ENZYMIC ASPECTS OF ACETYLCHOLINE METABOLISM

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SUMMARY

Investigations have been performed involving choline acetyltransferase (ChAc), the enzyme responsible for the synthesis of acetylcholine (ACh) and cholinesterase (ChE), the enzyme responsible for the degradation of ACh.

Attempts were made to purify ChAc in order to obtain a preparation which could be used to accurately characterise the enzyme. A comparison of preliminary purification procedures indicated that homogenisation in 0.1% Triton X-100/200 mM KCl was the best method for extracting ChAc from rat brain and human placenta. These studies were of value in obtaining enzyme preparations suitable for subsequent studies on the acetylation of various substrates by ChAc. Confirmation of earlier reports that inconsistent results are obtained on attempting to purify ChAc by ammonium sulphate fractionation suggested that this technique is of limited value in the purification of this enzyme. Purification of ChAc by the more specific technique of affinity chromatography was attempted but was unsuccessful.

A study was made of the acetylation of various substrates by ChAc. Several methods of determining ChAc activity were compared and it was found that there were some differences in the suitability of the various methods for the study of substrates other than choline. Complications which arose in the determination of ChAc activity, such as the formation of choline within ChAc preparations and the hydrolysis of the acetyl coenzyme A used in the determinations, were either explained or resolved so that reliable estimates of the acetylation of substrates by ChAc could be made.

Hemicholinium-3 (HC-3) and triethylcholine (TEC) were shown to be acetylated in vitro by ChAc preparations from rat brain homogenates. It was shown that a small percentage of the acetylation of TEC could probably be attributed to an enzyme other than ChAc present within the preparation. The possibility that carnitine acetyltransferase (CarAc) was involved in the acetylation of substrates by rat brain homogenates was investigated but the results suggested that, of the substrates tested, only carnitine was significantly acetylated by this enzyme. The acetylation by ChAc of edrophonium and of some analogues of hemicholinium was studied and this provided information on the possibility of a pre-synaptic component to the pharmacological actions of the compounds and also provided information on the structure/activity relationships between the various substrates with respect to their acetylation by ChAc.

Two potential pesticides were tested for and found to possess cholinesterase inhibitory activity which correlated with the in vivo toxicity of the compounds observed previously in mice. It was thought possible that their hydrolysis in water to an inactive product would provide a useful method of rendering the compounds harmless after they had achieved their desired effect as pesticides. However, their potency in inhibiting cholinesterase did not decline in parallel with their hydrolysis and the value of the compounds as pesticides is probably therefore limited.

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CONTENTS

INTRODUCTION

INTRODUCTION.

ACETYLCHOLINE AND ITS METABOLISM.

Distribution and function of acetylcholine.

Acetylcholine (ACh) is as its name implies, the acetyl ester of choline and in a similar manner to choline it occurs naturally in a wide variety of organisms (Whittaker, 1963). The most widely investigated function of ACh is as a neurotransmitter and as such it is found in the nervous tissue of numerous animal species (Hebb, 1963). The significance of its presence elsewhere is more vague but its wide distribution implies a greater significance than is suggested by the concept of ACh solely as a neuro-transmitter (Keyl, Michaelson and Whittaker, 1957).

ACh is formed by the bacterium Lactobacillus plantarum (Stephenson and Rowatt, 1947; Girvin and Stevenson, 1954) but does not appear to play an essential part in its metabolism (Alpert, Kisliuk and Shuster, 1966). ACh also occurs in some protozoa where its function is as yet obscure. (Bulbring, Lourie and Pardoe, 1949). The common nettle contains ACh and ACh appears to be responsible for the burning sensation produced by its sting (Emmelin and Feldberg, 1947). Other plants also contain ACh and it has been postulated that it acts as an ion permeability regulator in at least one species (Jaffe, 1970).

In the non-innervated human placenta a non-neurotransmitter role for ACh can also be envisaged. It has been known for some time that human placenta contains ACh in high concentration (Chang and Gaddum, 1933) and the presence of low levels of the ACh-synthesising enzyme, choline acetyltransferase (ChAc), in placenta from species other than human suggests that placental ACh may not occur only in humans (Welsch, 1974a).

However, the marked difference in placental ChAc levels of humans compared with other species suggests that the function of ACh in this tissue may vary between the species. A possible function of placental ACh has been suggested by Bull, Hebb and Ratkovic (1961) who showed that in human placenta ChAc, and presumably therefore ACh, is present in the chorionic villi in the cell layers separating maternal from foetal blood. From their results these workers postulated that the function of ACh in this tissue may lie in the active transport of materials across this placental barrier and this view has been supported by Welsch (1974b).

Acetylcholine as a neurotransmitter.

In a comprehensive review of his own work and that of his contemporaries Dale (1953) provides the experimental evidence which led to the concept of impulse transmission from nerve to nerve and nerve to muscle as a chemical rather than an electrical process. Acetylcholine and adrenaline were proposed as neuro-transmitters and Dale differentiated between systems utilizing ACh as transmitter and those thought to contain adrenaline by denoting them as cholinergic and adrenergic respectively. It was not until later that it was shown that it is not adrenaline but noradrenaline which in many animals is the main adrenergic transmitter (see review by von Euler, 1971).

The evidence described by Dale (1953) strongly suggested that ACh acted as a neurotransmitter at all preganglionic nerve endings of the autonomic nervous system, that is both sympathetic and parasympathetic, at postganglionic parasympathetic nerve endings and also at the junctions between voluntary nerves and striated muscle whilst adrenaline appeared to act at post-ganglionic sympathetic nerve endings. Dale (1953) also describes the evidence which led to the distinction of two types of action of ACh, one mimicked by muscarine and antagonised by atropine and the

other mimicked by nicotine and antagonised by curare.

Thus, over one generation, the foundations of the concepts of neurotransmitters generally and of ACh as a neurotransmitter in particular were firmly laid. However, the theories were not without their opponents and there are those who are still unconvinced by the experimental evidence supporting the idea of ACh as a chemical mediator of nervous transmission. The chemical transmission process is generally conceived as an intercellular process involving the diffusion of the transmitter substance from the pre-synaptic nerve endings to the post-synaptic effector cells where it causes a membrane depolarisation by altering the permeability of the membrane to ions. It is envisaged that for cholinergic mechanisms there is then a rapid removal of ACh by its hydrolysis to acetate and choline under the catalysis of acetylcholinesterase. The trans-synaptic diffusion process appears to Nachmansohn however as an imprecise and unlikely biological mechanism for the 'fastest and most precise function developed by nature'. His theory, the evidence for which he has fully reviewed (Nachmansohn, 1963) suggests that ACh, by its ability to affect membrane permeability to ions, is responsible for the transmission of axonal impulses and that its action is intra-cellular even at the synapse.

Proponents of the traditional, original theory of ACh as a neurotransmitter have to accept that as yet the presence of ACh within the axon has not been adequately explained even if the presence of the cholinergic enzymes within the axon can be accounted for by their axonal transport from their site of synthesis in the cell body to their site of action at the nerve terminal (Hebb and Morris, 1969). Even so, an axonal function of ACh would not negate the possibility of an intercellular transmission function and it may be that the true role of ACh will emerge from an integration of ideas from both theories.

It is noteworthy that the action of ACh as a regulator of membrane permeability is involved in all proposed functions of ACh, both neural and non-neural.

Feldberg (1945) like Dale before him (Dale, 1934) felt that the concept of ACh as a chemical transmitter in the central nervous system was a logical extension of the theory of its transmitter actions in the periphery. Using criteria similar to those used in identifying ACh as a neurotransmitter in the periphery a substantial body of evidence was obtained to suggest that ACh did act as transmitter across at least some central synapses (Feldberg, 1945). Since that time considerable weight has been added to the theory of ACh as a central transmitter so that Hebb and Morris (1969) were able to say that it is virtually certain that ACh has a central synaptic function and to date there has been no further evidence to suggest that they should revise this view.

Metabolism of acetylcholine in cholinergic nerve terminals.

Most work on the metabolism of ACh has centred around its function as a neurotransmitter and as such the present discussion is confined to the metabolism of ACh within cholinergic nerve terminals. The synthesis and degradation of ACh are considered in separate accounts of the enzymes which catalyse these reactions. It does however remain to describe the probable fate of ACh between its synthesis and degradation which consists of its storage within the presynaptic nerve terminal and its release from its storage sites.

Chang and Gaddum (1933) suggested that ACh was not freely diffusible in tissues since the high ACh concentrations present would result in large effects on many tissues and would also render the ACh susceptible to hydrolysis by cholinesterase. These workers therefore suggested that ACh might be stored in some special structure which prevented its free

diffusion throughout the tissue.

In 1952 Fatt and Katz reported the existence of spontaneous electrical discharges at motor end plates which had the character of miniature end-plate potentials (m.e.p.p.s.). Further work confirmed an initial suggestion that the release of ACh from the presynaptic nerve terminal may be responsible for these m.e.p.p.s. and led to the suggestion that the miniature potential represented a quantal unit of the normal end-plate potential (e.p.p.) (del Castillo and Katz, 1955). Around the same time de Robertis and Bennett (1955) described the existence of synaptic vesicles within nerve terminals and their cautious inference that these vesicles could have a role in the action of the neurotransmitter suggested the possibility that synaptic vesicles might be the morphological correlates of the quantal transmitter release observed in electrophysiological experiments.

The development of methods to isolate synaptic vesicles has provided evidence supporting a role of synaptic vesicles in the storage of ACh since the transmitter was found to be associated with the isolated vesicles (de Robertis, Rodriguez de Lores Arnaiz, Salganicoff, de Iraldi and Zieher 1963; Whittaker, Michaelson and Kirkland, 1963, 1964). The ACh content of synaptic vesicles has been estimated as being about 2,000 molecules per vesicle but it is not certain whether this correlates with the actual size of the quantum of ACh required to produce an m.e.p.p. since estimates of quantal size vary widely (Whittaker and Sheridan, 1965).

In addition to their storage function synaptic vesicles are thought to play an active part in the release of ACh which is envisaged as an all-or-none release of the contents of a particular vesicle into the synaptic cleft on collision with the presynaptic terminal membrane (see review by Hebb, 1972).

ACh release is complicated by the fact that in cat superior cervical ganglion (Collier, 1969; Collier and MacIntosh, 1969), rat diaphragm (Potter, 1970) and the electric organ of Torpedo (Durant, 1973) there appears to be a preferential release of newly synthesised transmitter. A possible explanation for this is the existence of different pools of ACh within the nerve ending. This could arise from a heterogeneous vesicle population of which only a fraction, probably those lying nearest the terminal membrane, would contain readily releasable ACh (Barker, Dowdall and Whittaker, 1972). An alternative explanation is that the released ACh is not derived from vesicles but represents a cytoplasmic or vesicle-bound pool of ACh (see reviews by Fonnum, 1973 and Marchbanks, 1975). The results of Hubbard and Kwanbunbumpen (1968) and Jones and Kwanbunbumpen (1970a,b) favour the hypothesis that released ACh is derived from vesicles since they showed a stimulation-dependent decrease in a small vesicle population localised near the external membrane at the neuromuscular junction.

CHOLINE ACETYLTRANSFERASE.

Choline acetyltransferase (ChAc, acetyl CoA: choline-O-acetyltransferase, E.C. 2.3.1.6) was originally defined as the enzyme responsible for the synthesis of ACh (Nachmansohn and Machado, 1943) but Korey, de Braganza and Nachmansohn (1951) realising that more than one enzyme is involved re-defined it more specifically as the enzyme responsible for the last stage of ACh synthesis; namely, the transfer of the acetyl group from acetylcoenzyme A (acetyl CoA) to choline.

The reaction catalysed by ChAc is shown in the equation below:-

$$\text{Acetyl CoA} + \text{Choline} \xrightleftharpoons{\text{ChAc}} \text{ACh} + \text{CoA}$$

The only natural synthetic mechanism for ACh known to date is that shown in the above equation and the parallel distribution of ChAc and ACh highlights this fact.

Source of acetyl CoA for ACh synthesis.

Acetyl CoA synthesis occurs within nerve terminals and appears to be mainly confined to the mitochondria. The synthesis requires an external source of acetyl groups and the main precursor candidates which have been suggested to supply these acetyl groups are acetate (Tucek, 1967 a,b) citrate (Tucek, 1967 a,b) and pyruvate (Browning and Schulman, 1968; Nakamura, Cheng and Naruse, 1970; Lefresne, Guyenet and Glowinski, 1973).

Hebb (1972) has reviewed the evidence which suggests that the acetyl CoA used for ACh synthesis is derived from that synthesised within mitochondria. Since it seems generally agreed that acetyl CoA does not readily permeate the mitochondrial membrane (Srere, 1965; Tucek, 1967b) the problem arises as to how acetyl CoA is translocated from its intramitochondrial site of synthesis to the extramitochondrial site of action of ChAc. Three possible mechanisms other than the direct transfer of acetyl CoA have been suggested for this process (Srere, 1965) as follows:- (1) Intramitochondrial acetyl CoA is hydrolysed by acetyl CoA hydrolase to acetate which after transfer to the cytoplasm is reconverted to acetyl CoA by acetyl CoA synthetase; (2) Acetyl CoA is converted by citrate synthetase to citrate which diffuses into the cytoplasm where acetyl CoA is produced under the action of citrate lyase; (3) Carnitine acetyltransferase converts the acetyl CoA to acetylcarnitine which permeates the mitochondrial membrane and is reconverted in the cytoplasm to acetyl CoA by the same enzyme.

This latter mechanism seems the least likely probability since carnitine acetyltransferase appears to be wholly associated with mitochondria (Beenakkers and Klingenberg, 1964; Barker, Fincham and Hardwick,

1968) and any bound enzyme appears to be connected with the inner mitochondrial membrane (Barker et al., 1968).

There is however evidence to suggest that citrate (Tucek, 1967a, Sollenberg and Sorbo, 1970) and to a lesser extent acetate (Tucek, 1967b) can serve as the acetyl donors, via acetyl CoA, for ACh synthesis. The greater synthesis of ACh from citrate has been attributed to the extra-mitochondrial localisation of ATP citrate lyase compared with the mainly mitochondrial localisation of acetyl CoA synthetase (Tucek, 1967a,b). There remain doubts as to whether these suggestions can wholly account for the acetyl CoA used for ACh synthesis. There are two reasons for this. Firstly, labelled acetate and citrate were found to be unable to serve as efficient precursors of acetyl CoA in slices of rat striatum (Cheng, Nakamura and Waelsch, 1967) although this has been attributed to permeability effects (Sollenberg and Sorbo, 1970). Secondly, the maximum activities of ATP citrate lyase and acetyl CoA synthetase were much lower than would be required to provide sufficient acetyl CoA for maximum activity of ChAc (Tucek, 1967b).

A full explanation of the source of acetyl CoA for ACh synthesis therefore awaits the results of further experimentation.

Source of choline for ACh synthesis.

Dross and Kewitz (1972) have suggested that choline derivatives can be synthesised in the brain of rats by the methylation of phosphorylated derivatives of ethanolamine. Although it is generally accepted that the synthesis of choline-containing compounds can occur in the liver (Bremer, Figard and Greenberg, 1960; Bremer and Greenberg, 1961; Artom, 1964) several workers disagree with Dross and Kewitz (1972) and suggest that this synthesis does not occur in brain (Bremer and Greenberg

1961; Ansell and Spanner 1967, 1971). The brain therefore requires an external supply of choline.

An external source of choline has also been shown to be necessary for the sympathetic ganglion to maintain its ACh stores and output during prolonged activity (Brown and Feldberg 1936; Birks and MacIntosh, 1961) and in support of this it has been shown that the ganglion can convert labelled choline into ACh (Friesen, Kemp and Woodbury, 1965; Collier and Lang, 1969).

Three possible sources, two of them external, of the choline used in the synthesis of ACh have been proposed. These are (1) free choline dissolved in the plasma, (2) blood-borne choline-containing phospholipids and (3) the re-uptake of choline released on the hydrolysis of ACh by cholinesterase.

Free choline.

Free choline has been reported to exist in blood but the reported choline levels in blood, as in brain, vary widely (Bligh, 1952). This can be attributed to the release of choline on the breakdown of phospholipids which can occur either enzymically or by the action of acids used as extractants (see Hebb, 1972 and p. 87). Ansell and Spanner (1971) could not detect the presence of free choline in the blood after the intra-peritoneal injection of (Me-^{14}C) choline to rats and this, among other results, led them to conclude that it is highly unlikely that the free base is the form in which choline is carried in the blood to the brain.

Schuberth and Jenden (1975) however, suggest that in the rabbit at least, free and derived choline in plasma are supplied to the brain in equal amounts.

Choline-containing phospholipids.

The alternative means of choline transport as suggested by Ansell and Spanner (1971) is as a lipid-bound form, possibly phosphatidylcholine or lyso-phosphatidylcholine and this suggestion has received support from Illingworth and Portman (1972) who showed the uptake of lysophosphatidylcholine into the brains of squirrel monkeys and its metabolism to phosphatidylcholine and choline. In support of their results both groups of workers have noted that the lipid-bound form of choline is more likely to penetrate the blood-brain barrier than is the more water-soluble free base.

Despite the delivery of choline to the brain as a chemically bound form it appears highly probable that extracellular free choline is the physiological substrate for ACh synthesis in nerve endings (Guyenet, Lefresne, Rossier, Beaujouan and Glowinski, 1973). In support of this is the demonstration of a high-affinity choline uptake system in rat brain synaptosomes operating at low choline concentrations and connected with the production of ACh (Yamamura and Snyder, 1972).

Re-uptake of choline.

The results of Perry (1953) suggested that free choline, derived from the hydrolysis by cholinesterase of released ACh, could be recaptured by the nerve terminal and re-synthesised into ACh. These results have since been confirmed by Collier and MacIntosh (1969) and Potter (1970) and whilst this re-uptake process appears to be less important than that operating in adrenergic nerve terminals it appears that nearly half the choline produced by transmitter hydrolysis in the rat phrenic nerve-diaphragm preparation can be re-used (Potter, 1970).

Localisation of ChAc.

Identification of neurones of the central nervous system as being cholinergic has relied heavily on histochemical methods for the detection of

acetylcholinesterase (AChE) (Silver, 1967). However the presence of ACh within a neurone cannot be taken as definitive evidence that the neurone is cholinergic since non-cholinergic neurones may also contain this enzyme (Silver, 1967). ChAc appears to be a more reliable marker of cholinergic mechanisms and the ultrastructural and intracellular localisation of ChAc would therefore be of great value in the mapping of central cholinergic mechanisms and in providing valuable information on the intracellular relationship between ChAc and ACh.

As indicated by Fonnum (1972, 1973) much of the information on the localisation of ChAc has been obtained with the aid of microchemical analyses coupled with microdissection techniques and techniques for the subcellular fractionation of tissues by differential centrifugation (Gray and Whittaker, 1963) and density gradient centrifugation (Whittaker, Michaelson and Kirkland, 1964).

Axonal ChAc.

ChAc is present within the axons of cholinergic nerves (Hebb, 1963) and it has been suggested that it is in transit from its site of synthesis in the cell body to the nerve terminal as evidenced by its accumulation proximally to a nerve ligature or section (Hebb and Waites, 1956; Hebb and Silver, 1961).

There appears to be some disagreement however as to whether the enzyme is transported in a soluble (Tucek, 1975) or membrane-bound (Hebb and Silver, 1961; Fonnum, Frizell and Sjostrand, 1973) form and whether all the enzyme within the axon is being transported (Tucek, 1975) or only a small percentage of it (Fonnum et al. 1973). There is also confusion concerning its transport velocity, which may be slow (Saunders, Dziegielewska, Haggendal and Dahlstrom, 1973; Tucek, 1975) intermediate or fast (Fonnum et al, 1973) and its direction of flow - either purely proximo-distal (Tucek, 1975) or partly retrograde (Fonnum et al. 1973).

As indicated by Kasa, Mann, Karcsu, Toth and Jordan (1973) it is probable that some or all of these discrepancies arise from the use of the functionally and morphologically different sciatic (Hebb and Silver, 1961; Saunders et al. 1973; Tucek, 1975) vagus and hypoglossal (Fonnum et al, 1973) nerves from different species - rat (Saunders et al., 1973) rabbit (Fonnum et al., 1973; Tucek, 1975) and goat (Hebb and Silver, 1961).

Intraterminal ChAc.

De Robertis, Rodriguez de Lores Arnaiz, Salganicoff, de Iraldi and Zieher (1963) reported an association of ChAc with synaptic vesicles derived from ruptured nerve endings of rat brain tissue subjected to differential centrifugation. However, Whittaker, Michaelson and Kirkland (1964) suspected that the synaptic vesicle fraction of de Robertis et al. was contaminated with small membrane fragments and also with intact and partially disrupted synaptosomes. Using hypo-osmotically ruptured synaptosomes from guinea-pig brain Whittaker et al. concluded that ChAc was localised in the soluble cytoplasm. McCaman, Rodriguez de Lores Arnaiz and de Robertis (1965) renewed the claim that ChAc was associated with synaptic vesicles and attributed the discrepancies between their results and those of Whittaker et al. to species differences. The results of Tucek (1966a) were to some extent in agreement with this, although his results suggested binding of ChAc to structures other than synaptic vesicles (Tucek, 1966b).

The work of Fonnum has done much to resolve the reasons behind the conflicting reports described above. He, like Tucek, reported an association of ChAc with non-vesicular particles (Fonnum, 1967) but found that this particulate form of the enzyme, the predominance of which was species dependent, was readily interchangeable with the soluble form of the enzyme depending on the pH and ionic strength of the suspending medium (Fonnum,

1967, 1968). Thus the conflicting results reported by previous workers probably stemmed from the use of different experimental conditions in addition to the use of different species.

The question of a possible physiological significance of the membrane affinity of ChAc has been approached by Fonnum (1968) and Fonnum and Malthe-Sorenssen (1972, 1973). They have suggested that part of the ChAc could be bound to the presynaptic axon membrane and thus be in a favourable position to acetylate choline entering the axon. This explanation could account for the reported coupled relationship between the uptake of substrates by synaptosomes and the acetylation of these substrates (Barker and Mittag, 1975). Also, the former workers have suggested that part of the ChAc could be attached to the outside surface of the synaptic vesicles thus providing a link between the synthesis and storage of ACh. The distribution of ^{3}H-ACh between the nerve ending cytoplasm and the synaptic vesicles after administration of ^{3}H-choline led Molenaar and Polak (1973) to conclude that at least a part of the vesicular ACh must be synthesised in a cytoplasmic subcompartment which is directly linked to the vesicles. It is possible that vesicle-bound ChAc constitutes this link.

If both the above suggested linking processes are in operation it is possible that ChAc is acting as a cytoplasmic carrier, acetylating choline as it enters the nerve terminal and transporting the ACh formed, in solution as an ACh - enzyme complex, to the synaptic vesicles. In this respect it is interesting to note that choline and ACh have been shown to have a solubilising effect on ChAc (Fonnum, 1968) although there is no evidence for a stable ACh-enzyme complex. There is insufficient evidence as yet to regard any of these suggestions as anything more than tentative and it should be noted that Fonnum (1970) has suggested that under physiological conditions the soluble form of ChAc will dominate and ACh synthesis will probably therefore take place within the cytoplasm of the nerve terminal.

Histochemical localisation of ChAc.

There has been a great demand for a ChAc localisation procedure based on histochemical techniques and although the development of a suitable method specific for ChAc has progressed during the last decade it has as yet met with only partial success.

The difficulties encountered are concerned with the specificity of the method and stem from the fact that the staining procedures are aimed at the coenzyme A (CoA) produced during the enzymatic reaction rather than at the enzyme itself. As such positive staining reactions will occur for any enzymes which are capable of hydrolysing acetyl CoA to acetate and CoA (Burt and Silver, 1973b). It is possible that ChAc can itself catalyse this hydrolysis in the absence of choline (Burt, 1969; 1970). A reduction in the non-specific hydrolysis of acetyl CoA can be obtained using diisopropylphosphofluoridate (DFP) but the formation of acetate is not completely abolished (Burt and Silver, 1973a). The methods also suffer from the fact that the CoA may diffuse away from its site of release prior to precipitation and the exact location of ChAc activity may not therefore be truly reflected (Burt and Silver, 1973b).

The main methods which have been used to visualise CoA are its precipitation as the lead mercaptide, which is subsequently converted to the brown-black sulphide (Burt 1969, 1970; Kasa, Mann and Hebb, 1970) or the reduction by CoA of ferricyanide to ferrocyanide, which is precipitated as an electron-dense manganous salt (Feigenson and Barrnett 1973). Recently, stains which produce a blue precipitate on the reduction via CoA of a tetrazolium salt have been described and these may be of value as histochemical stains for tissues. (Prince and Toates, 1973; Franklin, 1976).

Although the use of specific ChAc inhibitors have indicated that these CoA-staining techniques can be made reasonably specific for ChAc

(Kasa and Morris, 1972) a better approach would if possible be to direct the procedure directly at the enzyme. This can be done by using fluorescent antibody techniques but meets with problems due to the low antigenicity of ChAc (Malthe-Sorenssen, Eskeland and Fonnum, 1973). Eng, Uyeda, Chao and Wolfgram (1974) and McGeer, McGeer, Singh and Chase (1974) have however claimed success in developing ChAc-specific immunofluorescent techniques for the localisation of the enzyme but Rossier (1975) seriously questions the monospecificity of their antiserum and therefore questions the validity of their results.

It is clearly important that the limitations of the histochemical method should be fully understood before its experimental use results in the false and misleading interpretation of data. The reliability of the method should therefore, as emphasised by Burt and Silver (1973) be determined by the use of parallel analytical studies.

