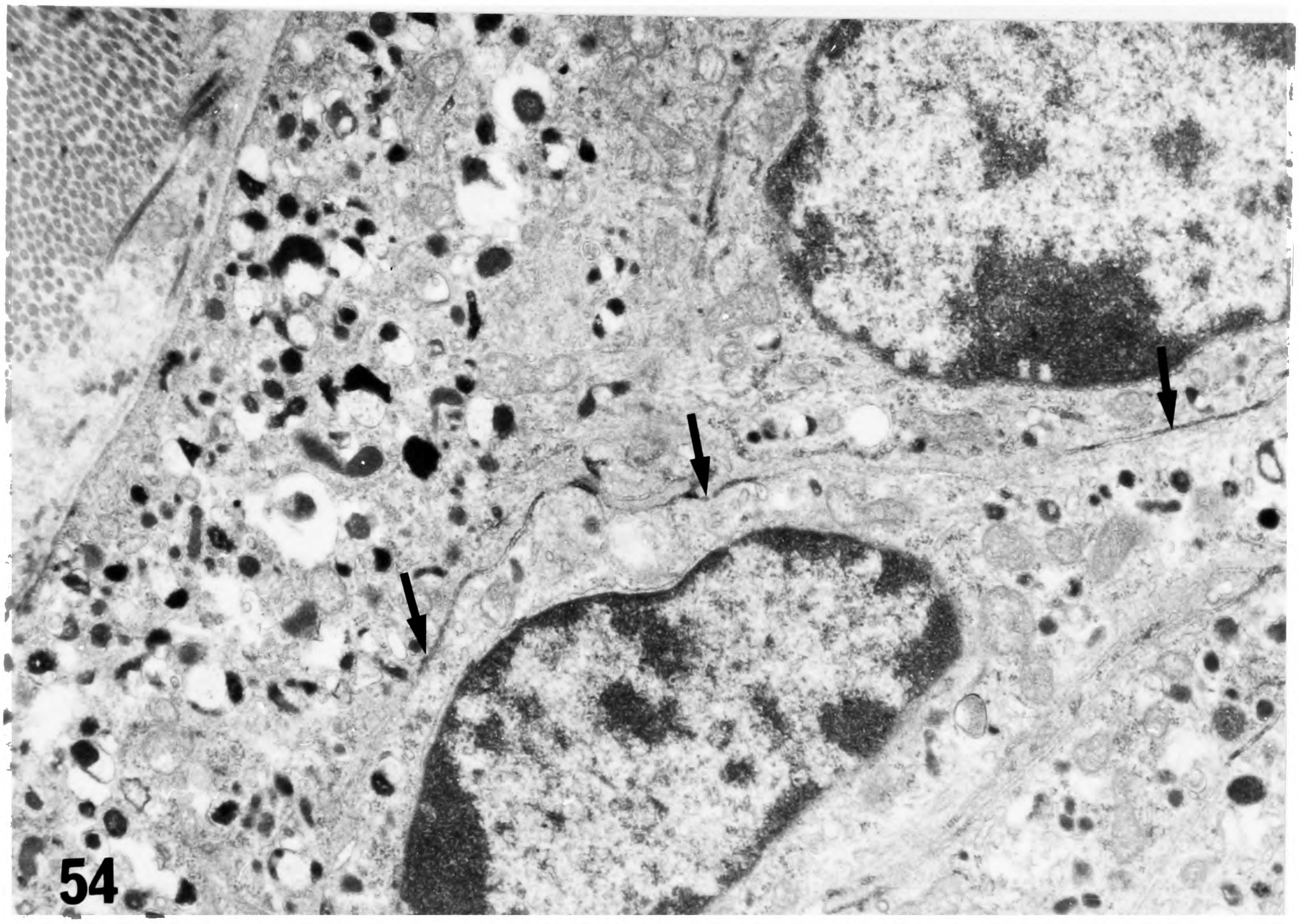


Fig. 52 : Survey picture of the nuclear region of an adrenaline-containing cell area. AChE activity is evident in the endoplasmic reticulumincluding the nuclear envelope while the Golgi system (G) shows only faint activity. End-product is also present between the chromaffin cells. Mag. X 10,000.





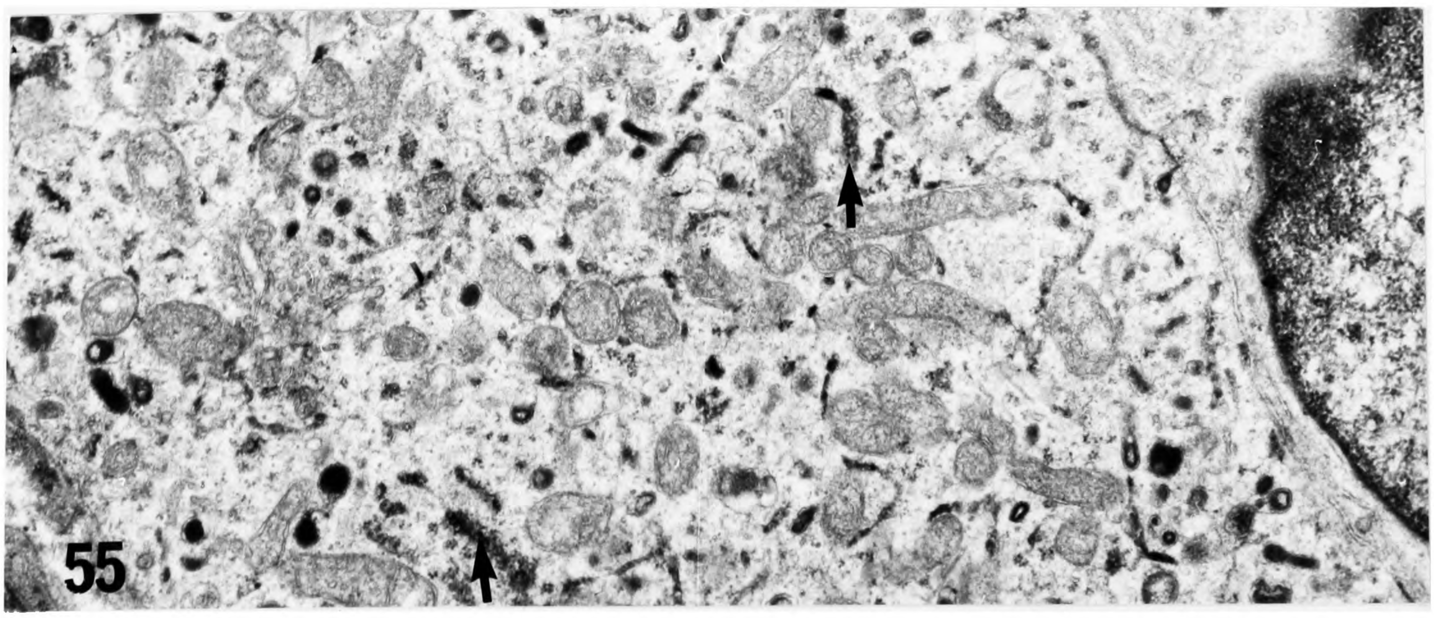
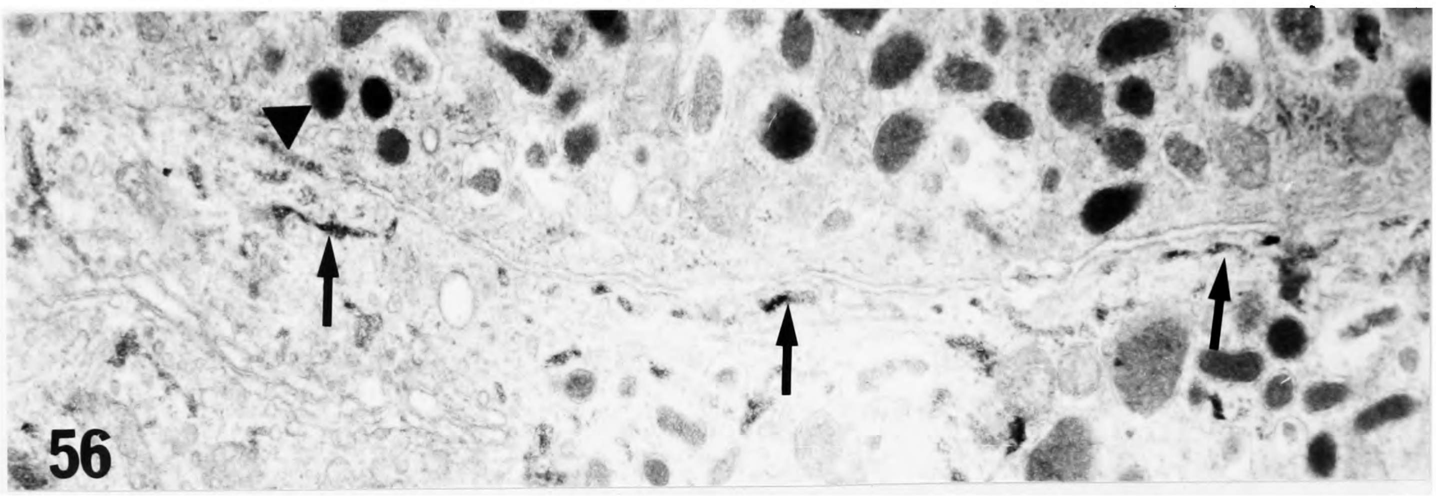


Fig. 53 : AChE positive Golgi (G). Mag. X 22,000. Figs. 54 and 55 : noradrenaline-containing cells with activity between the cells and in the ER. Mag. 54, X 19,000 ; 55, X 18,000.



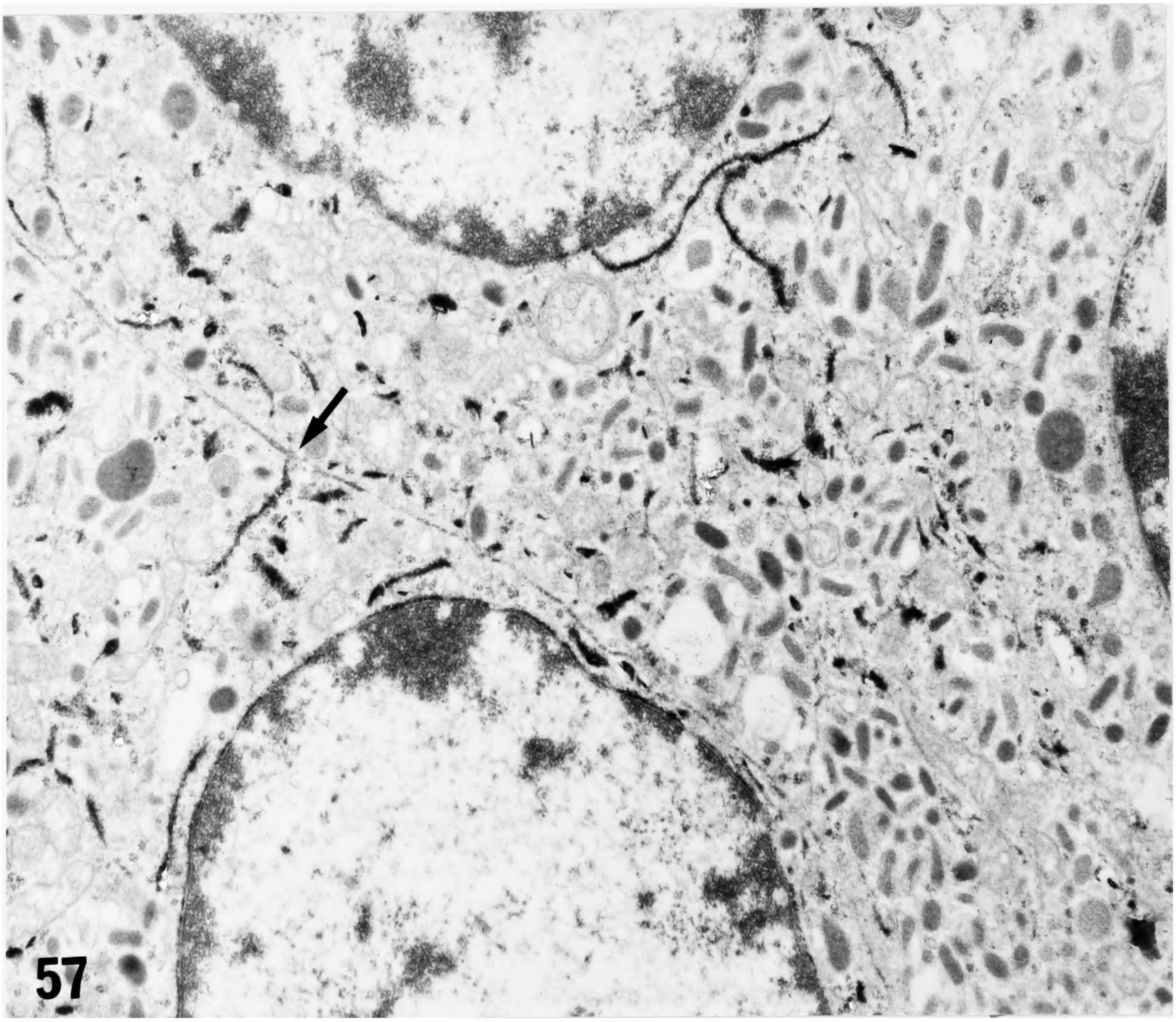


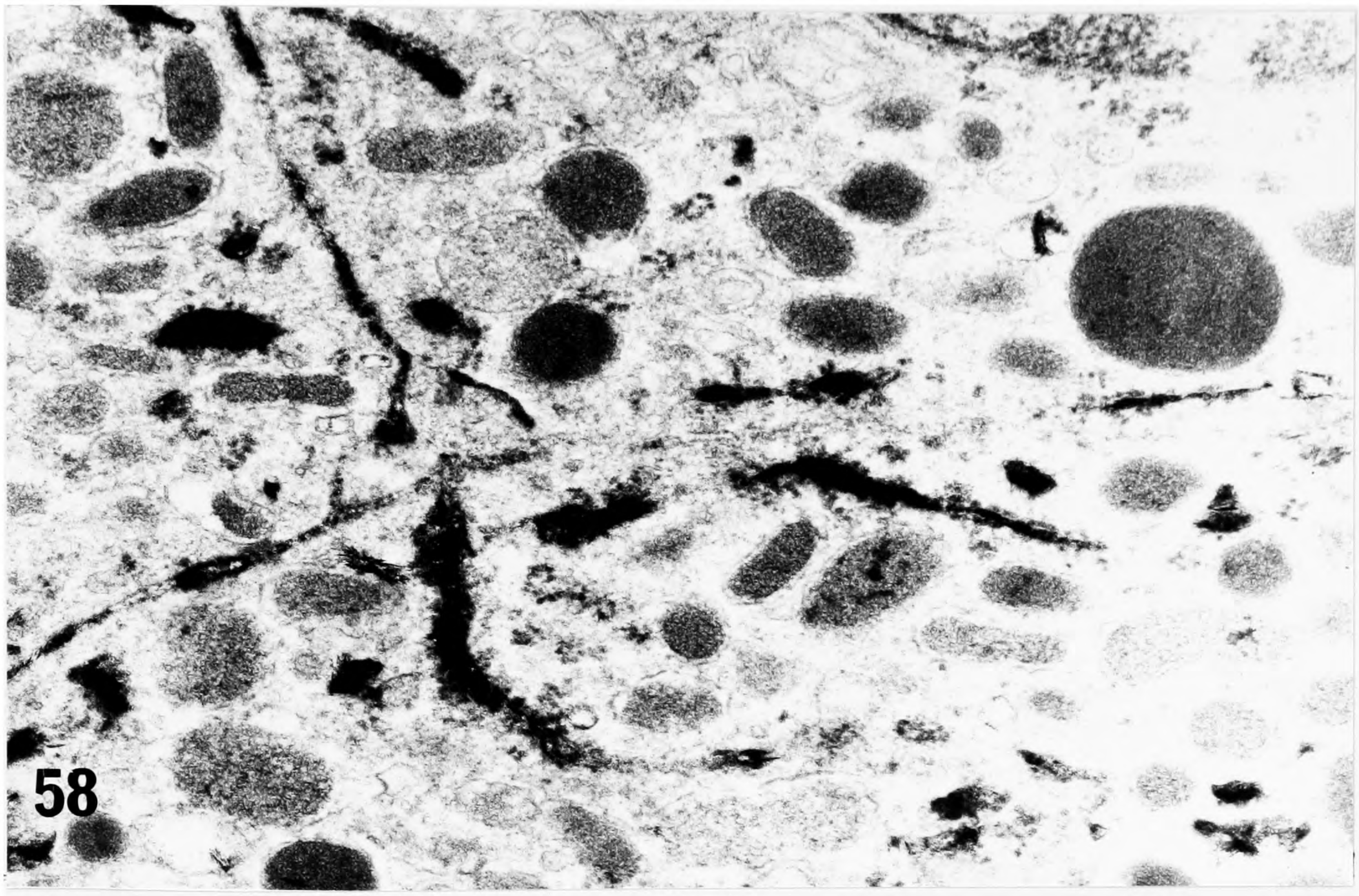
Fig. 56 : AChE-containing SER runs parallel with the plasma membrane (arrow). Golgi apparatus (G) is inactive. End-product between cells (arrowhead). Mag. X 24,000.

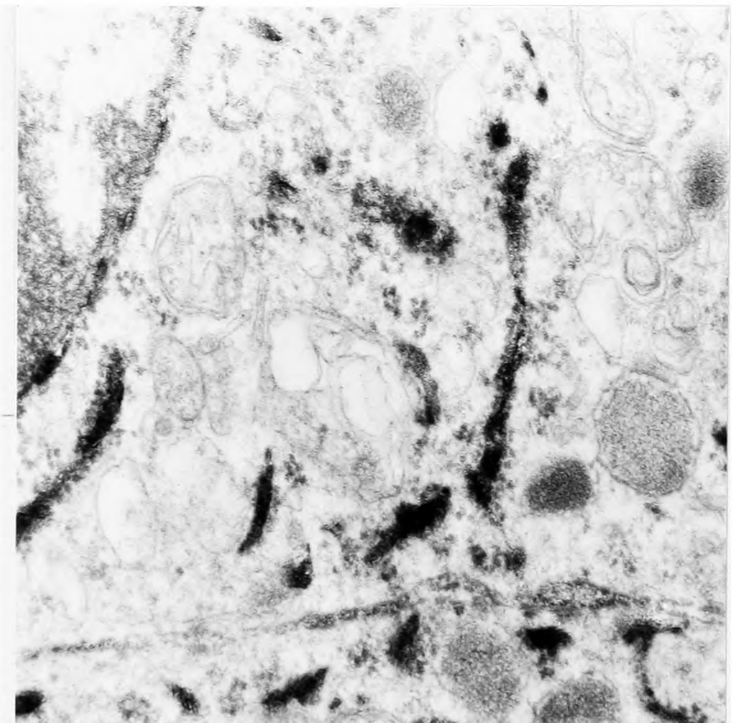
Fig. 57 : two adrenaline-containing cells with extensive AChE rich ER. Activity is also associated with plasma membranes and, at arrow, the internal and external activities seem continuous. Mag. X 15,000.

axons were found to contain this AChE-rich SER and, of those that did, some contained only one or two tubules while others (e.g. Fig.50) contained several. In the same axon not all the cisternae were active and sometimes the end-product was only present in a part of the length (Fig.49). When these tubules of SER lie close to the plasma membrane, the end-product within the cisternae often seems to be continuous with that outside the plasma membrane (Fig. 51).

So far as the chromaffin cells are concerned, end-product was found in both the adrenaline and noradrenaline cell types (identified by the characteristic appearance of the noradrenaline storage granules after fixation in glutaraldehyde as described by Coupland, Pyper and Hopwood, 1964). The activity, due to AChE, was found within the cisternae of both rough and smooth ER and in the nuclear envelope. For example, Fig.52 shows a survey picture of the nuclear region of an adrenaline cell area with extensive activity within the ER, between the cells and in the nuclear envelope. The Golgi system of one of the cells shows very faint activity (G). Fig.53 shows a higher-power micrograph of the Golgi system from a different adrenaline-containing cell and again faint activity can be observed (Arrow). Noradrenaline-containing cells are shown in Figs. 54 and 55. In common with the adrenaline-containing cells, they show activity within the ER (arrows) and between the cells (Fig. 54, arrow).

Elements of the ER were often observed running parallel to the plasmalemma (arrows) and fairly frequently they approached it like sub-surface cisternae (Fig. 56). A more extensive view of an adrenaline cell area also shows this parallelism of the plasma membrane and ER, but in addition it shows what appears to be a continuity of the end-product within the ER with that between the two cells (arrow, Fig. 57). If such an apparent





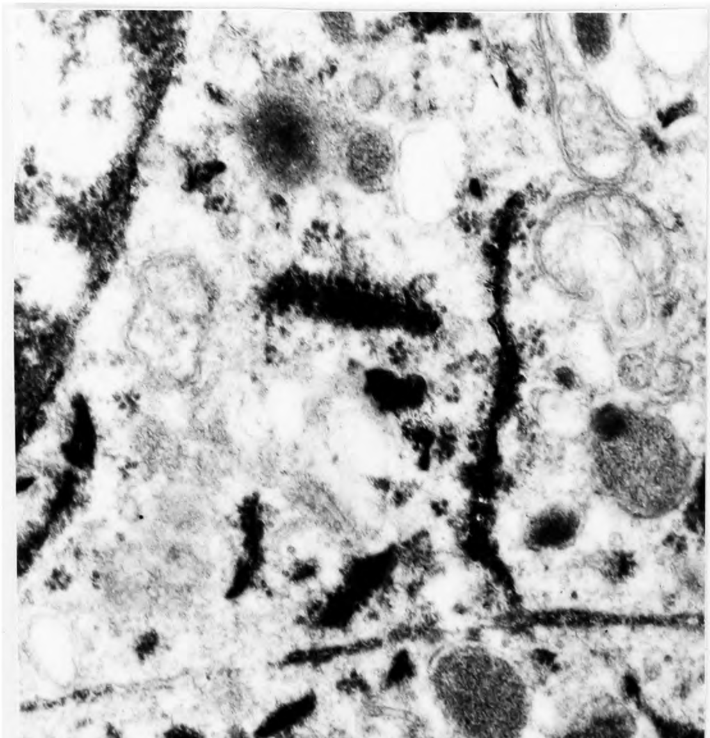


Fig. 58 : another example of end-product continuity within and without the ER of an adrenaline-containing cell. Mag. X 36,000.

Fig. 59 a and b : higher magnification, and serial section, of the "joined" area shown in Fig. 57. Mag. X 34,000.

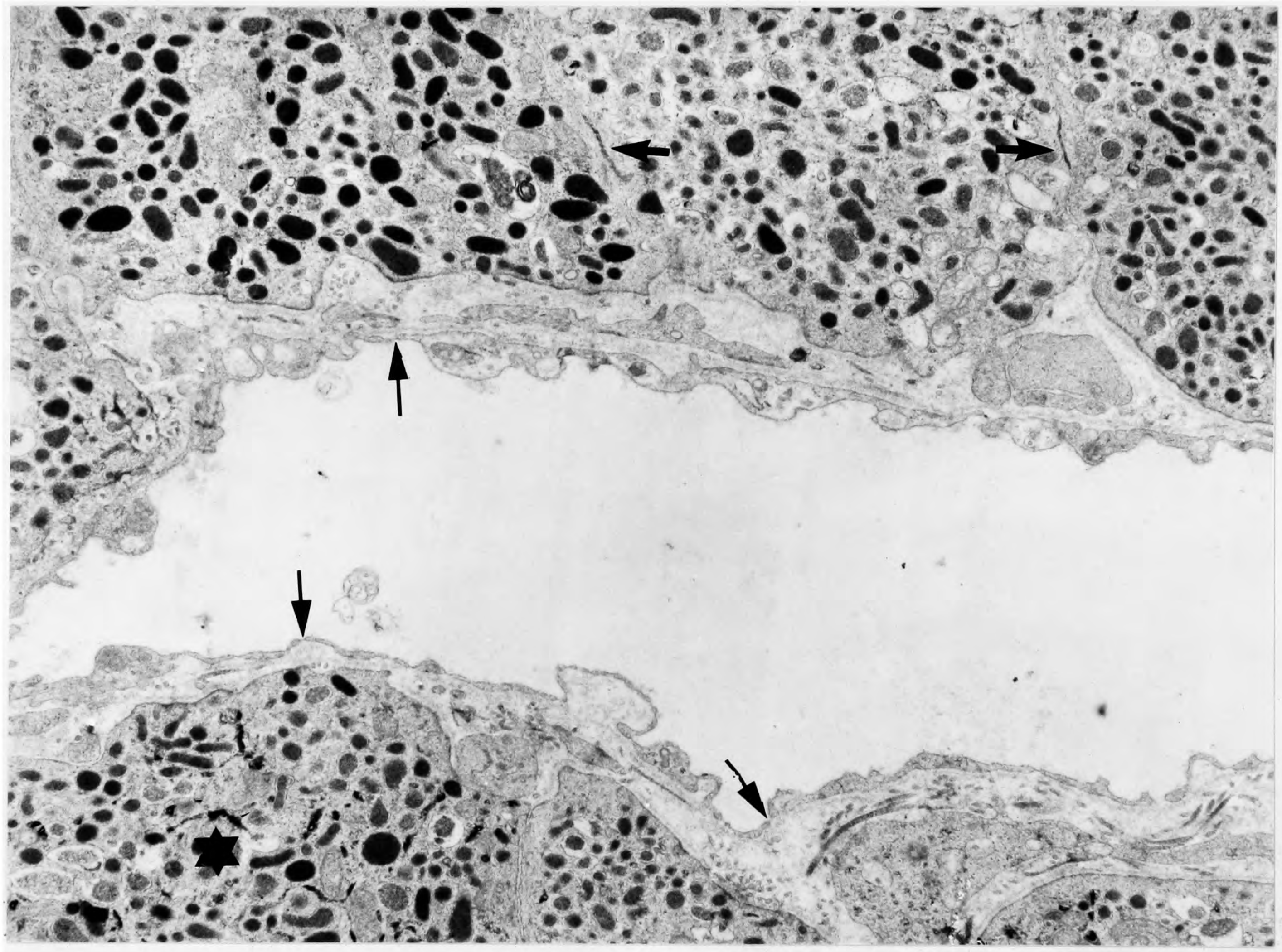


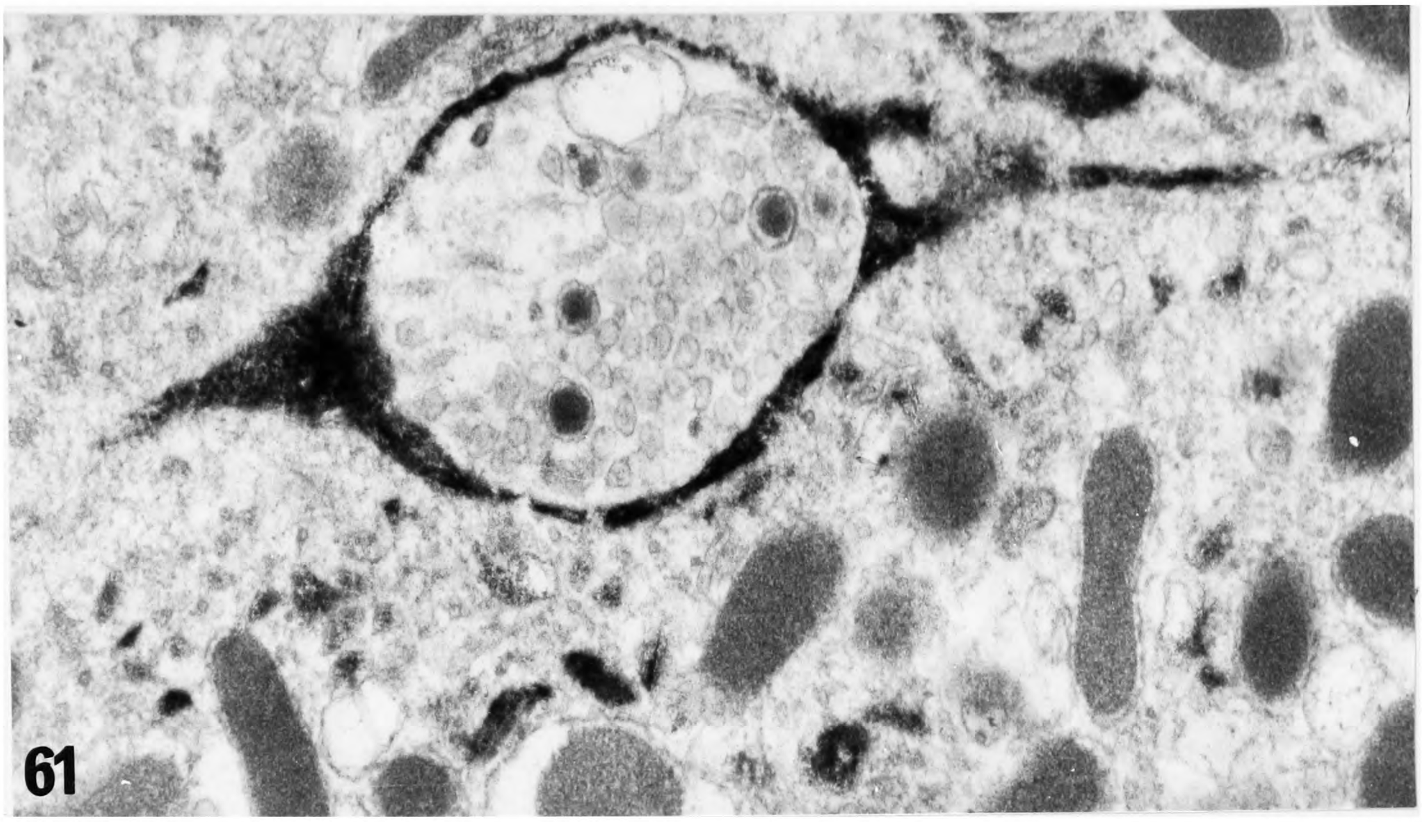
Fig. 60.

Adrenaline-containing cells are oriented towards a sinusoid which is surrounded with fenestrated endothelial cells (arrow). ER is not abundant in this part of the cell but when it is it contains AChE (asterisk). End-product can also be seen for short distances between the cells. Mag. X 7,700.

"joining" of the ER and plasma membranes is observed at higher power, the results shown in Figs. 58 and 59 are obtained. In Fig. 58 two adrenaline-containing cells are shown, both of which have activity in their ER, and both of which have elements of the ER running parallel to the plasma membranes of the cells. There is also end-product within the ER which is continuous with that between the cells. Figs. 59 a and 59 b show the results of a serial section through one of these "joins" - in fact, that shown in Fig. 57.

All of the results so far presented for the localization of the AChE within chromaffin cells have dealt with only one part of the cell, the ER-rich nuclear pole. At the other end, there is little ER but there are many chromaffin granules (Fig. 60) and the adrenaline-containing cells are oriented towards a sinusoid which is surrounded by fenestrated endothelial cells (arrows). When there is ER in this part of the cell, it also exhibits AChE activity; and the end-product can also be seen for short distances between the cells (arrowheads; Fig. 60).

To summarize the results of the experiments in which the localization of AChE in chromaffin cells was studied, it can be said that, of the two types, the AChE activity is more characteristic of the adrenaline-containing cell. Not only were a higher proportion of the adrenaline containing cells active, but they also showed a stronger reaction when the two types were present in the same section. Within the one adrenal gland, we found that not all the cells were active, and that those that were were usually situated in groups. This is unlikely to be due to lack of penetration of substrate because the groups of active cells could be found at any level within a given thick section.



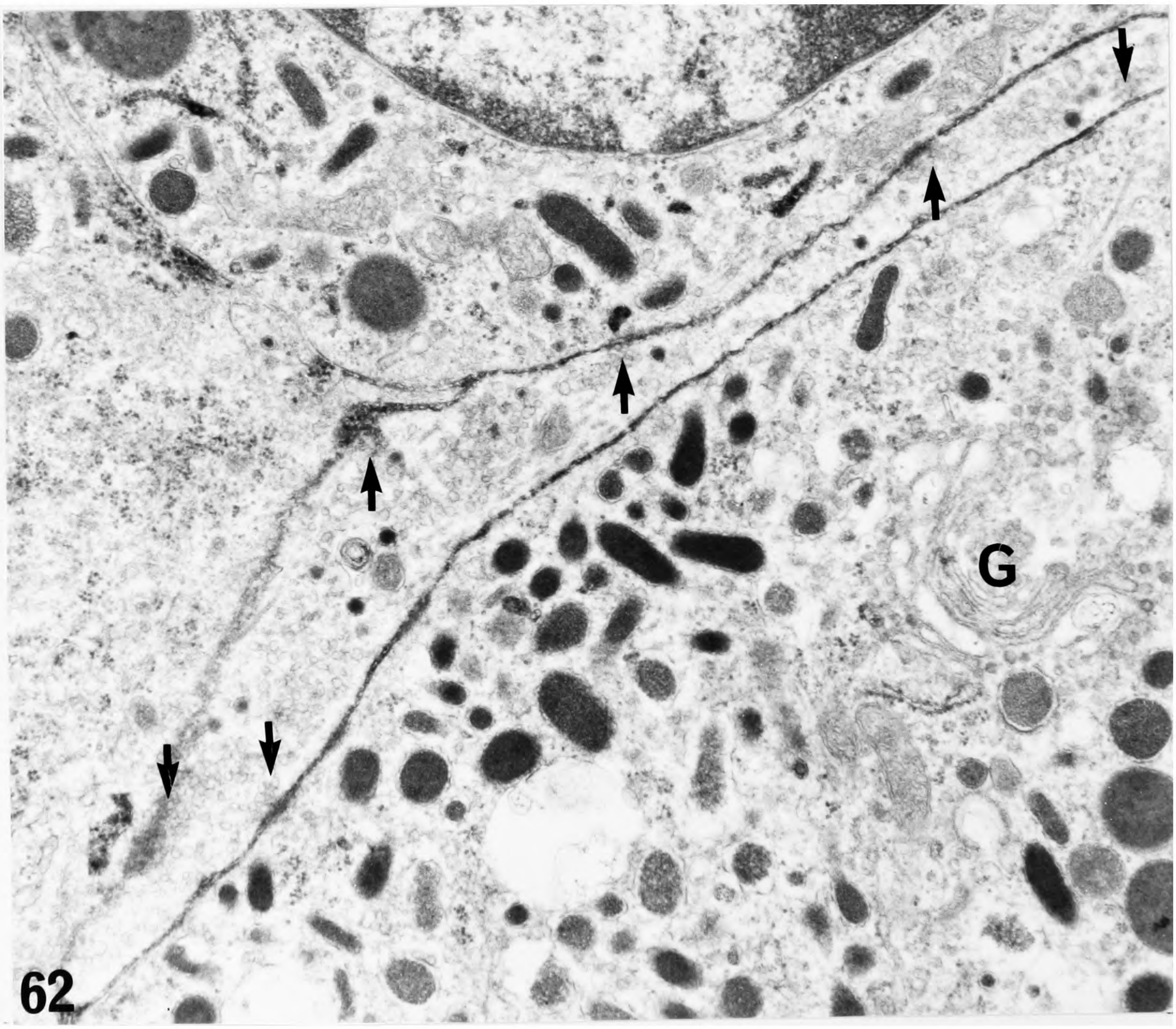


Fig. 61 : Preterminal axon between chromaffin cells. Mag. X 56,000.
Fig. 62 : AChE reaction in longitudinal section of nerve terminal running between three adrenaline-containing cells. Synapses shown by arrows, Golgi (G) negative, activity only between cells and in ER. Mag. X 23,000.

Activity can also be demonstrated between adjacent chromaffin cells, but this is only found when either or both of the cells show intracellular activity. Only very rarely can activity be shown within the Golgi system.

Nerve terminals can also be shown to have AChE activity around them. As shown in Fig. 61, however, none of this activity is within the nerve, it is all surrounding the unmyelinated preterminal axon shown in this micrograph. The end-product is localized between the plasma membranes of the two cells and this activity is continuous with that which is between the two chromaffin cells (Fig. 61). In those cases where the chromaffin cells show intercellular activity the continuity is always seen, but since, when there is no activity between the chromaffin cells, the axon is still surrounded by end-product, we suggest that this rather rare observation indicates that at least some of the activity is associated with the axon itself. Synaptic areas of the nerve terminals are shown in Fig. 62. The entire terminal is surrounded by reaction product and there is also some activity in the ER of a chromaffin cell (arrow). The synaptic areas as indicated by the vesicle clusters (arrows) do not show an AChE distribution which is significantly different from that found around the rest of the terminal. The axoplasm and the Golgi apparatus of the chromaffin cell (G) are both unreactive.

The findings we have made with this technique can be summarized as follows: (i) AChE is present in non-terminal nerve axons within the medulla, both intra-axonally in elements of the SER and on the axonal plasma membrane; (ii) the enzyme is also present within chromaffin cells, intracellularly in the ER and, probably, on the plasma membrane of the cells; (iii) of the two types of chromaffin cells, AChE is more characteristic of the adrenaline-containing cell; (iv) activity is only observable between chromaffin cells if either or both of the cells have activity within their ER; (v) the Golgi system is very rarely active with respect to AChE activity, and when it is, the activity is very weak; (vi) activity has never been observed in chromaffin granules or in any other discrete storage organelle; (vii) while nerve axons always show activity between the axonal and Schwann cell plasma membranes, this activity does not always seem to be in the form of one continuous zone of activity around the entire axon; (viii) we have never seen intra-axonal AChE activity in nerve terminals, even though there is always activity outside the ending.

DISCUSSION

From the earliest studies using light microscopic cytochemical techniques, together with surgical denervation experiments, it was concluded that the bulk of the AChE activity in the adrenal glands of various species was associated with the nerves innervating them (Koelle, 1951; Coupland and Holmes, 1958; Eränkö et al., 1959; Eränkö, 1967). This has never been in dispute, but the presence of AChE in the chromaffin cells is much more controversial. Koelle (1950) first reported that cat chromaffin cells contained cholinesterases, but later (Koelle, 1951) attributed the staining to a diffusion artifact. In cat, rabbit and rat adrenal glands the reaction in the chromaffin cells has also been attributed to a diffusion of the end-product (Coupland and Holmes, 1958) whereas in the hamster glands it was concluded that the cells did contain the enzyme (Eränkö et al., 1962). As mentioned in Chapter 1, the introduction of the electron microscope into the field has not resolved the problem of whether rat chromaffin cells, the only species to have been studied at this level, contain AChE (Lewis and Shute, 1969; Palkama, 1967).

The results presented in this Chapter show not only that bovine splanchnic nerve axons contain AChE, but also that the chromaffin cells contain considerable activity. It must be stressed, however, that the results only apply to bovine adrenal glands and, with the evidence mentioned above, but more specifically that of Palkama (1964), it is clear that species differences between adrenal medullae can be vast and that extrapolations from one species to another should be made only with extreme caution. We are indeed fortunate in this respect to have been able to do as much of the work as we needed (i.e. that described in Chapters 2, 3, 4 and 5) on the one animal.

There is no ready answer to the question of the seemingly different amounts of AChE in adrenaline- versus noradrenaline-containing cells. These results confirm earlier light microscopic observations that the two types of cell differ in some respects (location, vascularization, cell orientation and shape; Palkama, 1964; Hopwood, 1970) but structurally there were no observable differences. Since there is no reason to suspect that the differences in apparent enzyme content are due to technical faults in the procedure, the problem must be left unresolved.

To ask once more the question, is there an ultrastructural basis for the release of AChE in either the axons or the chromaffin cells? We are now much nearer to an answer, but before it can be properly given, the distribution of AChE which was found both within and near the cells must be closely analyzed.

The intramedullary axons can be characterized by their having a dual localization of AChE, one at the axon surface and another within the axonal SER, a pattern which seems to be a general characteristic of all nerves exhibiting AChE activity (see Chapter 1).

Kasa (1968) has shown that, in these typical cholinergic nerves, AChE-rich SER accumulates when the axons are ligated, a result which has also been found for the ligated calf splanchnic nerves (Somogyi, unpublished observations). But more importantly for this discussion, Kasa (1968) was able to observe an apparent connection between the SER tubules and the axolemma. He was able to distinguish three different types of connection, thus: (i) active tubule with inactive axolemma; (ii) inactive tubule with active axolemma and (iii) active tubule with active axolemma. In these experiments with the splanchnic axons we were only ever able to see an apparent join between an active tubule and an active axolemma (Fig.51) although it could, perhaps, be argued that the "connection" shown in Fig. 49 is by an inactive tubule. If these results mean that the AChE on the axon's surface is supplied from within the axon, via the endoplasmic reticulum, then there are many points opened for discussion.

One of these points, and the most relevant to this thesis, is the possibility that the axolemmal AChE might be supplied from within the axon by using the SER as first a transport and then a "release" organelle. If this were the case, more information about the process could be obtained by two means. The first would be morphological in that we could expect to see a decreasing proximo-distal gradient of SER; but unfortunately, there are too few active cisternae to allow a determination of whether such a gradient does occur. There does not seem to be any evidence in the literature for such a proposal either. However, it is noteworthy that we have never been able to show any AChE in any structure anywhere within the nerve terminals. Thus, we are obliged to conclude that, either the AChE is so rapidly removed from the terminal that none of it is ever stored there or, alternatively, that the enzyme never arrives there in the first place.

There is biochemical evidence that suggests the occurrence of decreasing proximo-distal gradients along axons of, amongst other things, AChE. Lubinska, Niemierko, Oderfeld-Nowak and Swarc (1963) analyzed the AChE level at different points along many nerve fibres of the dog, and they were able to show a linear decrease in enzyme activity the further away from the cell bodies that the sections were taken. The highest drop in activity,measured over long distances, but related to 10 mm so as to be able to compare longer and shorter nerves, was 5.7% per cm in the nerve innervating the gastrocnemius muscle; the lowest decrease was 0.4% per cm in the saphenous. Since neither the total protein concentration nor the fibre diameters, in unbranched axons, changed over the distances which were used for AChE estimation, it was concluded that the changes in AChE levels represented true changes in AChE concentration (Lubinska et al., 1963). Lubinska, Niemierko and Oderfeld-Nowak (1961) have also shown that it is possible to alter the shape of the gradient by treatment of the other contra-lateral (phrenic) nerve. Thus, the decreasing proximo-distal gradient in one nerve disappeared a few days after surgically sectioning the other and was replaced with a peak of activity in the middle of the nerve. Lubinska et al. (1961) raised the possibility that the change might be due to either an increased requirement for AChE due to the increased functional load, or to contralateral neurons affecting each others ability to synthesize proteins, or to both these reasons. But why, it could well be asked, would more of a supposedly membrane-bound enzyme, known not only to be present in vastly excessive concentrations at nerve terminals, but also to be extraordinarily efficient at its job (see Appendix), be required because of an increased load on the innervated tissue? The answer may well be that it is necessary because the enzyme is released from the nerve (Chapter 4) and that more is released during the increased "functional load" and hence more is required at the terminals. It is well known that other proteins, such as DBH and tyrosine hydroxylase, which are, respectively, released and regulatory in the amount of transmitter released from adrenergic nerves, both show increased concentrations in hyperactive neurons (see e.g. Thoenen, 1972). It would obviously be of interest to determine whether factors which can induce an increase in DBH and tyrosine hydroxylase levels in ganglia (cold stress, reserpine, swimming) can also induce a change in AChE levels, pre- and/or postsynaptically. It is hoped to commence experiments such as these in the near future.

If our results really mean that there is no AChE inside the splanchnic nerve terminals, and as has been mentioned, all negative cytochemical experiments must be treated with caution, then it seems unlikely that the axon is the source of the released AChE, i.e. that which appears in the perfusate. Even with due caution, we feel that the results can be interpreted in this way. It is not as though we were unable to detect any AChE in those sections which contain nerve terminals - they are cytochemically identical in every respect, containing both intra- and extra-cellular activity, to other sections. The consistent lack of demonstrable activity within the terminals cannot, therefore, be put down to purely technical problems. Of the two reasons which were put forward earlier to explain this dearth of enzyme the more reasonable would seem to be that none, or only very little, of the enzyme actually arrives at the nerve terminal. If the other alternative were correct, the terminal could be expected to show some intra-axonal activity, since it does not seem reasonable to suggest that the nerve could actually remove AChE more rapidly than it arrives. It is also noteworthy that the apical pole of the chromaffin cell, where most of the catecholamines and other proteins are released, is very poor in AChE-rich ER. A third, but highly unlikely alternative, for which there is no evidence at all, is that the AChE within the terminal is inhibited by some compound with which it comes into contact only in the synaptic region.

As to the reason why it seems unlikely that the axons are the source of the perfusate AChE, it is based mainly on the temporal relationship between the enzyme and the catecholamines as they appear in the solution. As shown in Fig. 31, the enzyme activity and amines appear virtually together. If the AChE was released from the axons, and there is a structural basis for suggesting this possibility (see e.g. Fig. 51; Kasa, 1968), then it would be expected that a macromolecule of its size would be so limited in its diffusion by the Schwann cell or myelin sheath that it would only slowly appear in the perfusate. Thus it is probable that the perfusate enzyme is derived from a source other than the nerve, although this is not meant to imply that the nerve is totally unable to secrete AChE.

The other major AChE-containing element in the adrenal medulla is the chromaffin cell, and it is apparent that the same ultra-structural basis for release is present in these cells as is present in the axon, i.e. tubules rich in AChE. Furthermore, it seems very likely that the AChE associated with the plasma membranes (the end-product between adjacent chromaffin cells?) is synthesized within one or both of the cells. AChE between the cells has never been observed unless at least one of them contains AChE-rich ER. The mechanism by which the intracellular enzyme gets between the cells could be as shown in Fig. 57, where there is an apparent joining of the membranes of the ER with the plasma membrane of the cell. These joins could represent temporary connections between the extracellular space and that within the tubule which, if the AChE is soluble within the tubule, could result in enzyme diffusing or being pumped outside the cell. Some of this enzyme could then diffuse through the intercellular space (with the aid of canaliculi, see Benedeczky, 1967; Grynszpan-Winograd, 1971) and eventually through the fenestrated endothelial cells. All of this seems reasonable, especially since there is no doubt that macromolecules can move in the opposite direction (see Holtzman and Dominitz, 1968).

Thus, there are reasons for supposing that AChE could be released from both the splanchnic axons and from the chromaffin cells that they innervate. But, as discussed earlier, the temporal relationship with catecholamines suggests that, at least in the isolated and perfused adrenal preparation, the chromaffin cells are the primary source of the released enzyme.

Chromaffin cells are well known secreting cells with catecholamines and various proteins, chiefly the chromogranins from chromaffin granules, are found in perfusates of isolated glands (see Smith and Winkler, 1972). It now seems reasonable to give this cell another property, that of protein secretion direct from the endoplasmic reticulum. Both types of secretion require a depolarization of the cell and are dependent upon the presence of Ca^{2+} ions; but there the similarities seem to end. The chromaffin granules are formed in the Golgi apparatus, a system which rarely shows any AChE activity and consequently this enzyme is never found in the chromaffin granules. Most of the chromaffin granules are in the supranuclear cytoplasm at the apical pole of the cell, whereas the AChE-rich endoplasmic reticulum is around the nucleus and along the lateral plasma membranes, i.e. close to the incoming nerve fibre. The chromaffin cell is, therefore, specialized not only in the type of protein that it secretes, but also in the spatial arrangement of the different secretory proteins within the cell. This is in addition, of course, to these proteins having quite distinct storage organelles.

Once again attention should be drawn to the similarities between AChE and the acid hydrolases. It has already been mentioned that these two types of enzyme might both be contained within the ER of the axons and both classes of protein are released from the adrenal gland by the same secretagogues. Whether or not the release of the acid hydrolases from the stimulated adrenal glands is another example of protein secretion from the ER, either from that within the nerve or that within the chromaffin cell, or whether it is a reflection of exocytosis direct from the lysosomes, is not known. Some clarification of this problem could perhaps be gained if it was known what relative proportions of the adrenal medullary acid hydrolases were localized in the nerve axons and the chromaffin cells, and of these values, what proportion was within the ER compared with the lysosomes of the two types of cell. Quantitative experiments of this type await much greater refinement in both centrifugal and cytochemical techniques.

The results reported in this Chapter also help toward our understanding of the seemingly insoluble problem of why adrenal glands whose splanchnic axons had been cut or ligated contained at least as much AChE as their contralateral controls. Part of the reason can now be attributed to the fact that the chromaffin cells, by far the largest single component in the adrenal medulla (see Coupland, 1965) are rich in AChE. From this observation and by using the quantitative data presented in Chapter 2, it can be calculated that the amount of AChE in the medulla which is derived from the nerve is likely to be only a small proportion of the total enzyme activity in the tissue. Thus, if we assume that there is no enrichment of AChE in the nerve terminals, an assumption which seems reasonable given the evidence discussed above, and that perhaps as much as 1% of the weight of the medulla is due to the presence of the nerve within the tissue, then the following calculation can be made. Since the average weight of the medullae used in these experiments was to the order of 1 g, then each would have contained about 10 mg of nerve tissue. As shown in Table 3, there are 1270 mU of AChE activity per gram of axons, or 12.7 mU in 10 mg. Table 3 also shows that the adrenal medullae of these animals contained 1200 mU of AChE per gram. The nerve, therefore, could be responsible for as little as 1.0% of the AChE activity in the adrenal medulla, an observation which offers an explanation for our inability to measure significant decreases in either AChE content or output from denervated glands.

Where then does all this information leave our working hypothesis? It is now in need of so radical a change that it should be completely rewritten. Thus: soluble and membranous AChE (but only isoenzymes 5 and 6) are both in elements of rapidly migrating axonal SER. Most of this enzyme is destined for the axon surface and only very little ever reaches the nerve terminals. In the postsynaptic cell there are extensive ER systems which are rich in AChE. These systems probably contain, in addition to a proportion of the $AChE_6$, all of the soluble isoenzymes, most of which are precursors of $AChE_5$ (an isoenzyme which could itself be the precursor of $AChE_6$). The remainder of the $AChE_6$, which is between the cells or on their plasma membranes, is supplied to this site either by an incorporation of the ER, whose membranes contain $AChE_6$, into the plasma membrane, or by a secretion of $AChE_5$ which, when bound by cell surface "receptor" sites, behaves biochemically and electrophoretically as $AChE_6$. Excess $AChE_5$ is that which is recovered in the perfusate.

A good deal of this latter paragraph is speculation, but it is presented as a working hypothesis. Some evidence for this hypothesis was obtained and the results will be presented in Chapter 7. But first I will describe experiments which were carried out in order to see whether extracellular fluid levels of AChE could be altered under more physiological conditions than those which were used for the _in vitro_ experiments described in Chapter 4. If our working hypothesis is correct, then all cholinergic cells, both neuronal and cholinoceptive, should have in common the property of AChE secretion. The results of experiments designed to examine this possibility are presented in the next Chapter.

CHAPTER 6

EXTRACELLULAR FLUID LEVELS OF ACETYLCHOLINESTERASE

INTRODUCTION

The experiments described in this Chapter fall into two distinct sections according to the two extracellular fluids studied. The first part contains the results of experiments in which the AChE content of bovine serum was analyzed, while in the second rabbit cerebrospinal fluid (CSF) was the fluid examined.

Our interest in bovine serum arose partly out of the results described in Chapter 4, but also because we had hoped, in other experiments, to stimulate the adrenal glands *in situ* via their splanchnic innervation, and to measure any consequent changes in the AChE level of the blood (plasma) as it left the gland. Before these latter experiments were started, blood samples were collected from various calves and the AChE content of the serum was estimated. As mentioned briefly in Chapter 4, there was considerable variation in the AChE levels of the serum; but a closer analysis with more data showed that the amount of AChE in the serum was influenced by the age of the animal. It is the results of these more detailed studies which are presented in the first part of this Chapter.

We were also interested in CSF as an extracellular fluid because it was known to contain AChE (see Davson, 1970). The experiments described in the second part of this Chapter, which were done with the collaboration of Mrs. Sally Goodman, were designed to try and alter the concentration of the AChE in the CSF by using physiological stimuli known to release acetylcholine from structures within the CNS. The experiments were based upon the premise that, if our working hypothesis is correct, all cholinergic synapses and structures share an ability to secrete AChE. The results of these experiments are discussed in the second part of this Chapter.

METHODS

Calf and sheep sera: These samples were prepared for us in Cambridge, as follows: blood was collected from the jugular veins of young and adult animals and allowed to clot. The serum was separated from the cells by centrifugation and was finally stored deep-frozen. The samples were collected and brought to our laboratory where they were thawed, centrifuged and assayed for AChE activity. Samples were also obtained, via indwelling cannulae, from bovine and sheep foetuses.

Rabbit CSF: The rabbits, all weighing between 2.5-3.0 kg, were anaesthetized with Urethane (25% w/v; i.v.; approximately 5 ml/kg) and their cisternae magnae were surgically exposed. Preliminary experiments were designed to determine the volume of CSF which could be removed before the animal showed obvious signs of ill-effect, and also to determine whether the removal of large volumes altered the AChE and protein levels of the fluid. For these experiments, a needle connected to a length of plastic tubing was inserted through the dura into the cisterna magna and the CSF allowed to drain out. The rate of drainage was roughly controlled by altering the height of the end of the tubing relative to the cisterna magna. Thus, if the tubing was lower, then the outflow was rapid, but this could be markedly reduced by raising the tubing. In this way we could control for experiments of long or short duration.

To sample the CSF during experiments either a needle connected to a syringe was inserted into the cisterna magna and a small volume of CSF (200-300 μl) was removed with gentle suction, or a needle with tubing attached was left in the cisterna magna and the fluid was removed at about 10 μl/min, with the aid of a pump.

To try and induce a change in the AChE level of the CSF, we stimulated either the sciatic or saphenous nerves. This was achieved by first exposing the nerves in the mid-thigh region so that, when necessary, the nerve could be stimulated (at 10 Hz and 4 V) for periods up to 5 min (see below). Blood pressure recordings and blood samples were obtained through cannulae in the femoral artery and vein respectively. When ACh was used to mimic the drop in blood pressure caused by nerve stimulation, 30 μg of base were injected at the same time intervals as the nerve had been stimulated, i.e. every second minute during 10 min.

The brains of some rabbits were removed and separated into several fairly gross anatomical regions. Thus the total and soluble AChE contents of whole brains, cortices, caudate nuclei, cerebella and olfactory lobes were estimated by analyzing and centrifuging 0.3 M sucrose homogenates of these tissues.

Electrophoresis after overnight dialysis against 5 mM phosphate buffer pH 7.0, AChE estimation, protein and LDH determinations were all carried out as described in other Chapters. Estimation of K^+ concentrations in the CSF were made using an Atomic Absorption Spectrometer (Unicam; SP 90).

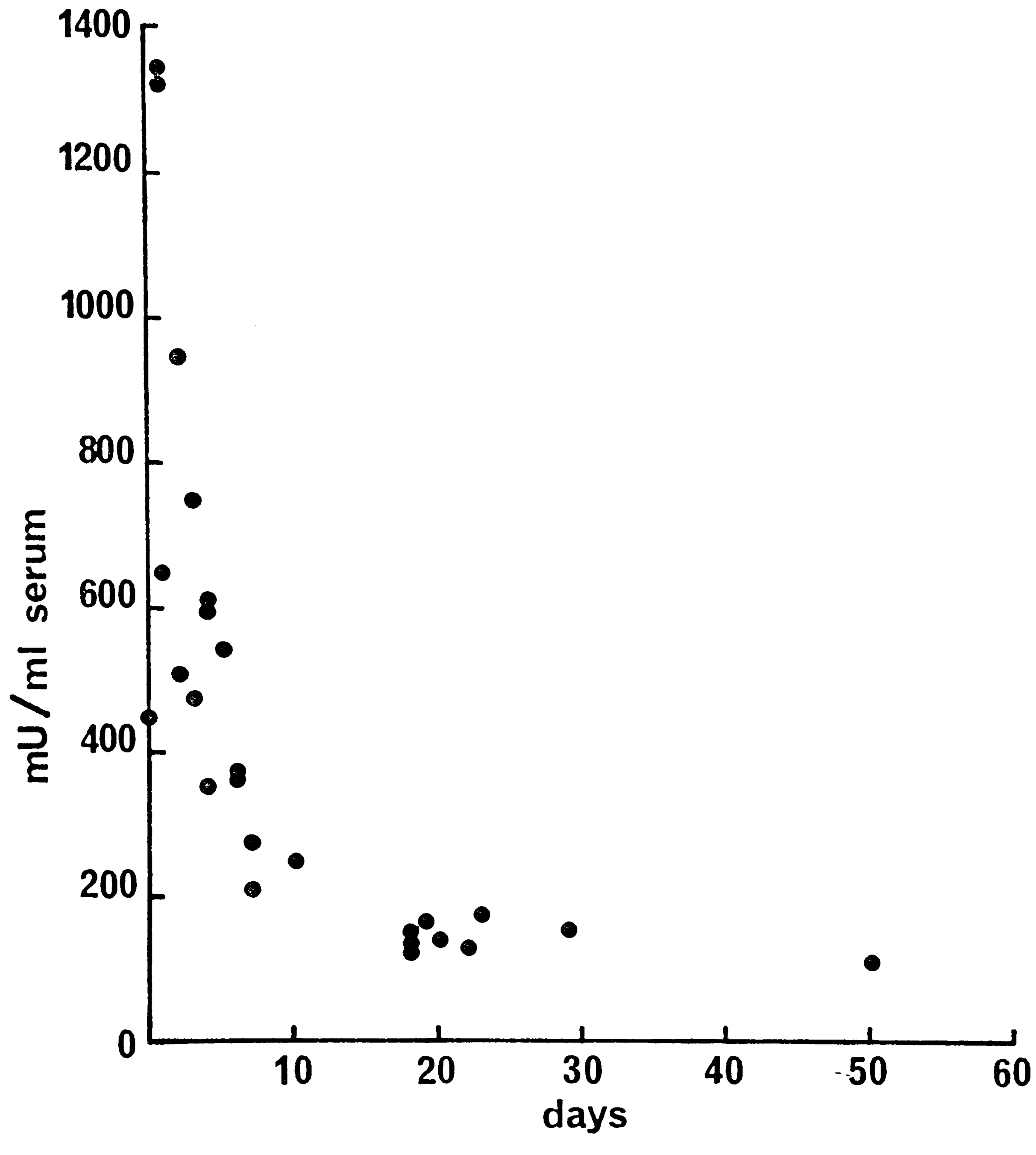


Fig. 63

Changes in the serum AChE levels of different calves at various times after birth.