Molecular properties of ChAc.

Molecular Weight of ChAc.

The first determinations of the molecular weight of ChAc were for the rabbit brain and human placental enzymes and were reported as 67,000 and 59,000 respectively (Bull, Feinstein and Morris, 1964). These determinations were obtained by sedimentation analysis and since then this technique has been used along with gel filtration to provide further estimates of the molecular weight of the enzyme. These estimates have been reported as 50,000 and 68,000 for rat brain ChAc (Potter, Glover and Saelens, 1968; Rossier, 1976); 35,000 or 70,000 for L. Plantarum ChAc and 100,000 for calf caudate nucleus ChAc (White and Cavallito, 1970); 65,000 for ox brain ChAc (Glover and Potter, 1971) and 62,000 for ChAc from human brain (White and Wu, 1973).

The unusually high value for the molecular weight of calf caudate nucleus ChAc reported by White and Cavallito (1970) prompted them to speculate that this could reflect aggregation of their enzyme. Chao and Wolfgram (1974) supported this assertion since they observed high molecular weight forms of ChAc, in enzyme preparations treated with ammonium sulphate, which could only have been formed by aggregation since they were not present in the initial extract. They attributed this aggregation to the ammonium sulphate treatment and a similarly produced aggregation of human placental ChAc has since been shown to occur (Banns, 1976). Recently Rossier (1976) has reported that under conditions of low protein concentration and in the absence of reducing agent aggregates with molecular weights as high as 400,000 daltons may be formed. His results also suggest that the monomer and dimer of rat brain ChAc are readily interchangeable. It is not known as yet whether aggregates of ChAc occur in vivo.

Another explanation which may account for some of the higher estimates of the molecular weight of ChAc is the existence of non-identical subunits of the enzyme. Thus Chao and Wolfgram (1973) have reported that an initially observed molecular weight greater than that of haemoglobin for bovine brain ChAc was due to the presence of two subunits with molecular weights of 51,000 and 69,000 daltons. Banns (1976) has also suggested the existence of subunits of human placental ChAc since the origin of ChAc fractions with molecular weights of 69,000 and 76,000 cannot be explained by aggregation.

Surface charge and isoenzymes of ChAc.

The tendency of ChAc to adsorb to negatively charged microsomes led Potter, Glover and Saelens (1968) to suggest that ChAc was more cationic than most proteins and that solubilisation of the enzyme might be due to

disruption of ionic linkages. Fonnum (1967, 1968) later confirmed that the membrane affinity of ChAc was due to its high positive surface charge which enabled it to bind to negatively charged membranes. He later showed that the different membrane affinities of ChAc from different species correlated well with their different surface charges (Fonnum, 1970).

A correlation has also been shown between the different membrane affinities and the isoelectric points of the isoenzymes of rat brain ChAc separated by isoelectric focusing (Fonnum and Malthe-Sorenssen, 1973). The evidence strongly suggests therefore that the membrane-binding properties of ChAc are due to its high positive surface charge.

Since Malthe-Sorenssen and Fonnum (1972) showed the existence of different molecular forms of ChAc in rat brain and cat brain, isoenzymes of ChAc from human brain (White and Wu, 1973) and bovine, mouse and monkey brain (see Malthe-Sorenssen, 1976) have been reported. ChAc enzymes from pigeon and guinea-pig (Malthe-Sorenssen and Fonnum, 1972), rabbit and Torpedo (Malthe-Sorenssen, 1976) and cockroach and horse shoe crab (Emson, Malthe-Sorenssen and Fonnum, 1974) have been reported to exist as single forms.

White and Wu (1973) have reported different kinetic properties of the isoenzymes of ChAc from human brain with respect to the natural substrates of the enzyme and its inhibition by naphthylvinylpyridine, although these differences may not be significant (Malthe-Sorenssen, 1976). Malthe-Sorenssen and Fonnum (1972) and Malthe-Sorenssen (1976) could however detect no such differences in the kinetic parameters of ChAc isoenzymes separated from smaller mammals and invertebrates and reported that the affinity for choline of the different molecular forms of ChAc within a species was the same. They suggested that this was an indication that the changes in amino acid composition of the different isoenzymes responsible for the charge differences were localised far away from the

active centre of the enzyme. Although it is possible that small differences in the kinetic behaviour of the different isoenzymes may have been masked by the interference of the ampholines used during electrofocusing the only criterion to date which conclusively distinguishes between isoenzymes of ChAc within a particular species is their difference in surface charge.

Substrate specificity of ChAc.

Acetylation with acetyl CoA analogues.

Berman, Wilson and Nachmansohn (1953) speculated that it would be biologically necessary for ChAc to possess a sharp specificity in order to prevent the formation of a whole series of choline esters which could have undesirable biological side effects. However, several naturally occurring choline esters other than ACh have been reported (see Whittaker, 1963) although the involvement of ChAc in their synthesis is probably limited, as suggested by the results described below.

The specificity of ChAc towards acyl CoA homologues has been studied using synthetic acyl coenzymes (Berman et al., 1953) or acyl coenzymes derived from a donor system. The donor system contained the carboxylic acid and CoA, from which the acyl CoA was synthesised via endogenous (Gardiner and Whittaker, 1954) or exogenous (Berry and Whittaker, 1959) acyl thiokinase. From these studies it appears that of several substrates tested only acetyl CoA and propionyl CoA were converted by ChAc to their corresponding choline esters, thus confirming the earlier results of Korey, de Braganza and Nachmansohn (1951) who showed the esterification of only acetic and propionic acids by a complete choline acetylating system. Later results using improved techniques demonstrated that ChAc could also catalyse the formation of butyrylcholine (Reisberg, 1957).

Butyrylcholine may normally be present in ox brain although some doubt surrounds this matter (see Berry and Whittaker, 1959). If this ester is present in this tissue it is conceivable that ChAc may be involved in its formation. ChAc may also be involved in the formation of propionylcholine which has been shown to occur in ox spleen (Gardiner and Whittaker, 1954). However the in vitro results discussed above suggest that it is unlikely that the in vivo formation of acylcholines other than the acetyl, propionyl and butyryl derivatives involves ChAc thus confirming the suspicions of Berman et al. (1953) of a high acyl group specificity of ChAc.

Acetylation of choline analogues.

The first evidence which suggested that ChAc might not be entirely specific for choline was obtained by Korey et al. (1951) who demonstrated the acetylation of dimethylethanolamine by an enzyme preparation from squid head ganglia. Although these workers did not observe any acetylation of monomethylethanolamine Burgen. Burke and Desbarats-Schonbaum (1956) using enzyme from rat brain were able to demonstrate the acetylation of both ethanolamine and its monomethyl derivative. These results indicate that possession of a quaternary nitrogen atom is not an absolute requirement of a substrate for its acetylation by ChAc.

Burgen et al (1956) also showed the acetylation of several quaternary choline analogues differing in their N-alkyl substitution and in general an increase in the size of the substituted alkyl groups was accompanied by a decreased acetylation. However, replacement of one or two methyl groups on the nitrogen atom by ethyl groups to give the compounds monoethylcholine (MEC) and diethylcholine (DEC) resulted in rates of acetylation greater than that of choline (143% and 102% respectively compared with choline) although the rate of acetylation of triethylcholine (TEC) was less than

that of choline (95% compared with choline). Re-investigation of the acetylation of these three compounds compared with choline was performed by several workers but produced conflicting results (Dauterman and Mehrota, 1963; Hemsworth and Morris, 1964). The reason for this discrepancy was resolved by Hemsworth and Smith (1970a) who showed that the relative rates of acetylation of the compounds was dependent upon their concentration since at high concentrations substrate-inhibition of the enzymatic reaction occurred. At lower concentrations the relative rates of acetylation were choline > MEC > DEC > TEC.

The pharmacological actions of TEC suggested that the compound may be of value in neurogenic spastic states (Bowman and Rand, 1961a) and this compound has since been studied quite extensively. Bowman and Rand (1961b) suggested that TEC might act at least partly by its uptake into cholinergic nerve terminals and its subsequent acetylation and release as a false transmitter. In addition to its ability to act as a substrate for ChAc TEC has been shown to accumulate in nerve ending fractions (Hemsworth and Bosmann, 1971) and acetyl TEC has been shown to be virtually devoid of ACh-like activity on cholinergic receptors (Holton and Ing, 1949). Ilson and Collier (1975) have shown that in the cat superior cervical ganglion perfused with ^{14}C-TEC, acetyl-[^{14}C]-TEC is released by nerve stimulation and there seems to be no reason to doubt their conclusion that TEC can act as a false, inactive, cholinergic neurotransmitter.

A similar role has been suggested for hemicholinium-3 (HC-3) and this is discussed in the Results section of this thesis where evidence of the in vitro acetylation of HC-3 by ChAc is described. A possible false transmitter role has also been ascribed to a series of dicholine compounds whose greater rate of acetylation with increasing distance between the two choline moieties paralleled their pre-junctional activity at the neuromuscular junction. (Hemsworth, 1971, 1975).

The importance of the distance between the quaternary nitrogen atom and hydroxyl group of choline analogues as a determinant for their activity as substrates of ChAc has been shown by the results of Burgen et al. (1956) and Dauterman and Mehrota (1963) who each tested two compounds with $\overset{+}{N}$-OH distances increased over that of choline by insertion of one or two methylene groups into the hydrocarbon chain. This increased $\overset{+}{N}$-OH distance abolished or greatly reduced the ability of the compounds to serve as substrates for ChAc. This result suggests that the $\overset{+}{N}$-OH distance in the choline molecule reflects the distance between the active site and binding site of the enzyme and the reduced acetylation of compounds with $\overset{+}{N}$-OH distances greater than that of choline can thus be explained by the impaired alignment of substrate and enzyme molecules.

The ability of compounds to serve as substrates for ChAc can also be affected by the possession of chemical groups which can sterically hinder access to the hydroxyl group. As such, α-carbon substituted compounds show a much reduced acetylation compared with choline (Hemsworth and Smith, 1970b) and compounds substituted on the β-carbon, i.e.. the carbon atom nearest the hydroxyl group, show a very low or non-existent degree of acetylation. (Burgen et al, 1956; Hemsworth and Smith, 1970b; Barker and Mittag, 1975). It would appear that β-carbon substitution and $\overset{+}{N}$-OH distance are more important than is N-alkyl substitution with respect to the ability of choline analogues to serve as substrates for ChAc (Barker and Mittag, 1975).

The lower specificity of ChAc towards analogues of choline compared with analogues of acetyl CoA results in the greater pharmacological significance of choline analogues with respect to ChAc and this is highlighted by the ability of some of these analogues to form false, cholinergic neurotransmitters.

Activation and inhibition of ChAc.

Activation of ChAc.

Although initial results were inconclusive in that NaCl was reported to inhibit (Korey, de Braganza and Nachmansohn, 1951), have no effect upon (Reisberg, 1957) and to activate (McCaman and Hunt, 1965) ChAc it is now well established that NaCl, along with many other salts, can activate most preparations of ChAc. This is evidenced by the salt activation which has been reported for ChAc from rabbit brain (Morris and Tucek, 1966), human placenta (Schuberth, 1966), rat brain (Potter, Glover and Saelens, 1968), ox brain (Glover and Potter, 1971), squid head ganglia (Prince and Hide, 1971) and snail, cockroach and horse shoe crab (Emson, Malthe-Sorenssen and Fonnum, 1974).

In general NaCl, KCl, $CaCl_2$ and $MgCl_2$ have an equal activating effect on ChAc up to salt concentrations of about 100 mM. At higher salt concentrations NaCl and KCl continue to activate up to 300-600 mM (Morris and Tucek, 1966; Potter et al. 1968) whereas $CaCl_2$ and $MgCl_2$ cause a marked inhibition of the enzyme (Morris and Tucek, 1966, Potter et al., 1968; Emson et al., 1974). Schuberth (1966) showed that different chlorides had an equal stimulating activity and concluded that activation was not due to the cation. The results discussed above suggest that at low salt concentrations this may be true but at higher salt concentrations it appears that the cation does have some effect.

Quantitative differences in the salt activation of ChAc have been noted between ChAc extracted from different species and also between preparations of ChAc differing only in their degree of purity (Glover and Potter, 1971) or age (Prince and Hide, 1971). It has been suggested that these differences could originate from the existence of several forms of ChAc with different sensitivities to salts (Prempeh, Prince and Hide, 1972) and presumably different sensitivities to purification and ageing.

The mechanism by which ChAc is activated by salts has not as yet been elucidated but Schuberth (1966) envisages a direct action on the enzyme.

Inhibition of ChAc.

ChAc can be inhibited non-specifically by certain cations (see above) and by thiol reagents directed against the sulphydryl group which appears to be present at the enzyme active site (Roskoski, 1974). However there has long been a requirement for a specific ChAc inhibitor in that it would be a valuable tool in characterising ChAc and in studying the biosynthesis of ACh. There are now two main groups of ChAc inhibitors, those derived from 4-stilbazole, including the styrylpyridine analogues (Smith, Cavallito and Foldes , 1967; Baker and Gibson, 1971, 1972) and the alkylaminoethyl esters (Persson, Larsson, Schuberth and Sorbo, 1967; Morris and Grewaal, 1969; Malthe-Sorenssen, Anderson and Fonnum, 1974).

Although many compounds make up these two groups of ChAc inhibitors only relatively few have been used biologically. Of those that have, chloro- and bromo-acetylcholine, 3-bromoacetonyltrimethyl ammonium (bromoketone) and one of the styrylpyridines have been useful in establishing the specificity of the histochemical localisation procedure for ChAc (Kasa et al., 1970; Kasa and Morris, 1972) and another of the styrylpyridine analogues has proved to be of some value in the purification of ChAc (Husain and Mautner, 1973).

Most interest has centred around the styrylpyridine analogues first studied by Smith et al. (1967) and particular interest has been shown in the most specific ChAc inhibitor of this group, 4-(1-naphthylvinyl) pyridine (NVP). The inhibition produced by these styrylpyridine analogues was shown by Smith and co-workers (1967) to be of a reversible, non-competitive nature in vitro but irreversible inhibition can occur on

prolonged incubation of these compounds with ChAc (White and Cavallito, 1970b). The precise mechanism by which these styrylpyridines inhibit ChAc is not known. However, they do not appear to affect the binding of acetyl CoA to the enzyme but do inhibit transfer of the acetyl group to choline and it has been suggested (White and Cavallito, 1970b) that they could act through an interference with the catalytic histidine moiety present at the active site of ChAc (see Currier and Mautner, 1974).

Interest has also been shown in the group of ChAc inhibitors studied by Baker and Gibson (1971, 1972)which, like the inhibitors studied by Smith et al. (1967), are derivatives of 4-stilbazole. It has been shown that this group of inhibitors exhibits irreversible non-competitive inhibition in vitro (see Malthe-Sorenssen et al., 1974) and the most specific inhibitor of the group, 3' chloro-4-stilbazole (CS), has been shown to inhibit irreversibly in vivo (Krell and Goldberg cited in Gibson and Baker, 1974).

Although a decrease in the levels of stored ACh might be expected from ChAc inhibition, in vivo experiments have shown that this does not occur after administration of either NVP or CS (Carson, Jenden and Cho, 1972; Glick, Mittag and Green, 1973; Krell and Goldberg, 1975) and in some cases NVP and CS have even been reported to increase endogenous ACh levels (Saelens, Simke, Schuman and Allen, 1974; Carson, Jenden, Cho and Green, 1976). It is unlikely that this lack of reduction in ACh levels is due to an inability of the compounds to gain access to ChAc since a marked inhibition of ChAc activity in the brains of mice and guinea pigs after administration of NVP and CS has been shown (Krell and Goldberg, 1975). These results, in addition to the evidence obtained by Krell and Goldberg (1975) that CS inhibits a pentobarbital-induced rise in mouse brain ACh levels and that pre-treatment with NVP decreases the brain ACh content of mice subjected to a swim stress, has led to the suggestion that

ChAc is not the rate-limiting step in ACh synthesis. (Krell and Goldberg, 1975; Carson et al., 1976). This theory implies that ChAc is present in considerable excess and that its partial inhibition would be without significant physiological effect since ACh synthesis would be unaffected.

Other results, however, do suggest that the responses to NVP administration are consistent with an inhibition of ACh synthesis (Glick et al. 1973; Saelens et al., 1974; Haubrich and Goldberg, 1975) although again there was no reduction in storage levels of ACh. This apparent anomaly of a reduction in synthesis of ACh without a reduction in its storage levels can be explained by the hypothesis of the preferential release of newly synthesised transmitter (see p. 6) which implies the existence of a small pool of transmitter with a high turnover rate. Thus inhibition of ChAc may affect this pool with consequent physiological effects whilst the storage pool of ACh remains unaffected.

Hemsworth and Foldes (1970) concluded from their results, obtained in physiological experiments, that the styrylpyridines might have other actions in vivo which mask their in vitro specific ChAc inhibitory activity. A lack of specificity of the styrylpyridines has also been noted by other workers in that in addition to their inhibition of ChAc these compounds also inhibit cholinesterase (Smith et al., 1967), choline uptake into synaptosomes (Barker and Mittag, 1973) and drug metabolising enzyme systems (Salama and Goldberg, 1975). This lack of specificity and the instability due to photoisomerisation of the styrylpyridines (White and Cavallito, 1970a) has led to a renewed interest in the alkylaminoethyl esters as ChAc inhibitors. (Rowell and Chiou, 1976a, b). The inhibition produced by two of these latter compounds has been shown to be reversible and uncompetitive with respect to both choline and acetyl CoA and it has been suggested that they have sufficient potency and specificity for use in biological studies.

As yet no reversible, competitive inhibitors of ChAc have been reported.

Kinetics and mechanism of action of ChAc.

Kinetic studies of ChAc provide information on the mechanism of action of the enzyme and also offer some indication of the possible methods by which ACh synthesis is regulated.

The kinetic data obtained by Schuberth (1966) for human placental ChAc suggested a "ping-pong" mechanism for the enzyme. This refers to a mechanism whereby one or more products are released before all the substrates have added to the enzyme (Cleland, 1963). Subsequent studies by several groups of workers have, however, suggested that both choline and acetyl CoA must add to the enzyme before any products are released, a mechanism designated by Cleland (1963) as "sequential". These basic types of mechanism can be distinguished by primary double reciprocal plots (Lineweaver and Burk, 1934) which if parallel suggest a "ping-pong" mechanism and if convergent suggest a "sequential" mechanism.

Sequential mechanisms are subdivided into four main types of mechanism namely, Ordered Bi Bi, Theorell-Chance, Random Bi Bi and Rapid Equilibrium Random Bi Bi. Ordered mechanisms require that the substrates add to the enzyme in an obligatory order and the products leave similarly and Random indicates that there is no such obligatory order (Cleland, 1963). The four types of mechanism can be distinguished by product-inhibition studies and a table summarising the kinetic requirements of each type of mechanism has been adapted from that produced by Cleland (1963) to suit the particular case of ChAc inhibition by ACh and CoA (Morris, Maneckjee and Hebb, 1971).

The Theorell-Chance mechanism, which is a limiting case of the Ordered Bi Bi reaction showing a very low steady-state concentration of the central enzyme-substrate complex, has been suggested for ChAc from most sources tested including calf caudate nucleus (White and Cavallito, 1970b), squid head ganglion (Prince and Hide, 1971), human placenta (Morris, et al., 1971; Sastry and Henderson, 1972), human and rabbit brain and sciatic nerve (White and Wu, 1973) and nervous tissue from snail, cockroach and horse shoe crab. (Emson, Malthe-Sorenssen and Fonnum, 1974). There is some doubt as to whether the mechanism of action of ox brain striate nuclei is Theorell-Chance or Ordered Bi Bi (Glover and Potter, 1971).

The Theorell-Chance mechanism requires that the substrates bind to the enzyme in an obligatory order and this brings into question which substrate binds first. Most workers favour acetyl CoA as the leading substrate and the main arguments in favour of this are the much higher affinity of acetyl CoA for the enzyme compared with choline (Sastry and Henderson, 1972; Emson et al., 1974) and the ability of acetyl CoA to bind to the enzyme independently of choline (White and Cavallito, 1970b; Prince and Hide, 1971; Prempeh, Prince and Hide, 1972; Roskoski, 1973).

With regard to the regulation of ACh synthesis the competitive inhibition shown by ACh with respect to choline suggests that ACh may, at least to some extent, control its own synthesis by feed-back inhibition (Kaita and Goldberg, 1969). It is also possible that CoA has a role in the regulation of ACh synthesis since product-inhibition by CoA has been shown to be much stronger than by acetylcholine (White and Cavallito, 1970b). Potter et al. (1968) however suggest that at concentrations likely to be reached in situ neither ACh nor CoA will inhibit ACh synthesis and these workers suggest that regulation of ACh synthesis by mass action involving the concentration of all substrates and products is more likely.

Purification and assay of ChAc.

These topics have been discussed along with other aspects of the enzyme in the appropriate sections of Results.

CHOLINESTERASE.

Cholinesterase was originally proposed as the term for the enzyme which hydrolyses esters of choline (Stedman, Stedman and Easson, 1932). The recognition that more than one enzyme is capable of catalysing this hydrolysis has led to an expansion of this definition to include all such enzymes. The specificity of the cholinesterases is not absolute and they also hydrolyse non-choline esters although the hydrolysis proceeds at a lower rate than for the choline esters (see Augustinsson, 1963). The cholinesterases also lack specificity with respect to different choline esters and this has enabled the distinction of two classes of cholinesterase which differ also in their distribution.

The cholinesterase found in nervous tissue and in erythrocytes was originally termed true cholinesterase but opposition to this nomenclature resulted in the suggestion of the term acetylcholinesterase (Augustinsson and Nachmansohn, 1949) and this name has gained general acceptance. Although acetylcholinesterase (AChE) is not specific for ACh or even for choline esters in general, it is most active towards esters whose structure most closely approaches that of ACh (see Augustinsson, 1963). The association of AChE with cholinergic nerves has led to the generally accepted view that its chief function is the hydrolysis of endogenous ACh. However its presence in non-cholinergic tissue, both nervous and non-nervous suggests that it has some additional function (see Hebb and Morris, 1969).

Cholinesterases other than AChE were originally designated pseudo-cholinesterases but it has been suggested that the enzymes should be named according to the substrates for which they are most specific. Thus an enzyme which specifically hydrolysed butyrylcholine would be termed butyrylcholinesterase, one splitting propionylcholine would be termed propionylcholinesterase, etc. However, the existence of cholinesterases of intermediate specificity renders even such an apparently clear-cut classification inadequate (Augustinsson, 1963).

Pseudocholinesterases are widely distributed in animal tissues including serum and although present in the nervous system a more general metabolic role is envisaged for these enzymes than for AChE (Hebb and Morris, 1969). However, a limited role for the pseudo-cholinesterases in the hydrolysis of ACh has not been ruled out.

Involvement of cholinesterases in the mode of action of pesticides.

Full accounts of the anticholinesterases have been given in the review by Holmstedt (1959) and in the comprehensive treatise edited by Koelle (1963). Some of these compounds, in particular the carbamate and organophosphorus compounds, have been shown to be lethal to insects and other invertebrates and as such have been used to control their numbers. According to Chadwick (1963) particular interest had been paid to this class of compounds as pesticides for two reasons. Firstly, there was a widespread assumption that the essentials of the mode of action of these toxicants were thoroughly understood and secondly, resistance to these compounds by the target organisms had been slower to build up and had been less extensive than for other classes of pesticide.

The theory behind the action of the carbamate and organophosphorus pesticides is that inhibition of AChE results in the accumulation of ACh at cholinergic synapses causing a lethal blockade of normal nervous activity. (Smallman, 1959). This theory implies the existence of

cholinergic mechanisms within the susceptible organisms and there is evidence in favour of this in that the presence of ACh, AChE and the enzymic system necessary for ACh synthesis has been shown in the nervous tissue of insects and other invertebrates, although their presence does not in itself establish any functional significance. (Smallman, 1959; Chadwick, 1963). Further evidence in favour of the theory is that ACh levels would be expected to increase after administration of the anti-cholinesterases and a number of studies have shown that this does occur. (see Chadwick, 1963).

With the mode of action of these compounds so apparently well defined it might be expected that it would be comparatively easy to produce new pesticides to suit specific requirements. However, Chadwick (1963) stated that out of over 50,000 organophosphorus anticholinesterase compounds produced only about 30 were on the insecticide market in 1963. This may in part be due to the fact that the general assumptions of the theory as to the mode of action of anticholinesterase pesticides are to some extent misfounded. Chadwick (1963) in the concluding paragraphs of his review states that whilst cholinesterase inhibition appears to be causally concerned with the toxic action of anticholinesterase pesticides there is reason to suspect that this toxicity is rarely exercised through what is ordinarily understood as a cholinergic mechanism.

Whether or not the action of carbamates and organophosphates is mediated wholly via cholinergic mechanisms it is now firmly established that cholinesterase inhibition forms the basis of their mode of action as pesticides.

MATERIALS

MATERIALS

Enzyme substrates.

Choline chloride. Hopkin and Williams Limited, Chadwell Heath, England.

DL-carnitine HCl. Sigma Chemical Co., Kingston-upon-Thames, Surrey, England.

Hemicholinium-3; 2,2' -(4,4'-biphenylene)-bis-(2-hydroxy-4, 4-dimethyl-morpholinium bromide). Aldrich Chemical Co., London, England.

Triethylcholine chloride. Ward Blenkinsop, London, England.

Acetylated derivatives of enzyme substrates.