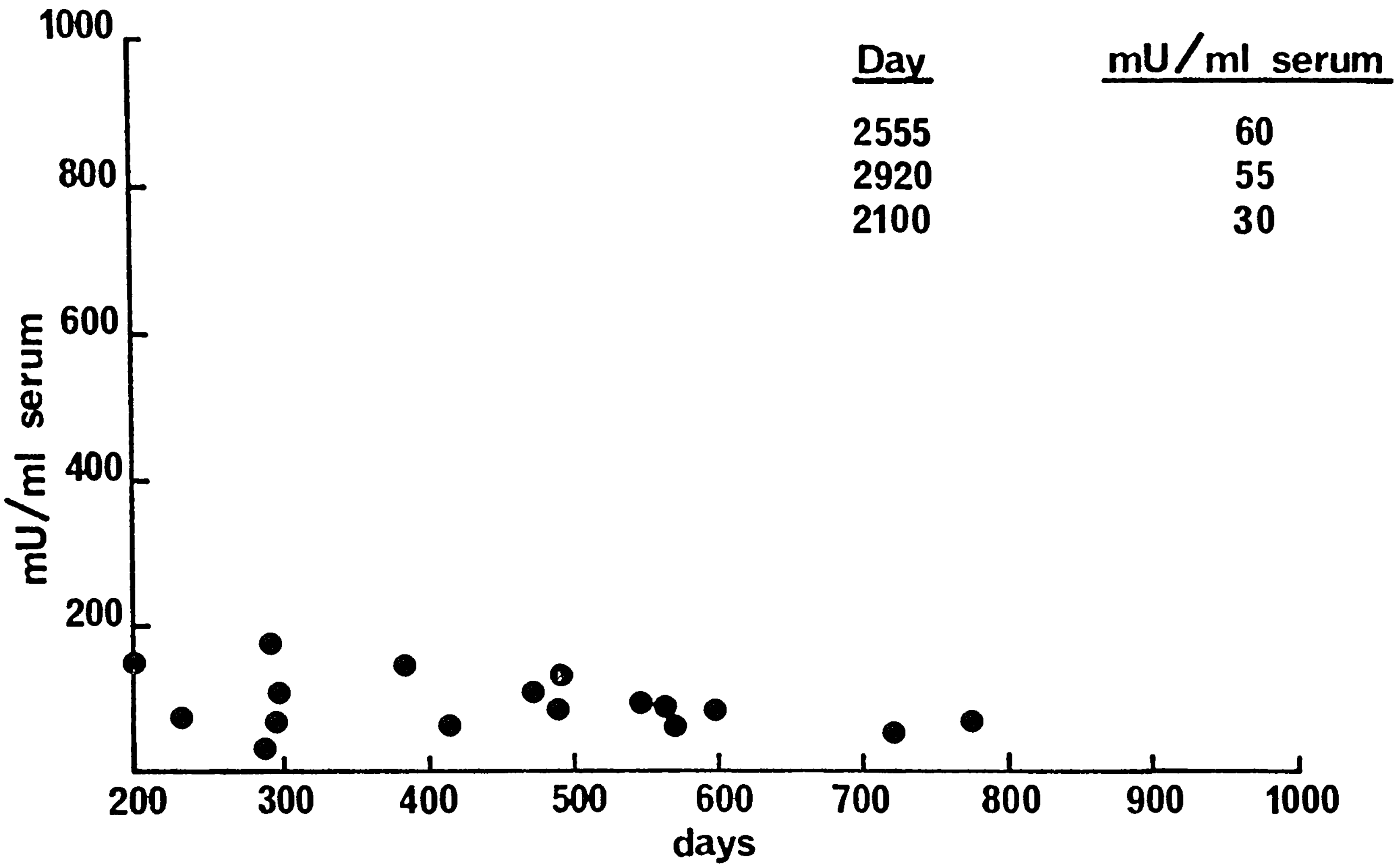


Fig. 64

Changes in the serum AChE content of different calves at various times after birth. The inset shows the AChE concentration in the serum of very old animals.

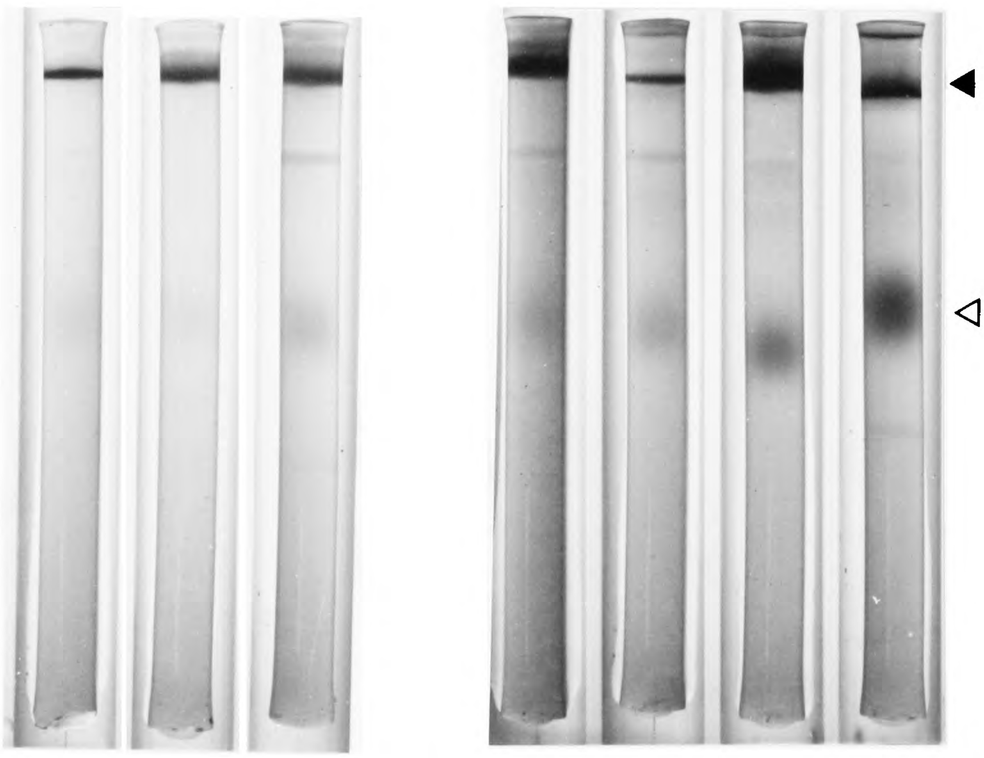


Fig. 65

Electrophoretic analysis of the AChE in the sera of calves of different ages. The samples used for these gels were taken from animals aged (reading left to right): 30 min, 7, 18, 29 and 50 days, 2 and 6 years. Each sample was diluted so that the same amount of AChE activity was applied to each gel. The band marked (◁) is not due to AChE activity but to material bound to serum albumin. Only the activity in the zone marked (◀) was inhibited by 10^{-4}M BW 284C51.

RESULTS

Calf and sheep sera.

The first samples analyzed were those from different calves of various ages and the results of these experiments are shown in Figs. 63 and 64. Soon after birth, there is a fairly rapid decline in the AChE concentration in serum (Fig. 63) and this continues, at a slower rate, until the animals are aged about 250 days (Fig. 64). The inset in Fig. 64 gives the AChE concentration in the serum of much older animals. From these results, which show considerably lower serum AChE levels in the older animals, it seems likely that there is a slow steady decrease in the AChE concentration in the serum, once the initial rapid phase is over. The lowest AChE levels we have ever measured were in samples from cows aged 15 years (30 mU/ml).

The results of an electrophoretic analysis of the AChE in the serum from calves of various ages is shown in Fig. 65. Some explanation of these results is necessary because the pattern obtained and shown in this Figure gives a false impression of the AChE in the samples. This is chiefly due to the fact that the same amount of AChE was run on each of these gels. For this purpose, it was necessary to greatly dilute the serum from the younger but not from the older animals, in order to keep the actual amount of AChE activity within the range given in the standard electrophoretic conditions in Chapter 2. There was, therefore, a vastly different amount of protein on each of these gels. An examination of each gel before staining showed that the fastest band on the Figure was not due to AChE activity, but rather to the tracker dye, bromophenol blue, bound to serum albumin. The next fastest, which was yellow in

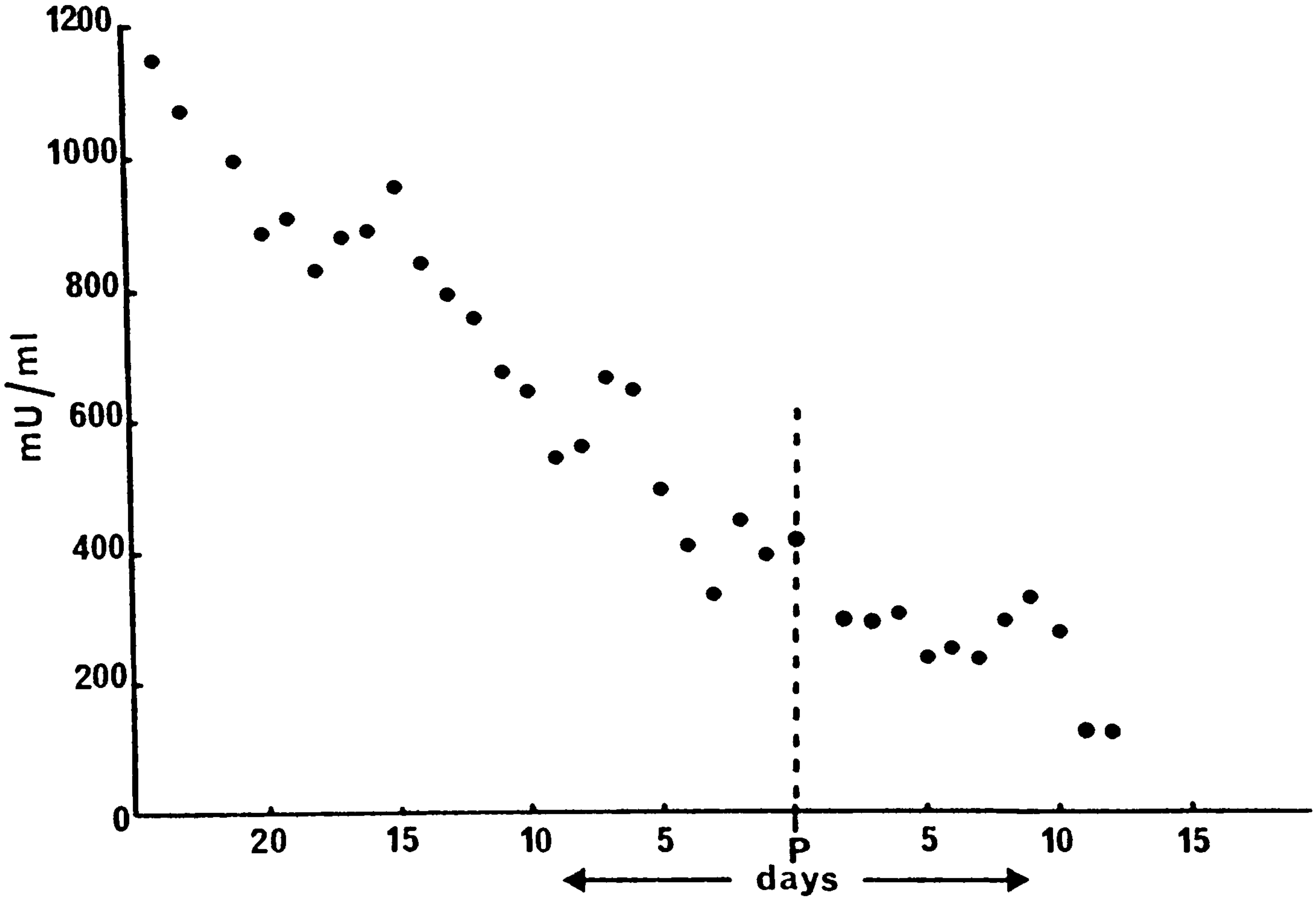


Fig. 66

Changes in the AChE content of foetal and neonatal calf serum (calf 37).

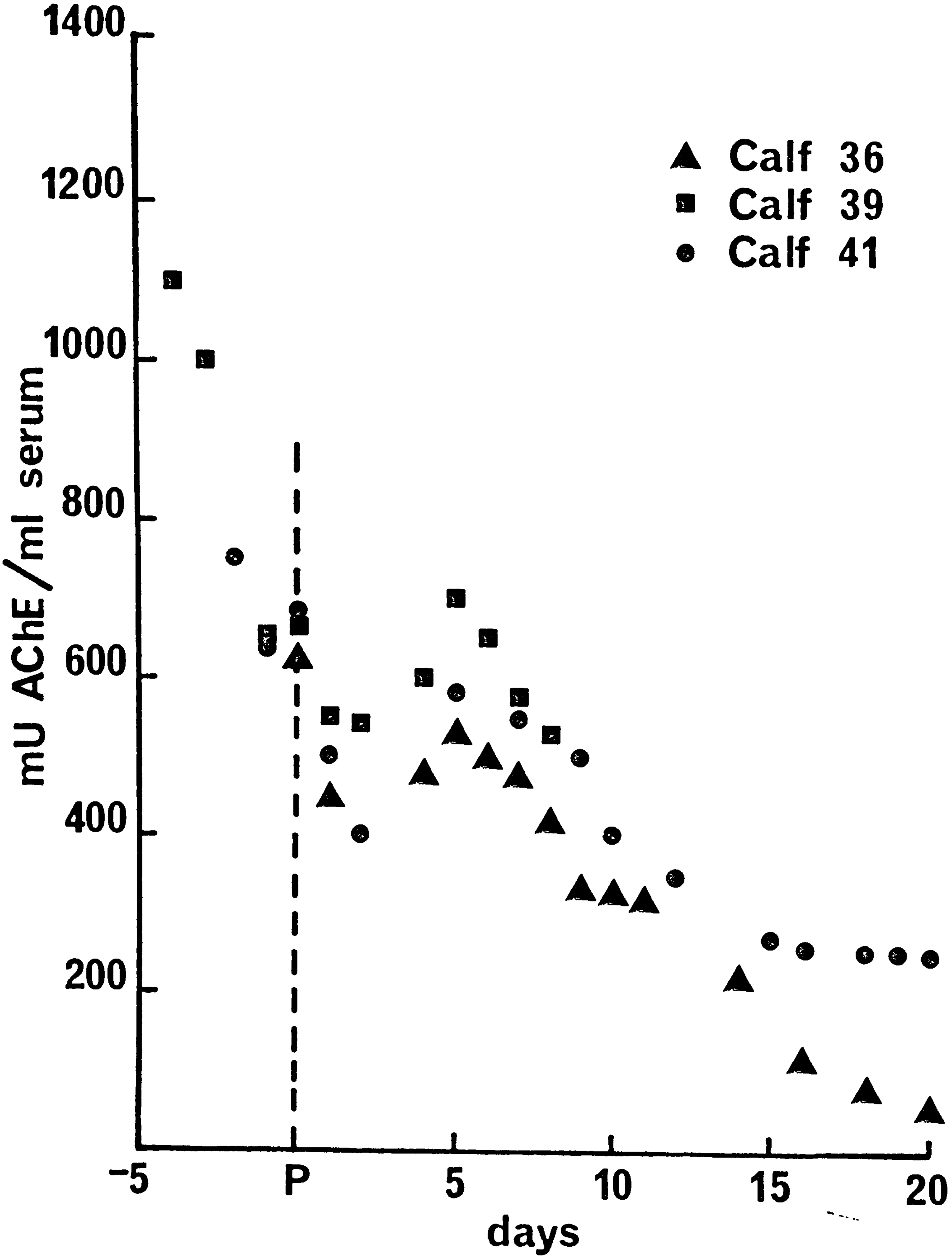


Fig. 67

Pre- and post-natal changes in the AChE concentration of bovine serum. The three animals whose values are shown here were all prematurely but normally born.

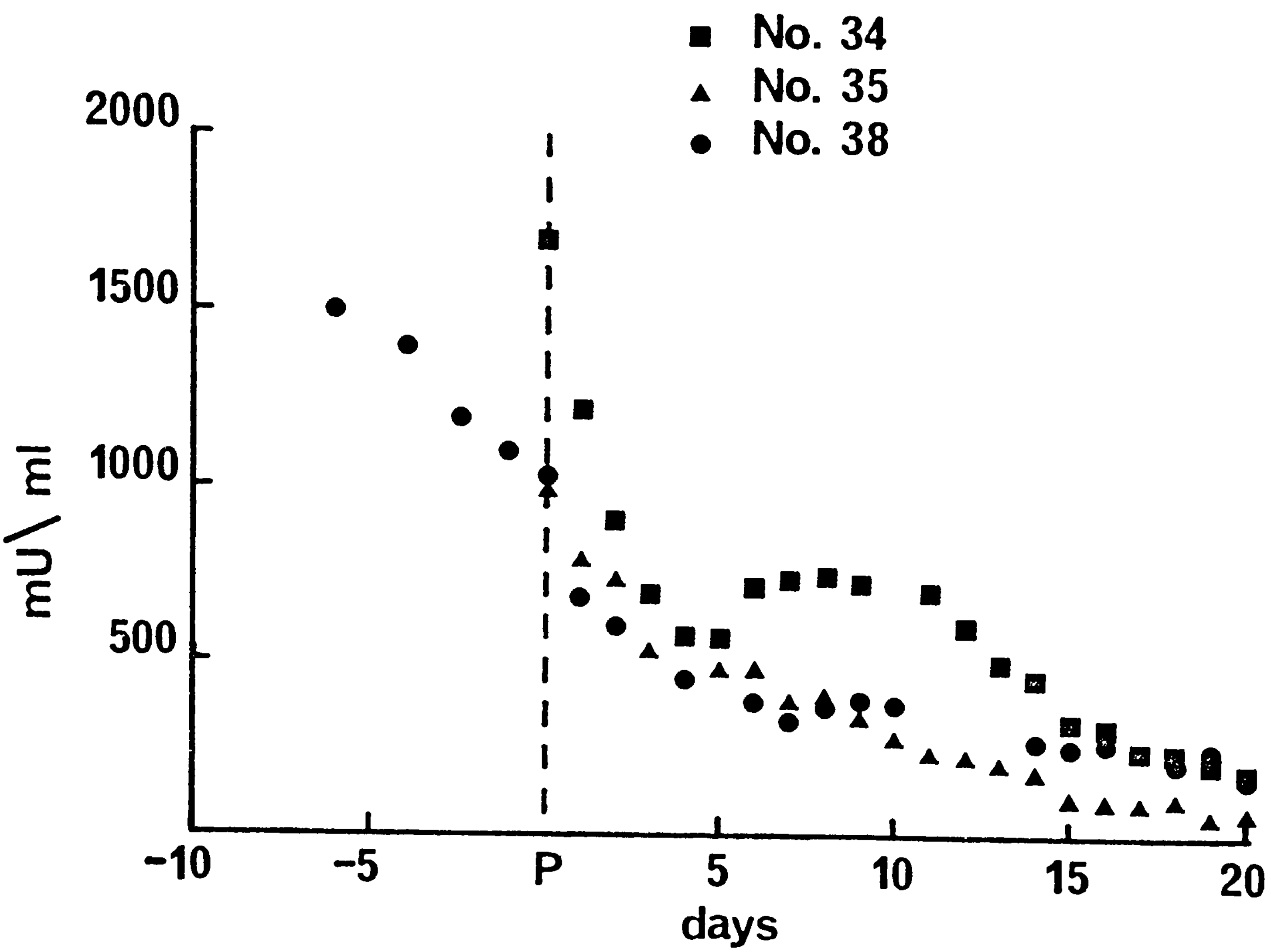


Fig. 68

The changes in the serum AChE concentration in different calves. The birth of all these calves was induced by the administration of dexamethasone to either the mother or the foetus.

<u>TABLE 5</u>

The serum AChE concentrations of different calves at birth.

Calf No	Gestation time (days)	Serum AChE (mU/ml)	Induced?
37	285	420	No
un-numbered	284	450	No
36	261	690	No
39	267	670	No
41	266	680	No
34	262	1700	Yes; Dexamethasone to mother
35	263	1000	Yes; Dexamethasone to foetus
38	257	1020	Yes; Dexamethasone to mother

colour, was haemoglobin, some of which is inevitably present in samples such as these. Since BW 284C51 inhibited only the marked band (arrow), it can be concluded that the changes in AChE activity during aging are a reflection of changes in the concentration of one isoenzyme (according to electrophoretic criteria also $AChE_5$) and not of a change in isoenzyme pattern.

At this stage it became possible for us to analyze the sera of both pre- and neonatal calves. These experiments yielded two different types of results: one which is analogous to the results mentioned above, and another which is noteworthy but not wholly related to the problem at hand. Some of the results of the first type are shown in Fig. 66. As can be seen in this Figure, the results obtained using calves of different ages are mere extensions of a phenomenon which commences during the later developmental stages of the foetus. Thus, 26 days before parturition (which, as shown in Table 5, occurred at full term, 285 days) a decrease in the AChE concentration of the serum starts and continues up to and beyond birth. The level of 400 mU/ml at parturition is still 8-fold higher than most adult AChE levels, although, as shown in Table 5, it is lower than in animals which were born prematurely. The results given in Fig. 67 were obtained from three animals which were born 18-24 days prematurely. The most striking difference between Figs. 66 and 67 is in the serum AChE concentration at birth, which is higher in premature calves. If dexamethasone is given to either the mother or the foetus to induce parturition after roughly the same period of gestation, then the AChE level is even higher. (Fig. 68). Thus, the serum AChE concentration at parturition falls into three categories: high if the birth is premature <u>and</u> induced, medium if premature but normal, and relatively low if the birth

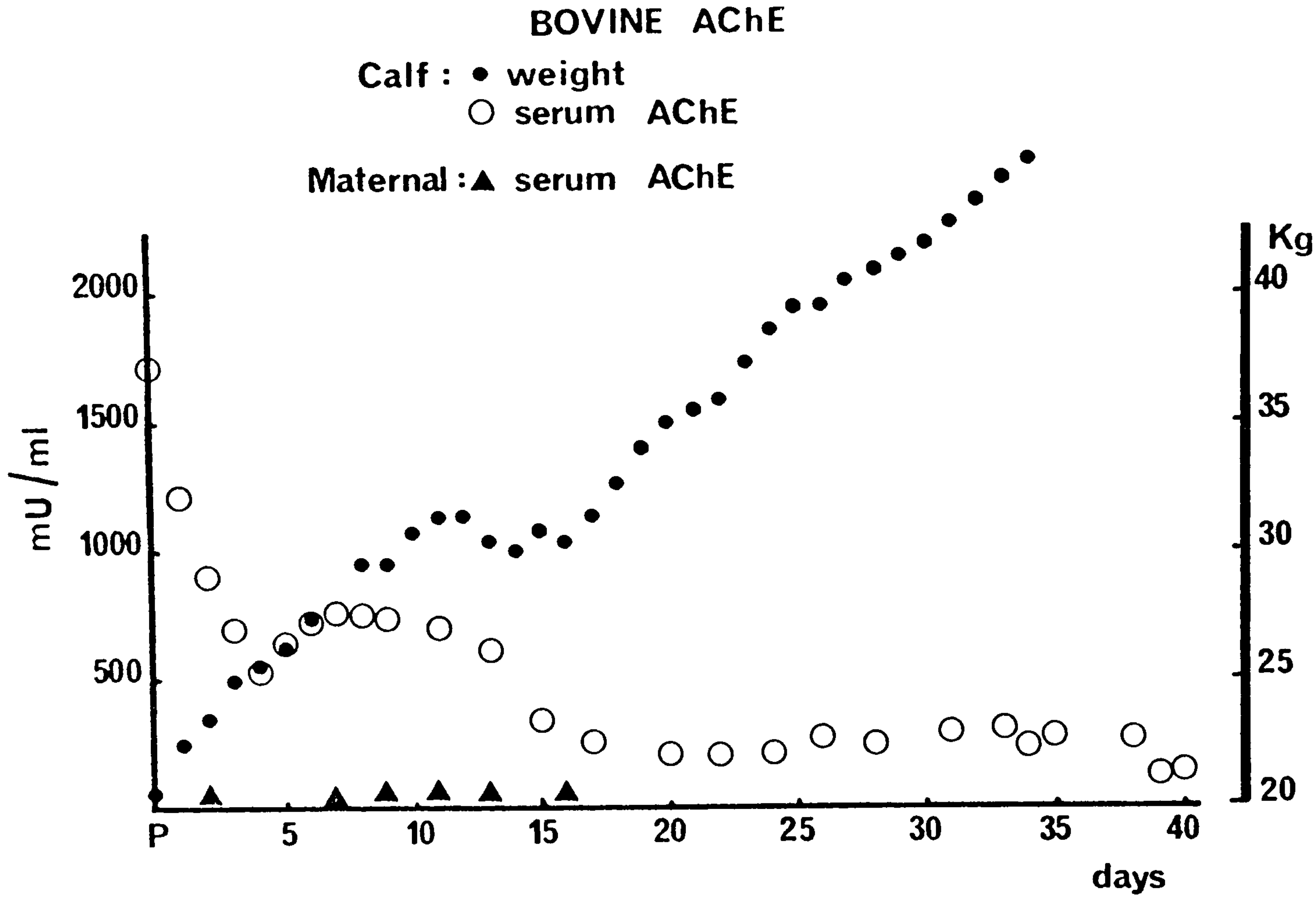


Fig. 69

Changes in the serum AChE and weight of calf 34 during its first 40 days. "P" represents the day of parturition.

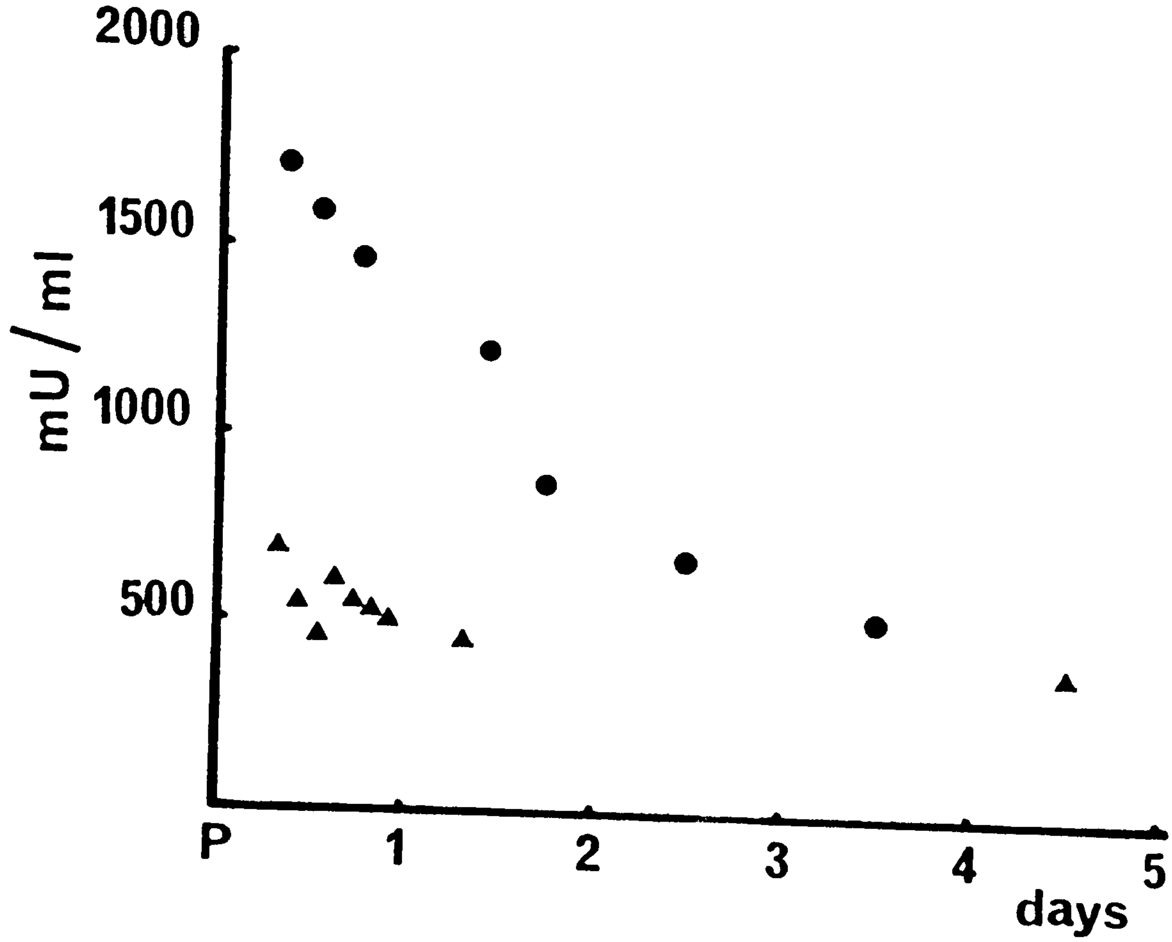


Fig. 70

Changes in the AChE concentration of bovine serum over short time intervals. Calf 34 (●); calf 41 (▲).

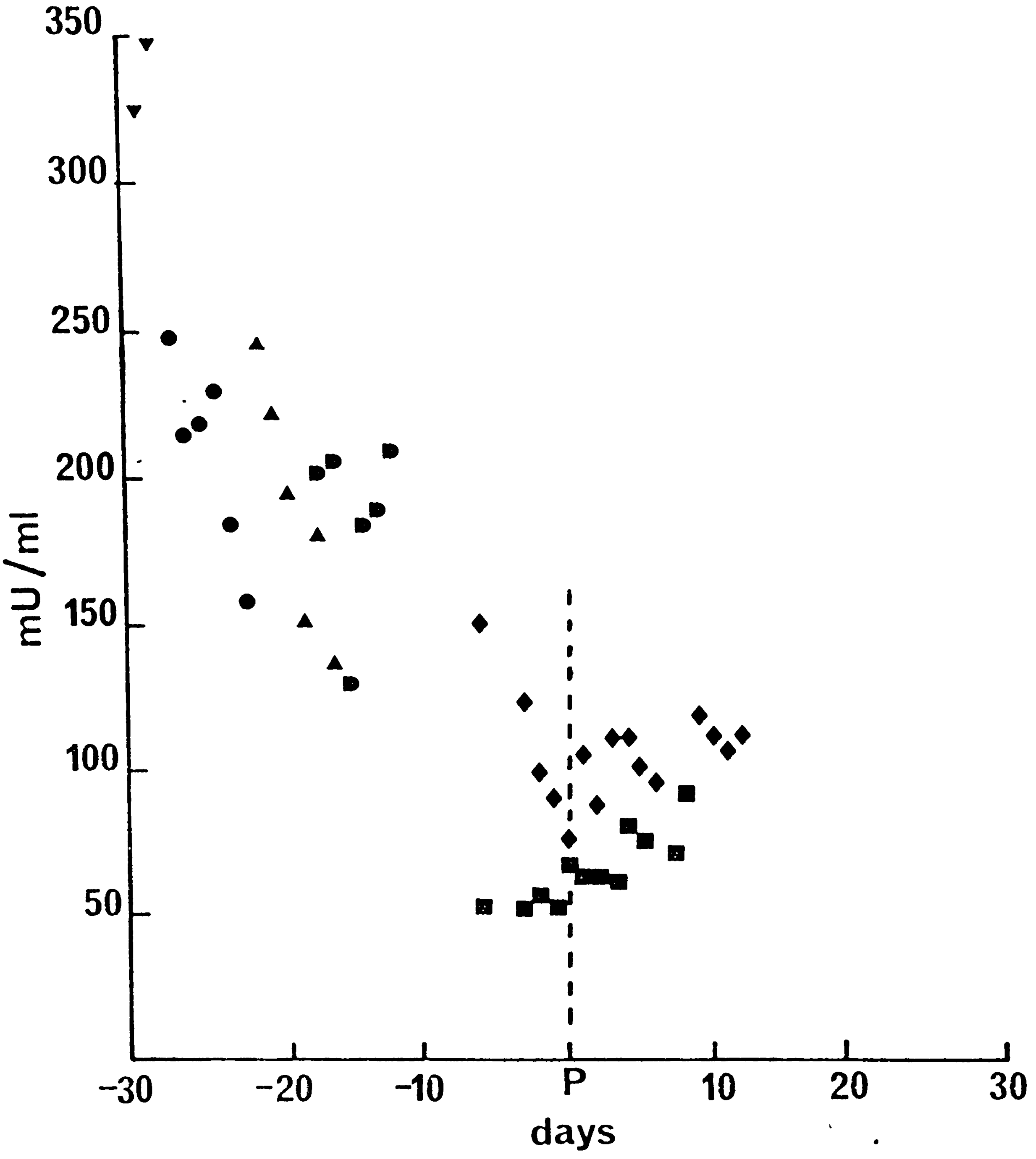


Fig. 71

The AChE concentration in various lamb and sheep sera. The only adult values are those represented by ■ which was the mother of the lamb ◆.

occurs normally and after a full gestational period (Table 5).

In many animals there is an increase in the AChE level of the serum between about 4 and 6 days after birth (Figs. 67, 68). This varies in extent from very slight (calves 35 and 38) through marked, to very large and prolonged (calf 34). It is at about this age that calves frequently suffer from infantile diarrhea and in one of these calves the illness was very marked (calf 34). As shown in Fig. 69, the increase in the AChE content of the serum precedes the weight loss due to the sickness by 4 or 5 days; the weight loss continues while the AChE level is high and then, as the animal begins to regain weight, the enzyme level falls again. During this time, the AChE level in the serum of the mother remains as shown, at a steady, low level.

One of the features of the changes in serum levels of AChE is the rate at which they seem to occur. An example of this is given in Fig. 70 which shows the results obtained when the enzyme activity in the sera of calves 34 and 41 (see Table 5) was measured over relatively short time intervals. It can be seen that there are marked changes in the AChE level, both up and down, within the space of twelve hours.

Comline and Silver (1966) have shown major differences in the relative stages of development of the adrenal medullae of sheep and cows at parturition. To complement the information that we had obtained for the calf sera, we also analyzed the AChE activity of sheep serum, foetal, neonatal and adult. As shown in Fig. 71, there are two notable differences between the species: the serum level at birth is much lower in the lamb than in the calf, about 100 mU/ml compared with 450 mU/ml, and the new-born lamb has an AChE level almost the same as that in the adult. Electrophoresis again shows that there is only one serum isoenzyme, irrespective

Fig. 72

The AChE isoenzymes in rabbit CSF (gel 1) and plasma (gel 4). Gels 2 and 3 were obtained by incubating plasma and CSF in the presence of 10^{-4} M BW 284C51.

TABLE 6

The distribution and percentage soluble of AChE in rabbit brain regions.

Brain region	mU AChE/g tissue			
	Total activity	Sedimentable	Soluble	% soluble
Whole brain	10150	9159	988	9.7
Cerebellum	6342	5627	715	11.3
Caudate nucleus	46303	43559	2729	5.9
Olfactory lobe	12990	12130	767	5.9
Cerebral cortex	10538	9382	1156	10.9

The soluble AChE was obtained by centrifuging each homogenate at 100,000 g for 60 min. The percentage of the enzyme which was soluble was calculated by assuming a 100% recovery of the activity after centrifugation. The actual recoveries ranged from 95-100%.

of the age of the animal from which the serum was obtained, but this isoenzyme has not been characterized further.

Rabbit CSF.

The first experiments on this fluid were designed simply to determine whether there was readily demonstrable AChE activity in the CSF of the rabbit. There were appreciable concentrations of AChE in the CSF and electrophoresis revealed that the activity was due to only one form of the enzyme (Fig. 72). Analysis of the plasma enzyme of the rabbit showed that, in common with ovine and bovine sera, the esterase which predominates is AChE. More than 95% of the activity was inhibited by BW 284C51 at a concentration of 1.5×10^{-6}M; the CSF enzyme was completely inactivated by this concentration of inhibitor. The electrophoretic pattern of rabbit plasma AChE is also shown in Fig. 72. Both CSF and plasma have an isoenzyme in common but the plasma has several others in addition, which comprise only a small proportion of the total enzyme activity. The amount of AChE in the plasma was found to be very variable but fell within the range 460 ± 70 mU/ml (mean $\pm$ S.E.M. ; n=6), which with a protein concentration of 65 ± 4 mg/ml (mean $\pm$ S.E.M. ; n=4) meant that the average specific activity of the AChE in rabbit plasma was 7.1 mU/mg.

The next experiments were designed to determine whether the AChE in the CSF was an isoenzymic form which was common to structures and regions within the brain and also whether there was any soluble AChE activity within the tissue. The results of these experiments are summarized in Table 6. Almost 10% of the AChE in a rabbit brain is soluble (the data are the mean of two experiments) and the data show that within the brain there are variations, not

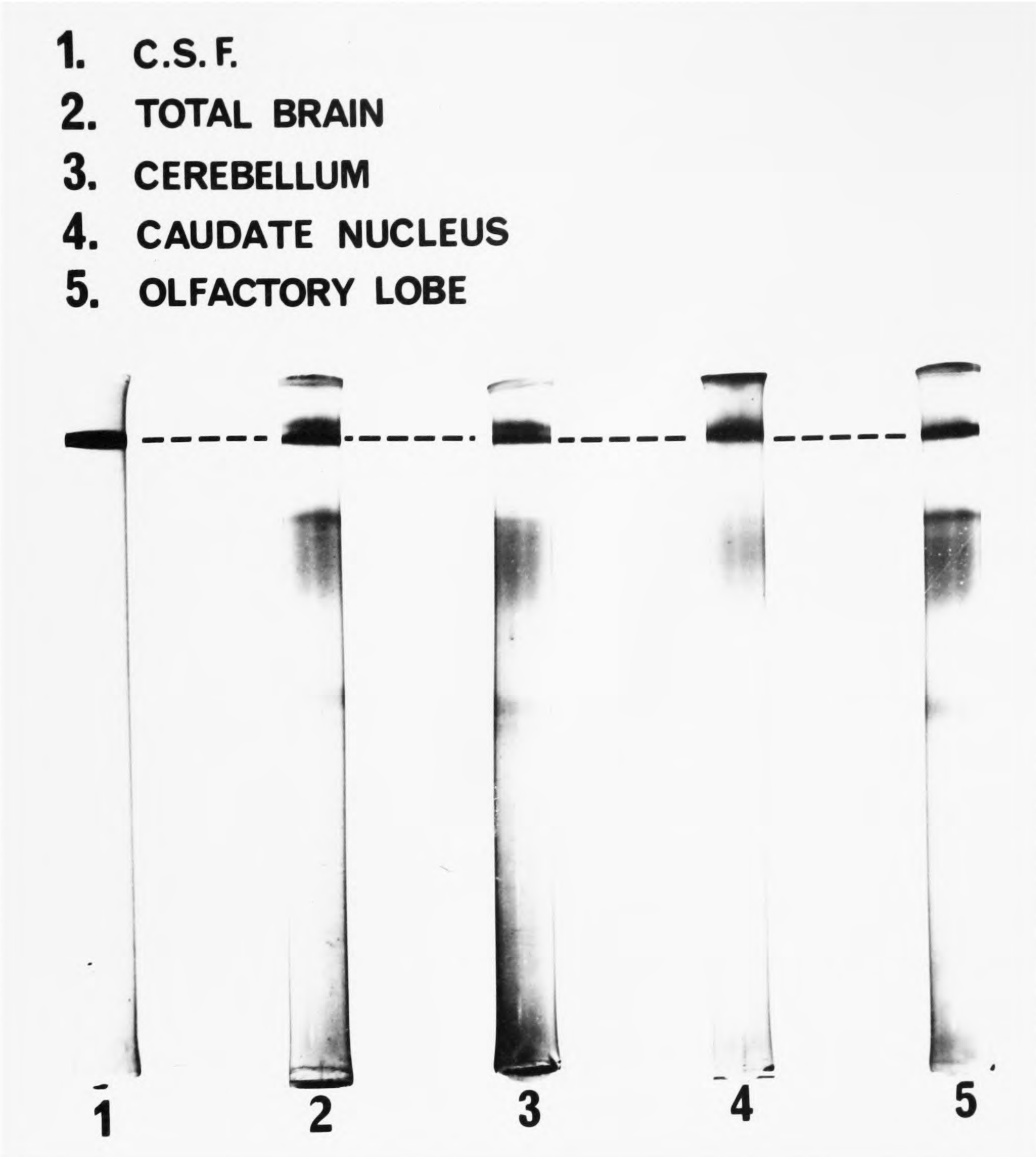


Fig. 73

Soluble isoenzymes of AChE in different regions of the rabbit brain compared with the isoenzyme in CSF.

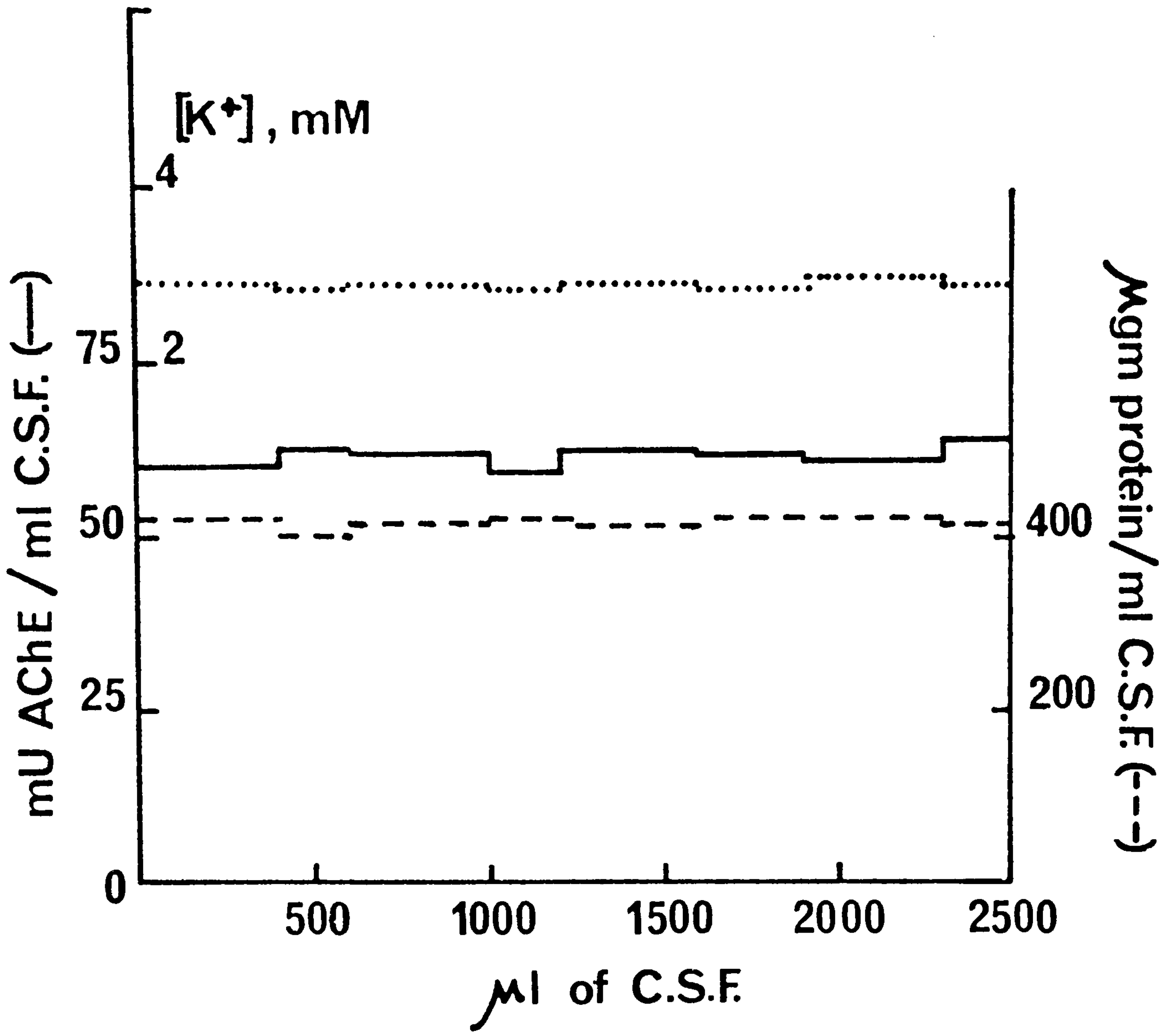


Fig. 74

Response of protein, AChE and K^+ concentrations to withdrawal of the maximum volume of CSF.

only in the amount of enzyme per gram of particular regions, but also in the proportion of the enzyme that is soluble. Thus, only 5.9% of the AChE of the caudate nucleus and the olfactory lobes is soluble, whereas almost twice as much, on a percentage basis, is soluble in the cortex. An electrophoretic analysis of the soluble AChE in various brain regions is shown in Fig. 73. These regions were chosen for this Figure to demonstrate the variations in isoenzyme pattern from region to region. In addition to the isoenzyme which they all have in common with the CSF, there are three other soluble isoenzymes which are not evenly distributed amongst the brain regions. A major soluble isoenzyme with a lower electrophoretic mobility than that present in the CSF was located in the cerebellum, whereas the olfactory lobe contained two faster migrating isoenzymes (the faint fast moving bands on all gels, except CSF, indicate some Cu^{2+} bound to serum albumin). The membrane-bound AChE was, in every case, a slowly migrating single isoenzymic species very similar in its electrophoretic behaviour to bovine $AChE_6$.

Before carrying out experiments to see whether the level of AChE in the CSF could be influenced by physiological stimuli, we first had to determine what volume of CSF could be safely removed from the rabbit, and how much could be removed without there being an effect on the AChE concentration of the fluid. From the result presented in Fig. 74, it is clear that a total of roughly 2.5 ml of CSF can be drained from the cisterna magna without altering the AChE, protein or K^+ level of the fluid. The only time that the concentration of any of these constituents changed (and also that of LDH) was when the animal died. After death, there was an almost immediate (within 1-2 min) increase

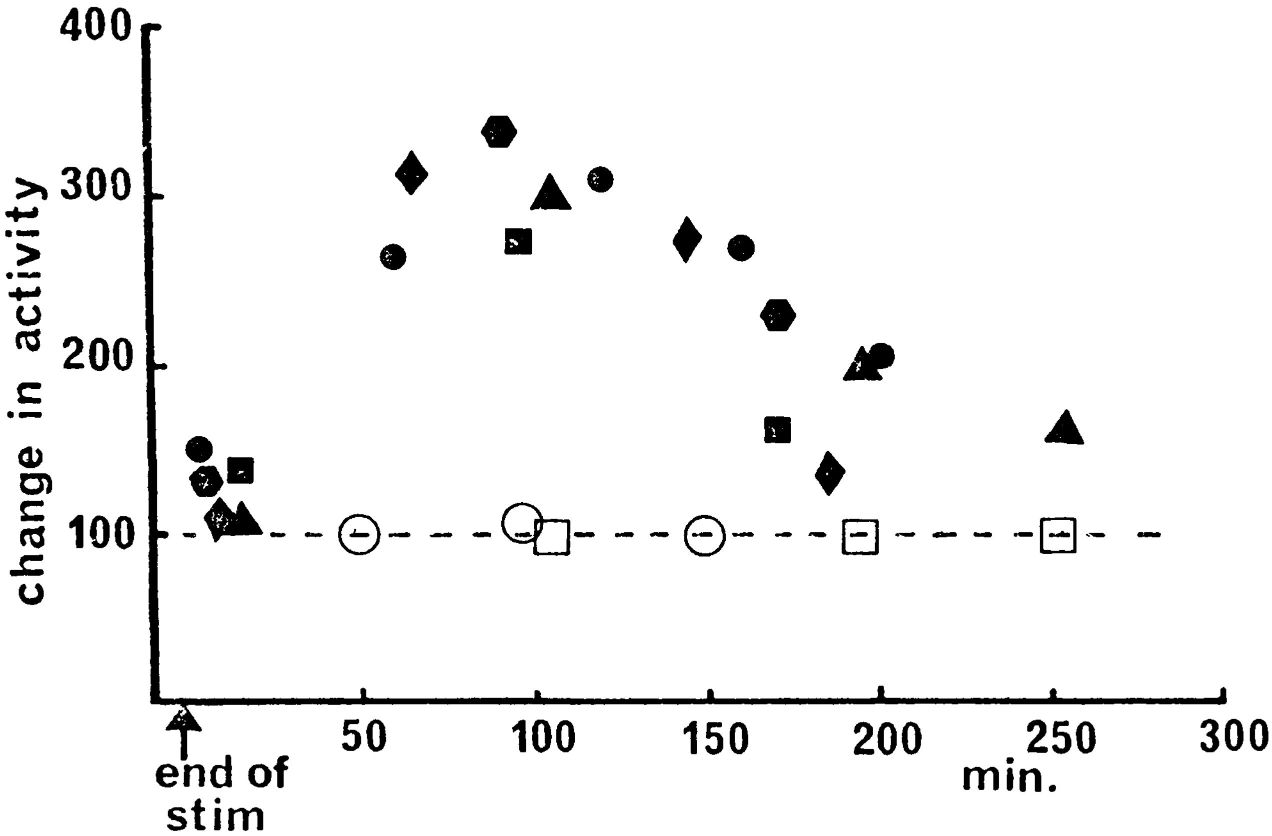


Fig. 75

The effect of peripheral nerve stimulation on the AChE concentration in rabbit CSF. Each type of symbol represents a different experimental animal.

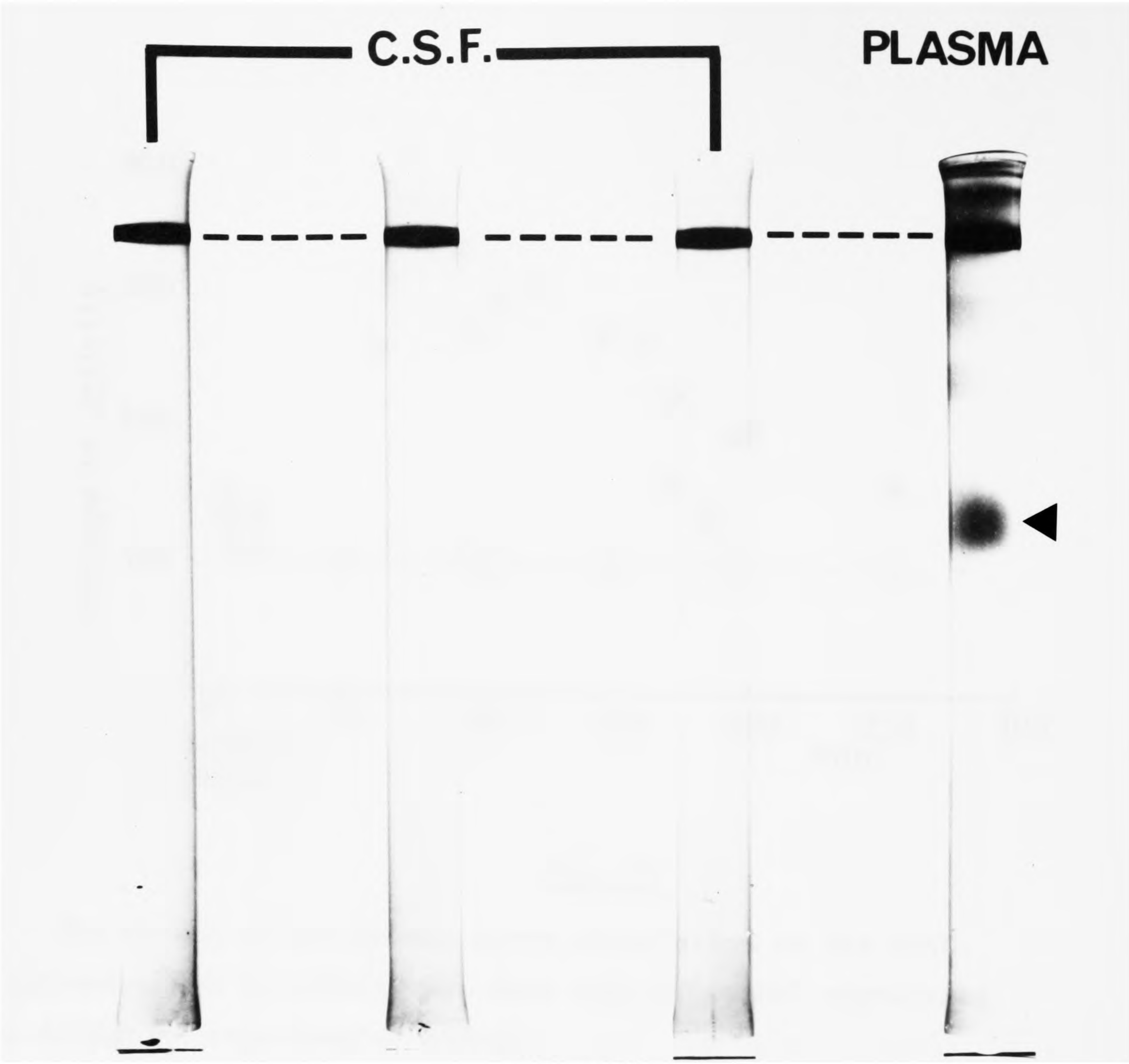


Fig. 76

The effect of peripheral nerve stimulation on the AChE isoenzyme in rabbit CSF. The three CSF gels are of samples taken before stimulation, at the peak of the increase, and after the return to normal levels. They can be compared with plasma taken from the same animal and then diluted so that the same amount of activity is run on all gels. The band marked (◀) in the plasma gel is due to Cu^{2+} bound to serum albumin and not to enzyme activity. All the activity on all four gels could be abolished by incubation in the presence of 10^{-4}M BW 284C51.

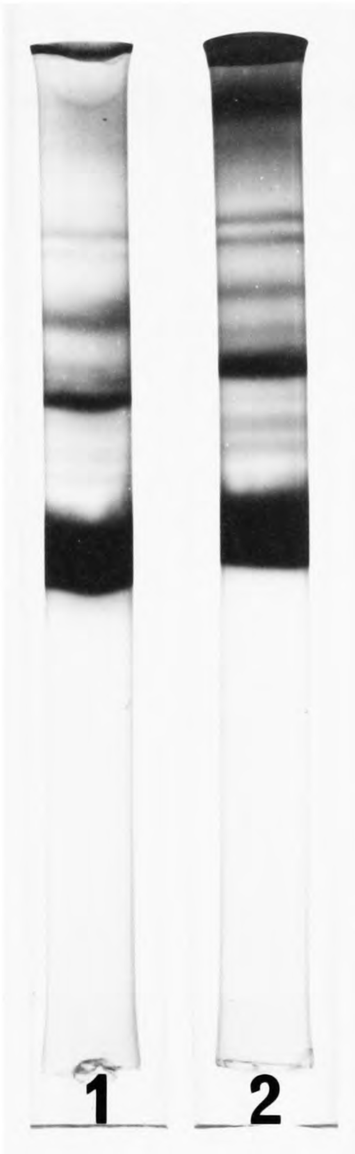


Fig. 78

The proteins in rabbit CSF (gel 1) and plasma (gel 2).

in all of the constituents of the CSF mentioned above.

When the peripheral nerves were stimulated, the effect on the AChE concentration in the CSF is shown in Fig. 75. In 5 experiments, each individually represented on this Figure, in which small volumes of CSF were removed with a syringe, the AChE activity per unit volume of CSF increased after stimulation: the peak of the increase being about 100 min after the final train of stimuli. The AChE activity at the peak of the increase ranged from 270 to 340 % of the activity before stimulation (as shown in Fig. 75, 100% = 61.3 $\pm$ 5.3 mU/ml). Within 3 to 4 h of the stimulation, the AChE level had returned to the ranges of the normal values. The control values (open symbols along the dotted line in Fig. 75) were obtained by either removing volumes of CSF equivalent to those taken during the course of the experiments but without stimulation of the nerves, or, alternatively, by stimulating the peripheral stump of a cut sciatic nerve. An electrophoretic analysis of the CSF taken from these rabbits is shown in Fig. 76. The CSF samples used to obtain these results were taken before stimulation, during the peak of the increase, and after the values had returned to normal. They show that the changes in AChE concentration are due to changes in the amount of the single isoenzyme normally present in CSF and not to the appearance of additional isoenzymes. If the CSF is compared with the plasma from the same rabbit, with the plasma diluted to give the same amount of activity on the gel as the CSF sample, it is also clear that there is not a contamination of the CSF with the plasma, which has a distinctively different isoenzyme pattern. Further evidence for this lack of contamination is given in Fig. 78, which shows the results of an electrophoretic comparison of the proteins in the CSF and the plasma - again diluted to give the same amount of protein on the two gels. The

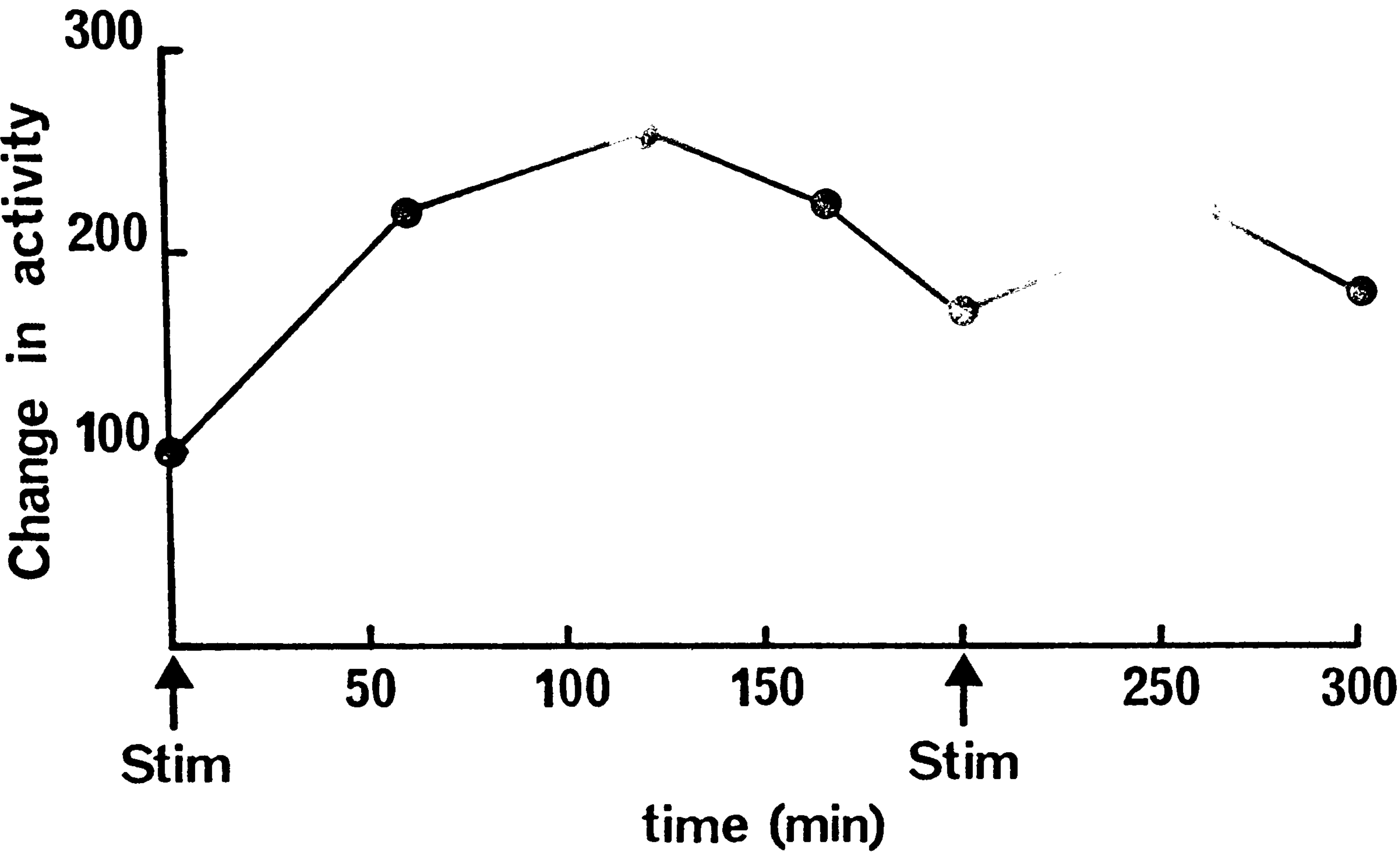


Fig. 79

The effect of two widely spaced trains of stimuli on the AChE concentration of rabbit CSF.