Acetylcholine chloride. Sigma Chemical Co., St. Louis, Mo., U.S.A.

Acetyl DL-carnitine HCl. Sigma Chemical Co., Kingston-upon-Thames, Surrey, England.

Acetyl hemicholinium-3; 4,4' biphenylene-bis-(2-oxoethylene)-bis-(2-acetoxyethyl-dimethylammonium bromide). Eastman Organic Chemicals, Rochester, New York, U.S.A.

ChAc inhibitors

4-(1-naphthylvinyl) pyridine hydrochloride. Calbiochem, San Diego, California, U.S.A.

N hydroxyethyl-4-(1-naphtylvinyl) pyridinium bromide. Calbiochem, Los Angeles, California, U.S.A.

Materials used for the isolation of ACh during ChAc determinations.

Ammonium reineckate; ammonium tetra thiocyanodiammonochromate. Schuchardt Chemical Co., Munich, Germany.

Sodium tetraphenylboron. BDH Chemicals Limited, Poole, England.

Butyl ethyl ketone. Koch-Light Laboratories Limited, Colnbrook, Bucks, England.

Mercuric potassium iodide. BDH Chemicals Limited, Poole, England.

Octan-2-one; n-hexyl methyl ketone. BDH Chemicals Limited, Poole, England.

Amberlite CG-50 ion-exchange resin (100-200 mesh). Mallinckrodt Chemical Works, St. Louis, Mo., U.S.A.

Commercial enzyme preparations

Acetyl cholinesterase; Acetylcholine hydrolase E.C. 3.1.1.7 from bovine erythrocytes. Used at an initial concentration of 5 mg/ml (16 units/ml). Sigma Chemical Co., St. Louis, Mo., U.S.A.

Cholinesterase from horse serum, Type IV. Used at an initial concentration of 5 mg/ml (35 units/ml). Sigma Chemical Co., St. Louis, Mo., U.S.A.

Carnitine acetyltransferase (Acetyl CoA: carnitine-o-acetyl-transferase; E.C. 2.3.1.7) from pigeon breast muscle. Crystalline suspension in 3.2 M $(NH_4)_2SO_4$ solution. pH approx 6. Sigma Chemical Co., St. Louis, Mo. U.S.A.

Radiochemicals.

Acetyl-β-methylcholine iodide (acetyl-1-^{14}C) (3.2 mCi/m mol diluted to 0.07 mCi/mmol with non-labelled Acβ-Me Ch) New England Nuclear, Winchester, Hants.

Acetyl-1-^{14}C choline iodide. (13.7 mCi/mmol diluted to 0.16 mCi/mmol with non-labelled ACh). New England Nuclear Corporation, Boston, Massachusetts, U.S.A.

^{14}C-1-acetyl choline iodide (2.43 mCi/mmol. Used at 3×10^{-3} M, 146.5 uCi/l). The Radiochemical Centre, Amersham, England.

(1-^{14}C) Acetyl coenzyme A. (58 mCi/mmol). The Radiochemical Centre, Amersham, England.

Butyryl choline iodide (Butyryl-1-^{14}C) 4.8 mCi/mmol diluted to 0.12 mCi/mmol with non-labelled BuCh). New England Nuclear Corp., Winchester, Hants.

Choline methyl - ^{14}C chloride (3.2 mCi/mmol. Used at 25 μCi/ml). New England Nuclear Corporation, Boston, Massachusetts, U.S.A.

Sodium acetate-1-^{14}C (2 mCi/mmol. Used at 6×10^{-4}M, 1.22 μCi/ml diluted with potassium phosphate buffer, pH 7.7). New England Nuclear Corporation, Boston, Massachusetts, U.S.A.

^{3}H-triethylcholine was the generous gift of Dr. B. Rennick.

Miscellaneous

Bovine serum albumin. Sigma Chemical Co., St. Louis, Mo., U.S.A.

p-chloromercuribenzoic acid. Sigma Chemical Co., St. Louis, Mo., U.S.A.

Edrophonium chloride; Tensilon. Roche Products Ltd., Welwyn Garden City, Herts, England.

EDTA; Ethylenediaminetetra-acetic acid. BDH Chemicals Limited, Poole, England.

PPO; 2,5 diphenyloxazole. Hopkin and Williams, Chadwell Heath, Essex, England.

POPOP; 1,4 di(2-(5-phenyloxazolyl)) benzene. Hopkin and Williams, Chadwell Heath, Essex, England.

Sephadex G-25, Sephadex G-50 and DEAE Sephadex. Sigma Chemical Co., St. Louis, Mo., U.S.A.

CNBr Sepharose. Pharmacia Fine Chemicals AB, Uppsala, Sweden.

METHODS

METHODS.

CHOLINE ACETYLTRANSFERASE ENZYME PREPARATION.

1. Initial extraction from the tissue.

a). Homogenisation.

The homogenisation media used were as described in Results.

Fresh rat brains were homogenised on ice in the homogenisation medium (10ml/g wet wt) using a glass homogeniser with a Teflon pestle.

Samples of full-term human placenta, either fresh and kept on ice or stored at -20°C, were homogenised using an Ultra-Turrax homogeniser.

b). Acetone-dried powders.

Acetone-dried powders were prepared from rat brains by a similar procedure to that used by Hemsworth and Morris (1964). Freshly dissected rat brains were placed in an ice-cold mortar and acetone, pre-cooled by the addition of dry ice, was added in large excess. The tissue was disintegrated in the mortar using a pestle and was then filtered under suction in a Buchner funnel. After washing with cold acetone the powder was left on the filter for 5 min. with suction and was then transferred to a desiccator and dried over P_2O_5 at 4°C. When dry the powders were resuspended in 200mM KCl.

c). Sonication.

Sonication of homogenates prepared as above was performed using a Soniprobe. The homogenate was surrounded by melting ice throughout sonication. In order to allow cooling of the homogenate sonication was usually performed intermittently in 10-minute periods up to a maximum of 100 mins.

For all three extraction procedures the resulting solutions were centrifuged at 20,000g for 20 min. and the supernatants, which were retained as the fractions containing ChAc, were either used immediately or stored at -20°C.

When ChAc preparations were required free from endogenous substrates they were passed through a Sephadex G-25 or G-50 column equilibrated with 200mM KCl.

2. Purification of ChAc preparations.

a). Ammonium sulphate fractionation.

An $(NH_4)_2SO_4$ fractionation range of 20%-30% (w/v) was used to selectively precipitate proteins from supernatants obtained by the procedures described above. Solid $(NH_4)_2SO_4$ was added to the supernatant to a concentration of 20% and the solution was mixed on ice for 1 hour. The solution was then centrifuged at 27,000g for 10 min. The pellet was resuspended in 200mM KCl for assay and $(NH_4)_2SO_4$ was added to the supernatant to increase the concentration to 30% (w/v). After mixing the supernatant solution on ice for 1 hour it was centrifuged at 15,000g for 10 min. and the resulting pellet, containing most of the ChAc (see Results), was resuspended in 200mM KCl.

b). Ion-exchange chromatography.

Ion-exchange chromatography was performed using a DEAE-Sephadex column equilibrated with 20mM sodium phosphate buffer containing 200mM NaCl. After addition of the enzyme preparation to the column, elution was performed with the same buffer and fractions of the eluate were collected using an LKB Ultrorac fraction collector.

The enzyme preparation used for this experiment was a rat brain homogenate partially purified by $(NH_4)_2SO_4$ fractionation.

The pellet obtained after the second centrifugation step of the $(NH_4)_2SO_4$ fractionation procedure was resuspended in phosphate buffer-NaCl and dialysed against 2x2l of the same buffer at 4^oC.

c). Affinity chromatography.

Organomercurial-sepharose resins.

These resins were prepared according to the method of Cuatrecasas (1970) and were equilibrated prior to use with 100mM KCl. After addition of the enzyme preparation to columns of these resins elution was performed with 10^{-1}M mercaptoethanol.

The enzyme preparation used was a sonicated rat brain homogenate in sucrose dialysed against 100mM KCl.

Styrylpyridine-sepharose resin.

p-aminobenzamidoethyl-Sepharose was prepared according to the method described by Cuatrecasas and Anfinsen (1971). An attempt was made to couple the ChAc inhibitor N-hydroxyethyl-4-(1-napththylvinyl) pyridinium bromide (NVPH) to this resin by allowing the resin to react with the inhibitor for 24 hours at 4^o in 0.25M sodium phosphate buffer pH 9.2. However, on performing the colour test with sodium 2, 4, 6-trinitro-benzenesulphonate (TNBS) (Cuatrecasas and Anfinsen, 1971) a red colour indicated that there had been no reaction.

DETERMINATION OF ChAc ACTIVITY.

1. Incubation system.

The rates of acetylation of choline and other substrates were estimated by incubation with acetyl-1-^{14}C coenzyme A and ChAc and by quantitive determination of the amount of labelled product formed.

The incubation mixture was placed in a plastic microfuge tube (Beckman) and contained 5ul ChAc, 10µl of distilled water, choline (2.5mM) or other substrate (2.5mM) and 10µl of a buffer solution containing acetyl-1-^{14}C coenzyme A (58mCi/mmol 63,800 dpm/10µl), 5 x 10^{-5}M; $MgSO_4$, 10^{-2}M; NaCl, 6 x 10^{-2}M; physostigmine sulphate, 4 x 10^{-4}M; EDTA, 2 x 10^{-4}M; albumin, 0.1mg/ml; and potassium phosphate (pH 7.7) 3 x 10^{-2}M. The total volume of the incubation mixture was 25µl. The mixture was incubated at 37°C for 15 min. over which time the reaction followed a linear time course (see Results).

Blank values were determined in which the enzyme was denatured by precipitation with trichloroacetic acid before incubation or in which the enzyme was replaced by distilled water.

For studies on enzyme inhibition the incubation mixture was modified by adding 5µl of substrate (5mM) and 5µl of inhibitor at the appropriate concentration.

When necessary, in order to ascertain that endogenous choline had been removed by passage of the ChAc through Sephadex G-50, incubations were carried out in which no exogenous substrate was added.

2. Isolation and identification of the acetylated product.

a). Reineckate precipitation.

The method routinely used was a modification of the procedure described by McCaman and Hunt (1965). After incubation, 5µl of 50% trichloroacetic acid (TCA) containing 2 x 10^{-1}M choline chloride as carrier was added to precipitate the enzyme. The tubes were centrifuged in a microfuge (Beckman) and 20µl of the supernatant was transferred to 50µl of a saturated ammonium reineckate solution in 0.5N HCl. The tubes were again

centrifuged and after removal of the supernatant the precipitate was washed with 50µl of 0.2N HCl. After recentrifugation and removal of the supernatant the precipitate was dissolved in 100ul of acetone and transferred to Whatman glass fibre filter discs. When dry the radioactivity was then determined by adding 5ml of a toluene-based scintillation fluor and counting in a liquid scintillation counter.

b). Extraction with sodium tetraphenylboron in butyl ethyl ketone.

After incubation, 20ul of the incubation was transferred to 7ml of 10mM sodium phosphate buffer (pH 7.4) containing 0.25mg of acetylcholine chloride according to the method of Fonnum (1969). 1ml of butyl ethyl ketone containing 15mg of sodium tetraphenyl-boron was then added and after thorough mixing the tubes were centrifuged to separate the layers. 0.5ml of the upper ketone layer was then transferred directly to 10ml of toluene based scintillation fluor and the radioactivity determined.

c). Extraction with mercuric potassium iodide in octanone.

The method used in the present experiments was a modification of that described previously by Glover and Green (1972). As in the reineckate method, the reaction was stopped by the addition of 5ul of 50% TCA containing 2×10^{-1}M choline chloride. One hundred ul of a solution containing 0.1M mercuric potassium iodide (K_2HgI_4) in octanone was added and mixed thoroughly. The tubes were then centrifuged in a microfuge to separate the two layers. The modification used in the present experiments consisted of removing 50µl of the upper octanone layer which was transferred to a Whatman glass-fibre filter disc. The filter disc was dried and was then counted in 10ml of toluene-based scintillation fluor.

In the method used by Glover and Green (1972) an aliquot of the octanone layer was added directly to scintillation fluor and counted for radioactivity.

d). Electrophoresis.

Electrophoresis was used both to isolate and to identify the acetylated products after incubation. The reaction was stopped by adding 5µl of acetic acid (1.5M)/formic acid (0.75M) buffer (pH 2) to the incubation medium. TCA was not used as it was found to interfere with the subsequent electrophoresis. After centrifugation, 20µl of the reaction mixture was applied to the mid-line of an electrophoresis paper strip. Four applications of 5µl were used and the paper was allowed to dry in between the applications in order to obtain a narrow band of sample on the paper. The strips were then subjected to electrophoresis at 500 volts for 1 hour in a V-type Durrum cell (Beckman) using 1.5M acetic acid/0.75M formic acid buffer as described by Potter and Murphy (1967). After electrophoresis, the strips were stained in iodine vapour whilst barely damp and the position of any stained band was marked in pencil.

Radioactive strips were passed through a Tracerlab 4π strip scanner to determine the position of the radioactivity. For a quantitative assessment of the radioactivity the strips were cut into 1cm portions and counted in 5ml of toluene-based scintillation fluor.

The position of the radioactive bands obtained after electrophoresis of incubation mixtures were compared with the stained bands of authentic compounds similarly subjected to electrophoresis. The authentic compounds were visualised by staining in iodine vapour or by spraying with Dragendorff's reagent.

Dragendorff's reagent.

Solution A. 0.8g bismuth subnitrate (bismuth oxynitrate) in 10ml glacial acetic acid + 40ml water.

Solution B. 40% (w/v) aqueous KI.

Dragendorff's reagent was prepared by mixing 1 volume Solution A, 1 volume Solution B, 4 volumes glacial acetic acid and 20 volumes of water.

The reagent was used as a spray.

e). Ion-exchange chromatography on Amberlite CG-50 (100-200 mesh).

A modification of the method of Diamond and Kennedy (1968) was used to isolate the acetylated products by ion-exchange chromatography.

After incubation 100µl of 50% ethanol containing 0.6mg/ml acetylcholine chloride was added to the tubes (Eppendorf 3810) which were then centrifuged for one min. in an Eppendorf 3200 centrifuge. 100µl of the supernatant was then transferred to a 2.5cm column of Amberlite CG-50 (100-200 mesh) prepared in distilled water and contained in a Pasteur pipette. The sample was allowed to run into the column which was then eluted 5 times with 0.5 ml sodium acetate (1mM) to remove unreacted acetyl-^{14}C coenzyme A. This eluate was discarded. The column was then eluted with HCl (IN) until a volume of 2ml had been collected. The radioactivity in this 2ml of HCl eluate was then determined using 10ml of a modified Bray's (1960) scintillation fluor.

Detection of Acetyl HC-3 and ACh in eluates from an Amberlite CG-50 column.

100µl of a solution of acetyl HC-3 (10^{-2}M) or ACh (2.5×10^{-2}M) was applied to the top of an Amberlite CG-50 column prepared

in a Pasteur pipette. Elution was then carried out using sodium acetate (1mM) and HCl (1N) as described above. This procedure was followed by elution with 2ml NaOH (1N).

To detect the presence of acetyl HC-3 in the eluates, two methods were used as described below.

1). Column eluates were passed directly through a Uvicord II spectrophotometer LKB 8303A type and their absorption at 280nm measured. Under these circumstances, larger volumes of eluants were used than described above.

2). Sodium acetate and HCl eluates were collected to a volume of 2ml. The NaOH eluate was collected in 1ml fractions. 100µl from each eluate fraction was then added to 100µl of a saturated ammonium reineckate solution in 0.5N HCl. The presence of acetyl or HC-3 in the eluate was indicated by the formation of a pink precipitate.

The reineckate precipitation method was also used for the detection of ACh.

For all the above extraction procedures the observed recovery of radioactive products was, when necessary, corrected for the fact that only part of the incubation medium was used.

CHOLINESTERASE ENZYME PREPARATION.

Fresh rat brains were homogenised on ice in 0.1% Triton X-100 containing 200mM KCl (10ml/g wet weight) using a glass homogeniser with a Teflon pestle. The homogenate was centrifuged at 20,000g for 20 min. and the supernatant containing the enzyme was used immediately or stored at -20°C.

DETERMINATION OF CHOLINESTERASE ACTIVITY.

The method used was the radiochemical procedure described by Siakotos, Filbert and Hester (1969).

1. Incubation system.

The rates of hydrolysis of ^{14}C-labelled substrates (see Results) were estimated by incubation with commercial cholinesterase or cholinesterase preparations obtained as described above and by quantitative determination of the radioactive product of hydrolysis.

The incubation mixture was placed in a 10ml graduated test-tube and contained 100µl of enzyme. 100µl of ^{14}C-labelled substrate (usually $3x10^{-3}M$) and 100µl of a buffer-salt-detergent mixture (containing NaCl 17.53g, NaH_2PO_4 2.62g, Na_2HPO_4 11-5g and 10 ml Triton X-100/1 distilled water adjusted to pH 7.4). The mixture was incubated at 37°C for 10 min. over which time the reaction followed a linear time course.

Blank values were determined in which the enzyme was replaced with distilled water.

For studies on cholinesterase inhibition the incubation mixture was modified by adding 70µl of enzyme and 30µl of inhibitor at the appropriate concentration. Controls contained 30µl distilled water in place of inhibitor.

2. Isolation and determination of the radioactive product of hydrolysis.

After incubation the reaction was stopped by the addition of 5ml of a resin-dioxane mixture (20g washed Amberlite CG-120, 200-400 mesh in 100ml dioxane). Dioxane was then added to the test-tube up to a volume of 10ml. After centrifugation 5ml of the supernatant was added to 5ml of modified Brays (1960) scintillation fluor and was then counted in a liquid scintillation counter.

LIQUID SCINTILLATION COUNTING.

Radioactivity measurements were made using a Beckman LS-230 liquid scintillation spectrometer.

Counting efficiencies were determined using internal standards.

Toluene-based scintillation fluor.

This fluor contained PPO (5g/litre) and POPOP (0.3g/litre) in toluene and was used for non-aqueous samples.

Modified Bray's fluor.

This fluor was based on that described by Bray (1960) and contained naphthalene (100g/litre) methanol (100ml/litre), PPO (4g/litre) and POPOP (0.2g/l) in 1,4 dioxan. This fluor was used mainly for samples containing water.

DETERMINATION OF PROTEIN.

Protein was determined by the method of Lowry, Rosebrough, Farr and Randall (1951) as described below.

Solution A. 10 parts 10% Na_2CO_3 in 0.5N NaOH + 0.25 parts 2% $CuSO_4$ + 0.25 parts 4% potassium tartrate + 5 parts distilled water.

Solution B. 10 parts distilled water + 1 part 2N Folin-Phenol reagent.

1ml of water was added to 20µl of test sample in a test-tube. 1ml of solution A was then added and after mixing the mixture was allowed to stand at room temperature for 10 mins. 3ml of solution B were then added and mixed thoroughly. After incubation at 37° for 20 mins. the absorption of the solution was measured on a Pye Unicam SP 500 spectrophotometer at 550nm. Blank determinations were obtained by substituting distilled water for the test sample.

Bovine serum albumen was used as a standard and the calibration curve is shown in Figure 1.

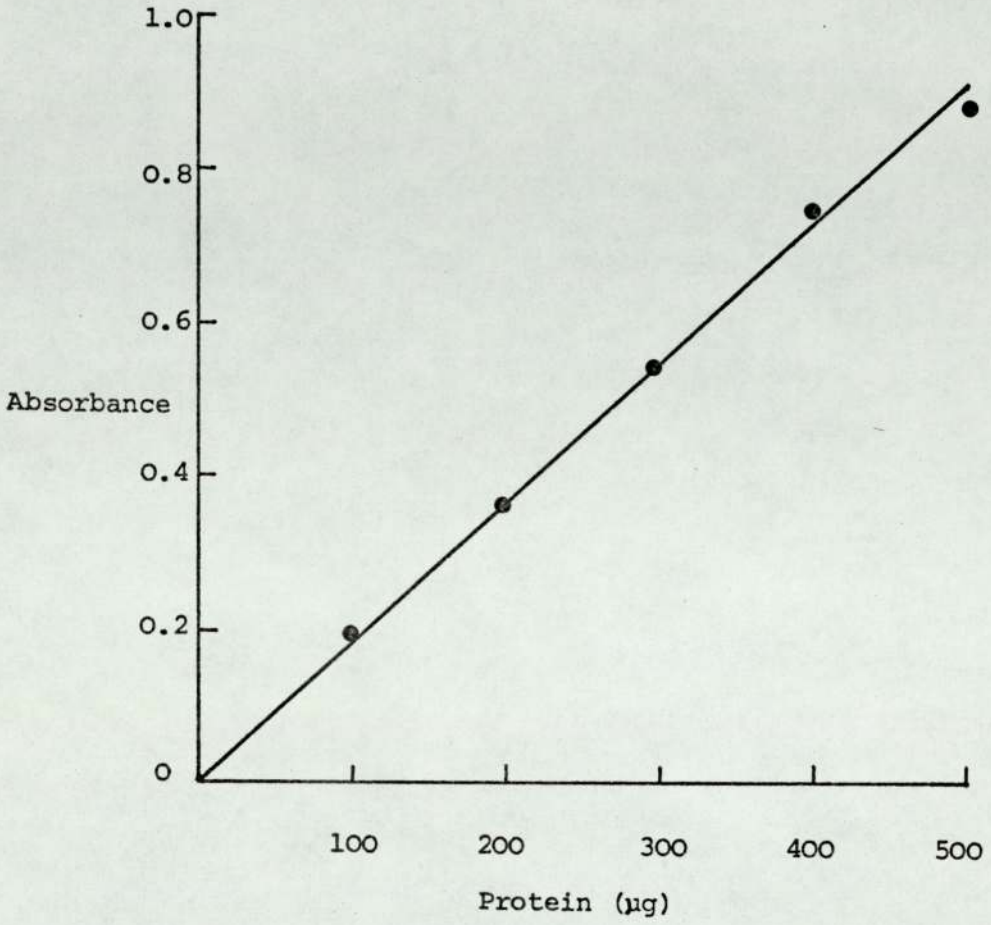


Fig. 1. Calibration curve of protein determination using bovine serum albumen as standard. Determinations were performed as described in the text. Each point represents the mean of 2 determinations.

RESULTS

CHAPTER 1.

PURIFICATION OF CHOLINE ACETYLTRANSFERASE.

INTRODUCTION.

Studies on an enzyme in situ are complicated by the many substances which compose its environment and the homeostatic mechanisms which control its actions. Therefore, in order to absolutely characterise an enzyme in terms of its physical and chemical properties it is necessary to obtain it in as pure a form as possible. Conditions can then be sought under which the enzyme best performs its normal role and which most closely approximate to the normal physiological environment of the enzyme as regards pH, temperature, and the presence of salts. From this basis a controlled study of the interaction of the enzyme with other agents can then be made.

Successful attempts to prepare active preparations of ChAc began in 1943 when Nachmansohn and Machado first demonstrated the synthesis of ACh by cell-free extracts of rat brain and electric organ. Their enzyme preparation had an activity of 35-100 µg ACh formed / g tissue / hour. Since then preparations of ChAc have been obtained from several sources and to varying degrees of purity. This can be seen in Table 1 which shows a summary of some of the attempts at purifying ChAc up to 1973. Recently Rossier (1975) reported a purification fold of 30,000 for rat brain ChAc leading to a preparation with a specific activity of 20 µmol / mg protein / min. Rossier estimates that a pure enzyme would have a specific activity of at least 100µmol/mg protein/min which means that a further 5-fold purification over that already obtained is required in order to obtain pure ChAc.

TABLE 1.

Summary of the purification of ChAc up to 1973.

YEAR	WORKERS	SOURCE OF ENZYME	FINAL SPECIFIC ACTIVITY	PURIFICATION FOLD
1943	Nachmansohn and Machado.	Rat brain	35-100 µg/g tissue/hour	
1948	Nachmansohn and Weiss	Squid ganglion	150 mg/g protein/ hour	
1953	Berman et al.	Squid ganglion	0.67-1.33 µmol /mg protein/min	10
1954	Kumagai and Ebashi	Guinea pig brain	0.5 mg/mg protein 1 hour	
1956	Burgen et al.	Rat brain	2.5-4.7 µmol/g powder/30 min.	
1957	Reisberg	Squid ganglion	0.5-1.33 µmol/g protein/min	10
1961	Mehrotra	House-fly brain	0.073 µmol/mg protein/min	20
1966	Schuberth	Human placenta	0.013 µmol/mg protein/min	65
1966	Morris	Human placenta	2.34 µmol/mg protein/min	100
1967	Prince	Squid ganglion	1.83 µmol/mg protein/min	
1968	Potter et al.	Rat brain	0.79 µmol/mg protein/min	645
1971	Glover and Potter	Ox brain	0.116 µmol/mg protein/min	40
1973	Husain and Mautner	Squid	116-666 µmol/mg protein/min	325

/contd.

TABLE 1 (continued).

YEAR	WORKERS	SOURCE OF ENZYME	FINAL SPECIFIC ACTIVITY	PURIFICATION FOLD
1973	Chao and Wolfgram	Bovine brain	0.146 µmol/mg protein/min	49
1973a	Rossier et al.	Rat brain	1.8 µmol/mg protein /min	2,700
1973	Malthe-Sorenssen et al.	Rat brain	4.0 µmol/mg protein /min	4,000

In all cases specific activity refers to the amount of ACh formed. Where possible the units of specific activity have been converted to µmol/mg protein/min for ease of comparison.

Details of the experimental techniques which have been used in attempts to extract and purify ChAc are given below along with an indication of their successes and shortcomings.

1. Initial extraction of ChAc from the tissue.

The release of ChAc from within its enclosing membranes depends on the disruption of these membranes and this can be brought about by mechanical or chemical means. Homogenisation alone is only mildly disruptive of membranes and this procedure can lead to preparations of isolated nerve endings (synaptosomes) which, after re-formation of the membranes remain 'pinched off' from the rest of the axon (Gray and Whittaker 1962). Membrane disruption produced by homogenisation is enhanced by the use of hypo-osmotic solutions which cause water to enter the cells with the result that they burst. This is shown by the release of lactate dehydrogenase, a cytoplasmic marker, on treatment of synaptosomes with sucrose hypo-osmotic to plasma (i.e., more dilute than 0.32 M) (Johnson and Whittaker, 1963). The addition of Triton X-100, a non-ionic detergent, to the homogenisation medium has also been shown to aid in the disruption of membranes and is effective in aiding the solubilisation of several enzymes (Fiszer and de Robertis, 1967; Lefresne, Guyenet, Beaujouan and Glowinski, 1975).

Most procedures aimed at extracting ChAc from tissues involve homogenisation followed by centrifugation but an alternative is the preparation of an acetone-dried powder from the tissue as described by Hemsworth and Morris (1964). Acetone-dried powders from rat brain (Nachmansohn and John 1944), squid ganglion (Nachmansohn and Weiss, 1948; Berman, Wilson and Nachmansohn, 1953; Reisberg, 1957; Prince, 1967), house-fly brain (Mehrota, 1961) and

human placenta (Schuberth, 1966; Morris, 1966) have been prepared in attempts to extract ChAc from these tissues. Morris (1966) however, points out that the use of acetone-dried powders is not suitable for some tissues such as human placenta as some enzyme activity is lost in the first acetone wash. Morris attributes this to the ease with which the enzyme is released from its binding sites within the placental tissue. However the procedure is suitable for preparing ChAc from other tissues such as rabbit brain as the enzyme in this tissue is released from its binding sites only with some difficulty.

ChAc released by the above methods occurs in both a particulate and a soluble form (Fonnum, 1967) the former resulting from an association of the enzyme with membrane fragments. The activity of both forms of the enzyme can be measured and this suggests that the active site of the enzyme is not involved in the membrane-binding of the particulate form (Fonnum 1968).

2. Purification of the released ChAc.

1. Ammonium sulphate fractionation.

Ammonium sulphate fractionation is a general and widely used method of purifying proteins and involves their selective precipitation by differing concentrations of ammonium sulphate. This method has been used extensively in the purification of ChAc but apprehension of the technique has been noted by Chao and Wolfgram (1973) on account of the irreproducibility of the results and the low recovery of enzymatic activity.

2. Gel filtration.

Gel filtration has been used to separate ChAc from other proteins on the basis of its molecular weight. Schuberth (1966)

used Sephadex G-100 to obtain a 4-fold purification of his enzyme preparation whilst Morris (1966), Potter (1968) and Glover and Potter (1971) obtained up to a 6-fold purification using Sephadex G-200.

3. Ion-exchange chromatography.

DEAE-Sephadex has been used by Morris (1966) and Chao and Wolfgram (1973) to remove anionic proteins from ChAc preparations, ChAc being cationic at pH 6 (Glover and Potter, 1971). In both cases a twofold purification of the enzyme was achieved over the previous step. CM-Sephadex has been used by Potter (1968) and Glover and Potter (1971) to obtain up to an 8-fold purification of ChAc over the previous step.

4. Affinity chromatography.

The procedures described up to now are general methods which are used in the purification of many proteins and are not specific for ChAc. Affinity chromatography (Cuatrecasas 1970) is a method which can be made specific for a particular protein or group of proteins and this method has been used in the purification of ChAc. Affinity chromatography relies on the attachment of a specific, reversible inhibitor to a column containing an insoluble polymer or gel such as sepharose. Proteins which show no significant affinity for the inhibitor pass straight through the column whilst the remainder are retained bound to the inhibitor. The bound proteins are eluted from the column under conditions in which the inhibitor does not bind to the enzyme, these conditions being brought about by changing the composition of the eluant.

Chao and Wolfgram (1973) having produced evidence that ChAc contained free sulphydryl (-SH) groups, took advantage of this fact by preparing an organomercurial-sepharose resin, from p-chloromercuribenzoate, which would retain -SH proteins. By passing a bovine brain extract through a column of this resin Chao and Wolfgram obtained a 3-fold purification of ChAc over the previous step with removal of 82% of the total protein from the extract.

Affinity chromatography was also used by Husain and Mautner (1973) who attached two different types of ligand to sepharose. The first ligand used was directed towards thiol groups on the enzyme since evidence had been reported that the active site of ChAc contained thiol groups (Roskoski 1973). The thiol-selective column was prepared by coupling succinylated amino-alkylamino sepharose using p-aminophenyl mercuriacetate and was used successfully to separate ChAc from non thiol-containing proteins. The second ligand used was an analogue of styryl pyridine as several analogues of this compound have been shown to be inhibitors of ChAc (Smith, Cavallito and Foldes, 1967; Cavallito, Yun, Smith and Foldes, 1969; Cavallito, Yun, Kaplan, Smith and Foldes, 1970). By using these two affinity resins in the purification of squid ChAc Husain and Mautner (1973) were able to obtain ChAc preparations with specific activities ranging from 116-666µmol/mg protein/min.

Although some success was obtained with the styrylpyridine resin the value of these inhibitors for affinity chromatography is limited by the non-competitive nature of their inhibition (Smith et al. 1967) as ideally a ligand used in affinity chromatography should show competitive inhibition. As yet no specific,

reversible, competitive inhibitor of ChAc suitable for affinity chromatography has been found and until an inhibitor of this type is found the value of affinity chromatography in the purification of ChAc seems limited.

5. Immunoabsorbance chromatography.

In an attempt to obtain antibodies to ChAc Rossier, Bauman and Benda (1973a) injected rabbits, hamsters, guinea pigs and roosters with partially purified ChAc preparations but were unable to elicit an immunological response to ChAc. However, antibodies were produced against some of the contaminating proteins and by polymerising these antibodies with glutaraldehyde they were able to produce an immunoabsorbance column which removed the immunogenic proteins. This step helped them to achieve a total purification-fold of 2,700 of a rat brain homogenate leading to an enzyme preparation with a specific activity of 1.87µmol/mg protein/min. A modification of this method has recently resulted in the 30,000 fold purification of rat brain ChAc mentioned earlier (page 46).

Similar results were obtained by Malthe-Sorenssen, Eskeland and Fonnum (1973) who obtained a 4,000 fold purification of rat brain ChAc using immunoabsorbance chromatography. However their procedure differed in that the antibodies to the contaminating proteins were coupled to sepharose rather than being polymerised with glutaraldehyde.

Antibodies have now been produced to ChAc from rat brain (Rossier, Bauman and Benda, 1973b), bovine caudate nuclei (Eng, Uyeda, Chao and Wolfgram, 1974) and human brain (Singh and McGeer 1974) and it seems only a matter of time before these antibodies are used to obtain further purification of ChAc, possibly leading to a pure enzyme.

Aims of the present work.

In the present work preliminary purification procedures have been compared and more advanced techniques attempted. The aim of the work was twofold. Firstly, it was hoped that partially purified preparations of ChAc from several sources could be prepared in order to compare their interaction with different substrates and inhibitors. The second aim of the work was to try and obtain a better purification of ChAc than had previously been achieved.

RESULTS.

Rat brain and human placenta were used as sources of ChAc and preliminary purification of the enzyme from both sources was attempted.

1. Initial extraction of ChAc from the tissue.

A. Homogenisation and centrifugation and the preparation of acetone-dried powders.

After excision of whole rat brains extraction of ChAc was performed by homogenisation followed by centrifugation in either 0.32 M sucrose or 0.1% Triton X-100 containing 200 mM KCl (TX-100/200 mM KCl). Homogenates prepared in sucrose were sonicated before centrifugation. Extraction of ChAc from rat brains was also performed by preparing acetone-dried powders of the tissue followed by resuspension in 200 mM KCl and centrifugation.

Extraction of ChAc from human placenta was performed by homogenisation in 0.25 M sucrose, 200 mM KCl or TX-100/200 mM KCl. As with rat brain homogenates those prepared in sucrose were sonicated before centrifugation. Acetone-dried powders of human placenta were not prepared since Morris (1966) indicated the unsuitability of this procedure for this tissue (See Introduction).

The different extraction procedures can be compared by considering 1). the distribution of recovered ChAc activity between the pellet and supernatant after centrifugation, 2). the removal of protein from the enzyme preparation during centrifugation, 3). the specific activity of the ChAc preparation and 4). the total activity of the ChAc preparation.

i). Distribution of ChAc activity after centrifugation.

Rat brain.

The majority of ChAc activity occurred in the supernatant after centrifugation for all three extraction procedures used. The supernatant with the highest percentage of ChAc activity was that from homogenates prepared in TX-100/200 mM KCl whilst the supernatant with the lowest percentage of ChAc activity was that from sucrose homogenates. (Table 2).

Human placenta.

As with rat brain homogenates the supernatant showing the highest percentage of ChAc activity was that from homogenates prepared in TX-100/200 mM KCl. (Table 4). Sucrose homogenates showed a lower percentage of ChAc activity in the supernatant compared with the supernatant from TX-100/200 mM KCl homogenates whilst homogenates of placenta prepared in KCl showed that most of the ChAc activity was associated with the particulate fraction and occurred in the pellet. (Table 4).

ii). Removal of protein from the ChAc preparation.

The amount of protein removed from the enzyme preparation is indicated by the percentage of total protein which occurs in the pellet after centrifugation. The amount of total protein removed during centrifugation depends on the procedure used for

extracting the enzyme and also on the source of the enzyme. For example, homogenisation of rat brain in sucrose followed by sonication and centrifugation removed only 25% of the total protein from the initial homogenate whereas 63% of the total protein was removed from acetone-dried powders of rat brain. (Table 2). The amount of protein removed from the homogenate into the pellet depends on the source of the enzyme and this is shown by the fact that homogenisation and centrifugation in TX-100/200 mM KCl removed 42% of the total protein from a rat brain homogenate but removed only 24% of the total protein from a human placental homogenate. Tables 2 and 4 show the amount of total protein removed by centrifugation using the different extraction procedures for rat brain and human placenta.

iii). Specific activity of the ChAc preparations.

Rat brain.

In the initial homogenates (or resuspended acetone-dried powder) the enzyme with the highest specific activity was that obtained on homogenising the brain in TX-100/200 mM KCl (Table 3). After centrifugation the enzyme in the supernatants showed an increase in specific activity for all three extraction procedures, the greatest percentage increase occurring in sucrose homogenates, which were sonicated prior to centrifugation. The supernatant with the highest ChAc specific activity was that obtained from acetone-dried powders of the brain although the specific activity of ChAc in the supernatants from TX-100/200 mM KCl homogenates was only slightly lower (Table 3).

Human placenta.

TX-100/200 mM KCl homogenates of human placenta contained ChAc of a higher specific activity than either 0.25 sucrose or 200 mM KCl homogenates. After centrifugation the specific activity of the enzyme in the supernatants showed an increase for TX-100/200 mM KCl homogenates and a decrease for homogenates in sucrose or KCl (Table 5). The specific activities of the enzymes in the supernatants from TX-100/200 mM KCl homogenates was similar for both rat brain and human placental preparations (Tables 3 and 5).

iv). Total activity of the ChAc preparations.

Rat brain.

In the initial homogenates the highest total ChAc activity occurred in those prepared in TX-100/200 mM KCl. (Table 3). However, after centrifugation, the highest total activity obtained was in the supernatants from sonicated sucrose homogenates in which a large increase in total activity occurred during sonication. The supernatants obtained after centrifugation of TX-100/200 mM KCl homogenates and resuspended acetone-dried powders showed some loss of ChAc activity. These results are summarised in Table 3.

Human placenta.

The total ChAc activity obtained in the initial homogenates of human placenta was greater than that obtained from the same wet weight of rat brain. (cf. Tables 3 and 5). The highest total activity was obtained from TX-100/200 mM KCl homogenates and the lowest from KCl homogenates. After centrifugation, the supernatants from all three extraction procedures contained less ChAc activity than the original homogenates. (Table 5).

TABLE 2.

Initial extraction of ChAc from rat brain.

Homogenisation procedure	Percentage of ChAc activity in pellet	Percentage of ChAc activity in supernatant	Percentage of protein in pellet	Percentage of protein in supernatant
0.32 M sucrose + sonication	14	86	25	75
TX-100/200 mM KCl	5	95	42	58
Acetone-dried powders	12	88	63	37

TABLE 3.

Initial extraction of ChAc from rat brain.

Homogenisation procedure	Specific activity of ChAc in original homogenate	Specific activity of ChAc in supernatant	Total activity of ChAc in original homogenate	Total activity of ChAc in supernatant
0.32 M sucrose + sonication	11	36	1577	3215
TX-100/200 mM KCl	27	46	2791	2609
Acetone-dried powders	18	51	1031	728

Units of specific activity are $\times 10^{-6}$ µmol/mg protein/min.

Units of total activity are $\times 10^{-6}$ µmol/g tissue/min.

TABLE 4.

Initial extraction of ChAc from human placenta.

Homogenisation procedure	Percentage of ChAc activity in pellet	Percentage of ChAc activity in supernatant	Percentage of protein in pellet	Percentage of protein in supernatant
0.25 M sucrose + sonication	42	58	33	67
200 mM KCl	65	35	32	68
TX-100/200 mM KCl	12	88	23	77

TABLE 5.

Initial extraction of ChAc from human placenta

Homogenisation procedure	Specific activity of ChAc in original homogenate	Specific activity of ChAc in supernatant	Total activity of ChAc in original homogenate	Total activity of ChAc in supernatant
0.25 M sucrose + sonication	30	26	2427	1638
200 mM KCl	24	9	1274	364
TX-100/200 mM KCl	36	50	3943	3761

Units of specific activity are 10^{-6} µmol/mg protein/min.

Units of total activity are 10^{-6} µmol/g tissue/min.

B. Sonication.

Sonication was performed on homogenates prepared in sucrose in order to enhance disruption of the cell membranes enclosing ChAc. Sonicated samples were found to heat up quite rapidly even when the samples were placed in a container of ice and so sonication was performed intermittently, the solutions being allowed to cool after every 10 minutes of sonication.

i). Sonication of rat brain homogenates.

Sonication of rat brain homogenates for a total of 100 minutes resulted in a 116% increase in the total activity of ChAc and a 209% increase in the specific activity of the enzyme.

ii). Sonication of human placental homogenate.

Samples of placental homogenate were taken at intervals during sonication and it was found that both the total activity and the specific activity of the enzyme showed an initial rise followed by a decline until after a total of 60 mins. there was only a 5% increase in total activity and a 21% increase in specific activity. The greatest increases in enzyme activity were obtained after 10 mins. sonication when the increase in the total enzyme activity was 44% and the increase in the specific activity was 42% over the initial homogenate.

2. Purification of ChAc preparations.

A. $(NH_4)_2SO_4$ fractionation.

Fractionation of ChAc preparations was performed using $(NH_4)_2SO_4$ concentrations of 20% and 30% (w/v). Proteins precipitating out of solution at 20% $(NH_4)_2SO_4$ were discarded, whilst those precipitating

between 20% and 30% $(NH_4)_2SO_4$ were retained. (See Methods).

i). Distribution of recovered ChAc activity.

In order to judge whether ChAc is being precipitated between concentrations of $(NH_4)_2SO_4$ of 20% and 30% the distribution of ChAc activity between the pellet and the supernatant at the two centrifugation steps must be measured. If ChAc does precipitate between 20% and 30% $(NH_4)_2SO_4$ a high percentage of ChAc activity should occur in the supernatant after the addition of 20% $(NH_4)_2SO_4$ and centrifugation. After the addition of 30% $(NH_4)_2SO_4$ and centrifugation the majority of the ChAc activity should occur in the pellet. The validity of using the protein fraction precipitating between 20% and 30% $(NH_4)_2SO_4$ as the fraction containing ChAc depends on the initial extraction procedure used as is evident from the results shown below.

Rat brain.

Sonicated homogenates of rat brain in sucrose retained only 25% of the recovered ChAc activity in the supernatant after the addition of 20% $(NH_4)_2SO_4$ and centrifugation. The percentage of recovered ChAc activity in the supernatant from TX-100/200 mM KCl homogenates and acetone-dried powders at this stage was about 75% (Table 6). The distribution of ChAc activity was more favourable after centrifugation at the 30% $(NH_4)_2SO_4$ stage than after centrifugation at the 20% $(NH_4)_2SO_4$ stage for all three extraction procedures. The percentages of ChAc obtained in the pellet at the 30% $(NH_4)_2SO_4$ stage were 91% from TX-100/200 mM KCl homogenates and 100% from sonicated sucrose homogenates and acetone-dried powders. (Table 7).

Human placenta.

Only placental homogenates prepared in TX-100/200 mM KCl were fractionated with $(NH_4)_2SO_4$. The percentage of ChAc activity which remained in the supernatant after centrifugation at the 20% $(NH_4)_2SO_4$ stage was low at 42% (Table 8) compared with rat brain homogenates in TX-100/200 mM KCl (Table 6). However 99% of the recovered ChAc activity was obtained in the pellet after the addition of 30% $(NH_4)_2SO_4$ and centrifugation. (Table 8).

ii). Removal of protein from the ChAc preparation.

The amount of protein removed refers here to the percentage of the total protein in the pellet after the 20% $(NH_4)_2SO_4$ stage and the percentage of the total protein which remains in the supernatant after the 30% $(NH_4)_2SO_4$ stage.

Rat brain.

The percentage of total protein removed varied between 40% and 80% depending on the $(NH_4)_2SO_4$ fractionation stage and the initial extraction procedure. In general less protein was removed at the 20% $(NH_4)_2SO_4$ stage than at the 30% $(NH_4)_2SO_4$ stage except for homogenates in sucrose from which about 80% of the total protein was removed at each stage. (Tables 6 and 7). At both fractionation stages the greatest percentage of total protein was removed from sucrose homogenates and the least percentage from TX-100/200 mM KCl homogenates. (Tables 6 and 7).

Human placenta.

As with rat brain enzyme preparations a greater percentage of total protein was removed at the 30% $(NH_4)_2SO_4$ stage than at the

TABLE 6.

Ammonium sulphate fractionation of ChAc preparations.

Rat brain. 20% $(NH_4)_2SO_4$.

Homogenisation procedure	Percentage of ChAc activity in pellet	Percentage of ChAc activity in supernatant	Percentage of protein in pellet	Percentage of protein in supernatant
0.32 M sucrose + sonication	76	24	80	20
TX-100/200 mM KCl	24	76	41	59
Acetone-dried powder	26	74	48	52

TABLE 7.

Ammonium sulphate fractionation of ChAc preparations.

Rat brain. 30% $(NH_4)_2SO_4$.

Homogenisation procedure	Percentage of ChAc activity in pellet	Percentage of ChAc activity in supernatant	Percentage of protein in pellet	Percentage of protein in supernatant
0.32 M sucrose + sonication	100	0	20	80
TX-100/200 mM KCl	91	9	47	53
Acetone-dried powder	100	0	32	68

TABLE 8.

Ammonium sulphate fractionation of ChAc preparations.

Human placenta. 20% - 30% $(NH_4)_2SO_4$.

$(NH_4)_2SO_4$ stage	Homogenisation procedure	Percentage of ChAc activity in pellet	Percentage of ChAc activity in supernatant	Percentage of protein in pellet	Percentage of protein in supernatant
20% $(NH_4)_2SO_4$	TX-100/200 mM KCl	58	42	39	61
30% $(NH_4)_2SO_4$	TX-100/200 mM KCl	99	1	18	82

20% $(NH_4)_2SO_4$ stage. (Table 8). Comparison of the percentage of total protein removed from TX-100/200 mM KCl homogenates from the two enzyme sources shows that a similar amount (about 40%) of the total protein was removed at the 20% $(NH_4)_2SO_4$ stage. However, a greater percentage of the total protein was removed from placental homogenates (82%) at the 30% $(NH_4)_2SO_4$ stage than from brain homogenates (53%).

iii). Specific activity and total activity of the ChAc preparation.

A phenomenon which occurred frequently but inconsistently during $(NH_4)_2SO_4$ fractionation of ChAc preparations was an increase of anything up to 250%, in the total activity of ChAc. This increased recovery of ChAc activity seemed to be independent of the original homogenisation procedure but was more evident during the 20% $(NH_4)_2SO_4$ stage. A consequence of the inconsistency with which this increase in total activity occurred is that the increase in specific activity observed, which is used as an indicator of purification of the enzyme, was also inconsistent, though always evident. The specific activity referred to is that of the enzyme in the resuspended pellet after the 30% $(NH_4)_2SO_4$ stage. The best purification achieved was from an acetone-dried powder of rat brain from which a 36-fold purification over the original resuspended powder was obtained, resulting in an enzyme preparation with a specific activity of 0.66 nmol/mg protein/min.

B. Ion-exchange chromatography.

Ion-exchange chromatography was attempted using the anion-exchanger DEAE-Sephadex in order to remove anionic proteins. Elution of the column was performed using 20 mM sodium phosphate buffer containing 200 mM NaCl

(pH 6.2) and 3 ml fractions of the eluate were collected. Each fraction was assayed separately for ChAc activity and the enzyme was found to be present in fractions 21-31 inclusive. These fractions were combined and the recovery of ChAc activity was determined as 46%. The amount of enzyme purification could not be calculated however as storage at -20°C prior to the determination of the total protein concentration resulted in a complete denaturation of the protein and consequent loss of ChAc activity.

C. Affinity chromatography.

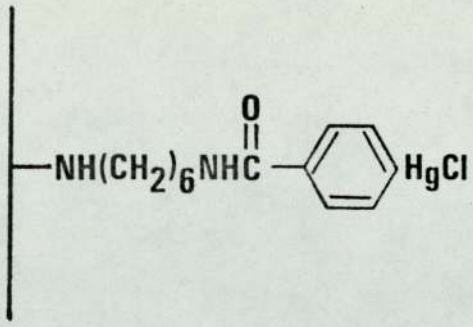
i). Organo-mercurial sepharose.

p-chloromercuribenzoate (p-CMB) was used to prepare two different affinity chromatography columns differing in the distance between the ligand (p-CMB) and the sepharose matrix to which it was bound. (Fig. 2. A and B). On passing a sonicated rat brain homogenate in sucrose through each column about 77% of the ChAc activity remained bound to the column and was not eluted with 10^{-1} M mercaptoethanol. However, 75% of the total protein was also retained on the column so that purification of the ChAc, had it been eluted, would have been minimal. Similar results were obtained with both organo-mercurial sepharose columns.

ii). Styrylpyridine sepharose.

The styrylpyridine analogue, hydroxyethyl naphthyl-vinylpyridine bromide (NVPH) (Figure 2C) has been shown to be a potent inhibitor of ChAc with a K_i of 10^{-6} M (Cavallito, Yun, Kaplan, Smith and Foldes, 1970). An attempt was made to couple this inhibitor to sepharose, for use as an affinity column, but the attempt was unsuccessful for reasons which are not readily apparent.

A.



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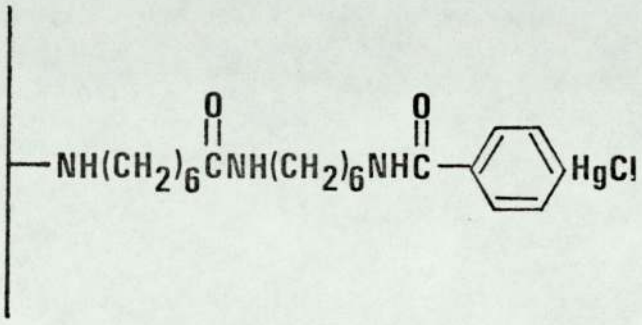


C.

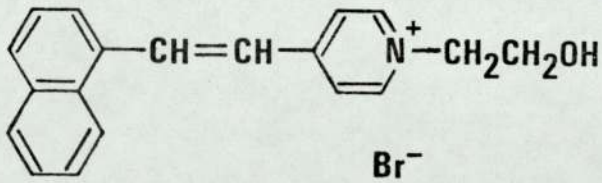


Fig. 2. Structural formula of A. Organomercurial-Sepharose 1, B. Organomercurial-Sepharose 2 and C. Hydroxyethyl naphthyl-vinyl pyridinium bromide (NVPH).

DISCUSSION.

1. Comparison of the procedures for the initial extraction of ChAc from the tissue.

1. Effect of Triton X-100 on the solubilisation and purification of ChAc.

Fiszer and de Robertis (1967) reported that Triton X-100 had no solubilising action on ChAc from rat brain. However, similar experiments performed by Lefresne, Guyenet, Beaujouan and Glowinski (1975) showed the progressive solubilisation of ChAc with increasing Triton X-100 concentrations. The first appearance of ChAc in the medium occurred at a concentration of Triton X-100 of 0.002% and corresponded to a disruption of the synaptosomal membrane. However, complete solubilisation was not achieved until a Triton X-100 concentration of 0.1% was reached and Lefresne et al. (1975) attributed this to a progressive disappearance with increasing Triton X-100 concentrations of interactions which exist between ChAc and large cellular fragments. In other words, Triton X-100 was converting ChAc from its particulate form to its soluble form. Fonnum (1967,1968) has shown that this conversion is also brought about by an increased pH and ionic strength and this may explain the discrepancy between the results of Fiszer and de Robertis (1967) and Lefresne et al. (1975). The former workers used conditions of low ionic strength so that although the enzyme may have been released from within its enclosing membranes it would still be associated with them and appear in the sediment on centrifugation. Lefresne et al. (1975) on the other hand used conditions of a higher ionic strength under which conversion of released ChAc to its soluble form would be aided.