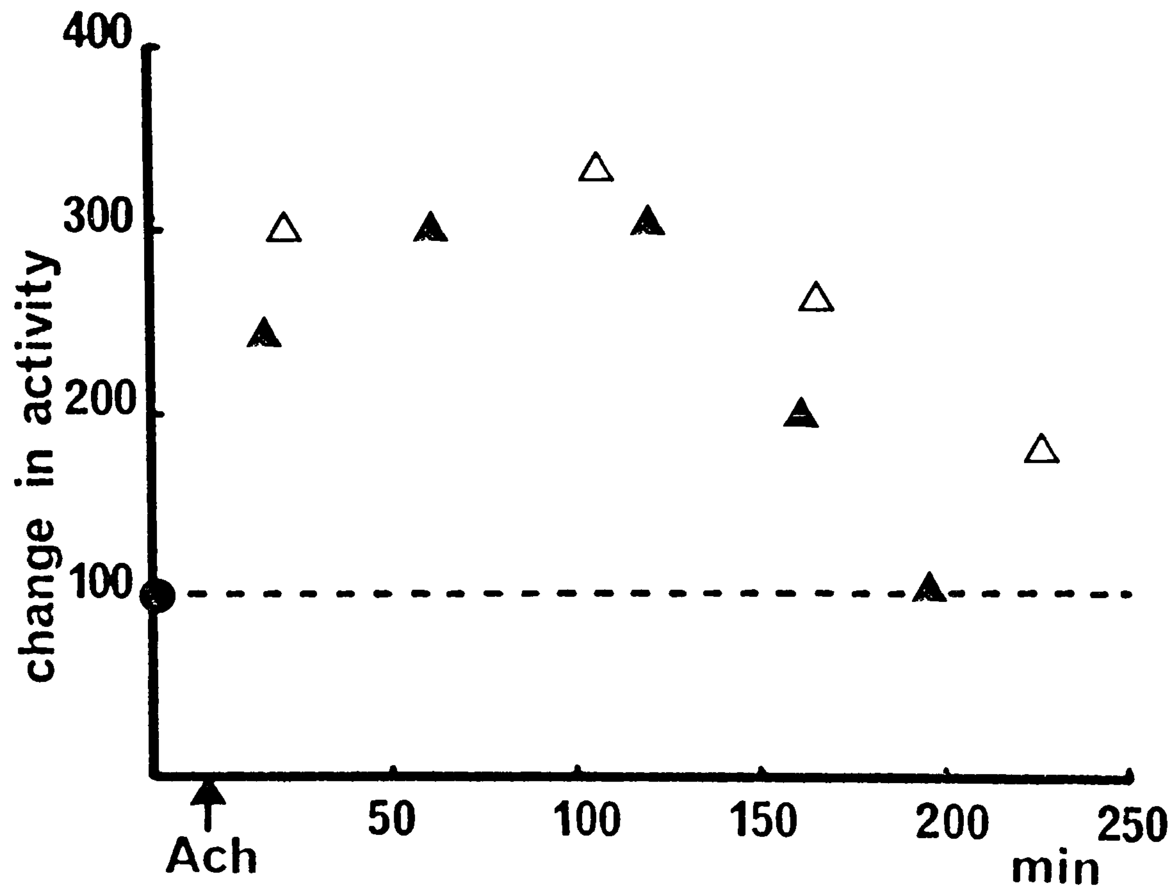


Fig. 80

The effect of injected ACh (i.v.) on the AChE concentration in rabbit CSF.

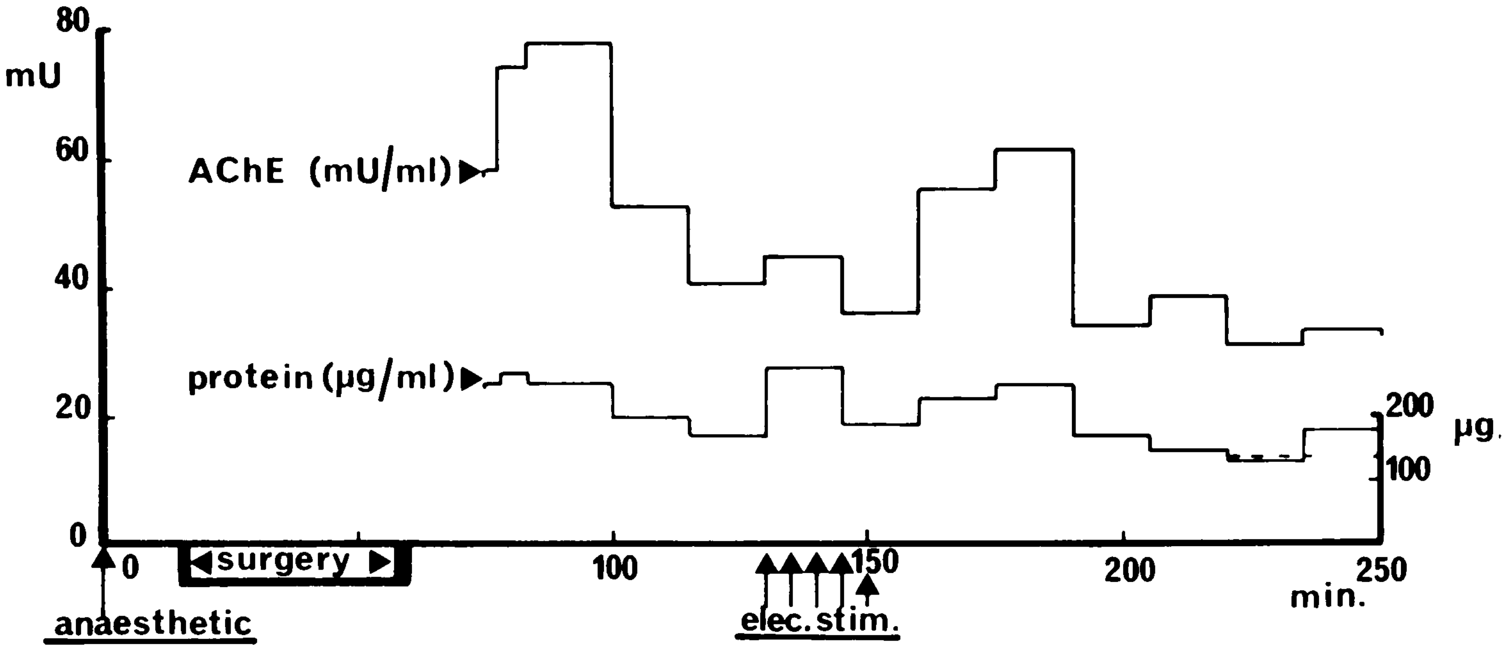


Fig. 81

The change in the AChE concentration of CSF which was continuously and slowly withdrawn from the cisterna magna. To change the concentration the sciatic nerve was stimulated as described in the text. The polyacrylamide gels show the type of AChE in the samples directly beneath each gel.

most striking difference between these two gels is the absence from CSF of the larger molecular weight proteins, which have a lower mobility in electrophoresis. In terms of AChE specific activity, the samples used for these Figures differed by a factor of 40; CSF 280 mU/mg protein, plasma 7.0 mU/mg protein.

If the sciatic nerve is stimulated twice, then it is possible to show two increases in AChE concentration in the CSF, provided that the trains of stimuli are sufficiently widely spaced (Fig. 79).

When the peripheral nerves were stimulated, there was a fall in the blood pressure of the animal and we decided to mimic this drop in blood-pressure by the (i.v.) injection of ACh. The results of these experiments are shown in Fig. 80, and they are almost identical to those obtained with nerve stimulation. The only difference is that, although the peak of activity is still some 100 min after the end of the injection period, the increase in the level of AChE in the CSF begins somewhat earlier.

Slightly more refined experiments were also performed, in which the CSF was continuously withdrawn from the cisterna magna at a rate comparable to its production which, according to Davson (1970), is to the order of 10μl/min. When, during this collection period, the sciatic nerve was stimulated, the results shown in Fig. 81 were obtained. About 15 min after the end of the stimulation period (the same as that used in the earlier experiments), there is an increase in the AChE concentration in the CSF. There is also a change in the protein content which is small and does not seem to be correlated with the change in the enzyme concentration. This observation of protein changes was also made during the earlier experiments, but again the protein and enzyme were not correlated. Throughout this experiment (i.e. Fig. 81) there was

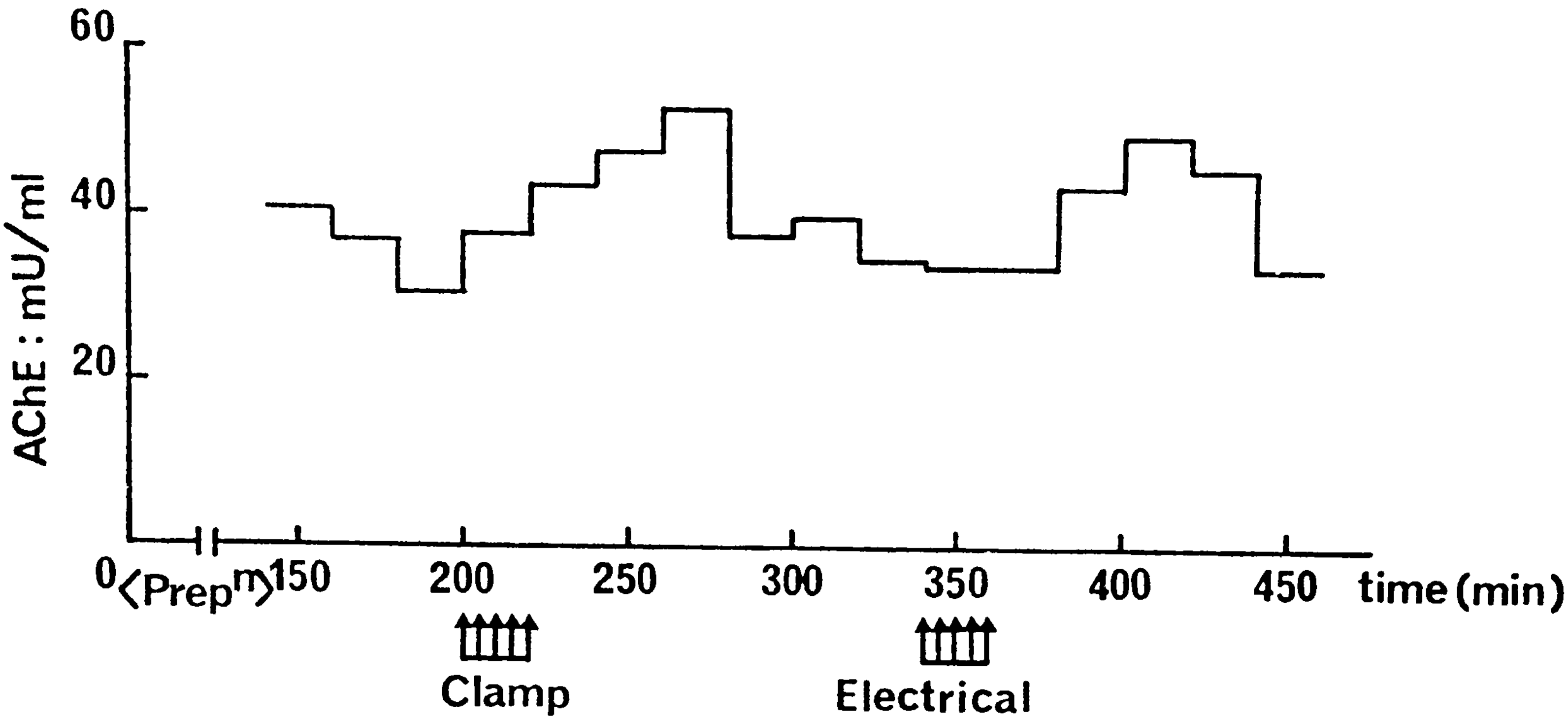


Fig. 82

The effect of foot clamp and electrical stimulation of the sciatic nerve on the AChE concentration of rabbit CSF.

no change in either the LDH activity or the K^+ content of the CSF. Also shown in Fig. 81 are the results of an electrophoretic analysis of various CSF samples, and again it is apparent that the change in AChE concentration reflects the change in the one normal isoenzyme.

An attempt was also made to determine whether a more natural stimulation of sensory fibres might provoke a secretion of AChE into the CSF. For this experiment, the animal was prepared, the cisterna magna tapped and the nerves exposed. However, instead of directly stimulating the sciatic nerve, we clamped the Achilles tendon for each second minute during 10 min. As shown in Fig. 82, the application of the clamp is followed soon after by a rise in the AChE level of the CSF. When the level has decreased, and if the nerve is stimulated, then another rise can be elicited.

DISCUSSION

Calf experiments: These results were included for two reasons: first to show that it is possible for the extracellular fluid levels of AChE to change dramatically within relatively short time periods, and secondly to demonstrate that the actual amount of AChE in the serum is in some way related to the physiological state and age of the animal (i.e. Fig. 69).

The origin of serum activity and the reasons why its concentration can vary, not only in the short term but also as a function of the age of the animal, are not known. We can only speculate that some of the changes in AChE concentration might be linked to the development of the nervous system.

Comline and Silver (1966) have shown major differences in the respective developmental stages of bovine and ovine adrenal medullae at parturition. Thus, the bovine adrenal medulla is not fully developed at birth, whereas the sheep glands can be "summarized by considering the new-born lamb as a miniature adult". Certainly a part of the under-development of the bovine gland is reflected in a far from complete innervation - stimulation of the splanchnic nerves results in a discharge of catecholamines which is only slightly higher than in the foetus and, in contrast to the adult, it consists of more noradrenaline than adrenaline. Without wishing to infer that the adrenal medulla is necessarily the origin of all the serum AChE (which we know not to be the case, because of the only marginal effects on serum enzyme levels caused by bilateral adrenal denervations), it is interesting that the two species have very different serum levels of the enzyme at birth; the animal with the more mature splanchnic innervation having a much lower serum AChE level. If this is coupled with a decreasing AChE content per gram of bovine adrenal medulla with age, then it seems possible that the changes in serum AChE with age might be a reflection of changes in the maturation of at least certain parts of the cholinergic nervous system.

If this suggestion is correct, the answer to the question of how the AChE arrives in the plasma may be found in the mechanism that has been proposed by Bunge (1973) and Bray (1973) for the growth of neurons. A microscopic analysis of nerve growth cones of cultured sympathetic neurons has established that these outgrowing parts of neurons have several unique features, one of which is an enrichment in SER. Bunge (1973) and Bray (1973) both suggest that the neurons grow by the incorporation of "vesicles" or tubules of SER into the tip of the cone, thereby adding to the surface membrane. Thus, the SER is thought to play a role in both neuron and membrane growth. In developing motor neurons tubules have been found which give the appearance of having fused or being in the process of fusing with the terminal, presynaptic membrane. The feature of these latter experiments is that, while this apparent fusion of tubule and membrane is fairly common in neonates, it is only very rarely observed in adult tissues (Kasa and Csillik, 1968). The tubules which fuse, however, are rich in AChE, just as the pre- and postsynaptic membranes are during development. It has already been postulated that AChE is released, from splanchnic nerve terminals possibly, but certainly from the adrenal medullae when the endoplasmic reticulum of these cells fuses with the plasma membrane. An increase in the amount of endoplasmic reticulum fusing during the developmental stage could give rise to the high extracellular fluid levels of AChE which were measured in these experiments. It is interesting that an analogous situation seems to exist in the plasma of embryonic and neonatal chickens. Wilson, Linkhart, Walker and Nieberg (1973) have shown that embryonic chicken plasma has a high AChE content which falls after hatching, so that in the adult there is only very little AChE activity. Wilson et al. (1973) showed that the plasma esterase isoenzyme pattern was similar to that in the sarcoplasm of the embryo muscle. This was an extension of their earlier observation (Wilson, 1972) that chick embryo muscles, growing and developing in culture, released relatively large amounts of AChE into the culture medium. Thus, these results strongly infer that the AChE of muscle, another postsynaptic cell, can also be released into extracellular fluids. Observations such as these are not confined to the chicken and calf because very similar results were obtained earlier by Barron et al. (1968), who studied developing muscles of the rat. Their two most relevant observations were that there is a greater amount of soluble AChE in neonatal than in adult muscles, and that the muscles from older animals lack an isoenzyme which is present in those of the neonates. This latter point is of some interest to us because of an observation which we have made but which is, as yet, unconfirmed. During the course of the experiments described in Chapter 4, we were given the adrenal gland from a very old cow. Perfusion and administration of K^+ to this gland did not result in any release of AChE; electrophoretic analysis of the AChE in this gland revealed that there was no, or only very little, $AChE_5$. Obviously, this observation has not been confirmed because of the difficulty in obtaining cows of sufficiently advanced age, but it is hoped that with the collaboration of Dr. Comline, we will soon be able to analyze tissues from such animals. To date, we can only speculate upon the apparent lack of this isoenzyme and wonder whether the decrease in the serum AChE levels in older animals is a reflection of this phenomenon occurring in the adrenal and in other tissues.

The other results presented in the first part of this Chapter will not be discussed in detail because they are concerned more with foetal and neonatal physiology than they are with the more functional aspects of AChE, which is the main theme of this thesis. Nevertheless, it is noteworthy that the illness in the animal shown in Fig. 69 was preceded by a marked and prolonged increase in the serum AChE and that recovery of normal growth coincided with the decrease in the enzyme activity. It is also notable that the animals with premature births had higher serum AChE levels than the normal, but that even these levels were surpassed if the birth was induced with dexamethasone (Table 5). These observations would seem to be of sufficient interest to be followed up by somebody able to correlate the physiological responses of the foetus and neonate to changes in the concentration of this enzyme.

These results can probably be best summarized by saying that they provide evidence that serum AChE is released by some structure(s) so that the levels can change significantly over fairly short time periods and that it seems not unreasonable to suggest that the serum concentration is a reflection of the state of development of the cholinergic nervous system and its postsynaptic elements (and even, possibly, the health of the animal).

Rabbit CSF: In trying to interpret changes in the concentration of AChE in the CSF, we must first consider the possibility of a non-neuronal origin of the enzyme, the most important being to exclude blood as a possible contaminant of the CSF. Fortunately, this is even more easily done with the CSF than it was for the adrenal perfusates. The specific activity of the AChE in the CSF is about 20 to 40-fold higher than that in the plasma taken from the same animals, and electrophoretic analysis of the proteins in the two fluids shows that, while the CSF contains a high proportion of serum albumin, it lacks most of the higher molecular weight proteins present in the plasma. It is interesting that the isoenzyme composition of plasma, if not identical to the CSF, is at least sufficiently similar for it to be concluded that the bulk of the plasma AChE activity is due to the presence of the same isoenzyme which is present in the CSF.

Therefore, the change in AChE concentration of the CSF cannot be due to a gross breakdown of the blood-brain barrier. We have also been able to gather evidence which suggests that we are not structurally damaging cells and hence inducing a breakdown of the normal intercellular relationship within the brain. Thus, removal of CSF did not alter the AChE, LDH, protein or K^+ levels, even when the entire possible volume of CSF was removed (see also Yaksh, Fedele and Yamamura (1973), who showed that removal of CSF from cat cisterna magna did not result in changes in the protein concentration of the fluid). Samples collected after stimulation had normal LDH and K^+ levels although they had elevated concentrations of AChE. Consequently, we were able to alter the enzyme concentration without evoking an increase in the amount of two constituents which could be used as indicators of a gross leakage of either small or large molecules from within the cell. It could be argued that any changes in either the K^+ or LDH would be too small to measure when diluted in a large volume of CSF. The answer to this criticism is basically that on some occasions we were able to measure changes in these two - in CSF taken post mortem. In these samples the AChE level was about the same as the peak increase found after nerve stimulation, but the K^+ and LDH levels had increased by the order of 20-fold. It is therefore valid to use measurements of K^+ and LDH as an index of gross cell leakage.

We can also argue against the possibility that a constant amount of AChE is being secreted into a decreasing volume of CSF and hence giving an "apparent" rise in AChE concentration. Davson (1970) has shown that the secretion rate of CSF in the rabbit is of the order of 10 μl/min. In the first series of experiments we withdrew, by the time of the peak AChE activity, three samples, each of which was 200-300 μl, i.e. 600-900 μl total. During this 100 min period, there would have been about another 1000 μl of CSF formed, and so the volume of the fluid was essentially unchanged. When a pump was used, we chose a flow rate of 10 μl/min so that, again, the volume at the end of the experiment was virtually unchanged from that at the beginning.

Given that the evidence described above excludes blood and structural damage as the source of the AChE, and that the possibility of a concentration of the macromolecules through loss of water is exceedingly unlikely, it must be concluded that the isoenzyme of AChE in the CSF is derived from structures within the brain. The results of the experiments in which the soluble AChE activity in brain homogenates was analyzed electrophoretically support this idea. Thus, there is one isoenzyme, which is unique to the soluble fraction, that has the same electrophoretic characteristics as the isoenzyme in the CSF. But this isoenzyme is only one of several found in high-speed supernatants of whole brain homogenates. This is strikingly similar to those observations described on the adrenal medulla in Chapter 4. A more detailed analysis of the brain shows that this isoenzyme is common to all areas studied but that the other isoenzymes are not evenly distributed amongst the different regions (the cortex, which is not shown in Fig. 73, was similar in composition to the caudate nucleus). Thus, the relationship between the AChE isoenzymes in the CSF and the brain is remarkably similar to that which exists between the adrenal and its perfusate enzyme, and it is this similarity which induced us to go further with these experiments.

To try and induce changes in the concentration of AChE in the CSF, we have used procedures that are known to evoke the release of ACh from various CNS structures, e.g., stimulation of peripheral sensory fibres. This field has been extensively reviewed by Pepeu (1973). Stimulation of peripheral nerves results in a release of ACh from the cerebral cortex (Mitchell, 1963; Szerb and Kanai, 1965) and a release of ACh from the caudate nucleus can be evoked by stimulation of the skin, the sciatic nerve or the contralateral caudate nucleus (Portig and Vogt, 1969). There are, therefore, reasons to suppose that provided ACh and AChE release are in some way related a stimulation of the peripheral nerves, as in these experiments, could result in a release of the enzyme.

All of the experiments presented in the second part of this Chapter show that different forms of peripheral stimulation increase the AChE content of the CSF. While this type of stimulation obviously initiates the release, we cannot be certain that it is the ultimate cause of the change in the AChE concentration. Stimulation of the type used in these experiments produces, in the rabbit anaesthetized with urethane, a pronounced but transient drop in the blood pressure. A similar rise in the AChE level in the CSF can be produced by injecting ACh i.v. to evoke a similar fall in the blood pressure. Whether the change in AChE concentration of the CSF is a reflection of direct stimuli reaching the brain from the sensory nerves in the sciatic (there was also a small drop in blood pressure in the rabbits whose feet were clamped), or indirectly caused by the drop in blood pressure, is not known. However, the exact reasons for why these peripheral stimuli are able to produce these changes in the AChE concentrations are not directly relevant to the main point of this discussion. These experiments were designed with the expressed aim of trying to determine whether the results of the in vitro experiments described in Chapter 4 could be reproduced under more physiological conditions using the brain and CSF of the rabbit. They were successful in that they show that AChE can be released from organs, into the fluids which bathe them, both in vitro and in vivo.

One final question needs to be answered and that is the one which asks whether it is reasonable to expect that proteins released from cerebral neurons would eventually arrive in the CSF. There are two reasons for supposing that they should: first, Brightman (1968) has shown that macromolecules which are introduced into the cerebral ventricles can cross the ependyma and enter the nerve terminals. But secondly, and more recently, Kreutzberg and Kaiya (1974) have shown that AChE itself injected into either the fore-brain parenchyma or the cerebral ventricles diffuses through the intercellular spaces at a velocity of "a few mm per hour". Their results are also very similar to those of Brightman (1968) in the respect that they also observed an uptake of AChE into the nerve cells. Thus, the observations of Kreutzberg and Kaiya (1974) confirm and extend those of Brightman (1968), and they add the vital piece of information that the rate of diffusion of exogenous AChE (electric organ; 240,000 daltons) around the intercellular spaces is only very slow: a "few mm per hour".

Is this slow rate of diffusion the reason why it takes about 100 min for the AChE activity to reach its maximum in those experiments where small discrete samples of CSF were taken? If the AChE was released from somewhere within the cortex, for example, it could take a considerable time for this enzyme to reach the CSF in sufficient amounts to significantly alter the concentration.

If this is so, then we would have to postulate that the early increase in enzyme concentration measured in the experiments in which the CSF was collected continuously occurs because it is released from a site which is much closer, or with more ready access, to the CSF. So far as ACh is concerned, release has been shown from a number of structures, some of which border the ventricles and release directly into them (e.g. Portig and Vogt, 1969), while the ACh released from the cortex can be collected from its surface (see Collier and Mitchell, 1966). It is not, therefore, inconceivable that two different methods of CSF collection might reveal the presence of two different release sites of the AChE, just as they seem to exist for ACh. Clarification of this point awaits further experimentation. It could, however, be briefly mentioned that neurons which directly come into contact with the CSF, both their axonal and synaptic processes, have been observed, although their exact type has not yet been determined (Vigh-Teichman, Vigh and Aros, 1973).

As was discussed briefly in Chapter 1, the presence of AChE in the CSF has long been known (Plattner and Hintner, 1930), but it has been largely ignored except in clinical studies of pathological conditions. For example, Plum and Fog (1960) reported that 69 out of 96 human patients with multiple sclerosis had "strikingly low" AChE concentrations in their CSF, while the remainder had relatively low levels when compared with "normal" controls. One of the first detailed analyses of the enzyme in CSF was that of Svensmark (1961) who compared the brain and CSF enzymes by the use of electrophoresis. Although his results clearly showed the presence of AChE (and ChE) in the CSF, the electrophoretic support used was paper, and the lack of resolution offered by this technique meant that he was unable to distinguish the presence of isoenzymes. However, not even this area of cholinesterase research is without controversy; Riekkinen and Rinne (1968) could find only ChE in human CSF. An additional, and as yet unexplained, observation was made by Johnson and Domino (1971) who, during the course of studies on the CSF of patients with various known neurological disorders, found that not only were both AChE and ChE present in appreciable concentrations, but also that there was a measurable choline acetylase activity. A study of the total protein content in these fluids showed that whereas the activities of AChE and ChE were not related in any way to the protein concentration, the choline acetylase varied in an exactly parallel manner. In our experiments it was also found that there was no direct relationship between the AChE and protein concentrations. Thus, the results of Johnson and Domino (1971) can be interpreted as showing that when there is a gross leakage of intracellular cytoplasmic constituents (measured by the choline acetylase activity in their experiments, LDH in ours), there is a concomitant change in the total protein concentration of the CSF. Johnson and Domino (1971) also found that patients with multiple sclerosis and myasthenia gravis had, respectively, low and high AChE concentrations in the CSF when compared with the fluid taken from "normal" people.

The observation that the CSF of patients with different diseases contain different amounts of AChE also points towards a possible functional correlation which cannot be ignored. In this respect, the CSF results and those obtained with calf sera point towards the same general conclusion: that the concentration of AChE in CSF taken from a completely rested individual could be a direct indication of his condition and health. It is noteworthy in this context that Croft and Richter (1943) have shown that the level of cholinesterase in human serum can be changed by muscular exercise. So it would seem worthwhile to investigate, and to try and correlate fully, the pathological and physiological condition of an individual with the concentration of not only AChE, but also any other macromolecule which is known to be associated with neurotransmitters and which can be found in the CSF. The results presented in this Chapter show that this rationale can be applied because, with the evidence that the AChE in the CSF is neuronally derived, its changes in concentration must be an index of nerve activity.

CHAPTER 7

THE RECOVERY AFTER DFP POISONING OF THE ACETYLCHOLINESTERASE IN THE SUPERIOR CERVICAL GANGLION OF THE RAT

INTRODUCTION

In Chapter 3 it was argued that because the AChE in the splanchnic axon terminals must turn over rapidly it was possible that the enzyme might be released from the nerve. This suggestion led to the experiments described in Chapter 4. However, since we now tend toward the hypothesis that most if not all of the AChE released into adrenal perfusates comes from the chromaffin cells, and that the nerve terminals contain very little of the enzyme, we must turn the argument of Chapter 3 in reverse. In other words, we must show that because the AChE of the chromaffin cells is released, at least a part of it is rapidly turning over. Thus a rapid turnover would offer support for our hypothesis whereas, if the rapidly turning over enzyme was found to be restricted to the axons, we could argue that , even though they might contain only a small proportion of the enzyme within the gland, the nerves were the sole source of the released AChE.

The experiments which are described in this Chapter approach the problem of where and how quickly AChE turnover occurs from a combined cytochemical and biochemical point of view. However, because of the amount of data required to properly analyze this problem we have had to turn to a species other than bovine and to use a tissue other than adrenal. For these experiments, tissues from rats were used and the superior cervical ganglion (SCG) was chosen rather than the adrenal because preliminary experiments had shown that this organ bore a distinct isoenzymic and cytochemical similarity to the bovine adrenal, whereas the rat adrenal gland was markedly different. Even so, it was known that there must be some differences between the rat SCG and bovine adrenal because, for example, denervation of the ganglion results in a 45% depletion in the AChE content (Klingman and Klingman, 1969) whereas section of the equivalent fibres running to the bovine adrenal did not result in a measurable fall in the AChE level of the gland (Chapter 4). Nevertheless, the similarities between the two tissues were considered sufficient to warrant these experiments and to allow a cautious extrapolation between the species.

METHODS

Young male Wistar rats weighing 175-200 g were injected sub-cutaneously with both diisopropyl fluorophosphate (DFP) (1.9 mg/kg) and atropine sulphate (6 mg/kg), the latter to protect against the toxic effects of the DFP. The two compounds were dissolved in the one solution which, because of the relative insolubility of DFP, was 0.9% NaCl and 1% (v/v) ethyl alcohol. The control animals were treated with the vehicle containing atropine sulphate at the same dose used for the experimental animals. At different times after injection, the animals were killed and their SCG, some blood and in some experiments a length of preganglionic trunk were removed. The tissues were first desheathed and then sometimes weighed before being homogenized, in 1.0 ml of 0.3 M sucrose, in a small hand-driven all glass homogenizer. This homogenate was used to estimate the total AChE activity in the ganglion or trunk and, after freezing and thawing once, followed by centrifugation at 100,000 g for 60 min, its supernatant was used to estimate soluble AChE activity. The blood was allowed to clot, it was then centrifuged and the serum collected. It, like the tissue samples, was stored frozen before assay.

The left SCG of some rats was denervated by removing a section of the preganglionic trunk (usually about 0.5 cm of the nerve) from animals under nembutal anaesthesia (37 mg/kg; i.p.) The wound was sutured and 72 h later the ganglia were removed and treated as described above.

Estimation of AChE activity, electrophoresis and the cytochemistry were all done as described in other Chapters. When the ganglia were analyzed electrophoretically it was decided to run the same equivalent amount of ganglia on each gel, in order to be able directly to compare ganglia from different animals. This meant, however, that the absolute amount of AChE activity varied widely, as can be seen in the relevant Figures, but it allowed us to estimate qualitatively whether there were selective changes in any individual isoenzymes during the recovery period. The changes in AChE level during the recovery period were analyzed statistically using Student's t-test.

RESULTS

Total and soluble AChE activities in ganglia.

The AChE activity of control ganglia was found to be 31.1 ± 1.1 mU/ganglion (Table 7) while that of the preganglionic trunk was only 1.0 ± 0.1 mU/cm (mean ± S.E.M.; n = 6). Since the average weight of each ganglion was 1.8 mg and that of the trunk was 0.2 mg per cm length, these results can be used to calculate that there are 17,300 mU/g wet weight of ganglia and 5,000 mU/g wet weight of nerve. While it is accepted that the measured

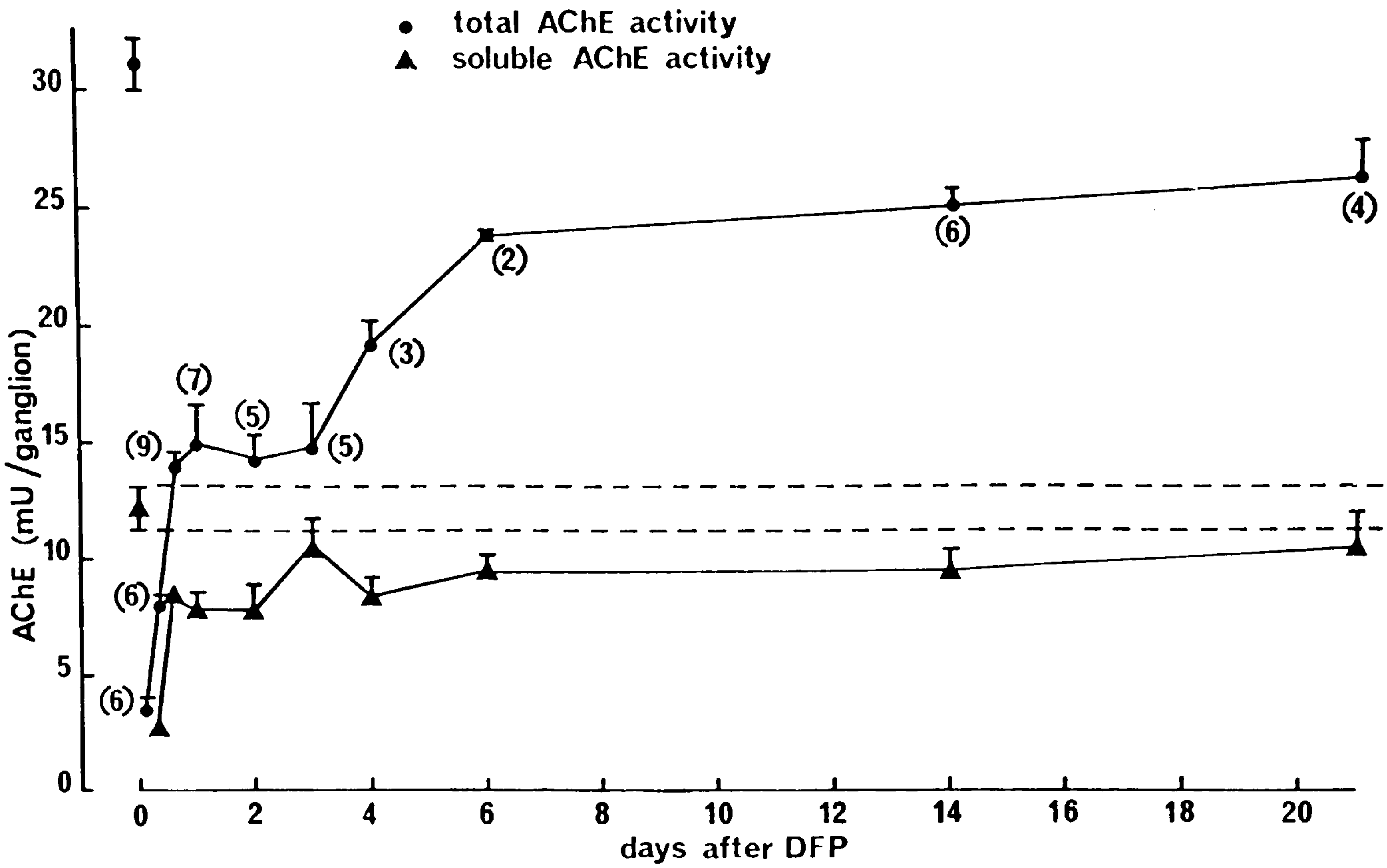


Fig. 83

The recovery of total and soluble AChE in rat ganglia after treatment with DFP. The 16 h, 1, 2 and 4 day soluble AChE concentrations are statistically different from the amount of soluble enzyme in control ganglia at the $P < 0.05$ level.

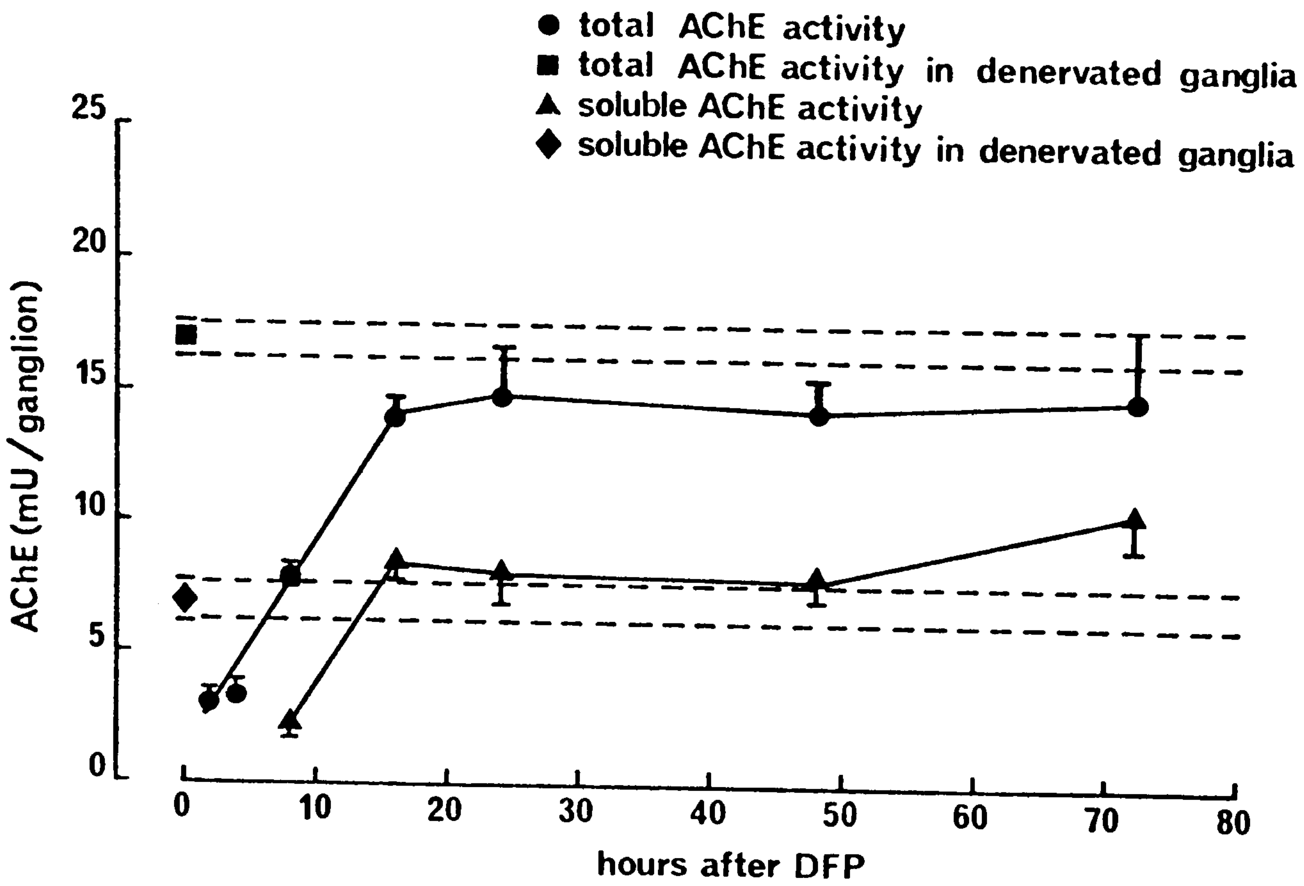


Fig. 84

The changes in the AChE levels in rat ganglia at short time intervals after DFP poisoning. None of the experimental points up to 48 h differ significantly from the "control" values. The amount of soluble activity after 72 h differs from the control level at the $P < 0.05$ level. The controls shown are the enzyme levels in ganglia denervated 72 h prior to analysis.

weights are possibly subject to considerable error, these results are very similar to those of Klingman and Klingman (1969) who obtained values of 18,500 and 7,400 respectively.

To find empirically the best dose of DFP to use for these experiments, we examined the tissues of several rats after they had been treated with different amounts of the inhibitor. The highest survival rate (>95%) with the lowest AChE level after 2 h was obtained with 1.9 mg/kg. Use of 2.0 mg/kg resulted in only a marginally greater inhibition of the AChE but a much higher mortality rate, whereas with 1.5 mg/kg, there were no mortalities but the AChE content of the ganglia was still quite high (7.5 mU) 2 h after the treatment. One final check on the procedure was made by testing the possibility that some DFP might have been left in the tissues, or plasma, which after homogenization could reach and inhibit newly synthesized AChE. When homogenates of normal ganglia, or normal serum, were mixed with tissues taken from animals treated with DFP the measured activity was always equal to the sum of the individual components in the mixture. It was, therefore, unlikely that residual DFP would prove to be a complicating factor in these experiments.

When the AChE levels of various ganglia were estimated at times ranging from 2 h to 21 days after DFP treatment, the results shown in Figs. 83 and 84 were obtained. The curve drawn in Fig. 83 is complex and can be broken down into at least two component parts. It can be seen in Fig. 83 that after the initial rapid phase and its subsequent plateau, there is another rapid increase in the AChE concentration which starts between 3 and 4 days and proceeds up to about 6 days after the treatment. Thereafter, there is a slow steady increase in the AChE level so that by 21 days, the

TABLE 7

The effect of denervation (72 h) on the total and soluble AChE activity in the superior cervical ganglion.

	mU/ganglion	S.E.M.	n	% soluble
Total activity:				
Control	31.1	1.1	17	
Denervated	16.9	1.7	6	
Soluble activity:				
Control	12.2	1.2	15	39.2
Denervated	6.9	1.7	6	40.1

treated ganglia do not differ significantly from the control values. The second phase does not seem to be closely paralleled by changes in soluble AChE activity, although there too it is apparent that there is a slow steady increase starting from 3 to 4 days and going on up to 21 days after DFP.

However, since the aim of these experiments was not a detailed study of the recovery of ganglionic AChE after DFP treatment, but rather to determine whether pre- or postganglionic AChE turns over rapidly, most of the rest of this Chapter will be restricted to the results obtained during the initial period of between 2 and 24 h after the administration of DFP. The results obtained from ganglia analyzed during this period (and up to 72 h) are shown in detail in Fig. 84, where they can be compared with the AChE levels, both soluble and total, found in ganglia which had been chronically denervated 72 h before analysis. There is a rapid and linear increase in the total AChE content of the ganglion from DFP-treated rats, which starts from as early as 2 h and proceeds up to 16 h. The line drawn is described by the equation $y = 0.81\ x + 1.16$ (correlation coefficient 0.992; $P < 0.001$) from which it can be calculated that the rate of recovery is 0.81 ± 0.03 mU/h during this early and rapid phase. As can also be seen in Fig. 84, the rapid recovery is a reflection of increases in both total and soluble AChE activity, although there does seem to be a short lag phase before the soluble enzyme begins to accumulate. The levels attained by 16 h for both soluble and total AChE are very similar to their respective levels in the denervated ganglia (Table 7); statistically there is no difference between the total activities from 16 up to 72 h, while the soluble activities are not different from between 16 and 48 h. The soluble

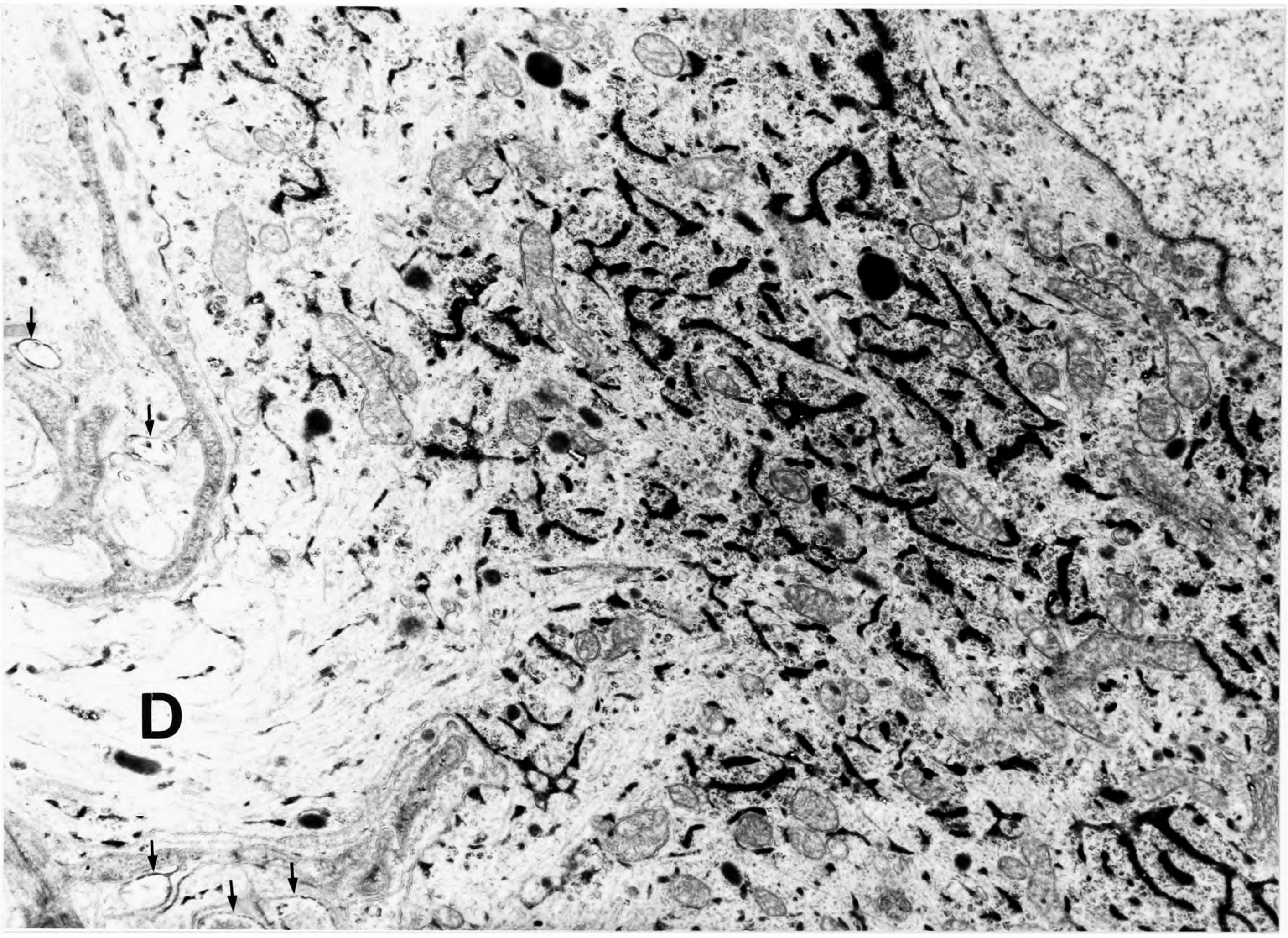


Fig. 85

AChE reaction in an untreated ganglion cell of the rat. There is extensive activity within the endoplasmic reticulum. When the endoplasmic reticulum enters the dendrite (D) it is aligned parallel to the membrane of the cell. Several small preterminal axons also react positively for AChE (arrows). Mag. X 10,400.

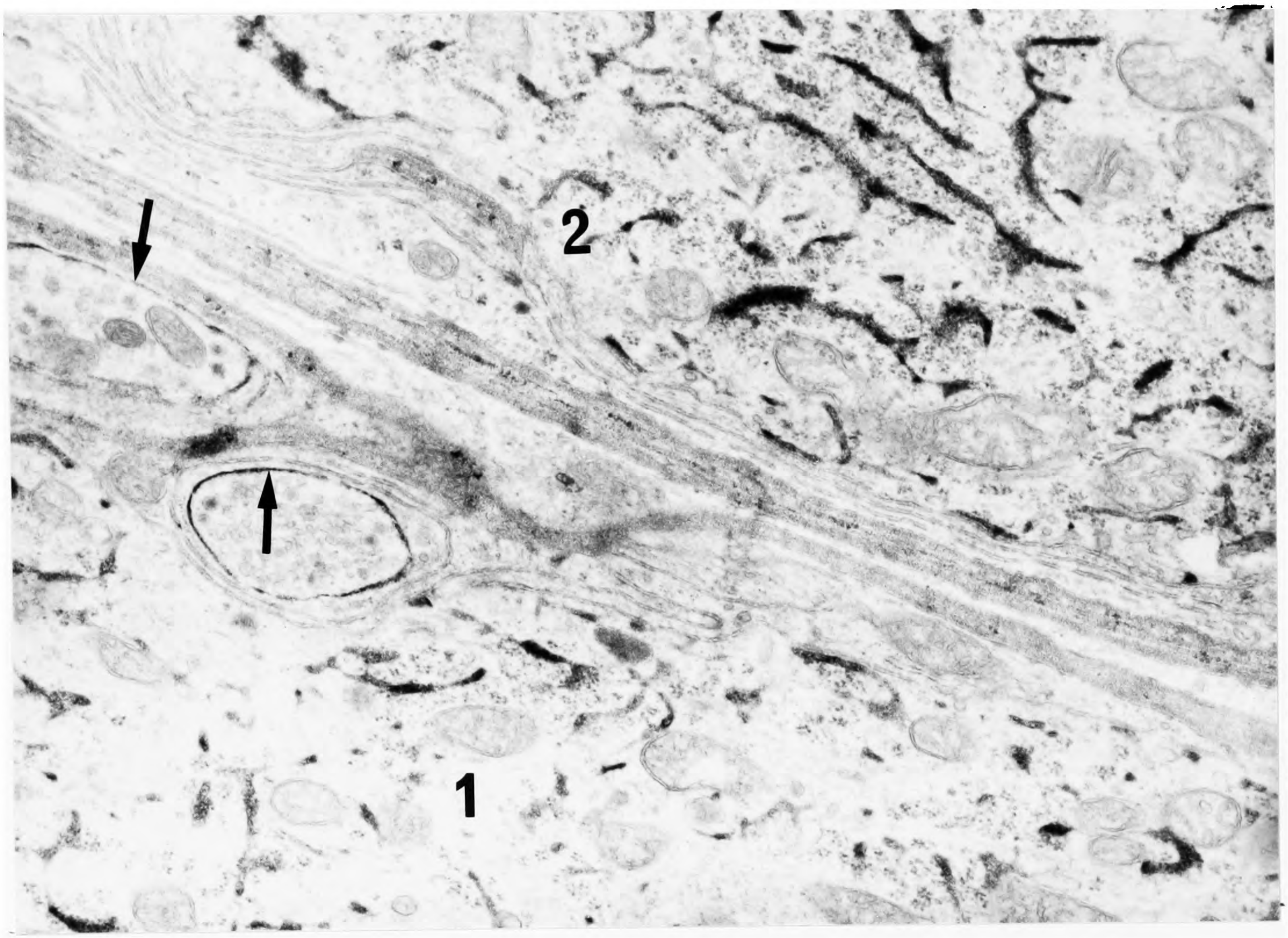
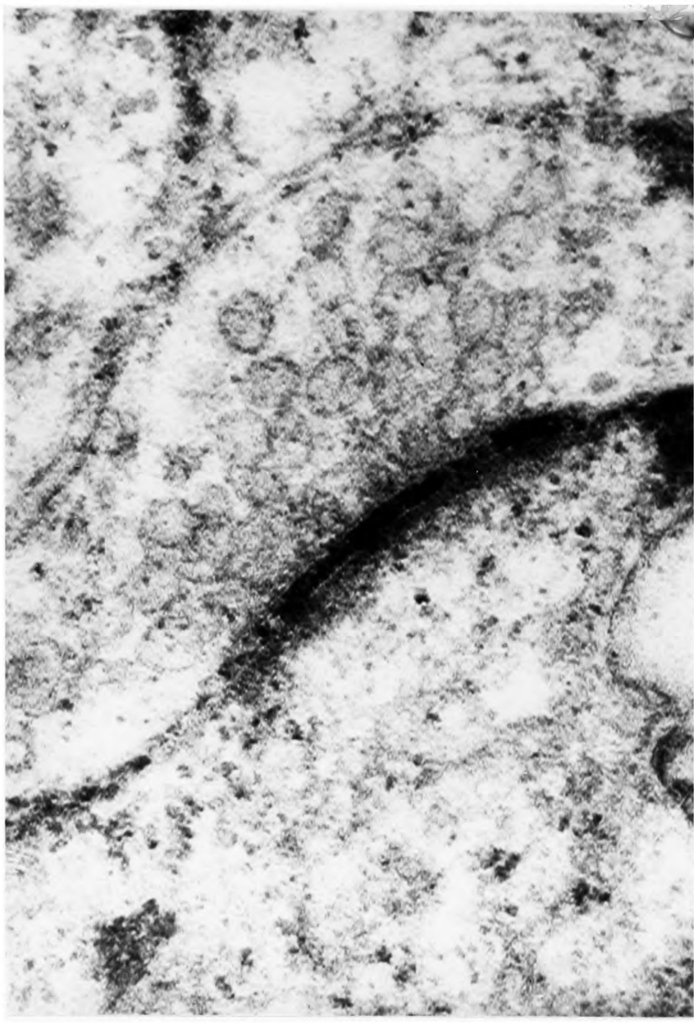


Fig. 86

AChE reaction in untreated ganglion cells of the rat. Two ganglion cell bodies (1 and 2) both with activity within the endoplasmic reticulum, and two preterminal axons each with activity associated with the axolemmal membrane (arrows). Mag. X 23,500.



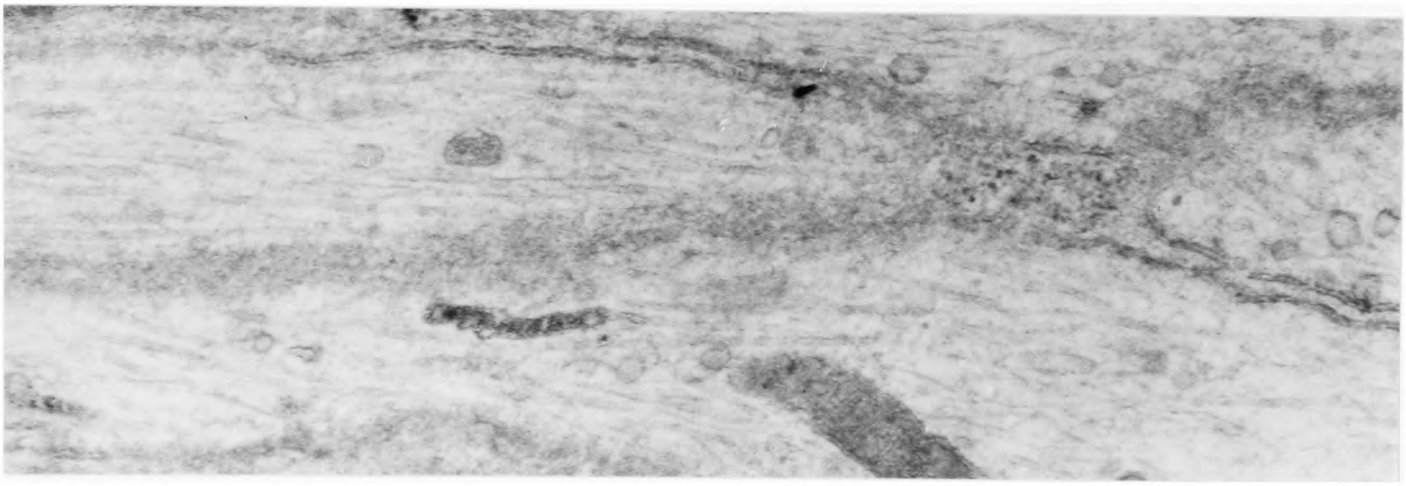


Fig. 87: A synaptic area in an untreated rat superior cervical ganglion. There is activity around the nerve, particularly at the synapse, but there is no activity within the nerve ending. Mag. X 90,500.

Fig. 88: AChE reaction in postganglionic axons. The activity can be seen to be within structures which are similar to those found in cholinergic axons. Mag. X 35,200.

activity at 72 h does differ significantly from the denervated "control" level but it is not known whether this is a result of variation in the values or an indication that this time is a critical period in the overall recovery phase (see also Fig. 83).

An attempt was also made to estimate the AChE concentration in the preganglionic trunk both before and after DFP treatment. As mentioned earlier, it was possible to estimate the enzyme in control trunks but after administration of DFP the activities were so low that no accurate assessment of their AChE content could be obtained; by the time we were able to measure the enzyme level in the trunk (14 days after DFP) we could not detect any significant differences from the control values.

Cytochemical studies.

To complement the biochemical studies, the ganglia from normal and DFP-treated animals have also been examined and analyzed cytochemically. Four parts of the ganglia have been examined and examples from these areas in untreated control animals are given in Figs. 85-88. First, in ganglia cell bodies, appreciable activity can be found within the ER of the cells (Fig. 85) and, if this activity is near a dendrite, the ER is arranged in such a way that it runs parallel to the plasma membrane (arrow). Small preterminal axons can also be seen in Fig. 85 which react positively for AChE. An area which contains both cell bodies and axons is shown in more detail in Fig. 86; again there is AChE activity within the ER of the cells, but the only observable activity associated with the axons is that around their plasma membranes. A third area, a synapse, is shown in Fig. 87, where it can be seen that there is activity around the nerve terminal, particularly concentrated in the actual synaptic region, but that the intra-

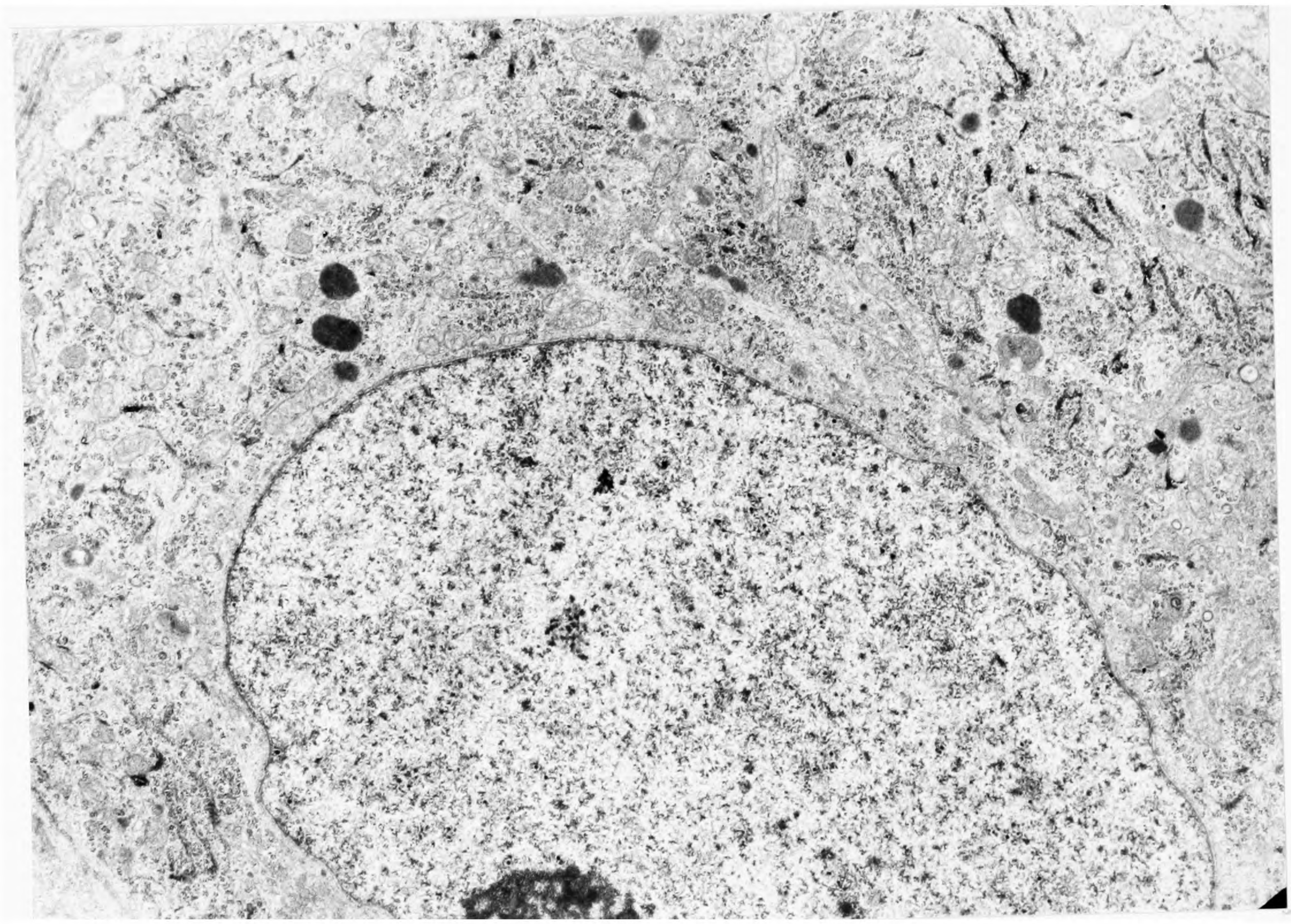


Fig. 89

Rat ganglion cell 2 h after DFP. 2 h after administration of DFP faint activity can be seen in the endoplasmic reticulum of the ganglion cells. Mag. X 7,200.