These previous studies on the solubilising action of Triton X-100 on ChAc were performed on preparations fractionated on sucrose gradients. In the present experiments the solubilising action of Triton X-100 on ChAc has been studied on initial homogenates and on enzyme preparations obtained at subsequent stages during purification by ammonium sulphate fractionation. In the initial homogenates extraction of ChAc into the homogenisation medium was enhanced in the presence of Triton X-100 for both rat brain and human placental enzymes as judged by the higher total ChAc activity of Triton X-100-containing homogenates. Triton X-100 also increased the percentage of ChAc occurring in the soluble form as indicated by the higher percentage of ChAc obtained in the supernatants of Triton-containing homogenates after centrifugation. These results support those of Lefresne et al. (1975) and suggest that Triton X-100 enhances membrane disruption and also aids in the conversion of ChAc from the particulate form to the soluble form.

A disadvantage of the addition of Triton X-100 to homogenates is that, due to the non-specific nature of the solubilising effect, a greater percentage of the total protein is associated with the ChAc than in homogenates not containing the detergent. This was evident both during the initial extraction of the enzyme and also during the ammonium sulphate fractionation stages. The result of this is that the purity of the enzyme as judged by its specific activity is reduced to some extent although Triton-containing homogenates still produced ChAc of a higher specific activity than homogenates not containing Triton X-100.

2. Comparison of the use of acetone-dried powders with the use of homogenates for the initial extraction of ChAc.

Nachmansohn and John (1944) reported that ChAc prepared from an acetone-dried powder of rat brain was twice as pure as the enzyme prepared from fresh tissue, but they did not indicate the total amount of ChAc released. The present results are similar in that acetone-dried powders of rat brain gave rise to an enzyme with a higher specific activity than did homogenates. However, the total ChAc activity from acetone-dried powders was much lower than that obtained from homogenates. This suggests that either the enzyme is not released into solution because of a lack of disruption of the enclosing membranes or there is some loss of the enzyme in the acetone wash as described by Morris (1966) for human placenta. If the latter is the case it would seem that ChAc is released from its binding sites within rat brain and human placenta with equal facility. Of the ChAc that is released and recovered the majority is in the soluble form and appears in the supernatant after centrifugation of the resuspended powder.

The greater purity of the soluble ChAc derived from acetone-dried powders as compared with that derived from homogenates seems from the present results to stem not from a greater release and recovery of ChAc from the tissue but from the removal of a greater percentage of the total protein. This greater removal of protein suggests that there may be less mechanical disruption of membrane protein during the preparation of acetone-dried powders resulting in an association of the protein with larger membrane fragments which are removed on centrifugation.

3. Effect of sonication on the solubilisation and purification of ChAc.

Homogenates in sucrose and KCl show a lower total ChAc activity than homogenates containing Triton X-100 and this is probably a reflection of the lesser membrane disruption which occurs in the absence of Triton X-100. Further evidence for this is that sonication of rat brain sucrose homogenates, a procedure which is aimed at enhancing membrane disruption, results in a large increase in the total amount of measurable ChAc, most of which corresponds to the soluble form of the enzyme. This increased total ChAc activity is not accompanied by a similar increase in the total protein with the result that the specific activity of the enzyme is greatly increased. However, this increase in the specific activity is not as great as it might be since sonication also results in the solubilisation of a high proportion of the total protein, a fact which suggests that a high degree of membrane disruption has occurred.

Sonication has less effect on the release of ChAc from human placenta and this may be due either to a greater resilience of the non-neural membranes to sonication or possibly to a greater susceptibility of the placental enzyme to the denaturation caused by sonication.

4. Conclusions regarding the initial extraction of ChAc.

An initial extraction procedure is judged not only on the purity of the enzyme produced but also on the amount of enzyme released from the tissue. It has been seen that a high enzyme specific activity does not necessitate the solubilisation of a high proportion of the total ChAc within the tissue but depends more on the amount of contaminating protein removed. However, for an

efficient extraction procedure it is essential to obtain a high solubilisation of the enzyme if it is intended to proceed to further purification since the yield of the enzyme can decrease dramatically as purification progresses. Of the extraction procedures compared in the present work it is suggested that homogenisation in Triton X-100/200 mM KCl gives the best balance between enzyme purity and recovery and is the method of choice for extracting ChAc from both rat brain and human placenta.

2. Purification of choline acetyltransferase.

1. Ammonium sulphate fractionation in the purification of ChAc.

Ammonium sulphate fractionation has been used in most attempts at purifying ChAc. The fractionation range used has varied between workers but has most commonly been about 15%-30% (w/v). However, Miroshnichenko, Popova and Severin (1971) suggested that a broader range of saturation was better having shown that there was as much ChAc activity in fractions precipitating between 30% and 45% as in fractions precipitating between 15% and 30%. In the present work the concentration range used was 20%-30% and both sediments and supernatants of ammonium sulphate fractions were tested for ChAc activity so that any failure to recover the full enzyme activity in the required fraction would come to light.

The distribution of recovered ChAc activity between sediments and supernatants was found to be influenced by the initial extraction procedure. However, in general, of the recovered enzyme activity, the majority was obtained in the required fractions, the greatest loss of activity occurring by precipitation at the 20% saturation stage. If ammonium sulphate fractionation is to be used therefore in the purification of ChAc it would seem from the present

results that a saturation range of 20%-30% is satisfactory but that a decrease in the lower concentration would probably be advantageous to the recovery of the enzyme, although not necessarily to its purity.

A complicating factor during ammonium sulphate fractionation was the increase in total ChAc activity which occurred frequently but inconsistently. This phenomenon has also been reported by Polsky (1973) who after using ammonium sulphate fractionation in the purification of ChAc from squid head ganglia found that the total amount of enzyme activity was 110%-260% as high as it had appeared to be immediately after extraction. The reason for this increased activity is not clear but it could be due either to the removal of endogenous enzyme inhibitors from the preparation, or to an activation of the enzyme elicited perhaps by some conformational change possibly increasing the number of active sites or making such sites more accessible.

Chao and Wolfgram (1973) expressed a distrust of conventional enzyme purification techniques such as ammonium sulphate fractionation and ion-exchange chromatography on the basis of the low recovery of enzymatic activity and the irreproducibility of the results. These workers later obtained a quantitative recovery of ChAc on fractionation with ammonium sulphate, whilst reporting the formation of high molecular weight aggregates of ChAc in enzyme fractions treated with ammonium sulphate (Chao and Wolfgram, 1974). The inconsistencies reported in the present work support the assertions of Chao and Wolfgram and suggest that ammonium sulphate fractionation in the purification of ChAc is of limited value.

2. Affinity chromatography in the purification of ChAc.

Two types of affinity chromatography resin, organomercurial-sepharose and styrylpyridine-sepharose have been used in attempts to purify ChAc by affinity chromatography. In the present work the use of organomercurial-sepharose met with only partial success in that rat brain ChAc was retained by the resin but was associated with a large percentage of the total protein. Since these experiments were performed organomercurial-sepharose has been used with greater success by Chao and Wolfgram (1973) and Husain and Mautner (1973) to increase the purity of ChAc from bovine brain and squid-head ganglia respectively. The improved success of these workers may be attributed to different experimental conditions from those used in the present work or possibly to the species differences of the enzyme preparations. These species differences may be reflected in differing affinities of ChAc and total protein towards the resin and these could result from a species difference in the number of sulphydryl groups per ChAc molecule and a difference in the number of sulphydryl group-containing proteins within the preparations.

An attempt to prepare a styrylpyridine-sepharose resin by coupling NVPH to sepharose was unsuccessful. However, other styrylpyridine analogues have since been coupled to sepharose by other workers (Husain and Mautner, 1973; Habal, 1974) but as yet only Husain and Mautner have achieved any useful purification of ChAc using this method since Habal failed to recover any enzyme activity in the protein fractions eluted from his resin.

3. Stability of partially purified preparations of ChAc.

The instability of partially purified ChAc preparations has been well documented (Morris, 1966; Miroshnichenko, Popova and Severin, 1971; White and Wu, 1973a) and was observed during the

present work in eluates from a DEAE-Sephadex column after storage for one week at -20°C and also in fractions collected from an isoelectric focusing column.

A clue as to the reason for this instability of purified ChAc preparations comes from experiments using gel filtration, a procedure which incurs particularly heavy losses of enzyme activity from preparations which are otherwise very stable (Malthe-Sorenssen and Fonnum, 1972; Chao and Wolfgram, 1973). Chao and Wolfgram (1973) have correlated this loss of activity with a dissociation of bovine brain ChAc into two non-identical sub-units which occurs on removal of haemoglobin from the preparation. These workers note the similarity between their experiments and those of White and Cavallito (1970a) who observed a drastic loss of ChAc activity after chromatography using Sephadex G-100 unless bovine plasma albumen was present.

It is not known whether the instability of ChAc preparations purified by methods other than gel filtration is due to a similar dissociation of the enzyme into sub-units but White and Wu (1973a) have reported the stabilising influence of bovine albumen on ChAc eluted from an isoelectric focusing column. Since a high salt concentration does not protect the enzyme from the dissociation (Chao and Wolfgram, 1973) it appears that it may be necessary to add albumen to purified ChAc preparations in order to stabilise them. Hopefully this will not hinder studies on the enzyme, since White and Wu (1973a) state that albumen does not influence the kinetic properties of ChAc from human brain.

4. Conclusions regarding the purification of ChAc.

Progress in the purification of ChAc has been rapid especially since the application of immunological methods to the problem. These methods however are highly consumptive of animals and time and this fact placed these techniques out of the scope of the present work. Affinity chromatography at present seems to be the only rival to the immunological approach but still awaits the discovery of a suitable specific inhibitor of ChAc. These factors resulted in a re-direction of the present work away from the purification of ChAc. However, the present results were used to advantage in the preparation of both crude and partially purified ChAc preparations which were used to study the enzymic acetylation of various compounds.

SUMMARY.

1. Several methods have been used for the extraction and purification of ChAc from rat brain and human placenta.

2. A comparison of the different procedures used for the extraction of ChAc from the tissues suggests that homogenisation in Triton X-100/200 mM KCl is the method of choice for the extraction of ChAc from both rat brain and human placenta.

3. Ammonium sulphate fractionation has been used during the purification of ChAc but its value appears to be limited; the reasons for this are discussed.

4. Purification of ChAc by affinity chromatography has also been attempted but the lack of a suitable ChAc inhibitor limits the purification which can be achieved using this technique.

5. The greater instability of ChAc preparations which have been partially purified was shown, and this is discussed in relation to previously documented results.

6. The results achieved by other workers who have applied immunological techniques to the purification of ChAc, suggest that pure preparations of the enzyme may be obtained in the near or not too distant future.

CHAPTER 2.

REMOVAL OF ENDOGENOUS CHOLINE FROM PREPARATIONS OF CHOLINE ACETYLTRANSFERASE.

INTRODUCTION.

Choline acetyltransferase preparations derived from homogenates of rat brain contain free endogenous choline which can interfere with studies on the substrate specificity of the enzyme through a competition between the two substrates for the active site of the enzyme. In the past, the rate of acetylation of a substrate in the presence of endogenous choline has been determined by subtracting the rate of acetylation of endogenous choline from the total rate of acetylation which occurs in the presence of both substrates (Barker and Mittag 1975). However, in view of the possible competition between the two substrates, which could affect their acetylation rates, it is essential to first remove the endogenous choline so that the other substrate can be studied without complication. The removal of choline from preparations of ChAc has been performed by gel filtration using Sephadex G-25 or Sephadex G-50 (Hemsworth 1971b, 1972; Mann and Hebb, 1975) and Hemsworth (1971b, 1972) has used the resulting choline-free enzyme to study the acetylation of various substrates.

The present experiments were performed because ChAc preparations, which had been passed through Sephadex G-25 or G-50, as a preliminary to substrate specificity studies, still appeared to contain choline.

RESULTS.

1. Removal of endogenous choline from choline acetyltransferase preparations by gel filtration.

Passage of a rat brain homogenate through a Sephadex G-25 or

G-50 column should remove any endogenous substrate present within the preparation. However, preparations which had been subjected to gel filtration using Sephadex still gave values higher than blank values when ChAc determinations were performed in the absence of added substrate. It seemed unlikely that this result was due to some non-specific reaction not involving ChAc since the inclusion of 10^{-3}M NVP in an incubation mixture not containing added substrate reduced the amount of recovered radioactivity during assay almost to blank values. A possible explanation for the results was that choline was not fully removed by gel filtration of the enzyme preparation and so this possibility was investigated with the use of ^{14}C-labelled choline.

^{14}C-choline was added to a rat brain homogenate which was applied to a Sephadex G-50 column and then eluted with 200 mM KCl. Fractions of the eluate were collected and the radioactivity and protein content of each fraction was determined. A clear separation of the ^{14}C-choline from the main protein peak was obtained as shown in Figure 3. It should be noted that the fractions corresponding to those normally collected as the most appropriate for ChAc activity were fractions 6, 7 and 8 in which no radioactivity could be detected suggesting a complete separation of ^{14}C-choline and ChAc within these fractions.

2. Formation of choline in preparations of choline acetyltransferase.

Previous workers have reported that choline levels of intact brain and brain homogenates can increase with time even in preparations kept at -4°C . (See Discussion). In order to determine whether an increase in the level of choline could occur in the short time elapsing between elution of a ChAc preparation from a Sephadex column and assay

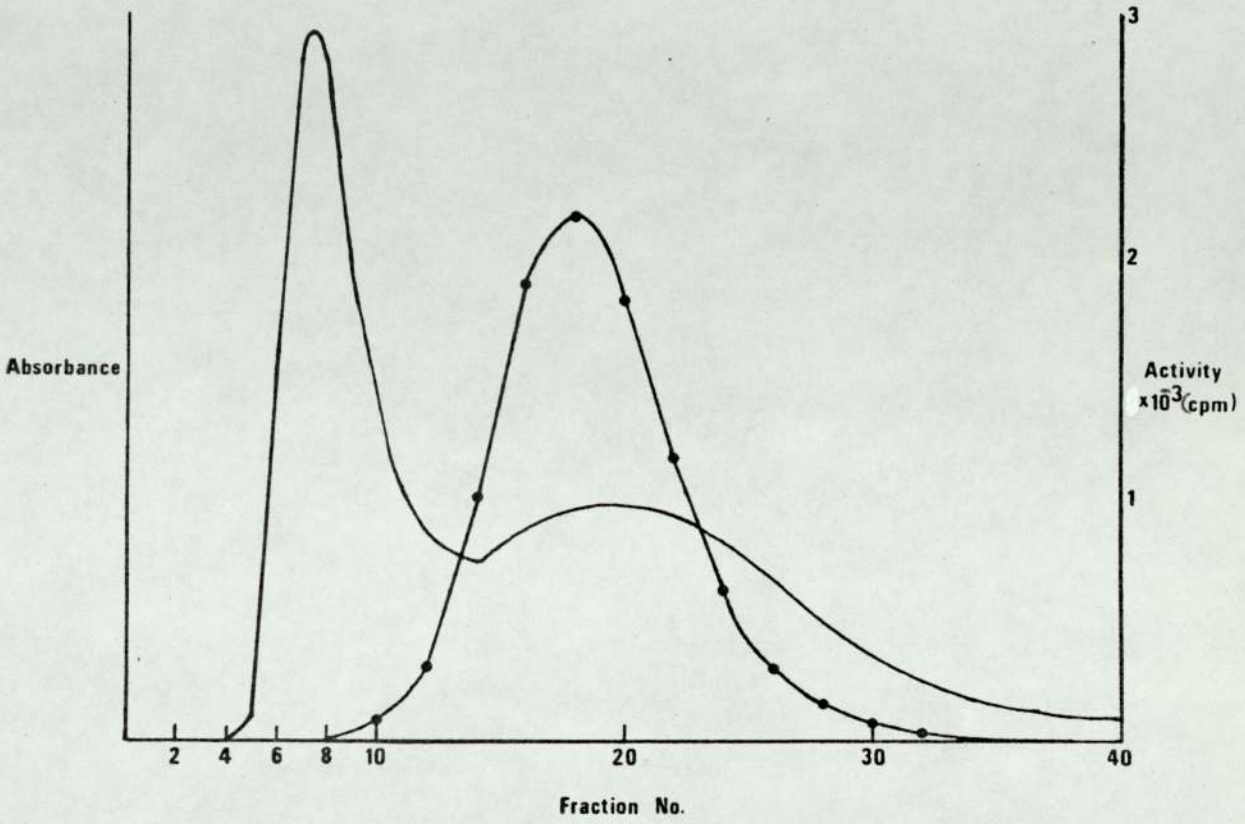


Fig. 3. Separation of choline from the main protein fraction of a rat brain homogenate using Sephadex G-50. The protein elution pattern (—) was determined by measuring the U.V. absorption of the column eluate at 280 nm. The 14-C choline elution pattern (●) was determined by adding 10 ml of Bray's fluor to 100 µl samples from each fraction of eluate and determination of the radioactivity using a liquid scintillation counter.

of the preparation, a time study of the rate of formation of choline in a rat brain homogenate was performed.

A rat brain homogenate was passed through a Sephadex G-50 column and assayed immediately and then again after 10 minutes and 3 hours, the preparation being kept in ice throughout. After 3 hours the enzyme preparation was stored at -20°C for a further 21 hours and then re-assayed. The concentration of choline in the homogenate at the various times was estimated by comparing the amount of recovered radioactivity on assay with ^{14}C AcCoA but no added substrate, with that recovered when the same enzyme preparation was incubated with ^{14}C AcCoA and various concentrations of choline (Figure 4). Values for Figure 4 were obtained from assay of the enzyme preparation some time after gel filtration and were therefore obtained by subtracting the counts per minute obtained without added substrate from the counts per minute obtained with the various concentrations of choline. Some small error will have been incurred by this procedure since the graph of choline concentration v. counts per minute (Figure 4) is not absolutely linear over the relevant concentration range. However, the error will only be slight and the procedure is useful as it enables absolute values for the amount of choline formed to be estimated. These values are plotted on Figure 5 and indicate a rapid increase in the choline concentration of the homogenate even when the preparation was stored at -20°C. The rates of choline formation for the three periods 0-10 minutes, 10 minutes - 3 hours, and 3 hours - 24 hours, were calculated as being 3.5, 0.9 and 0.6 nmol/g tissue/min respectively.

3. Inhibition of the formation of endogenous choline in homogenates.

Mann and Hebb (1975) reported that rat brain ChAc preparations stored at -18°C did not show an increase in the level of endogenous

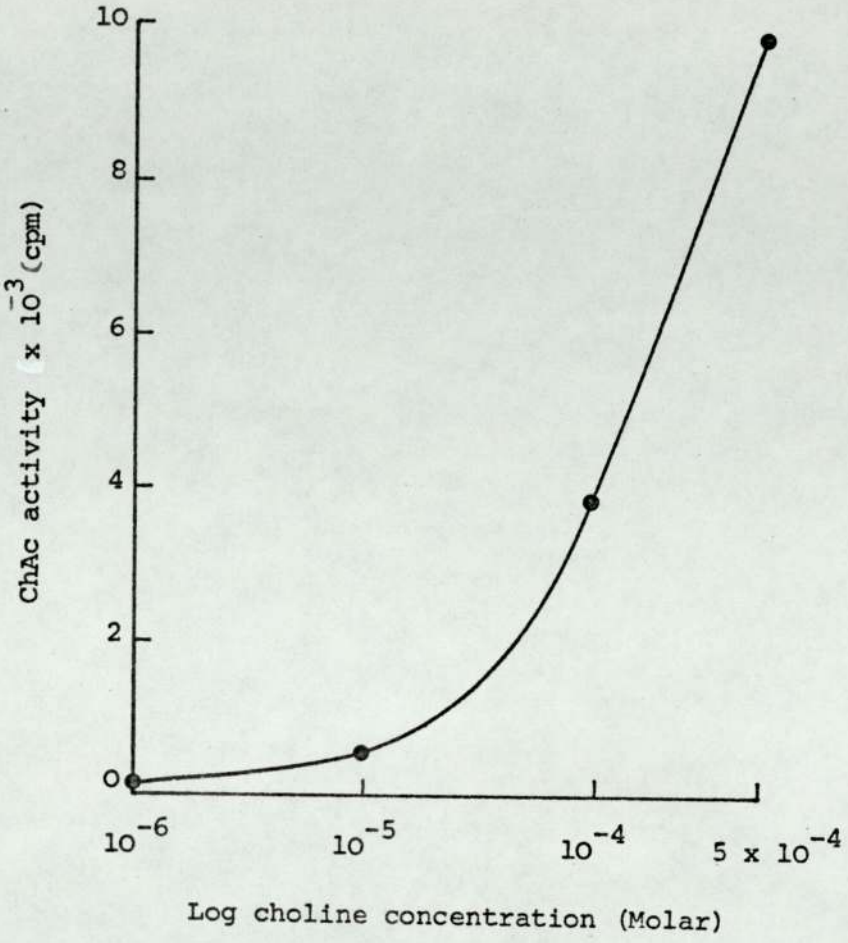


Fig. 4. Effect of choline concentration on the ChAc activity of a rat brain enzyme preparation. ChAc determinations were performed as described in Methods. The results have been corrected for activity due to the presence of endogenous choline within the enzyme preparation.

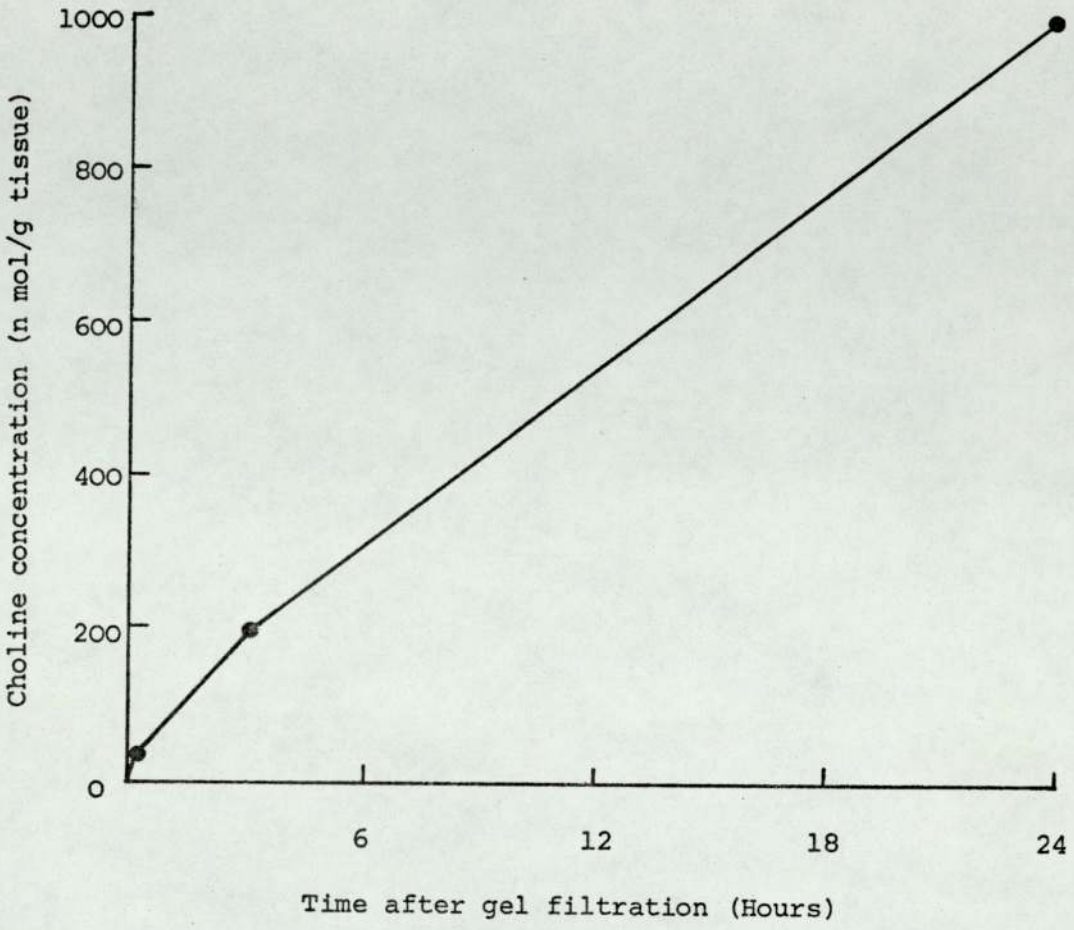


Fig. 5. Formation of choline within a rat brain homogenate after gel filtration using Sephadex G-50. The enzyme preparation was kept in ice for the first 3 hours after gel filtration and was subsequently stored at -20°C.

choline and attributed this to an inhibition of L-3 glycerylphosphoryl choline glycerophosphohydrolase (E.C. 3.1.4.2) by the 1 mM EDTA which the extracts contained. In the present experiments endogenous choline levels of homogenates increased even in the presence of 1 mM EDTA although a possible influence of the EDTA on the rate of formation of choline cannot be ruled out.

DISCUSSION.

Reported values for brain endogenous choline levels vary widely as noted by Schuberth, Spark and Sundwall (1970a) and Shea and Aprison (1973), and range from 25.3 nmol/g tissue (Shea and Aprison, 1973) to 600 nmol/g tissue (Smith and Saelens, 1967). This wide variation in reported choline levels can be attributed at least in part to the rapid post-mortem formation of choline which has been shown to occur both in intact brains and homogenates (Schuberth, Spark and Sundwall, 1970b; Dross and Kewitz, 1972; Shea and Aprison, 1973; Eade, Hebb and Mann, 1973; Present observations), a fact which makes the interval between death and determination of the choline concentration a critical factor.

The post-mortem formation of choline probably occurs through a breakdown, either spontaneous or enzymatic, of choline-containing phospholipids present within the tissue. Illingworth and Portman (1972) have shown that lysophosphatidylcholine present in plasma is a possible precursor of brain choline and if so the reaction probably proceeds via the deacylation of lysophosphatidylcholine to glycerophosphorylcholine (Dross and Kewitz, 1972; Illingworth and Portman, 1972) from which free choline is released. This latter step is catalysed by a diesterase present in brain (Webster, Marples and Thompson, 1957; Illingworth and Portman, 1972) which Mann and Hebb (1975) have reported is inhibited in the presence of EDTA. In the present work, choline was formed in homogenates even in the presence of

EDTA but this may have been due to a non-enzymatic breakdown of the phospholipid, or the breakdown of phospholipids other than glycerophosphorylcholine, such as phosphorylcholine or cytidinediphosphate choline (CDP-choline) (Dross and Kewitz, 1972).

Indications as to the rate of formation of choline in intact brains and homogenates have been given by several workers. Dross and Kewitz (1972) have shown that in intact rat brains the rate of formation of endogenous choline is most rapid during the first few minutes after decapitation and is more rapid at 38°C than at 22°C. They also reported that homogenates showed an increase in the level of choline but the rate of formation (10 nmol/g tissue/minute) was lower than in intact brains, although the rate was constant over 2.5 hours. Other rates which have been reported for the formation of choline in rat brain homogenates are 2.4 nmol/g tissue/minute over 2 hours at +4°C (Schuberth et al., 1970b) and at least 9.7 nmol/g tissue/minute during the first 4 minutes after the death of rats at -4°C and 6.1 nmol/g tissue/minute over the next 2.5 min (Shea and Aprison, 1973). The present results show a rate of choline formation over the first 10 minutes of 3.5 nmol/g tissue/minute which declines to an average rate of 0.9 nmol/g tissue/minute for the next 3 hours. Storage of the preparation at -20°C resulted in only a slight lowering of this rate of choline formation to 0.6 nmol/g tissue/minute.

Gel filtration of a ChAc preparation using Sephadex G-50 was shown to remove labelled choline completely from the eluted fraction of the preparation normally used as containing the enzyme. It would seem therefore that the present observations that rat brain ChAc preparations which have been passed through Sephadex G-50 still contain choline, can be accounted for by a rapid formation of choline within the homogenate rather than an inability of the Sephadex to separate choline from the enzyme. This formation of choline is rapid enough to be of significance even if

the interval between elution from the Sephadex column and assay of the preparation is only a few minutes. There is no evidence of any choline remaining in the preparation when the enzyme is used immediately on elution from the column.

The rapid formation of choline within homogenates presents a problem in that a particular enzyme preparation can only be used once before it has to be re-subjected to gel filtration. This is a disadvantage because comparison of the results obtained in separate experiments may not be possible since gel filtration is accompanied by a dilution of the enzyme which does not necessarily correspond with the dilution of the total protein which occurs and this results in a change in the specific activity of the enzyme. As a result extra controls have to be used during experiments and often relative rather than absolute values have to be used as an indication of the degree of acetylation of various substrates by ChAc. Another possible method of overcoming the problems associated with the formation of choline in ChAc preparations used for substrate specificity studies of the enzyme, may be to prevent the formation of choline by extraction of the lipid from the preparation.

SUMMARY.

1. It is necessary to remove endogenous substrate from ChAc preparations which are to be used for substrate and inhibitor studies and this should be easily achieved by gel filtration. However, preparations of ChAc which had been passed through Sephadex G-50 appeared to still contain endogenous choline.

2. Gel filtration of rat brain homogenates containing ^{14}C-choline was shown to achieve a clear separation of the choline from the protein fraction.

3. A rapid formation of choline was shown to occur in rat brain homogenates and this is discussed in relation to previously reported results. This rapid formation of choline probably accounts for the results described in 1). above.

4. EDTA did not appear to inhibit the formation of choline in homogenates as had previously been suggested.

CHAPTER 3.

DETERMINATION OF CHOLINE ACETYLTRANSFERASE ACTIVITY.

INTRODUCTION.

Determination of the activity of ChAc preparations is performed by incubation of the enzyme with its substrates, acetyl coenzyme A (acetyl CoA) and choline, and measurement of the amount of ACh or coenzyme A (CoA) formed in the reaction shown below.

$$\text{Acetyl CoA + choline} \xrightleftharpoons{\text{Choline acetyltransferase}} \text{ACh + CoA}$$

Most ChAc assays now use acetyl CoA, radioactively labelled on the acetyl group of the molecule, and this gives rise to labelled ACh which can be detected by scintillation spectrometry. Earlier assays however used non-labelled compounds and the ACh formed was detected by bioassay, usually on the eserinised frog rectus abdominis muscle preparation (Nachmansohn and Machado, 1943).

Whether or not the acetyl CoA is labelled there are two methods for providing its presence in the incubation mixture. One method is the use of a coupled enzyme system (Schuberth, 1963; Fonnum, 1966; Morris and Grewaal, 1971) and the other is the addition of commercially available synthetic acetyl CoA to the incubation mixture. The former method was the method first used and enzymes which have been used to produce acetyl CoA are phosphate transacetylase (E.C. 2.3.1.8.) and acetyl CoA synthetase (E.C. 6.2.1.1.) which catalyse the following reactions.

$$\text{CoA + acetylphosphate} \xrightleftharpoons{\text{Phosphate transacetylase}} \text{AcCoA + orthophosphate.}$$

Acetyl CoA synthetase

$$\text{ATP + acetate + CoA} \rightleftharpoons \text{AMP + pyrophosphate + acetyl CoA}$$

The coupled enzyme systems for the production of acetyl CoA have the advantage that constant rates of ACh synthesis can be obtained during fairly long incubations. However, two enzyme reactions and two incubation periods are necessary to ensure formation of sufficient acetyl CoA for the reaction with choline to proceed at a maximal rate (Schrier and Shuster, 1967) and most workers now seem to favour the use of commercially available ^{14}C acetyl CoA. Assays differ therefore mainly in the method used for separating the ^{14}C ACh synthesised during incubation from the residual ^{14}C acetyl CoA.

McCaman and Hunt (1965) recovered the labelled ACh by precipitation with ammonium reineckate, leaving acetyl CoA in solution. This method has been criticised on the basis of the laborious washing of the precipitate which has to be performed to remove trapped acetyl CoA. Also, since the washing procedure does not remove all the acetyl CoA, the method gives rise to high blank values. Blank values obtained in radiochemical ChAc assays usually refer to the amount of recovered radioactivity which occurs in the absence of active enzyme and the main cause of this is the recovery of labelled acetyl CoA, or in some cases the recovery of products formed from acetyl CoA, such as acetate. Lower blank values were obtained by Goldberg, Kaita and McCaman (1969) who precipitated ACh using a periodide. A further advantage of periodide precipitation over reineckate precipitation was stated by Goldberg and co-workers (1969) to be the greater solubility of periodide precipitates in toluene-based scintillation fluors which meant that the assay was more adaptable to larger scale analyses in which greater quantities of precipitate are formed.

Another method involving the recovery of ACh by precipitation was described by Fonnum (1966) who used sodium tetraphenylboron (Kalignost) as the precipitating agent. Fonnum later improved the method by making the process one of liquid cation-exchange, by using the sodium tetraphenylboron in solution in butyl ethyl ketone (Fonnum, 1969 a, b). The reaction is shown by the following equation.

$$Na(Ph)_4B + ACh^+ \rightleftharpoons ACh(Ph)_4B + Na^+ \quad \text{(Fonnum 1969b).}$$

This modification simplified the assay since the method does not involve the production of a precipitate whereas in previous methods a precipitate was formed and required washing. The assay has an added advantage in that it produces low blank values since only 0.1% of the radioactivity of the original acetyl CoA contaminates the ACh fraction. A disadvantage of the assay is its use of organic chemicals although Fonnum has recently improved the technique by performing the assay in a closed scintillation vial, thus reducing the exposure of the experimenter to the hazardous materials (Fonnum, 1975).

A similar procedure to that used by Fonnum was reported by Glover and Green (1972) who extracted ACh from incubation mixtures using a solution of potassium mercuric iodide in octanone. This method was reported to be simpler than that of Fonnum (1969) but produced much higher blanks.

The electrophoresis of ACh and acetyl CoA has been described by Potter and Murphy (1967) and this technique has been used to separate the two compounds during ChAc assays (Potter, Glover and Saelens, 1968; Glover and Potter, 1971; Giller and Schwartz, 1971). The technique has an advantage in that both ACh and acetyl CoA can be recovered and this has proved to be a valuable asset during the present work. The separation of ACh from choline, although less effective than the separation of ACh and

acetyl CoA, can also be achieved by electrophoresis and this enables the use of labelled choline as substrate as an alternative to labelled acetyl CoA (Giller and Schwartz, 1971).

Separation of ACh and acetyl CoA can also be achieved by ion-exchange chromatography and this method was first applied to ChAc assays by Schrier and Shuster (1967) who used Dowex anion-exchange resins which retained acetyl CoA but allowed ACh to pass straight through. A similar procedure, but using the cation-exchange resin Amberlite CG-50 to retain ACh was used by Diamond and Kennedy (1968), the ACh being subsequently recovered by elution of the resin with HCl.

In addition to the main methods for the determination of ChAc activity described above, another method which has been used is the spectrophotometric measurement of the CoA formed during the incubation using Ellmann reagent (Schuberth, 1966; Prince, 1967; Potter et al, 1968) or 4,4^{1} dithiodipyridine (4-PDS) (Chao and Wolfgram, 1972). Chao and Wolfgram (1972) point out that their procedure, though lacking in sensitivity, is ideal for determining the activity of ChAc during its purification, due to the rapidity of this method.

In the present work five different extraction procedures have been used to recover ACh from incubation mixtures, and an attempt has been made to resolve the several complications which arose. This section deals only with the use of these assays in studying the acetylation of choline; the differing adaptabilities of the assays to the study of substrates other than choline is dealt with in another section. (See 'Acetylation of hemicholinium-3 by choline acetyltransferase' page 117).

RESULTS.

The ChAc assays used differed only in the methods used for separating ACh and acetyl CoA; the incubation mixture and incubation period were the

same whichever extraction procedure was subsequently used to recover the ACh.

1. Procedures used for the recovery of ^{14}C ACh formed in the incubation mixture.

a). Reineckate precipitation.

This method of extracting ACh was used routinely for the assay of ChAc preparations and the modifications of the original procedure (McCaman and Hunt, 1965) which were used were found to be satisfactory. In particular, it was found to be unnecessary to allow 30 minutes for completion of precipitation after addition of an aliquot of the incubation mixture to the ammonium reineckate solution since the amount of radioactivity recovered from a particular incubation mixture was the same regardless of the time allowed for precipitation.

The reaction occurring in the incubation mixture was linear with respect to time for the duration of the incubation period (Figure 6) and was proportional to the enzyme concentration up to recovered c.p.m. of at least 17,500. (Figure 7). Enzyme preparations which gave rise to recovered radioactivities greater than 17,500 c.p.m. were diluted and their activities re-determined.

b). Sodium tetraphenylboron-butyl ethyl ketone extraction.

This method was used as initially described by Fonnum (1969) except that a different scintillation fluor was used. Blank values were low confirming Fonnum's initial report that only a small percentage of the original acetyl CoA contaminated the ACh fraction. In the present work, the assay was complicated by fluorescence of the mixture contained in the scintillation vial. (See 'Percentage

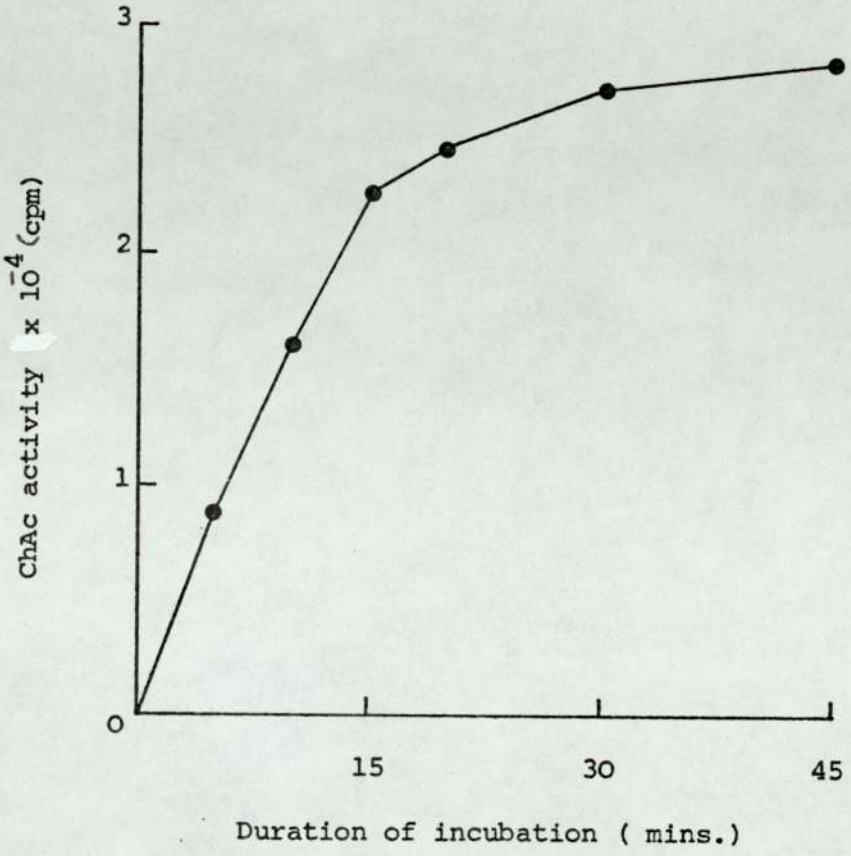


Fig. 6. Determination of ChAc activity. Activity v. duration of incubation. The enzyme preparation used was the supernatant from a rat brain homogenate prepared in 0.1% Triton X-100/ 200mM KCl and centrifuged at 20,000g for 20 minutes.

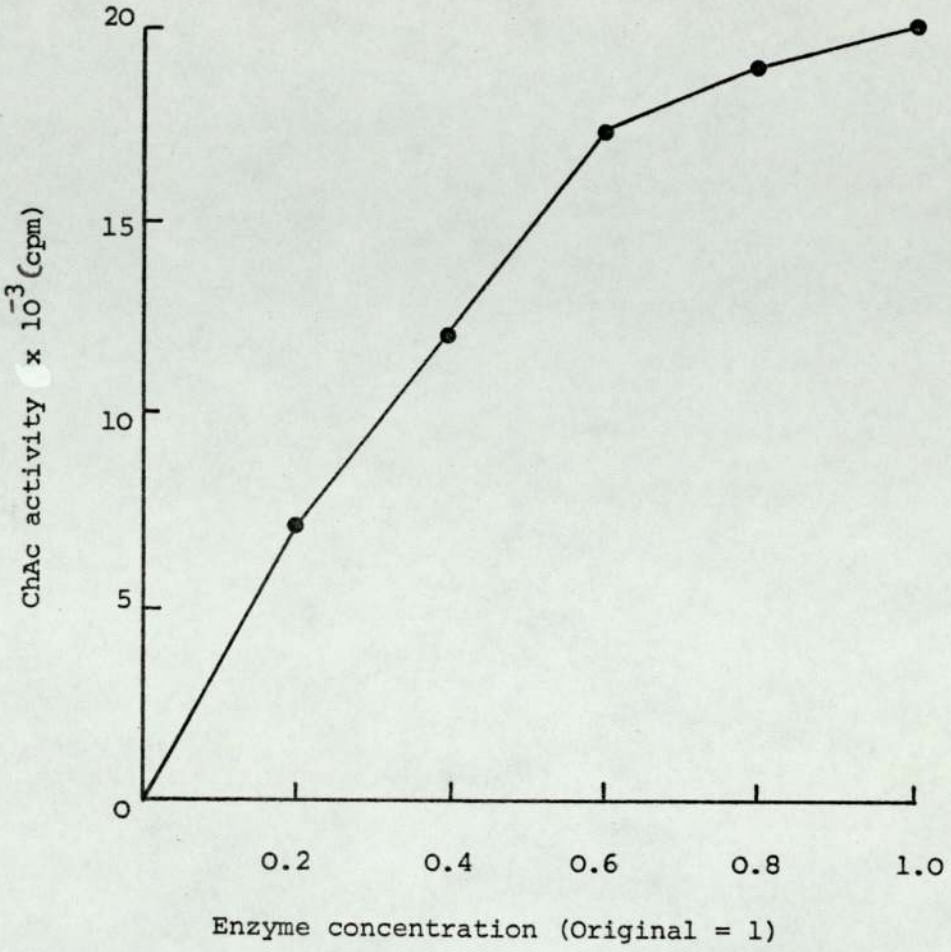


Fig. 7. Determination of ChAc activity. Activity v. enzyme concentration. The enzyme preparation used was the supernatant from a rat brain homogenate prepared in 0.1% Triton X-100/ 200mM KCl and centrifuged at 20,000g for 20 mins.. Dilutions of the enzyme were made using Triton X-100/ 200mM KCl.

recovery of ACh using the different extraction procedures' page 106).

c). Potassium mercuric iodide-octanone extraction.

This method was used initially exactly as described by Glover and Green (1972). However, extraction of incubation mixtures using K_2HgI_4- octanone consistently gave higher values than when similar incubations were extracted by reineckate precipitation. This fact led to the investigation detailed below which resulted in a slight modification of the original method.

In order to determine whether the high recovery of radioactivity after organic extraction was due to recovery of some of the original ^{14}C acetyl CoA, extraction of an incubation mixture was performed without prior incubation. The low recovery of radioactivity obtained suggested that the initial observation was not due to recovery of ^{14}C acetyl CoA, but was more probably due to the recovery of a radioactive product formed from the acetyl CoA during the incubation. This presumed reaction was shown to be dependent on the presence of active enzyme since inclusion of boiled enzyme in the incubation mixture gave rise to a low recovery of radioactivity similar to blanks.

As it was possible that the product formed from ^{14}C acetyl CoA during incubation might be ^{14}C acetate the ability of the K_2HgI_4- octanone extraction procedure to recover acetate was investigated. This was performed by extraction of a solution of ^{14}C sodium acetate using K_2HgI_4- octanone and determination of the distribution of the acetate between the organic and aqueous layers, by measurement of the amount of radioactivity in the two layers. By this method, approximately 50% of the sodium acetate was found to be extracted into the organic phase.

Further evidence suggesting that acetate may be responsible for the high recovery of radioactivity after an incubation was obtained by allowing the octanone layer to dry on filter paper before counting, as opposed to its addition directly to scintillation fluor. This drying procedure resulted in a marked reduction in the amount of radioactivity recovered. Thus, the recovered radioactive product is volatile and the most likely candidate is ^{14}C acetate formed during the incubation from ^{14}C acetyl CoA. Using the above modification of the original method, any non-volatile products formed during incubation, such as ^{14}C ACh, remain on the filter paper and their radioactivities can be determined.

Possible sources of acetate formed during incubation are the hydrolysis of acetyl CoA or the hydrolysis of ACh formed from acetyl CoA during the incubation. Hydrolysis of ACh as a source of acetate seemed unlikely since the incubation mixture contained the anticholinesterase eserine and also incubations containing no substrate gave high values when extracted with K_2HgI_4-octanone. Also, the reduction of recovered radioactivity when the octanone layer was dried first on filter paper, compared with its addition directly to scintillation fluor, was approximately the same whether distilled water, hemicholinium-3, or choline was added to the incubation mixture (Table 9) and this reduction in recovered radioactivity was also the same for incubation mixtures in the presence and absence of ^{14}C ACh (Table 10).

The results strongly suggest therefore that ^{14}C acetate is formed during incubation of ^{14}C acetyl CoA with an active enzyme preparation, probably as a result of enzymic hydrolysis of the ^{14}C acetyl CoA, and that this ^{14}C acetate is recovered on extraction with K_2HgI_4-octanone but not by reineckate precipitation. Drying

TABLE 9.

Mercuric potassium iodide/octanone extraction procedure.

1. Effect of drying organic layer on filter paper prior to counting.

SUBSTRATE	cpm Octanone layer added directly to scintillation fluor (A).	cpm Octanone layer dried on filter paper prior to counting (B).	A-B
Distilled water	3986	58	3928
Hemicholinium -3 (1 mM).	5048	1178	3870
Choline (1 mM)	9290	5114	4176

ChAc, from which the endogenous choline had been removed, was incubated with acetyl-^{14}C coenzyme A and either distilled water, HC-3, or choline. After incubation the incubation mixture was extracted with 0-1 M K_2HgI_4 in octanone.

For an enzyme preparation of similar activity but not subjected to gel filtration the value for A-B was 3,189 cpm.

TABLE 10.

Mercuric potassium iodide/octanone extraction procedure.

2. Incubations performed in the presence and absence of ^{14}C ACh.

	cpm Octanone layer added directly to fluor. (A).	cpm Octanone layer dried on filter paper prior to counting. (B).	A-B
1. No added ^{14}C ACh.	6680	1470	5210
2. With added ^{14}C Ach	10634	5288	5346

1. contained 5 ul ChAc, 10 ul distilled water and 10 ul buffer. (See Methods).

2. contained 5 ul ChAc, 5 ul distilled water, 5 ul ^{14}C ACh and 10 ul buffer.

Endogenous choline had not been removed from the enzyme preparation.

Incubation was for 15 minutes at 37°C followed by extraction with 0.1 M K_2HgI_4 in octanone.

of the octanone layer on filter paper before counting overcomes the problems associated with this recovery of acetate.

(d) Electrophoresis.

Potter and Murphy (1967) have reported the electrophoresis of acetycholine and related compounds and the large difference in the mobilities of acetyl CoA and ACh as reported by these workers renders the procedure suitable for their separation during ChAc assays. In the present work the mobilities of ACh and acetyl CoA were determined as 7.8 cm/h and 0.7 cm/h respectively.

Electrophoresis allows the recovery of both ACh and acetyl CoA and thus the total recovery of radioactivity can be calculated. When this calculation was performed it was found that there was some loss of radioactivity, the degree of which depended on the incubation time and whether or not the enzyme preparation had been subjected to gel filtration (Table 11). These results are probably accounted for by the enzymic hydrolysis of the ^{14}C-acetyl CoA leading to the formation of ^{14}C acetate which evaporates and is lost. (See Discussion). The addition of choline to the incubation mixture was found to markedly reduce the amount of radioactivity lost whilst hemicholinium and edrophonium reduced the loss of radioactivity only slightly. (Table 12).

A disadvantage of electrophoresis was the lower counting efficiency which was obtained during the determination of the amount of radioactivity on a strip of electrophoresis paper. This was clearly seen when the counting efficiencies obtained for compounds dried on electrophoresis paper were compared with those obtained for the same compounds dried on glass fibre filter discs prior to counting. (Table 13). The c.p.m. obtained on counting

TABLE 11.

Post-incubation recovery of radioactivity using electrophoresis.

1. Effect of incubation time and gel filtration.

ENZYME	INCUBATION TIME	cpm not recovered	Percentage of cpm not recovered.
Original homogenate	15 mins	13,367	48.5
Original homogenate	60 mins	18,820	68.3
Sephadex G-50 eluate	15 mins	4,844	17.6

Total cpm added to incubation mixture - 27,539.

All incubations were performed in the absence of added substrate.

The incubation mixture and its subsequent electrophoresis were as described in Methods.

TABLE 12.

Post-incubation recovery of radioactivity using electrophoresis.

2. Effect of the presence of exogenous substrate.

SUBSTRATE	cpm not recovered	Percentage of cpm not recovered
Distilled water	11,855	37.4
Hemicholinium-3	11,489	36.2
Edrophonium	10,576	33.3
Choline	6,363	20.1

Total cpm added to incubation mixture - 31,727.

Endogenous substrate had been removed from a ChAc preparation which had been partially purified by $(NH_4)_2SO_4$ fractionation.

The incubation mixture and its subsequent electrophoresis were as described in Methods.

TABLE 13.

Counting efficiencies of ^{14}C ACh, ^{14}C-choline, and ^{3}H-TEC dried on glass fibre filter discs or electrophoresis paper strips prior to counting.

Compound	dpm added	cpm counted on filter discs.	cpm counted on electrophoresis paper.	Counting efficiency on filter discs.	Counting efficiency on electrophoresis paper
^{14}C ACh	7,333	7,028	5,467	95.8%	74.6%
^{14}C-choline	20,000	19,160	15,123	95.8%	75.6%
^{3}H-TEC	Not known	12,601	2,036		

^{3}H-triethylcholine (^{3}H-TEC) indicate that the decrease in counting efficiency obtained on drying this compound on electrophoresis paper rather than on glass fibre filter paper, is greater than the decreased counting efficiency of ^{14}C ACh and ^{14}C-choline under similar conditions. This may be due to TEC being labelled with tritium rather than ^{14}C since ^{14}C ACh and ^{14}C choline showed similar reductions in counting efficiency (Table 13).