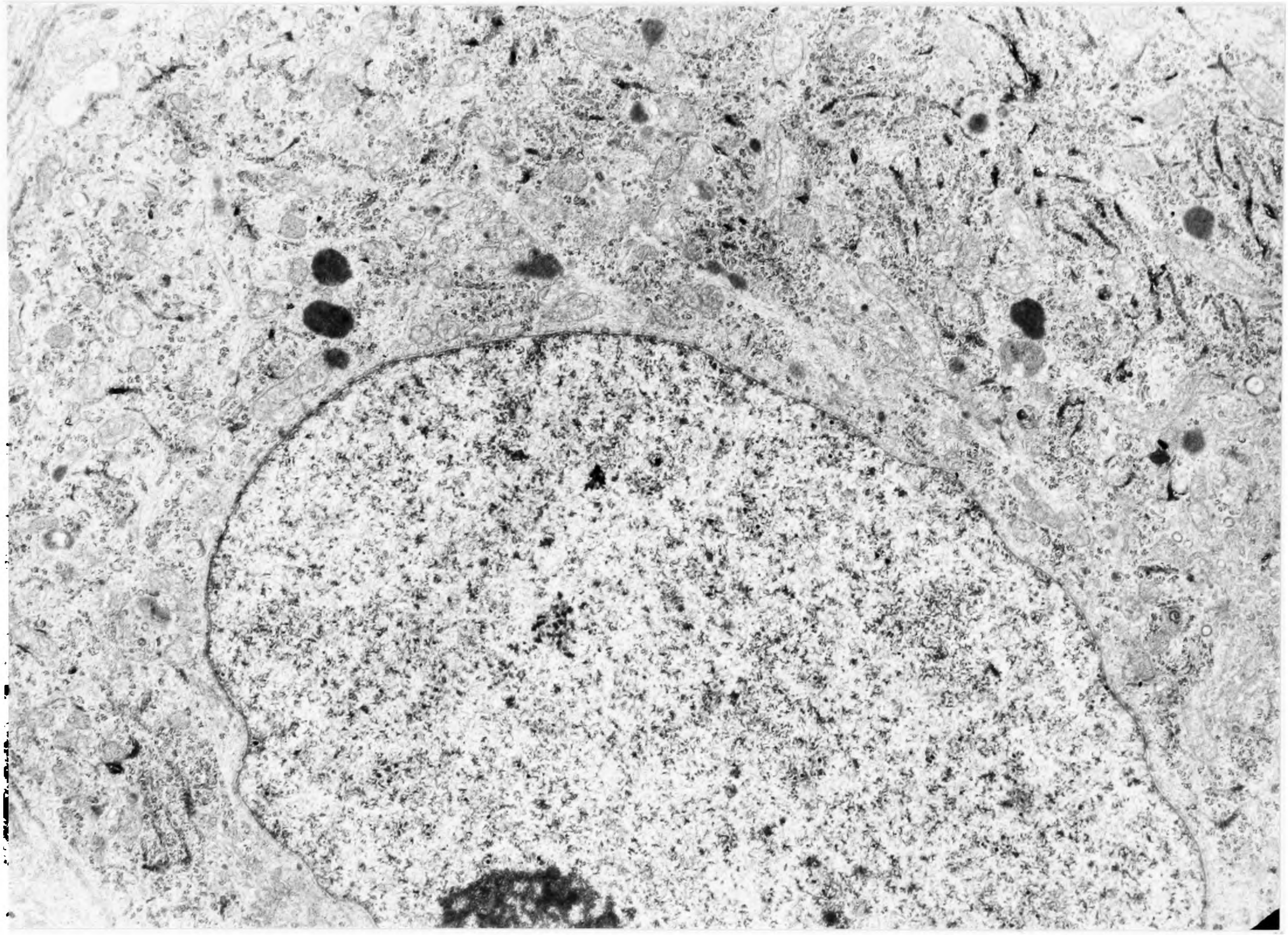


Fig. 39

Rat ganglion cell 2 h after DFP. 2 h after administration of DFP faint activity can be seen in the endoplasmic reticulum of the ganglion cells. Mag. X 7,200.

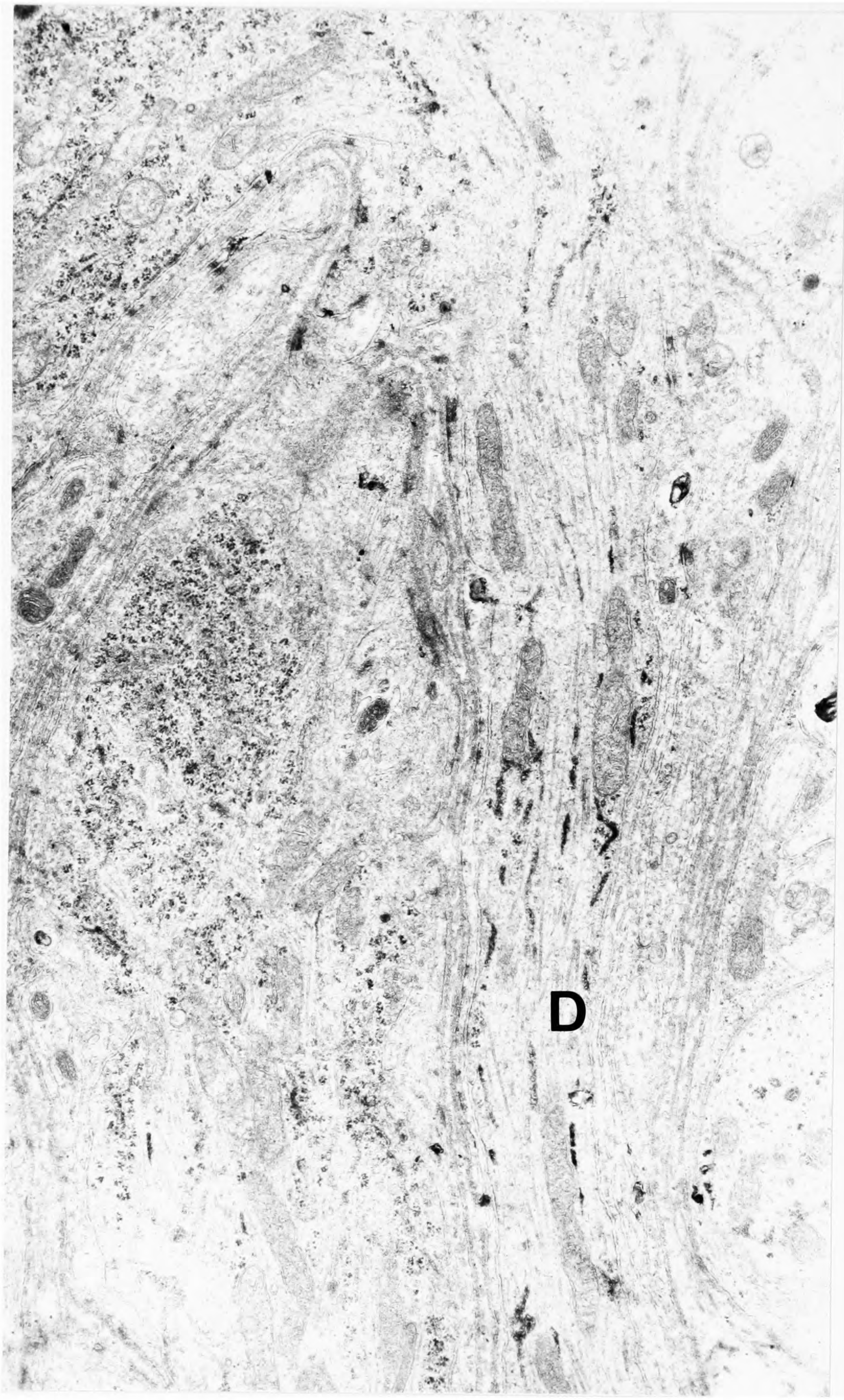


Fig. 90

A rat ganglion cell and dendrite 2 h after treatment with DFP. By this time activity can be observed within tubules which are within the dendrite (D) and a parallel to the plasma membrane of the cell. Mag. X 10,200.

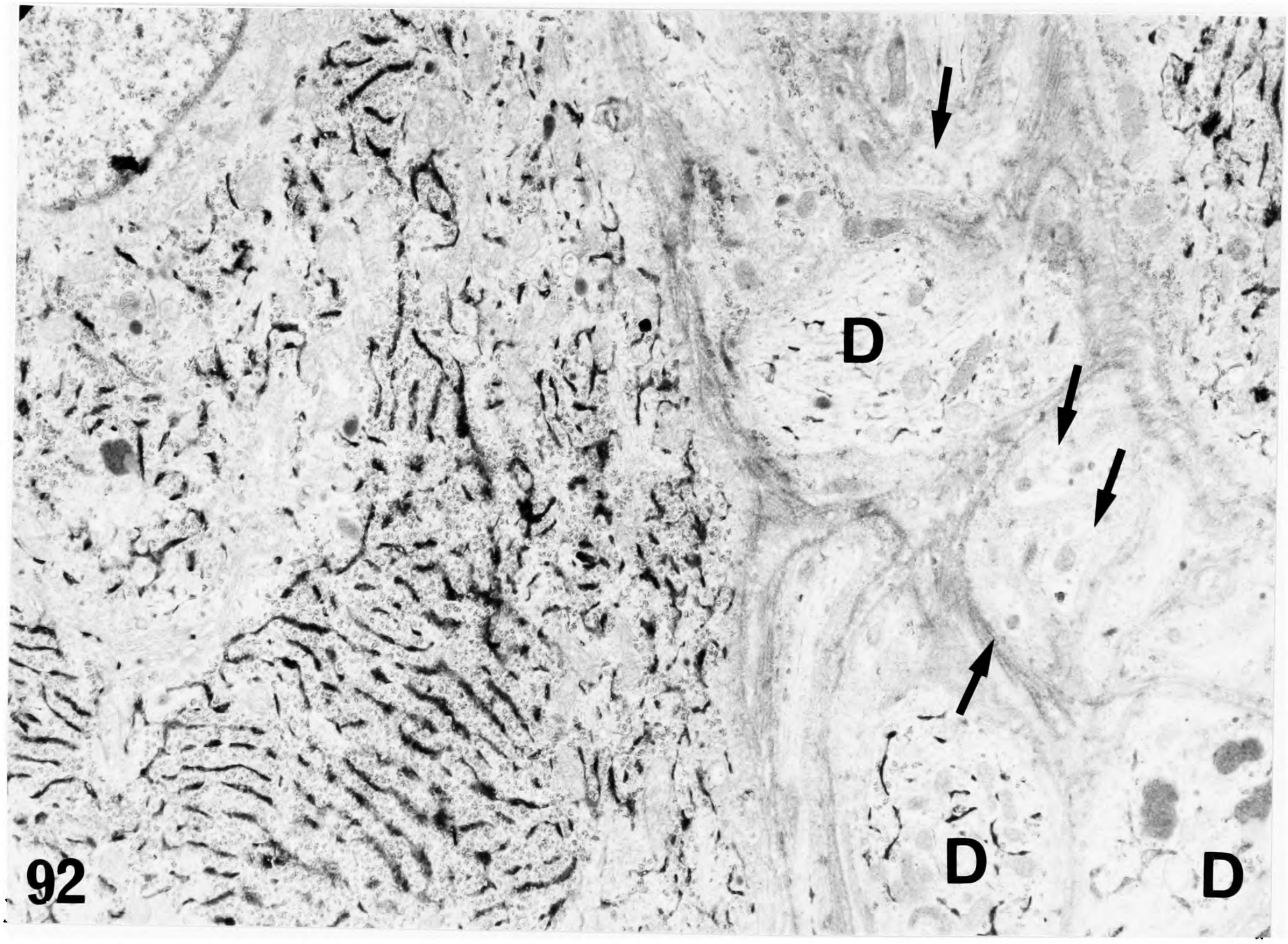


Fig. 91: AChE-rich endoplasmic reticulum within postganglionic axons 8 h after DFP. Mag. X 70,800.

Fig. 92: Ganglion cells, dendrites and preterminal axons 16 h after treatment with DFP. One ganglion cell (C) with extensive activity associated with the endoplasmic reticulum and several dendrites (D) are all active. Several preterminal axons (arrowed) are devoid of activity. Mag. X 7,300.

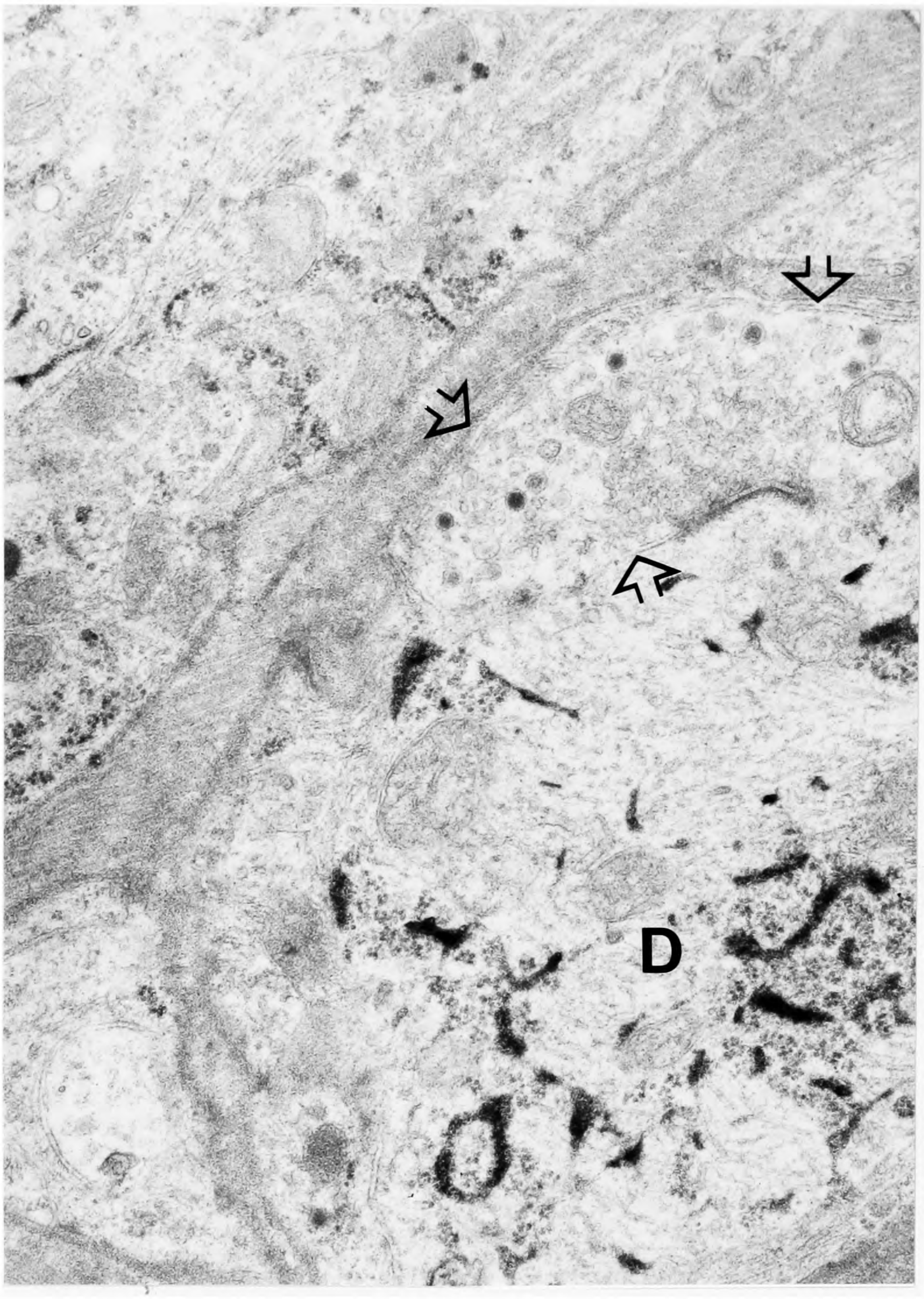


Fig. 93

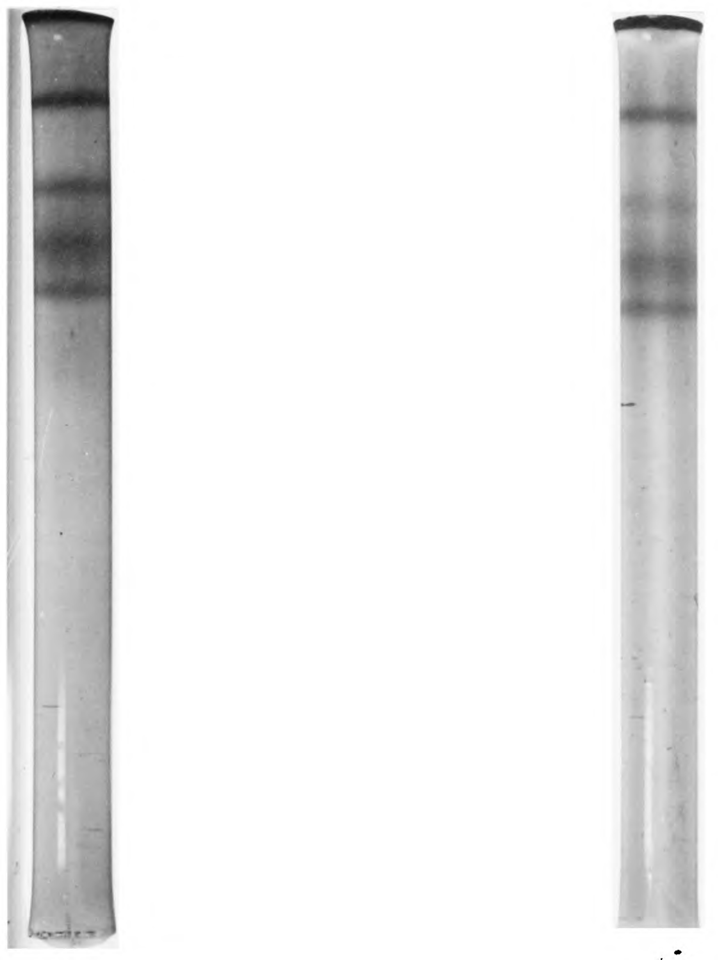
Dendrite and nerve terminal 16 h after DFP treatment. There is activity within both rough and smooth endoplasmic reticulum in the dendrite (D) but the nerve ending (arrowed) is devoid of activity. Mag. X 25,800.

terminal axoplasm is totally devoid of activity. Finally, as shown in Fig. 88, there are elements of SER in postganglionic axons which are rich in AChE.

The results obtained when ganglia were examined at different times after DFP are shown in Figs. 89- 93. A general view of part of a cell body shows that within 2 h of DFP treatment, there is demonstrable AChE activity within the ER of the cell (Fig. 89). If an area comparable to that shown in Fig. 85 is examined 2 h after treatment it can be seen that not only does the cell have activity within the ER after this time, but it is also exporting AChE-rich ER into the dendrite (Fig. 90). By 8 h tubules rich in AChE can also be found within the postganglionic axons (Fig. 91) so it would seem that the AChE in all compartments of the ganglion cells, i.e. cell bodies, dendrites and axons, has at least partially recovered as soon as 8 h after DFP treatment. By 16 h, when both total and soluble AChE activities are back to "control" levels, there is extensive activity throughout the cell body and its dendrites, but nowhere, as shown in Fig. 92, is there any enzyme associated with the preterminal axons (arrows; Fig. 92). Nerve terminals examined 16 h after DFP were found to be completely devoid of of activity (Fig. 93; cf. Fig. 87) although the cell with which it makes synaptic contact has intense activity within its ER system (Fig. 93). Thus, the cytochemical evidence is that, when all of the AChE activity which might be considered to be associated with the postganglionic cells has returned (Fig. 84), no enzyme activity can be observed which is attributable to the preganglionic fibres, it is all within the nerve cell bodies.

Isoenzymes of AChE.

The types of isoenzymes within the ganglia have also been examined. The bulk of the AChE activity, which is membrane-bound,



1 2

Fig. 94

Soluble isoenzymes of AChE in untreated (gel 1) and chronically denervated (72 h; gel 2) rat ganglia.

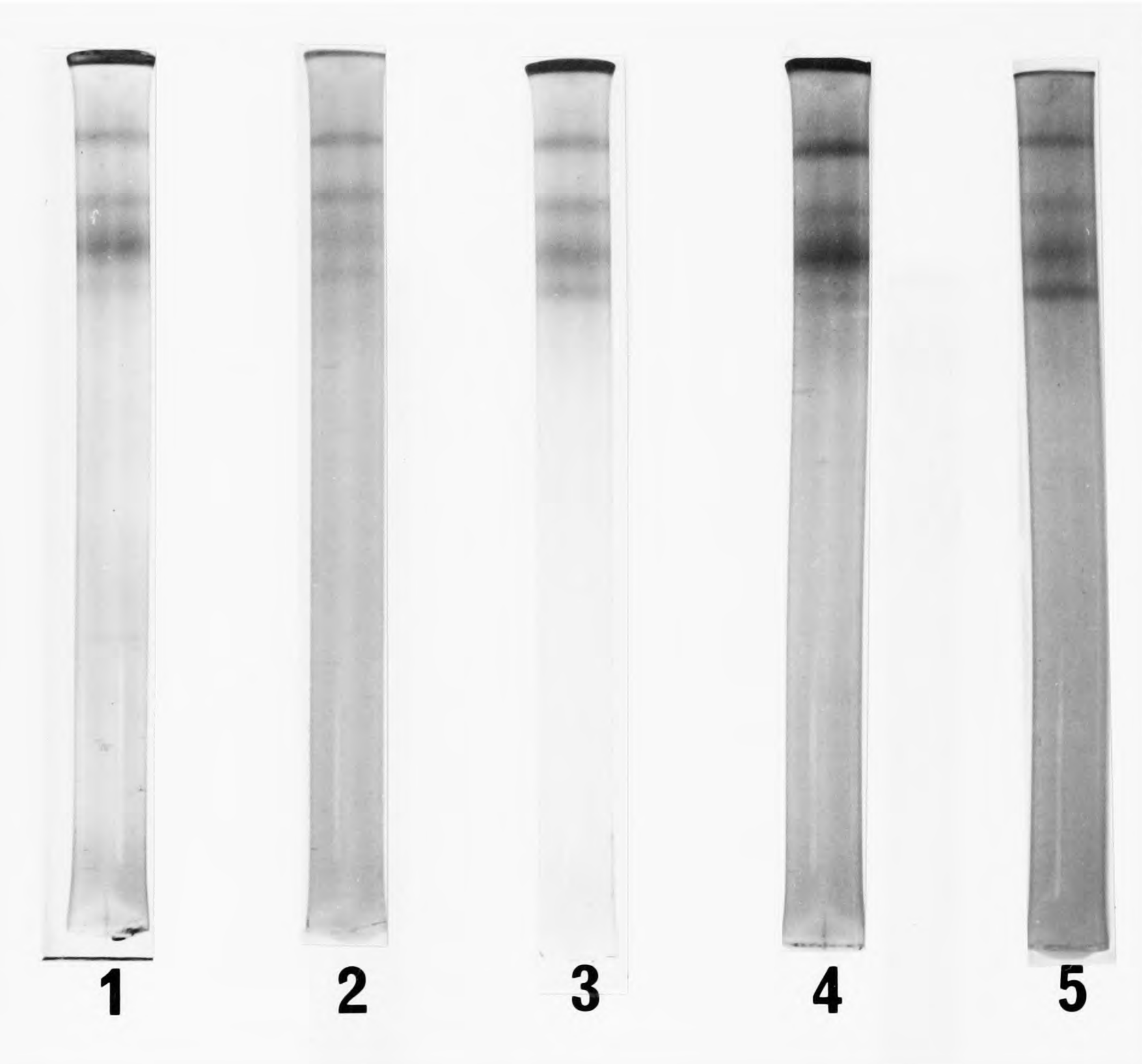


Fig. 95

The soluble isoenzymes of AChE present in the ganglia at different times after DFP treatment. Gel 1, 8 h; 2, 16 h; 3, 24 h; 4, 48 h; 5, 72 h.

behaves as a single isoenzyme when Triton X-100 dissolved membranes are electrophoresed on polyacrylamide gels. This isoenzyme, which is the slowest migrating of all those present in the ganglia, has very similar electrophoretic characteristics to the $AChE_6$ from bovine adrenal medullae and splanchnic nerve axons. The soluble AChE has a more complex isoenzymic composition because, as shown in Fig. 94, the total activity is made up of 4 electrophoretically separable isoenzymes (see also Gisiger, Venkov and Gautron (1974), who obtained a similar result). These isoenzymes have not been characterized further nor has an attempt been made to establish any of their properties. As also shown in Fig. 94, there is no qualitative difference in the isoenzyme pattern of ganglia which had been denervated 72 h before analysis.

When the soluble AChE in ganglia treated with DFP was examined electrophoretically it was found that from the earliest time at which there was sufficient activity to analyze on the gels, there were no qualitative differences between the isoenzymes in control and treated tissues (Fig. 95). Thus, the changes in soluble AChE activity probably represent changes in the amount of all of the soluble isoenzymes and not selective changes in one or other form.

Because of the very low AChE activity, even in normal preganglionic nerve trunks, there was some difficulty in obtaining good results when the isoenzymes in the preganglionic trunk were studied. However, the evidence is that only the slowest moving soluble isoenzyme is present in the trunk, the faster migrating enzyme forms are absent.

DISCUSSION

Before discussing the results obtained in these experiments it is essential to consider the evidence which suggests that reappearance of AChE activity after DFP treatment is the result of the synthesis of new enzyme molecules and not of a spontaneous or even enzymatic dephosphorylation of the "old" AChE. Experiments carried out in vitro using AChE from a variety of sources have shown that there is no spontaneous reactivation of the inhibited enzyme (Hobbiger, 1951; Davison, 1955; Wilson and Meislich, 1953; Blaber and Creasey, 1959). There is also a phenomenon termed "ageing" which renders DFP-inhibited AChE resistant to dephosphorylation by any of the available nucleophilic reactivators (see e.g. Cohen and Oosterbaan, 1963) and this has been shown to be caused by the formation of a very stable mono-isopropylphosphoryl-AChE (Berends, Posthumus, van der Sluys and Deierkauf, 1959). This latter highly stable compound can only be hydrolyzed under fairly drastic alkaline conditions (Berends et al., 1959) and so it seems likely that under normal circumstances there would not be any appreciable reactivation of inhibited AChE. So far as an enzymatic reactivation of the AChE is concerned, this too seems unlikely. Davis and Agranoff (1968) incubated DFP-inhibited brain homogenates for extended periods and did not observe any increases in AChE activity. Given the unstable nature of DFP in aqueous solutions (Koenig and Koelle, 1961), there is little likelyhood of a phosphorylation/dephosphorylation reaction being set up during an experiment such as that carried out by Davis and Agranoff (1968) and so it also seems unlikely that there is an enzymatic reactivation of the AChE. The explanation first put forward by Burgen and Hobbiger (1951) for the reappearance of AChE activity is therefore still the most likely; it represents newly synthesized enzyme molecules.

Turnover of AChE: In view of the points mentioned above, the results presented in this Chapter provide evidence that post-ganglionic AChE turns over fairly rapidly. If it is accepted that the first 15 mU of activity to return are entirely within the postsynaptic cells, then it can be calculated that the turnover time for the total AChE in the ganglion cells is 18.5 h (calculated using the measured rate of recovery of AChE activity, 0.81 mU/h). The only other study in which a similar type of calculation has been made seems to be that of Davis and Agranoff (1968), who estimated that only one of several AChE isoenzymes in rat retina turned over rapidly (half-life 3.2 h). To arrive at this figure, Davis and Agranoff (1968) made use of quantitative densitometry; as the zones of AChE activity were developing, they repeatedly scanned and hence quantitated the enzyme activity on their gels. In my hands, densitometry has not proved to be useful in estimating the relative turnover times of the isoenzymes. The area produced by a scan of my gels bore no relationship to the known amount of activity on the gels and the variation was totally random. Thus, I was left with no option but to rely on qualitative estimates of the proportion of the total soluble AChE which is attributable to each individual isoenzyme.