(c) Ion-exchange using Amberlite CG-50.

The method of Diamond and Kennedy (1968) which uses ion-exchange to recover ACh from the incubation mixture of a ChAc assay, was scaled down to suit the present requirements. The efficiency of the resin in separating ACh and acetyl CoA was tested using ^{14}C ACh and ^{14}C acetyl CoA. Sodium acetate was found to elute ^{14}C acetyl CoA but not ^{14}C ACh from the column and complete elution of acetyl CoA was achieved using 2.5 ml of sodium acetate. Elution of the column with 0.5 ml volumes of IN HCl resulted in elution of ^{14}C ACh in the first four acid fractions.

A disadvantage of this method compared with the other extraction methods used was that radioactive quenching occurred in the counted solutions thus reducing the observed c.p.m. (Table 14) and making the assay less reliable for ChAc determinations of enzyme preparations of low activity.

2. Percentage recovery of ACh using the different extraction procedures.

The counting efficiencies and percentage recoveries of ACh obtained using the different extraction procedures are shown in Table 14. The recovery of ACh was determined by adding a known amount of ^{14}C ACh (5,500 d.p.m.) to an incubation mixture which was then extracted by the

different methods. Counting efficiencies were determined using internal standards.

The electrophoretic extraction produced an apparent ACh recovery of 123% (Table 14). However, this is probably due to the observed counting efficiency being lower, when calculated using an internal standard, than is the actual value during an experiment. This is probably at least partly due to the addition to the scintillation vial, of an extra strip of electrophoresis paper containing ^{14}C ACh as the internal standard. It may also be partly due to a change in the physical properties of the electrophoresis paper during electrophoresis since the counting efficiency obtained after adding ^{14}C ACh to electrophoresis paper previously soaked in 1.5 M acetic acid/0.75 M formic acid showed a slight increase (2.2%) compared with the counting efficiency obtained after adding ^{14}C ACh to unsoaked electrophoresis paper. (See Discussion).

An apparently high recovery of ACh was also initially observed with the $NaPh_4B$-butyl ethyl ketone extraction procedure. It was, however, observed that the c.p.m. obtained with this extraction procedure decreased steadily with time. This is probably due to fluorescence within the scintillation mixture and may be the reason that Fonnum (1969) chose a different scintillation fluor. The problem is overcome by leaving the samples in the dark and re-determination of their activities until they are constant, a simple but time-consuming procedure. The recovery of ACh using the $NaPh_4B$/butyl ethyl ketone extraction procedure was determined by this method to be 87.5%. (Table 14).

TABLE 14.

Counting efficiency and recovery of ACh using different extraction procedures.

Extraction procedure	cpm recovered	Counting efficiency	dpm recovered	Percentage recovery of ACh.	
				Present	Reported by original workers
Reineckate precipitation	4886	92.16	5303	95.1 ± 5.5	97 - 102
$NaPh_4B$/butyl ethyl ketone	4363	89.47	4877	87.5 ± 1.2	95
K_2HgI_4/ octanone	3946	88.80	4448	79.8 ± 3.5	>90
Electrophoresis	4621	67.49	6853	123.0 ± 5.0	Not stated
Ion-exchange	3516	74.09	4758	85.3 ± 0.7	94

Amount of ^{14}C ACh added to incubation mixture 5575 dpm.

Results represent the mean or mean ± sem from three determinations.

Counting efficiencies were determined using internal standards.

DISCUSSION.

1. Comparison of the different extraction procedures used to recover ACh from incubation mixtures.

ChAc assay methods depend primarily on the incubation mixture being of a suitable composition to ensure that the enzymic reaction is linear over the ranges of time and enzyme concentration employed, and the incubation mixture used in the present work was found to be satisfactory in this respect. The second consideration is the extraction procedure used to recover ACh from the incubation mixture and the present experiments and the results described here make a comparison between five different established extraction procedures.

Familiarity with the reineckate precipitation extraction and the lack of complications with its use led to the use of this method as the reference method whenever a complication was suspected in any of the other extraction methods. The percentage recovery of ACh using the reineckate procedure was of a similar order to that reported by the original workers (McCaman and Hunt, 1965).

The $NaPh_4B$-butyl ethyl ketone extraction method was technically easier than the reineckate method and resulted in lower blanks. However, fluorescence of the mixture contained in the scintillation vials lengthened the otherwise very rapid procedure and this disadvantage outweighed its benefits over reineckate precipitation. The problem could probably be overcome by the use of a different fluor system which would result in this assay method being the easiest and quickest of those used.

Of the five extraction methods used, only the K_2HgI_4-octanone procedure showed any evidence of the recovery of ^{14}C acetate formed during incubation. This recovery of acetate is probably the reason for

the high blanks reported in the original description of the assay (Glover and Green, 1972) but the problem may easily be overcome by the simple modification described in Results.

Electrophoresis as used in the present work is not ideally suited for the assay of a large number of samples since the apparatus used had a maximum capacity of eight electrophoresis strips and thus limited the number of samples which could be assayed to eight per hour. The method does however have advantages in that both ACh and acetyl CoA can be easily recovered and also it is possible to identify the labelled products of an incubation, by comparison of their mobilities with those of authentic compounds. This has been of particular advantage in the study of the acetylation by ChAc of compounds other than choline. (See later sections).

A problem which arose with the electrophoretic extraction procedure was in the determination of the counting efficiency of compounds dried on electrophoresis paper prior to counting. The problems arise from a self-absorption of β-energies resulting from the diffusion of the labelled molecules into the absorbent filter paper and this is more apparent when compounds are counted on electrophoresis paper than when they are counted on glass fibre filter discs. (Dyer, 1974). Dyer also mentions that the counting of ^{3}H-labelled materials on chromatography paper is less reliable than for those labelled with ^{14}C, and the present results, comparing the counting of ^{3}H-TEC with the counting of ^{14}C ACh and ^{14}C choline, confirm this.

The results for the percentage recovery of ACh by electrophoresis suggest that the counting efficiency obtained during an experiment was higher than that calculated using an internal standard. Two possible explanations could account for this. Firstly, the use of an internal

standard requires the addition of a second strip of electrophoresis paper to the scintillation vial, thus possibly increasing the absorption of labelled materials. The observed counting efficiency using an internal standard would therefore appear to be low. Secondly, during an experiment, the absorption of labelled materials by the filter paper may be reduced due to a change in the physical properties of the paper as a result of being soaked for one hour in formic acid-acetic acid and subjected to an electric current. The counting efficiency obtained during an experiment might therefore be higher than that obtained using an internal standard. The present results provide some evidence that soaking the electrophoresis paper in formic acid-acetic acid does increase the counting efficiency of ^{14}C ACh, but the effect of the electrophoresis procedure as a whole on the counting efficiency of ^{14}C ACh cannot readily be determined without the prior knowledge of the percentage recovery of ACh. In view of the difficulty in determining the true percentage recovery of ACh by electrophoresis, this method is perhaps best used as a qualitative procedure, in conjunction with quantitative assessments made by a different extraction procedure.

The most laborious and time-consuming extraction method used was the ion-exchange method of Diamond and Kennedy (1968), although greater experience with the method would probably result in improvements. The use of an anion-exchange resin as used by Schrier and Shuster (1967) is probably simpler than the use of a cation-exchange resin since ACh will pass straight through the resin, and thus there will be no necessity for the prior removal of acetyl CoA by washing. This latter method seems to be highly favoured by Hebb (1972) and it would appear that the method can be used to process up to 40 samples in one hour.

In summary, all the extraction procedures used permit the assay of ChAc and the study of the acetylation of choline. Their advantages and disadvantages seem to depend somewhat on personal preference but all the methods do possess some inherent shortcomings. However, these shortcomings need present little problem if the correct extraction procedure is chosen to suit particular circumstances.

2. The hydrolysis of acetyl CoA during incubation.

The hydrolysis of acetyl CoA with the production of free acetate has been shown to occur during incubation of acetyl CoA with an active rat brain enzyme preparation (Kasa, Mann and Hebb, 1970; Present observations). The mechanism by which acetyl CoA is hydrolysed is not clear as yet but it has been suggested that ChAc, in formalin-fixed tissue and under incubation conditions used for the histochemical localisation of the enzyme, can catalyse the hydrolysis of acetyl CoA in the absence of added choline (Burt, 1969, 1971).

Kasa, et al. (1970) suggested the alternative or additional possibility that the hydrolysis of acetyl CoA was catalysed by acetylcholinesterase present within the tissue since Burt (1969) did not include eserine in his incubation mixture. Kasa and co-workers (1970) supported their assertion by showing the eserine-sensitive hydrolysis of acetyl CoA by a commercial preparation of bovine erythrocyte acetylcholinesterase. Using techniques for the histochemical localisation of ChAc, Kasa and co-workers (1970) showed that eserine reduced but did not abolish the hydrolysis of acetyl CoA in tissue sections and they also showed that cells from parts of the nervous system shown by biochemical methods to contain no ChAc also showed some acetyl CoA hydrolysis. These experiments indicated therefore that some hydrolysis of acetyl CoA occurred which was independent of both ChAc and cholinesterase. These workers also showed an eserine-sensitive hydrolysis of

acetyl CoA in a rat brain homogenate in addition to an eserine-insensitive hydrolysis which they ascribed to the action of esterases other than cholinesterases. However, ChAc would be present within the homogenate and could possibly account for some or all of the eserine-insensitive hydrolysis. Heading and Buckley (1971) have reported that the eserine-sensitive acetyl CoA hydrolysis reported by Kasa et al. (1970) occurs via the production of acetylcholine and that it is actually the hydrolysis of ACh which is inhibited by eserine. The acetyl CoA hydrolysis observed by Burt (1969) could also have proceeded via ACh since the tissues used in his experiments would contain endogenous choline from which ACh could be formed.

The previously reported results discussed above indicate that there are at least four possible ways in which acetyl CoA can be hydrolysed to produce free acetate. These are 1). by the catalytic action of ChAc, 2). by the catalytic action of cholinesterases, 3). by the catalytic action of esterases which are not inhibited by eserine or sodium fluoride but which are inhibited by di-isopropylfluorophosphate (DFP) (Kasa et al., 1970), and 4). via the production and subsequent hydrolysis of ACh.

In the present experiments eserine was present in the incubation mixture and it was shown that the source of acetate was not ACh. Therefore the hydrolysis of acetyl CoA during incubation is likely to have been a result of catalysis by ChAc and/or esterases other than cholinesterases. That choline was shown to reduce the hydrolysis of acetyl CoA suggests that ChAc may be involved and that there is competition between choline and water for the enzyme active site. Substrates with a lesser or non-existent affinity for ChAc, namely hemicholinium-3 and edrophonium (see later results pages 117 and 160) reduced acetyl CoA hydrolysis to a lesser extent than did choline.

However, this protective effect of choline on acetyl CoA hydrolysis was only evident during the electrophoresis experiments and was not observed during experiments using the K_2HgI_4-octanone extraction procedure, which measured the amount of acetate produced. Also, gel filtration of an enzyme preparation appeared to reduce the amount of acetyl CoA hydrolysed, as judged by the electrophoresis experiments, whilst appearing to increase the amount of acetate produced as judged by the K_2HgI_4-octanone procedure. These latter observations are only qualitative and cannot be quantitative since dilution of the enzyme during gel filtration does not permit an accurate comparison of the results obtained before and after gel filtration.

These results suggest that although the amount of acetate produced from acetyl CoA is unaltered by gel filtration or the presence of choline, the total amount of labelled volatile product formed is affected. This can be explained if it is assumed that a labelled volatile product other than ^{14}C-acetate is formed, and that this product, which could possibly be $^{14}CO_2$, is not extracted by K_2HgI_4-octanone. An explanation of the observed results would then require that the formation of this second product was inhibited by choline, and was affected by gel filtration, in the second case possibly due to a removal of an enzyme involved in the reaction. If this explanation is correct it is unlikely that ChAc is involved in the production of acetate (Figure 8A) since choline does not appear to reduce acetate formation, but it is possible that ChAc is involved in the formation of the second volatile product (Figure 8B) since this reaction or series of reactions does appear to be choline-sensitive. However this choline sensitivity does not necessitate the involvement of ChAc since the presence of choline in the incubation mixture will produce a third reaction involving the production of ACh (Figure 8C) and it may be therefore, that there is a preferential

A.

14-C acetyl CoA ⟶ 14-C acetate + CoA

Reaction possibly catalysed by non-cholinesterase esterases which are not removed from the preparation by gel filtration. 14-C acetate is recovered by the K_2HgI_4-octanone extraction procedure.

B.

14-C acetyl CoA ⟶ Second labelled, volatile product. Possibly 14-C CO_2.

Possibly a series of reactions in which ChAc may be involved. Some or all of the enzymes catalysing the reaction are removed from the preparation by gel filtration.
The labelled, volatile product is not recovered by the K_2HgI_4-octanone extraction procedure.

C.

14-C acetyl CoA ⟶ 14-C ACh + CoA

Reaction catalysed by ChAc.

Note. The presence of eserine in the incubation mixtures used for ChAc determinations removes the possibility of the formation of acetate via the hydrolysis of either acetyl CoA or ACh by cholinesterases.

Fig. 8. Possible reactions involving acetyl CoA and occurring in the incubation mixtures used for ChAc determinations.

involvement of acetyl CoA in this third reaction which reduces the production of the second volatile product whether or not ChAc is involved in this latter reaction.

The results discussed above suggest that the breakdown of acetyl CoA in incubation mixtures of ChAc assays, is a rather more complicated process than might at first appear, and further work is required for the full elucidation of the mechanisms by which this breakdown takes place. In the meantime, care must be taken to ensure that products other than ACh formed during incubations for ChAc determinations, are not recovered by the extraction procedure used to recover ACh.

SUMMARY.

1. Five different extraction procedures have been used to recover the ACh formed during the determination of ChAc activity, and their relative merits and shortcomings have been discussed.

2. All the extraction methods used were successful in that they permitted the assay of ChAc preparations and a study of the acetylation of choline.

3. Attempts have been made to either explain or resolve complications which arose with the use of the different extraction procedures.

4. The breakdown of acetyl CoA to products other than ACh, which was shown to occur during ChAc determinations, has been discussed with reference to the present results and to results previously reported by other workers.

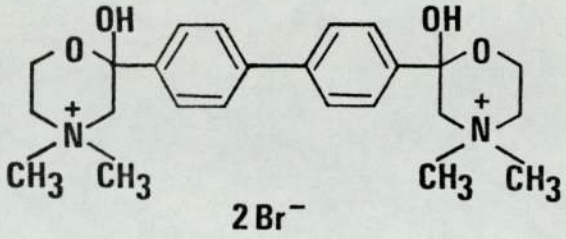
CHAPTER 4.

THE ACETYLATION OF HEMICHOLINIUM-3 BY CHOLINE ACETYLTRANSFERASE.

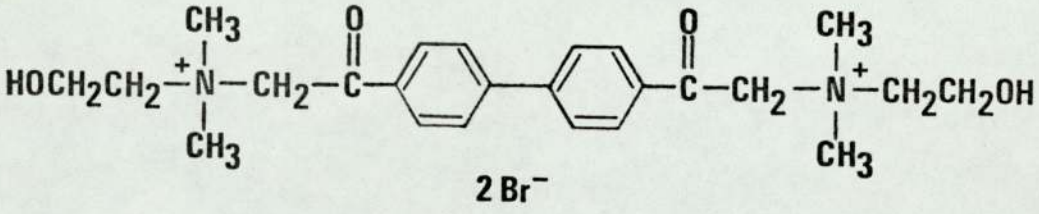
INTRODUCTION.

A series of bis-quaternary ammonium compounds derived from $\alpha\alpha'$ dibromo 44' bisacetophenone was synthesised by Long and Schueler (1954) and Schueler (1955). Some of the compounds from this series were named the hemicholiniums on the basis of their structure which contains one or two choline-like moieties which are generally considered to be held in a hemi-acetal configuration, although they may also exist in a straight chain form. The structural formulae of the hemi-acetal and straight-chain forms of hemicholinium-3 (HC-3) are shown in Figure 9; HC-3 being the most potent of the hemicholiniums in causing respiratory paralysis in several species (Schueler, 1955).

A characteristic of HC-3 is the slow rate of onset of the respiratory depression which it causes - described by Schueler (1960) as its latent toxicity. The transmission blockade produced by HC-3 in isolated nerve-muscle preparations also shows a slow rate of onset and in addition is dependent upon the frequency of electrical stimulation of the preparation, the inhibition being more marked at higher stimulation frequencies. (Reitzel and Long, 1959). It is now generally considered that low doses of HC-3 act presynaptically at cholinergic nerve terminals to inhibit the synthesis of ACh and that the slow rate of onset and frequency dependency of the inhibition are due to the fact that no effect is observed until the pre-formed stores of ACh are depleted. The exact nature of the presynaptic effect of HC-3 has not as yet been fully elucidated but several possibilities have been investigated as detailed below.



Cyclic hemi-acetal HC-3



Open chain (Seco) HC-3

Fig. 9. Structural formula of Hemicholinium-3.

HC-3 has been shown to inhibit the formation of ACh in membrane-enclosed systems of cholinergic nerves (MacIntosh, Birks and Sastry, 1956; Birks and MacIntosh, 1961) but appears to be without effect when the membranes have been disrupted by ether treatment (Gardiner, 1961). This dependence on intact membranes suggested that the inhibition of ACh synthesis produced by HC-3 was not due to a direct action on choline acetyltransferase (ChAc). This suggestion, plus the fact that HC-3 inhibition is reversed by choline (Schueler, 1955) led to the proposition that the main effect of HC-3 is to inhibit the uptake of choline into the presynaptic nerve terminal (Gardiner, 1961; Diamond and Kennedy, 1969; Hemsworth, Darmer and Bosmann, 1971; Guyenet, Lefresne, Rossier, Beaujouan and Glowinski, 1973) as originally suggested by MacIntosh and co-workers (MacIntosh et al., 1956; MacIntosh, 1959). However, interference by HC-3 with other aspects of choline and ACh metabolism has also been suggested.

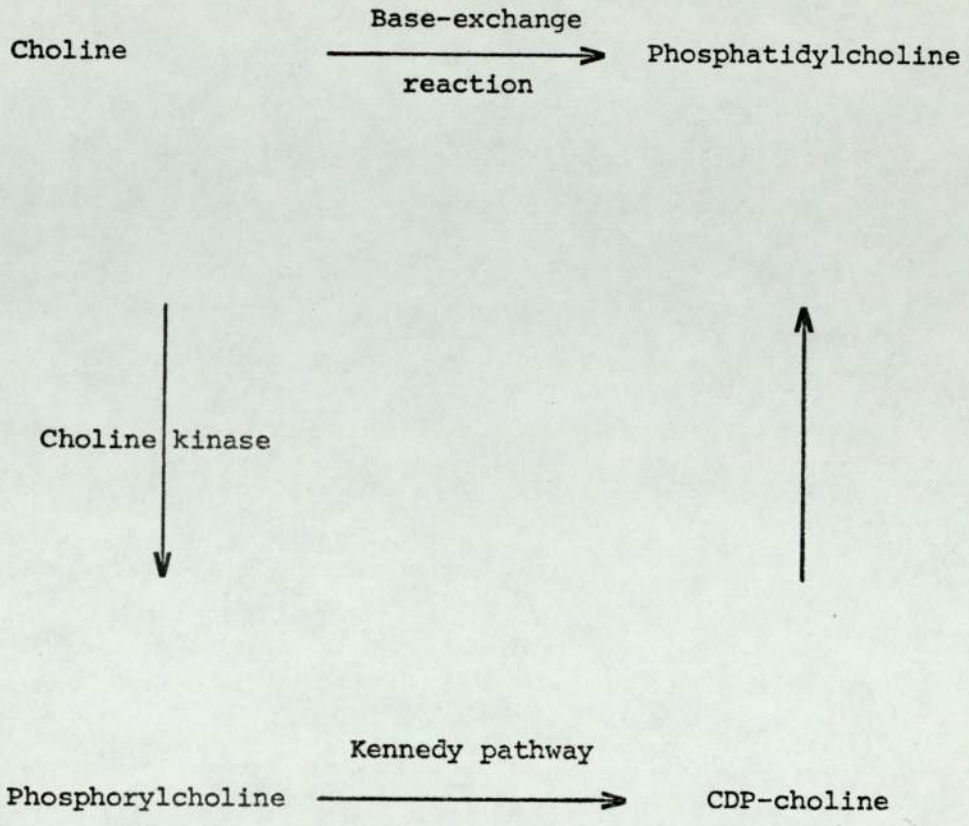
Gomez, Domino and Sellinger (1970 a,b) reported that HC-3 increased the incorporation of choline into phosphorylcholine, cytidine diphosphocholine (CDP-choline) and phosphatidylcholine in vivo in the caudate nucleus of the dog. The work of Ansell and Spanner has confirmed the increased conversion of choline to phosphatidylcholine in rat brain but their results suggested that the phosphorylation of choline to phosphorylcholine was inhibited (Spanner and Ansell, 1973; Ansell and Spanner, 1975). Diamond and Kennedy (1969) using guinea-pig synaptosomes also reported a reduced phosphorylation of choline. Since the chief pathway for the formation of phosphatidylcholine is considered to be the Kennedy pathway (Figure 10) it seems a contradiction that an inhibition of phosphorylcholine formation can be associated with an increased formation of phosphatidylcholine. However, Ansell and Spanner (1975) explain this on the basis

that HC-3 inhibits choline kinase (Ansell and Spanner, 1974) thus reducing the formation of phosphorylcholine, and that a consequence of this may be that there is a stimulation of the formation of phosphatidylcholine by another route; namely an exchange of the base moiety of a phospholipid with free choline (Figure 10). This hypothesis does not explain the increased formation of phosphorylcholine observed by Gomez et al. (1970 a, b) but species differences may possibly account for the discrepancies in the results.

It is not clear if the altered rate of incorporation of choline into phospholipids discussed above affects the conversion of choline to ACh but it may reduce the latter through a reduction in the amount of choline available for acetylation.

Other postulated mechanisms by which HC-3 could affect ACh metabolism include an inhibition by HC-3 of the transfer of ACh from the cytoplasm into the synaptic vesicles as suggested by Collier (1973), and the acetylation of HC-3 by ChAc in place of choline.

The acetylation of HC-3 has been investigated by several workers and has produced conflicting results. It has been proposed by Rodriquez de Lores Arnaiz, Zieher and de Robertis, (1970) and Hemsworth (1971a) that for HC-3 as for triethylcholine (TEC), a compound with pharmacological actions similar to HC-3 (Bowman, Hemsworth and Rand, 1962), that acetylation may take place followed by release of the acetylated product as a false transmitter. These workers have reported that HC-3 is acetylated in vitro by ChAc but that the acetylation is only about 25% as efficient as the acetylation of choline. This suggestion has been disputed however by Diamond and Milfay (1972) who could find no evidence for the acetylation of HC-3 by soluble rat brain ChAc in vitro. In addition, Collier (1973) showed a lack of release of accumulated ^{14}C HC-3 upon nerve stimulation of a cat superior cervical ganglion preparation.



Hemicholinium-3 increases the conversion of choline to phosphatidylcholine probably by increasing the base-exchange reaction shown above. This increased base-exchange reaction occurs in addition to or possibly as a result of the inhibition by hemicholinium-3 of choline kinase.

Fig. 10. The effect of hemicholinium-3 on the metabolism of choline.

The present work was performed in an attempt to resolve the controversy surrounding the acetylation of HC-3 and used several established methods to recover any acetylated product that might be formed during incubation.

RESULTS.

Five different methods were used for estimating the rate of acetylation of choline and HC-3 by ChAc, by using various methods for isolating the radioactive acetylated products of incubation. The percentage recovery of ACh formed during incubation has been previously shown to depend on the extraction procedure used for its isolation. (See Determination of ChAc activity page 106). It was not possible to determine the recovery of acetyl HC-3 as it was not possible to obtain any labelled compound; also calculations showed that spectro-photometric determinations of the acetyl HC-3 recovered would not be possible due to the large amount of extractant which would be necessary to permit a reliable comparison with the radiometric determinations. For this reason it has not been possible to convert cpm of acetylated product to μ mol of acetylated product and a comparison of the results obtained with the different methods has been made by considering the percentage acetylation of HC-3 compared with that of choline at the same concentration. On this basis the different extraction methods gave very different estimates of HC-3 acetylation and the results are shown in Table 15.

Isolation of radioactive acetylated products of incubation.

(a) Reineckate precipitation.

When either HC-3 or choline was used with acetyl-^{14}C-coenzyme A as a substrate for ChAc, a radioactive substance was precipitated during

the isolation procedure by ammonium reineckate. ChAc enzyme preparations from brain homogenates generally contain endogenous choline partially from plasma and possibly from breakdown of phospholipids. (See 'Removal of endogenous choline from ChAc preparations' page 81). The absence of endogenous choline substrate in the ChAc enzyme preparation after passing through the Sephadex G-50 column was evident from incubations in which no exogenous substrate was added; such an incubation gave values for acetylated product which were similar to blank values.

HC-3 was acetylated at a rate of 27.9% compared with the acetylation of choline at the same concentration (Table 15). In the presence of endogenous choline with HC-3 as substrate the recovered radioactivity was greater than when no HC-3 substrate was added also indicating an acetylation of HC-3.

(b) Sodium tetraphenylboron/butyl ethyl ketone extraction.