There seem to be only two reports in which the recovery of AChE after DFP has been studied in the SCG. The species used in these studies was cat and the time intervals at which the AChE levels were examined ranged from 1 to 15 days (Koenig and Koelle, 1960, 1961). One of the most interesting features of the studies by Koenig and Koelle (1961) is the level of AChE in the ganglion at the earliest time interval studied (1 day). From between 1 and 3 days after DFP treatment the AChE level in the ganglion was only 20% of the control value. It is unlikely to be coincidental that preganglionic denervation of the cat SCG also results in only 20% of the original activity remaining in the ganglion (Chapter 1). Thus, the results of Koenig and Koelle (1961) infer that all of the AChE which is probably attributable to the postganglionic cells of the cat had returned within 24 h of the DFP treatment, just as it had in the experiments reported in this Chapter on rat ganglia. Interestingly, Koenig and Koelle (1961) also found that the AChE content of the ciliary ganglion (in which, according to Koelle and Koelle (1959), all of the ganglion cells as well as the preganglionic trunk are rich in AChE) had recovered to almost 90% of control levels by 15 days after DFP treatment. Moreover, the return of activity was not biphasic as it was in my experiments and those on the cat SCG (Koenig and Koelle, 1961), it followed a single smooth curve.

One other result has been reported which also offers support for my conclusions. When Glow, Rose and Richardson (1966) studied the recovery after DFP of AChE in rat adrenals, they found that the activity was already very high within 24 h of treatment and that it had returned to normal levels within 5 days. In fact, Glow et al. (1966) showed that there was a consistent overshoot in the AChE activity so that by 5 days after treatment the adrenals had an AChE content which was 120% of the control level. It is not known with any certainty whether rat chromaffin cells contain AChE (see Chapter 1), although Somogyi, working in our laboratory, has preliminary evidence that both the chromaffin cells and the intramedullary ganglion cells are rich in the enzyme. It remains to be shown whether either or both of these types of cell contribute to the rapid recovery of the AChE.

An interesting comparison can be drawn between ganglia and muscle in their respective responses to DFP poisoning. From the results presented in this Chapter, there seems little doubt that sympathetic ganglion cells can synthesize AChE; the presence of an intact nerve supply is probably only necessary for a complete return to normal levels. For the AChE of muscle the case seems to be different. Filogamo and Gabella (1966) have shown that muscles of chickens treated with DFP recovered their AChE levels only if a normal innervation to the muscle was maintained; if the nerve was cut at the time of DFP administration there was no recovery of the enzyme. This is despite the fact that Salpeter (1967) has shown that as much as 85% of end-plate AChE is localized in the junctional fold region, and as little as 10% is associated with the nerve terminal. Thus, the nerve is necessary for the appearance of AChE on the postsynaptic membrane (Incidentally, Rose and Glow (1967) have shown that it is not simply the ACh released from the nerve which is required for the return of the AChE activity). Whether the nerve induces the muscle to synthesize AChE, or whether it supplies the postsynaptic enzyme by a process similar to that discussed in Chapters 4 and 8 is not known. However, it is noteworthy that muscles from embryonic chickens, grown in tissue culture, recover from DFP poisoning in a markedly different way from the adult muscles discussed above. The work of Barron et al. (1968), Guth (1968) and Wilson and Walker (1973) not only shows that foetal and neonatal muscles can rapidly recover from DFP poisoning, it adds the possibility that these tissues cannot "store" the AChE that they synthesize. Wilson and Walker

(1973) and Wilson et al. (1973) have shown that newly synthesized AChE (after DFP) is released into the tissue culture medium. Thus, the foetal and neonatal muscles have the ability to rapidly synthesize AChE independently of the nerve supply, but they seem unable to "store" it; adult muscles are less able to synthesize the enzyme, but what they have they can retain (see also the discussion of Chapter 6). The different aged muscles seem to behave almost as though they were different tissues. The evidence suggests that neonatal muscles bear a striking resemblance to adult sympathetic ganglia: they each have a high proportion of their AChE in a soluble form (Barron and Bernsohn, 1968; Koelle et al., 1970) whereas the bulk of the AChE in adult muscles is particulate-bound (Koelle et al., 1970); only foetal and neonatal muscles have cytochemically demonstrable AChE within the muscle cells (Wilson et al., 1973), adult muscle cells are devoid of activity (Koelle, 1963); ganglion and young muscle cells can both synthesize AChE more readily than adult tissues. Is it possible that different cholinergic tissues all develop in a similar way and it is only later that they take on different properties to suit their different functions?

Localization of AChE in ganglia: The combined biochemical and cytochemical experiments presented in this Chapter show that all of the AChE activity which is present in the SCG at short time intervals after DFP treatment is restricted to the postganglionic cells. It must take a considerable time for the cell bodies of the preganglionic neurons to synthesize and then transport by whatever means, that proportion of the enzyme which they contribute to the ganglion. These results also show that the axolemmal AChE must be derived from the axon, just as was the splanchnic axonal enzyme. In ganglia after DFP, when the cells which the preganglionic axons run between or innervate are rich in AChE, there is no enzyme associated with the axons. Conversely, the splanchnic axons always show AChE-rich axolemma even when they are observed between chromaffin cells devoid of enzyme.

Rat SCG and bovine adrenal medulla have several similarities which we can assume to be characteristic of preganglionically innervated neurons: there are several soluble isoenzymes of AChE in the cells, while their innervating nerve fibres seem only to contain one; a considerable proportion of the AChE within the cells is in the ER and this seems to migrate away from the nucleus toward the plasma membrane (and in ganglion cells into the dendrites and postganglionic axons) and there appears to be no activity within the nerve terminals of the incoming preganglionic fibres. The chief difference seems to be in the higher proportion of the AChE which is lost from SCG compared to the bovine adrenal medulla when the tissues are denervated.

While the similarities between the tissues might be expected because they both contain essentially postganglionic cells innervated with preganglionic fibres, can the difference between them be explained? Probably it can be partly accounted for by the observed differences in the specific activity of the AChE between the tissues and their respective innervating fibres. Thus, on a weight basis, the SCG has 3.5-fold more AChE than the preganglionic trunk (17,500 versus 5,000 mU/g) while the bovine tissues have the same concentration of enzyme in each. Under normal circumstances the increased concentration of AChE within the ganglion would be put down to an accumulation of

AChE within the nerve terminal. But our observations entitle us to ask: where does the enzyme accumulate? Never have we seen AChE activity within the nerve ending which is where activity could be expected to accumulate if AChE is supplied to the terminal by axonal transport mechanisms. And since we now know that post-ganglionic cells show appreciable AChE activity we are entitled to ask the question whether it is necessary to postulate that the enrichment in the AChE concentration of the ganglion is due to an increase in the amount of activity associated with the nerve endings.

What is the evidence for this postulate? Principally the whole case rests upon the disappearance of AChE from chronically denervated ganglia. Denervation results in the degeneration of the preganglionic nerves (see Chapter 1) and so the loss of AChE is thought to be due to this disappearance of the nerve fibres (McLennan, 1954; Dhar, 1958; Klingman and Klingman, 1969). However, in experiments that I have carried out with Dr. M.R. Matthews, it was found that the rate of disappearance of AChE from denervated ganglia was extremely rapid. Within 16 h of the operation, the AChE content in the ganglion had started to fall and had reached its minimum level (55% of normal) by as soon as 24 h. This rate of decrease would seem to be too fast if it were simply dependent upon a degeneration and loss of the axons (see Matthews, 1973) and so we must postulate that there must be an alternative, or at least an additional reason for the loss of AChE activity upon denervation.

Is there more conclusive evidence against the idea that pre-ganglionic nerve fibres contribute a major portion of the ganglionic AChE? First, let us consider the quantitative data in much the same way as it was considered for the bovine adrenal medulla and splanchnic axons. If as much as 1% of the weight of the average ganglion was due to the nerve, then the weight of nerve within the tissue would be about 18 μg. As mentioned earlier, 200 μg of nerve contain 1 mU of enzyme activity and therefore 18 μg of unenriched nerve would contain 0.09 mU of AChE activity. However if, as discussed earlier, 45% of the AChE in a ganglion is derived from the nerve (i.e., 14.0 mU) this would require an increase in the AChE concentration of the fibres to the order of 155-fold. This should certainly have been observable cytochemically. We are left, therefore, with the alternative idea that the bulk, but not necessarily all, of the ganglionic AChE is derived from the postganglionic cells and that the changes in enzyme level after denervation are due either to an alteration in the rate at which the protein is synthesized or to a difference in the amount of AChE which is removed or transported from the ganglion, or to a combination of both factors. It is pertinent, therefore, that the proportion of a ganglion's AChE which is soluble remains virtually constant after denervation (Fig. 84). We have already suggested that, because of the rapid recovery of the soluble enzyme after DFP treatment, most of it is probably localized within the cell bodies, and thus a reduction in amount of the enzyme, induced by denervation, must mean that the lack of an incoming fibre and hence, probably, nerve activity, influences the amount of AChE in the postganglionic neurons. I would therefore like to suggest that the effects of denervation on the AChE content of ganglia (and adrenal medullae) are more complex than originally proposed; while not disputing that some AChE activity is associated with the preganglionic axons, it would seem that once these fibres enter and reach the tissues that they innervate, their content of AChE is diluted within a far larger pool. If this is so, then the second increase in total activity shown in Fig. 83 may not represent the return of presynaptic AChE but could be a reflection of a conversion of soluble into membrane-bound enzyme which, because of the almost fully saturated stores of the soluble ènzyme, does not result in a decrease in the concentration of the latter form. Synthesis can keep up with demand. A final resolution of these problems awaits further experimentation, as does the reason for the slow steady increase in soluble AChE from about 4 days up to 21 days after DFP.

The results reported in this Chapter provide evidence that the AChE in postganglionic cells can turn over rapidly. Thus, they offer support to our hypothesis that these cells could release AChE. It is noteworthy that the DBH in sympathetic ganglia, known to be released from the nerve terminals of the ganglionic neurons, has been shown to have a turnover of 5-6 h (De Potter, 1975).

CHAPTER 8

QUESTIONS ANSWERED AND QUESTIONS ASKED.

A GENERAL DISCUSSION OF ALL THE RESULTS.

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A GENERAL DISCUSSION OF ALL THE RESULTS.

In Chapter 1, when describing the type of work that was carried out for this thesis, I expressed the hope that my work would answer some of the questions surrounding this field of research and, possibly more importantly, that it would raise many more. We are now in a position to decide whether these aims have been achieved. In this Chapter, I will briefly describe the questions which I feel have been answered and discuss how future work could answer any questions which have been raised by these studies.

There seems little reason to doubt that homogenization of cholinergically innervated tissues will always result in the appearance of truly soluble AChE. The circumstantial evidence that elements of SER, broken during the homogenization, liberate their internal, soluble enzyme, while retaining the type associated with their membranes, is strong, and these structures have been found in every cholinergic fibre so far examined. Thus, the first established fact is that cholinergic nerves do contain soluble AChE and so AChE cannot be considered to be a totally membrane-bound enzyme. This also appears to be the first report that the membrane-bound and soluble AChE are composed of different isoenzymic forms. In earlier reports this has been suggested (Davis and Agranoff, 1968), doubted (Maynard, 1966) and denied (Barron and Bernsohn, 1968). But the implications of my finding in the understanding of, for example, centrifugation experiments are very clear. Without this knowledge, centrifugation of tissue homogenates produces a confusing result, an almost equal distribution of AChE between particulate and soluble fractions. With it, however, it is clear that at least so far as the nerve axons are concerned, there are two principal isoenzymes, each with its own unique localization, one in the particulate fraction and one in the high-speed supernatant. The confusion surrounding the centrifugation experiments can thus be resolved. The tissues which contain a more complex isoenzyme composition than the axons are in themselves more complex than the relatively simple axonal system. The three organs which have been used for the experiments described in this thesis, bovine adrenal medullae, rat SCG and rabbit brain, all have more than one soluble isoenzyme of AChE. But they all differ from the axons in that they contain, in addition to nerve fibres, both nerve cell bodies and nerve terminals (considering, of course, that the chromaffin cell is a type of postganglionic neuron). The work presented in this thesis suggests that these extra soluble isoenzymes are exclusively derived from the cell bodies. I would like to suggest that these isoenzymes are related in that they could be smaller molecular weight precursors of both $AChE_5$ and $AChE_6$ (for simplicity, I will always use the terms $AChE_5$ and $AChE_6$ to describe, respectively, the soluble isoenzyme with the slowest electrophoretic mobility and the membrane-bound form, even when the tissue under discussion does not have that number of isoenzymes). The relationship between $AChE_5$ and $AChE_6$ is harder to explain. One possible explanation, and one which seems fairly likely, is that $AChE_6$ is really $AChE_5$ which is simply attached to a membrane in such a way that there is no significant alteration in its apparent size, but that there is an alteration in electrophoretic mobility. To postulate an attachment of this type is not unreasonable since it is known that the membrane-bound AChE at motor end-plates can be detached from its normal site and solubilized by enzymatic means. Thus, a motor nerve/muscle preparation treated with both collagenase and protease yields soluble AChE and a separated nerve and muscle (Hall and Kelly, 1971; Betz and Sakmann, 1971). This treatment had not resulted in a disintegration of the tissue and hence a release of the enzyme from intracellular sites; the nerve could still conduct action potentials and the muscle respond to ACh. Morphological analysis showed that both tissues had a completely normal appearance (Betz and Sakmann, 1971). AChE at the motor end-plate is not, therefore, an integral part of the membrane of either cell, it is attached, presumably, to the muscle (Salpeter, 1967) in such a way that it can be detached by treatment with relatively non-specific proteolytic enzymes. Thus, it seems unnecessary to suggest that the $AChE_6$ in any of the tissues studied in this work is an integral part of any membrane, it could simply be attached to it through some type of "receptor" site which is present on the membrane surface. If this is so, it could explain a puzzling observation made during the course of this work - the widespread occurrence of $AChE_6$. This isoenzyme of AChE is the only membrane-bound form present in all of the tissues studied. This relative simplicity of composition could be explained if all tissues have a common "receptor" site and if $AChE_5$ is the only soluble isoenzyme able to combine with this "receptor", then they would all contain $AChE_6$ and only $AChE_6$. It is noteworthy in this context that in one place where the enzyme is considered to be a constituent protein of the membrane (bovine erythrocyte, see Augustinsson, 1963), I have found that it is a totally different isoenzymic species from $AChE_6$ (unpublished work).

We were also able to establish the fate of $AChE_5$; it is released from cells. However, the reasons why and the actual site of release are still open to debate. As remarked in earlier Chapters, our current working hypothesis is that the perfusate AChE, in the experiments described in Chapter 4, is derived from the chromaffin cells. This is not meant to imply that nerve axons cannot release the enzyme and perhaps more meaningful discussion of this point can be carried out if the reasons why the enzyme is released are considered along with the question of the site of its release. At the ultrastructural level there is little doubt that the immediate source of the released enzyme is the ER of the cells, and we have been able to observe an apparent fusing of the ER with the plasma membranes of both axons and chromaffin cells. Is it possible that the tubules fuse with the plasma membranes to provide either new membranes or new AChE? Either possibility seems reasonable and clearly there is one mechanism which could fulfill both roles. If the membranes of the ER are precursor plasma membranes, as postulated by Bray (1973) and Bunge (1973) then a fusion of these tubules with the cell membrane would result in not only new plasma membrane but also one with the AChE (6) already attached and in its correct position. Excess $AChE_5$, in position but not yet associated with the membrane, would be secreted and disposed of as unwanted material.

Alternatively, the released $AChE_5$ could either diffuse away from the site of release and occupy more distant "receptor" sites, or reach the blood to play another but unknown role. Which of these several alternatives is the most likely to occur is not known. We must wait until it is determined whether it is $AChE_6$ or $AChE_5$ that is most required by the cells. It also remains to be seen whether the postulated "receptor" can combine with the $AChE_5$ by a non-enzymic mechanism. This seems the only way in which the second alternative described above could be brought about since it would occur in the extracellular space.

Are there arguments against the possibility that the ER is precursor plasma membrane? Probably the only one of real importance is that of Teichberg and Holtzman (1973) who showed that the membranes of axonal SER were some 20-25% thinner than the membrane of the axon itself. However, as shown by Pfenninger (1972), new plasma membrane takes some time to mature. This conclusion was based upon the observation that the plasma membrane of nerve growth cones had many fewer "particles" than did those of more mature axons. It was suggested that it took some considerable time for particles to be added to the membrane matrix, and hence for maturity. It has already been mentioned that the plasma membrane of nerve growth cones seems to be supplied by a mechanism similar to that which I proposed above for more mature axons, so it is conceivable that differences in thickness are not evidence against the possibility of a relationship between the two types of membrane.

The possibility that extracellular fluid levels of AChE are an index of nerve activity was also examined and to some extent confirmed. Certainly, there does not seem to be much doubt that stimulation of peripheral nerves results in an increase in the AChE concentration of CSF, although the reasons why and from which structure the protein is released are not known. However, the relationship between the brain and its extracellular fluid, the CSF, and that which exists between the adrenal medulla and its perfusate are strikingly similar. Each fluid contains only one isoenzyme, and changes in the concentration of AChE reflect changes in the amount of this isoenzyme, while both tissues have several soluble forms of AChE stored within them. Thus, we must add a third fact to those already known about the proteins which are secreted, not only are they stored within different structures and spatially oriented differently within the cell, but there is a system by which only presumed mature proteins are released. But at least knowing the structure within which the AChE is localized makes envisaging this latter point relatively straightforward. If the AChE behaves in an analogous fashion to the immunoglobins, it would be reasonable to suspect that the further along the ER the protein is situated, the more mature and fully developed it has become. For example, as the last carbohydrate is added to the immunoglobin chain, it finds itself at the end of the ER tubule, and it is this form which is eventually released (Williamson, 1969). Immature forms of the protein are not released extracellularly.

Finally, it was shown that postganglionic AChE can be synthesized fairly rapidly, at a rate of about 5% of the stores per hour. The only real complication in the results provided by these experiments is how to interpret the short lag phase between the start of the accumulation of total AChE and that of the soluble enzyme. If the suggestions discussed above are correct, it is necessary that soluble isoenzymes are produced first, for they in turn give rise to the membrane-bound form of AChE. However, it seems that in the initial recovery phase after DFP poisoning, the first to accumulate is the enzyme associated with the membranes. Can this be explained in terms of the above hypothesis? It can, if we assume that the isoenzymes with the fastest electrophoretic mobility turn over more rapidly within the cell than do $AChE_5$ and $AChE_6$. A situation such as this could, in the early stages, result in an accumulation of $AChE_5$ and, because there are more of the hypothetical receptor sites available than there is normally enzyme, this is rapidly bound and hence transformed into $AChE_6$. This is admittedly a speculation, but one possibility.

There are two main questions raised by this work: first, why is AChE released and secondly, what is the importance of AChE inside adrenergic cells? An answer to either question will probably not be easy to obtain but there are several points which are worth discussing.

It now seems beyond doubt that AChE plays an important part in the termination of the effects of ACh at both ganglionic and neuromuscular synapses. It is not possible to consider that diffusion of ACh away from the receptors is the only mechanism by which the action is stopped (Ogston, 1955). But this is not the real problem. We must ask why it is necessary for a cell, either pre- or postsynaptic, to release AChE when, for example, as much as 85% of neuromuscular AChE is already on the end-plate (Salpeter, 1967) and almost all of the functional AChE in ganglia is already on the neuronal cell membrane (Koelle, 1971). With a turnover number as high as 195,000-295,000 (molecules ACh/min/active site; Berry, 1951; Cohen and Warringa, 1953) and an estimated 1-2 x 10^7 active sites per synaptic region (Hubbard, 1973) it would seem reasonable to examine reasons other than those related to ACh metabolism for the release of AChE.

When considering the release of AChE or, for that matter, any protein from cells, the discussion must include the possible implications of there being local but probably quite high concentrations of these macromolecules in the extra-cellular space at the site of release. In so far as the tissues used in my studies are concerned, we know that a considerable number of proteins can be released from chromaffin cells (it remains to be seen whether similar proteins can be released from central neurons) and in addition, there is the possibility of there being other, unnamed macromolecules released from the cholinergic fibres (Musick and Hubbard, 1972). Quite apart from the actual number of proteins released from the chromaffin cells (7 or 8 chromogranins; Schneider et al., 1967; 4 acid hydrolases; Schneider, 1968) the remarkable fact about these cells is, as pointed out by Smith (1968), that they secrete, on a weight basis, as much protein as they do catecholamine. Yet we are no nearer today to understanding why these large amounts of protein are released than we were in 1965, when the phenomenon was first shown (Banks and Helle, 1965). We don't know how much AChE is released in terms of the amount of protein, but we do know that we have never seen a protein band on polyacrylamide gels which corresponds to the zone of enzyme activity. It can also be calculated, by using the specific activity of the most highly purified AChE (12,500μmole/mg protein/min; Leuzinger, 1969), that the average of 34 mU of AChE activity secreted from the adrenal gland is the responsibility of only about 3 ng of actual protein. We are clearly not dealing with a secretion of the chromogranin proportions. But in terms of the total amount of $AChE_5$ in the adrenal medulla (estimated to be about 90 mU; Chapter 3), as much as 35% can be secreted under the experimental conditions used in Chapter 4. Thus, while the actual amount of material may only be small, it is nevertheless a significant proportion of the total available activity and hence, presumably, of the protein.

There is a precedent for AChE secretion and, more interestingly, it is one which does not seem to be related to cholinergic function. Parasitic nematodes are a variety of intestinal worms which infect, notably, sheep and they have a number of interesting properties. First, different species of nematodes have widely different proportions of soluble AChE (6-64%; Ogilvie, Rothwell, Bremner, Schnitzerling, Nolan and Keith, 1973). Secondly, if the worms were kept in saline solutions, a large amount of AChE activity was found in the bath, the amount being unrelated to the amount in the tissues (Ogilvie et al., 1973). Thirdly, the highest amount of activity secreted was by immediately post-larval worms (Rothwell, Ogilvie and Love, 1973) and fourthly, there were different soluble isoenzymes of AChE (Hogarth-Scott, Watt, Ogilvie and Rothwell, 1973). A remarkable series of similarities with many of the experiments described in this thesis. The only major difference is that the AChE might be stored in pre-formed secretory granules (Lee, 1970) although this has not been subsequently confirmed. The main point, however, is that a worm secretes AChE for reasons totally removed from aspects of cholinergic transmission. The protein must have other functions, but these are not known with any certainty. (Ogilvie et al., 1973).

One more point that I would like to make is that marked and rapid changes in AChE tissue levels can also be detected. Thus, AChE is known to have an age-dependent circadian rhythm in the brain (Mondgil and Kanungo, 1973; Mohan and Radha, 1974), rapid changes in the cerebral cortex in response to asphyxia (Stastny and Sedlacek, 1973) and a rapid decrease and restoration activity in the hypothalamus in response to the suckling stimulus (Oba, Ota and Yokoyama, 1971). Of the available mechanisms by which the AChE content of a tissue might rapidly decrease, the work presented in this thesis suggests the possibility of an excretion of the protein. An increase or restoration of the AChE level, which also occurs rapidly, can be accounted for by the very fast rate of synthesis of the protein, as shown in Chapter 7. If the protein is released, where does it go? To determine whether there is a circadian rhythm in the AChE concentration of extracellular fluids might supply the answer.

So far as the second question raised by this work is concerned, the importance of AChE in adrenergic cells, little can be said. The possibility that the chromaffin cells might release the enzyme has been discussed in earlier Chapters, but we do not know if adrenergic neurons also release AChE. However, we do know that these cells transport AChE down the axons, probably within a similar structure to that which contains the enzyme in cholinergic nerves. We also know that the apical catecholamine-secreting pole of the chromaffin cells also contain sparsely distributed but still demonstrable AChE-rich endoplasmic reticulum profiles. Is it possible that both these types of cells actively transport AChE-containing tubules to the area from which they release their better known secretory products? If it could be shown that adrenergic sympathetic neurons also release AChE (from their terminals?), then many fascinating possibilities would be raised. Not least of these is the one which could mean that AChE has other roles in neuronal function that are just as important as its ability to hydrolyze ACh. It is even possible that this latter ability could prove to have been a hindrance to our understanding of the functions and roles of AChE. It seems to me that it is not enough to assume that, because there is sound evidence <u>for</u> cholinergic transmission, <u>all</u> the AChE is present to hydrolyze the acetylcholine. For example, we must ask why postganglionic adrenergic neurons, and chromaffin cells, contain AChE. We must ask why these adrenergic neurons transport AChE not only into their dendrites, but also down their axons. <u>And</u> we must ask why there never appears to be AChE activity anywhere within the presynaptic cytoplasm, although the axons which give rise to these synapses are known to transport the enzyme in their direction.

<u>A new working hypothesis</u>

Considering all the evidence presented in this thesis, I would like to propose the following working hypothesis for the role, function and behaviour of AChE in the autonomic nervous system:

(i) Axons: At least a part of the AChE within the axoplasm is contained within elements of the smooth endoplasmic reticulum. The activity is associated with both the internal phase, where it is probably soluble, and the membranous phase of these tubules. This enzyme is destined for the axolemmal surface and is supplied to this site by a mechanism which involves a fusion of the endoplasmic reticulum with axonal plasma membranes. Whether there follows an active expulsion of the internal, soluble enzyme or whether the prime objective of the fusion is to incorporate the tubule membrane into the plasma membrane is not known. The net effect of this process, however, is to replace old external AChE with new enzyme. Thus, it is not likely that there is any marked change in the enzyme activity from one end of the axon to the other since, although these tubular structures are migrating down the axon, they only serve to replace an equivalent amount of AChE; activity is not lost during the fusion process. There are two problems associated with this proposal. First, we don't know with certainty the rate of flow of the AChE-rich tubules and secondly, we don't know what proportion of the total enzyme activity they contain. However, since it is unlikely that an appreciable amount of the soluble AChE is located within the soluble cytoplasm, I propose that the bulk of the AChE is associated with the SER and that this flows at a rate not markedly different from the one calculated in Chapter 3.

To explain the dearth of AChE in the nerve terminals is not possible with the current information. But we know that the synaptic plasma membrane must be reconstituted after a release of neurotransmitter, so it is possible that when it is known how this is done, we will find the answer to the question why there is no AChE within the synapse, even though there is activity associated with the membrane. Perhaps the synaptic plasma membrane is renewed at a site close to the synapse but which is still preterminal axon.

(ii) Postganglionic cells: The major proportion of the AChE in ganglia (including adrenals) is derived from the cells originating in these tissues. This can be used to explain the relative simplicity of the isoenzyme pattern in axons when compared with that of the cells. Actively synthesizing cells show all stages in the development of the protein molecule, but they only export "mature" enzyme. Thus, $AChE_5$ and $AChE_6$ can be regarded as being finished products of the smaller molecular weight forms.

From one result obtained with DFP, i.e., where it was found that intracellular activity had returned but that there was no activity associated with the synaptic area, I suggest that the AChE within the cells has little to do, at least directly, with the hydrolysis of ACh within the ganglion. This property is restricted to the enzyme which is around the synapse and which must be supplied by the nerve itself. Thus, in the terms used by Koelle and Koelle (1959) the functional AChE is that associated with the synaptic terminals. It remains to be shown whether there is sufficient AChE associated with these fibres to hydrolyze the amount of ACh liberated during nerve activity.

APPENDIX

When I started writing this thesis I intended to use the appendix not only as a means of presenting the sources of my materials and redéfining the abbreviations used, but also to gather together some of the properties of acetylcholinesterase. However, having arrived at the stage to do this, I find that most of the relevant properties have already been mentioned at different points in the text. Perhaps the only property which still needs to be discussed is the actual mechanism behind the hydrolysis of the substrate.

The active centre of the enzyme is thought to consist of two parts, an anionic site which gives the cholinesterases their specificity for cationic substrates, and an esteratic site. It is at this latter site that the actual hydrolysis of the substrate, bound to the enzyme through the anionic site, takes place. The dual binding site is also used to explain the observed substrate inhibition of the enzyme - a molecule bound to each site as could occur at high substrate concentrations, would mean that neither of them was hydrolyzed. Acetylcholinesterase is also one of the most rapidly reacting enzymes, with both a high turnover number (see Chapter 8) and a high specific activity. The highest specific activity has been found for acetylcholinesterase obtained from the electric organ - 730,000 μmole substrate hydrolyzed/h/mg protein (Leuzinger, 1968).

Materials

With only the following exceptions, all reagents used were at least of "Analar" purity and were obtained from British Drug Houses, Poole, Dorset.

Acetylthiocholine and 5,5' dithionitrobenzoic acid (DTNB) were both obtained from Boehringer Mannheim GmbH. The DTNB was always recrystallized, from water, before use. Eserine sulphate, β-diphosphopyridine nucleotide (reduced form) and the standard proteins were obtained from Sigma Chemical Corp., London. The reagents used for microscopy were purchased from TAAB Laboratories, Reading. BW284C51 was bought from Wellcome reagents, Kent, and the DFP (Dyflos B.P.C.) was purchased from Boots Pure Drug Co., Nottingham.

Abbreviations

ACh	acetylcholine
AChE	acetylcholinesterase
ATCh	acetylthiocholine
ChE	non-specific cholinesterase
CNS	central nervous system
CSF	cerebrospinal fluid
DBH	dopamine β-hydroxylase
DFP	diisopropylfluorophosphate
DTNB	5,5' dithionitrobenzoic acid
ER	endoplasmic reticulum
LDH	lactate dehydrogenase
SCG	superior cervical ganglion
SER	smooth endoplasmic reticulum

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