Acetylation of both HC-3 and choline was also evident with this method of extraction. The percentage acetylation of HC-3 compared with choline was shown to be 11.0% (Table 15). When HC-3 was used as substrate in the presence of endogenous choline, i.e. when the ChAc enzyme had not been passed through the Sephadex G-50 column; the amount of radioactive product recovered after extraction with sodium tetraphenylboron was greater than when no HC-3 substrate was added indicating that acetyl HC-3 was synthesised by ChAc in the presence of endogenous choline.

(c) Mercuric potassium iodide/octanone extraction.

Using the present modification in the method of Glover and Green (1972) (See Determination of ChAc activity page 98) HC-3 was found to be acetylated at a rate of 22.7% compared with the acetylation of choline at the same concentration (Table 15).

TABLE 15.

Acetylation of HC-3 and choline by ChAc.

Extraction procedure	Acetylation of HC-3 ($\times 10^4$ cpm/mg protein/min).	Acetylation of Choline ($\times 10^4$ cpm/mg protein/min).	Percentage acetylation of HC-3 compared with choline.
Reineckate precipitation (n = 7)	1.50	5.93	27.9 $\pm$ 2.7
$NaPh_4B$/Butyl ethyl ketone (n = 7)	0.89	8.35	11.0 $\pm$ 0.6
K_2HgI_4/octanone (n = 7)	1.32	5.74	22.7 $\pm$ 1.2
Electrophoresis (n = 2)	0.98	6.04	16.3 $\pm$ 0.2
Ion-exchange Amberlite CG-50 (n = 3)	0	4.63	0

Conditions for the determinations were as described in Methods. Endogenous choline had been removed from the ChAc by passage through Sephadex G-50. HC-3 and choline substrates were added to the incubation system to obtain a final concentration of 1 mM.

The results shown in the first two columns represent typical results for one determination for each extraction procedure. Those in the third column represent the mean $\pm$ s.e.m. from a number of determinations.

(d) Electrophoresis.

Electrophoresis of an incubation mixture containing ChAc which had not been passed through Sephadex G-50, and to which no substrate was added showed the formation of two labelled quaternary products of incubation. Fig. 11 shows the record from the strip scanner of the results of paper electrophoresis of such an incubation. Peaks B and C represent the two labelled quaternary products of the incubation. Peak C was shown to correspond in position to the iodine stained band of authentic ACh paper electrophoresed in a similar manner. Peak B was shown to correspond to acetyl carnitine which was also subjected to the same conditions of paper electrophoresis. Peak A represents the residual ^{14}C acetyl CoA.

When a ChAc enzyme preparation which had previously been passed through Sephadex G-50 was used in the incubation without added choline or HC-3 substrate, there was no labelled quaternary product detected by electrophoresis (Fig. 12). This result demonstrated that all endogenous choline and carnitine substrate had been removed from the ChAc enzyme preparation by passage through the Sephadex column.

When HC-3 was used as substrate in the incubation system, the radioactive peak observed after electrophoresis was detected 6.8 cms from the origin and this peak corresponded in position to an iodine stained band of authentic bi-acetyl HC-3 similarly subjected to paper electrophoresis (Fig. 13).

Fig. 14 shows the scanning trace of the electrophoresis paper of the products of an incubation where choline substrate was added to the incubation system. The radioactive peak corresponded in position to the iodine stained band of an electrophoresis strip to which ACh had been applied which moved a distance of 7.8 cms from the origin.

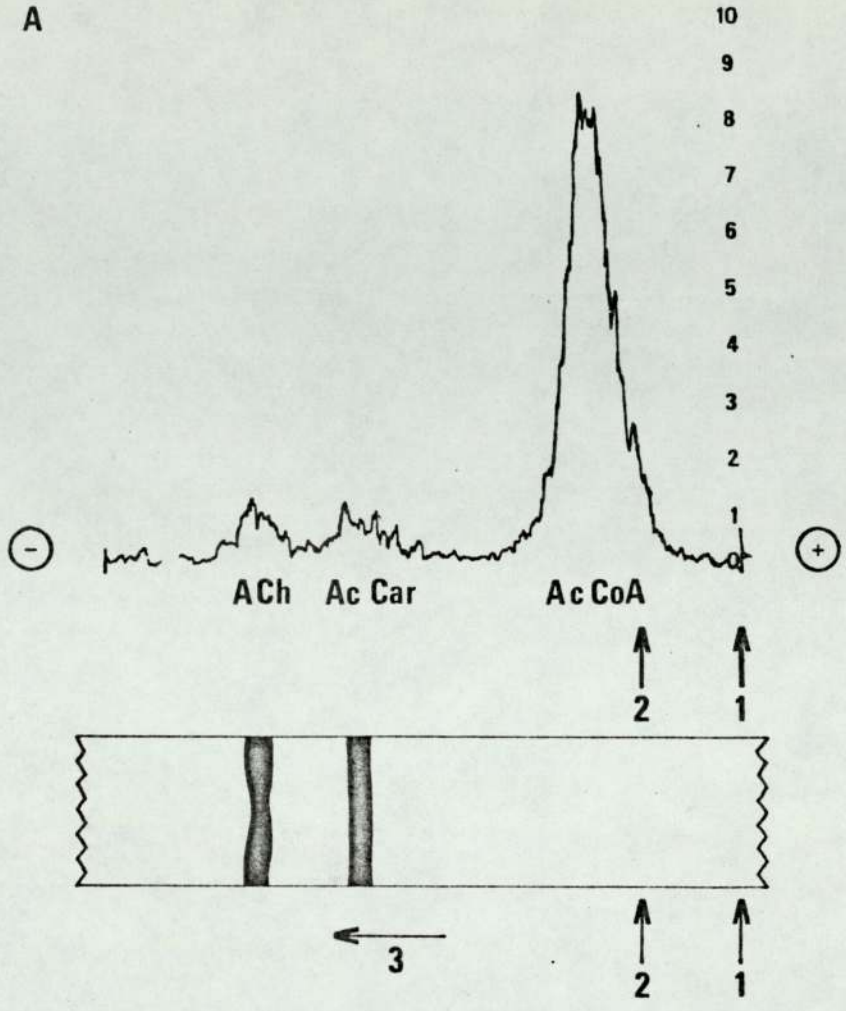


Fig. 11. Determination of radioactive peaks on paper electrophoresis strip passed through Tracerlab strip scanner. ChAc which had not been subjected to gel filtration was used in the incubation mixture with no added substrate. Paper electrophoresis was undertaken as described in Methods. The arrows represent (1) the origin of scanning, (2) the origin of electrophoresis on the paper strip and (3) the direction of the applied voltage from anode to cathode. Peak A (upper record) represents unreacted 14-C acetyl CoA. Peaks B and C represent acetylcarnitine and ACh respectively and correspond to the movement of authentic acetylcarnitine and ACh on paper electrophoresis visualised by staining with iodine vapour or Dragendorff's reagent (lower record). Full scale deflection on the abscissa, 0-10, corresponds to 1,000 cpm.

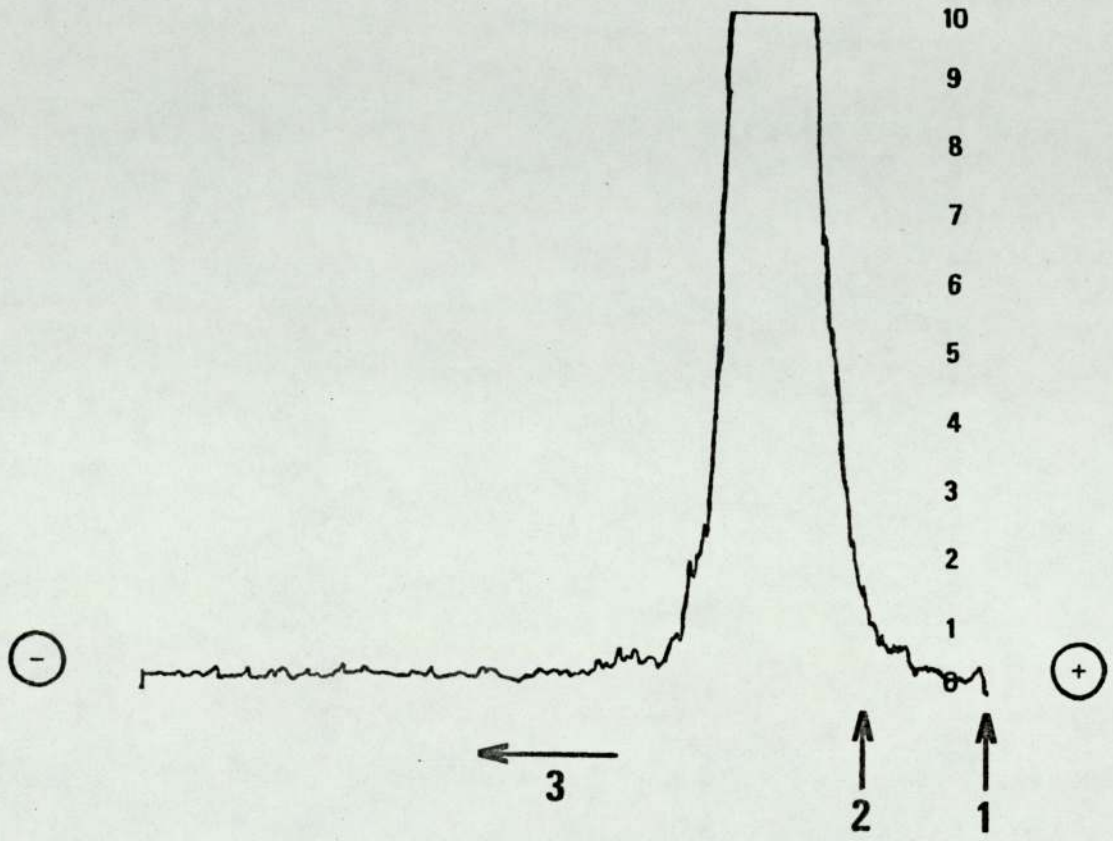


Fig. 12. Electrophoresis paper strip scanned for radioactive peaks. ChAc which had been passed through Sephadex G-50 was used in the incubation with no added substrate. The arrows represent the same conditions as those described in Fig. 11. The peak represents unreacted 14-C acetyl CoA. Full scale deflection corresponds to 1,000 cpm.

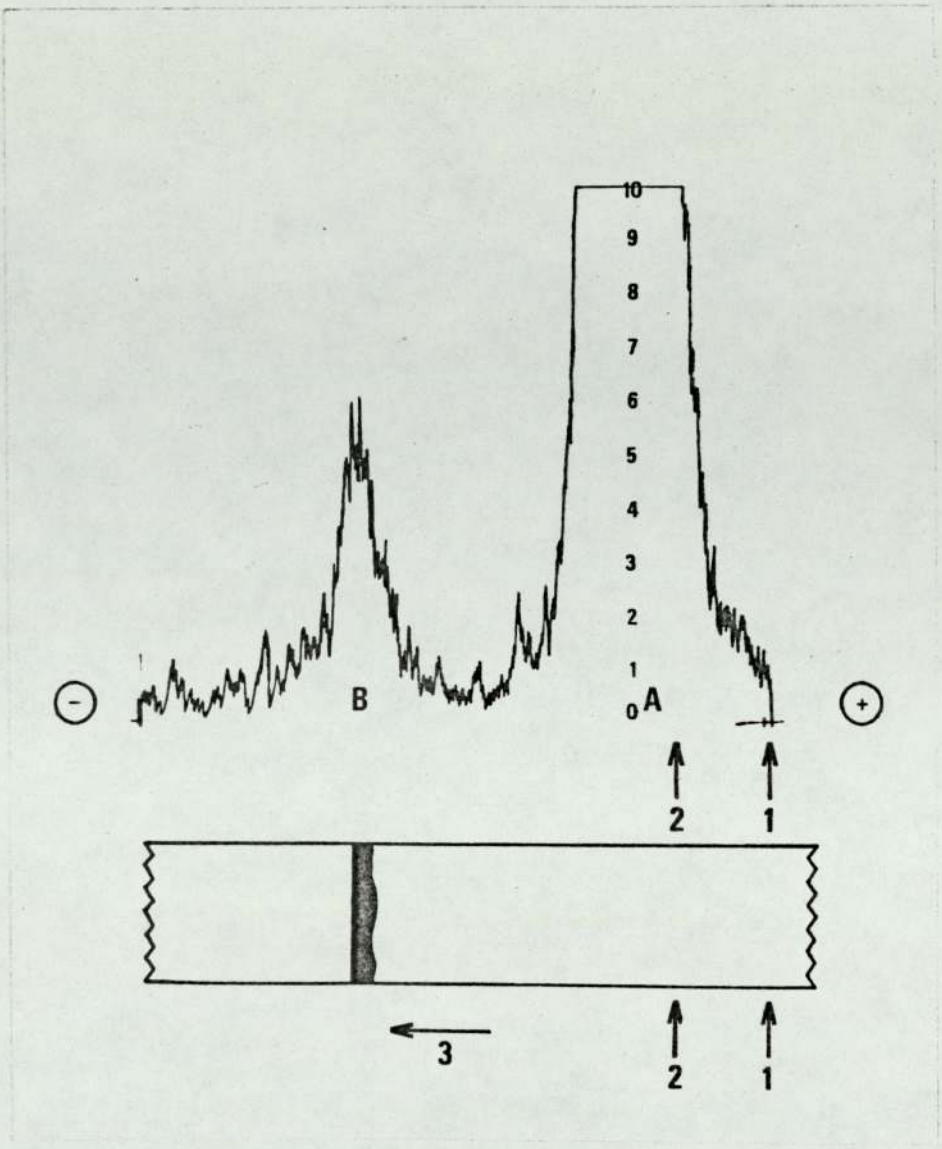


Fig. 13. Electrophoresis paper strip scanned for radioactive peaks. ChAc which had been passed through Sephadex G-50 was used in the incubation with HC-3 as substrate. The arrows represent the same conditions as those described in Fig. 11. The upper record shows the peaks of A, unreacted 14-C acetyl CoA and B, acetyl HC-3. Peak B corresponds to the iodine-stained band on the paper electrophoresis strip of authentic acetyl HC-3 (lower record). Full scale deflection corresponds to 250 cpm.

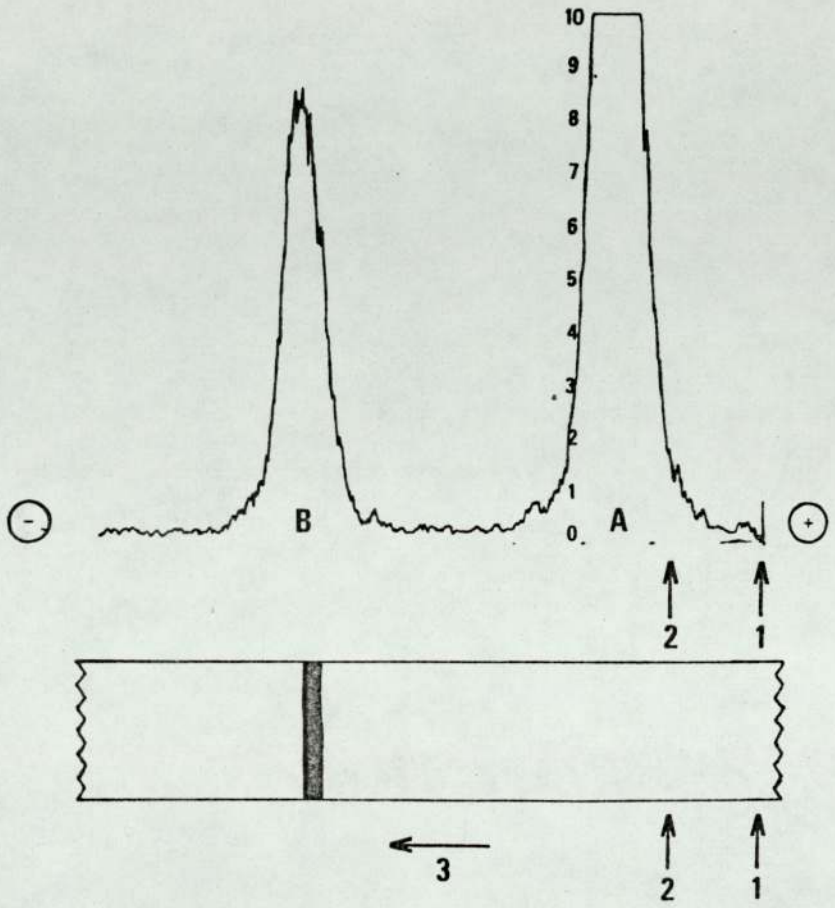


Fig. 14. Electrophoresis paper strip scanned for radioactive peaks. ChAc which had been passed through Sephadex G-50 was used in the incubation with choline as substrate. The arrows represent the same conditions as those described in Fig. 11. The upper record shows the peaks of A, unreacted 14-C acetyl CoA and B, ACh. Peak B corresponds to the iodine-stained band on the paper electrophoresis strip of authentic ACh (lower record). Full scale deflection corresponds to 1,000 cpm.

A quantitative analysis of the acetylation of HC-3 and choline was made by counting the radioactivity in 1 cm strips of the electrophoresis paper in the liquid scintillation counter. This method showed that HC-3 was acetylated 16.3% compared with the acetylation of choline (Table 15).

(e) Ion-exchange on Amberlite CG-50.

Table 16 shows the results of the acetylation of choline and HC-3 substrates in the presence of endogenous choline, i.e., when the ChAc enzyme had not been passed through a Sephadex G-50 column. No acetylation of HC-3 was observed. The total cpm of radioactive product, when HC-3 was present in the incubation, was lower than when no HC-3 was added. When 1 mM choline was added to the incubation system ^{14}C ACh was recovered in the HCl eluate from the Amberlite CG-50 ion exchange column. These results may be compared directly with those obtained by Diamond and Milfay (1972).

When a ChAc enzyme was used which contained no endogenous choline substrate an incubation of such choline-free ChAc with added HC-3 or choline substrate showed similar results to those shown in Table 16. Expression of these results of acetylation in terms of cpm/mg protein/min are shown in Table 15.

Since the other extraction methods used in our experiments indicated that HC-3 was acetylated by ChAc two possible reasons for this discrepancy were considered. Firstly any acetylated HC-3 formed during the incubation might not bind to the ion-exchange column. Alternatively any acetyl HC-3 might be bound to the column but might not be eluted from the ion-exchange column by solutions which elute ACh. These two possibilities were therefore investigated.

TABLE 16.

Acetylation of HC-3 and choline and isolation of the radioactive acetylated products of incubation by ion exchange chromatography.

Substrates present	cpm
Endogenous substrate	1947
" " + HC-3 1 mM	1577
" " + choline 1 mM	24534

Incubation conditions as described in the Methods. Radioactive products were isolated by ion-exchange chromatography on Amberlite CG-50 as described in the Methods.

Acetyl HC-3 was shown to bind to the Amberlite CG-50 ion exchange resin but the compound was not eluted by 1N HCl. Larger volumes of 1N HCl in excess of the 2 ml normally applied for elution purposes also had no effect on the binding of acetyl HC-3 to the column. HC-3 was however shown to be detected in the eluate when 1N NaOH was used for elution of the ion-exchange column. Detection of the acetyl HC-3 in the solutions applied to and eluted from the column was made by U.V. absorption at 280 nm and by the use of ammonium reineckate (see Methods).

ACh was found to be removed from solution by passage through the Amberlite CG 50 ion-exchange column and elution of the ACh was made with 1N HCl.

These results show therefore that both acetyl HC-3 and acetylcholine bind to the ion exchange column but that unlike ACh, acetyl HC-3 is not eluted by 1N HCl.

DISCUSSION.

The present results indicate that when HC-3 is used as a substrate for ChAc, together with acetyl CoA, a product is formed which can be recovered by a number of procedures which have been used previously for the isolation of the radioactive products of acetylation by ChAc. Identification of this material by electrophoresis suggests that the product is the acetylated form of HC-3. It is not certain whether it is the mono-acetylated or bi-acetylated form of HC-3 or a mixture of both since the mono-acetylated compound was not available for the determination of its electrophoretic mobility. However, the mobility of bi-acetyl HC-3 was the same as that of the product formed in the incubation medium. The present results show that the observed percentage acetylation of HC-3 (1 mM) compared with choline (1 mM) varied between 0 and 28% depending on the

method used to recover the acetylated product. It is suggested that this variation in percentage acetylation is caused by the different recoveries of acetyl HC-3 and ACh with respect to each other and with respect to the different methods.

The method of paper electrophoresis first used by Potter and Murphy (1967) has the advantage that the products of acetylation may be identified. It is suggested that because of this identification procedure the electrophoresis method was the best of the extraction procedures used for investigating the acetylation of substrates other than choline by ChAc. The present data using electrophoresis shows that both ACh and acetylcarnitine are formed on incubation of brain homogenates in vitro with ^{14}C acetyl CoA and therefore both choline and carnitine are present as endogenous substrates in brain fractions. Passage of the enzyme preparation through a Sephadex G-50 column removes all endogenous choline and carnitine.

The results using the ion exchange resin do show some agreement with the results reported by Diamond & Milfay (1972) in that no acetylated radioactive product of incubation is observed when HC-3 is used as substrate. The present interpretation of this result differs from that of Diamond and Milfay (1972) however, and the reason for this difference stems from the fact that acetyl HC-3 is bound to the Amberlite CG-50 resin but is not eluted by HCl whereas ACh is bound to the resin and is eluted by 1N HCl. Diamond & Milfay (1972) do not give an indication of the recoveries of ACh and acetyl HC-3 obtained with their ion-exchange resin but seem to have assumed that acetyl HC-3 and ACh will react with and elute from the ion-exchange resin in a similar manner. The reason for the resistant binding of acetyl HC-3 to the column is not known but 1N NaOH was shown to remove the quaternary compound. It would be expected that acetyl HC-3 would bind to the column in a similar manner to ACh, however this does not occur and it may be that the acetyl HC-3 forms some covalent bonding with the matrix

of the ion exchange resin. The ion-exchange procedure therefore appears to be less suitable than other methods for studying the acetylation of choline analogues.

Another possible difference in the interpretation of the results of Diamond and Milfay (1972) and of the present results is that the former workers used an enzyme preparation in which endogenous choline had not been removed. It is possible therefore that competition between choline and HC-3 for the active site of the enzyme might occur. This might have produced data in which the acetylation of the endogenous choline and HC-3 was indistinguishable since identification of the products was not made. However the present results indicate that HC-3 is acetylated even in the presence of endogenous choline and our results are in agreement with those obtained previously. (Rodriguez de Lores Arnaiz et al., 1970; Hemsworth 1971a).

The discrepancy between the results of Diamond and Milfay (1972) and those of Rodriguez de Lores Arnaiz et al. (1970) and Hemsworth (1971a) seem therefore to stem from the use of different methods to detect the acetylated product, rather than the presence or absence of endogenous choline.

A variety of choline analogues have previously been shown to be acetylated by ChAc in vitro (Burgen, Burke and Desbarats - Schonbaum, 1956; Dauterman and Mehrota, 1963; Hemsworth and Morris, 1964; Hemsworth and Smith, 1970 a, b) and it appears that HC-3 can be included among these. It also seems possible that the acetylation of HC-3 might take place in vivo since it has already been shown that HC-3 is taken up into cholinergic nerve terminals (Sellinger, Domino, Haarstad and Mohrman, 1969) although not necessarily by the choline uptake mechanism (Slater and Stonier, 1973) and the present results show that the acetylation occurs even in the presence of endogenous choline. The importance of this possibility has yet to be determined but Slater and Stonier (1973) suggest that a competition between

choline and HC-3 at an intracellular site may be a more important factor in the inhibition of ACh synthesis by HC-3 than is the impairment of choline uptake and it is possible that such a competition occurs for acetylation by ChAc. Mann and Hebb (1975) also reported evidence suggesting that HC-3 could be acetylated in vitro and they indicated that the low free choline levels in rat brain increases the possibility that the in vivo inhibition of ACh synthesis by HC-3 depends on its action as a competitive substrate of ChAc.

Mann and Hebb (1975) also make the valid point with reference to the acetylation of TEC, that it is not necessary for any acetyl TEC formed to be released for failure in transmission to occur and this applies equally to the action of HC-3. Thus the observation of Collier (1973) that accumulated ^{14}C HC-3 was not released on nerve stimulation does not negate the possibility that acetylation of HC-3 had taken place.

Although most evidence strongly suggests that HC-3 inhibits the uptake of choline into presynaptic nerve terminals (Guyenet et al., 1973; Haga and Noda, 1973; Snyder, Yamamura, Pert, Logan and Bennett, 1973) Mann and Hebb (1975) have cast doubt on whether a reduction in the intraterminal choline uptake automatically reduces the synthesis of ACh. However, Barker and Mittag (1975) provided evidence that the rate-limiting step in the biosynthesis of ACh in situ is choline transport and suggested that the high affinity transport system is in some way coupled to ChAc. Thus compounds taken up by this mechanism would probably be acetylated.

The two main theories postulated to explain the action of HC-3, namely the inhibition of choline uptake and competition with choline for ChAc, need not therefore be mutually exclusive and it may even be that they are mutually dependent. However, further work is necessary in order to assess the relative importances of the various mechanisms ascribed to HC-3 by which it may inhibit the formation of ACh in vivo.

SUMMARY.

1. The acetylation of hemicholinium-3 (HC-3) by choline acetyltransferase (ChAc) from a rat brain homogenate has been investigated using several different methods to recover the acetylated product.

2. Identification of the incubation product by electrophoresis showed that HC-3 was acetylated although it was not established whether the product was the mono- or bi-acetylated form.

3. The observed acetylation of HC-3 (1 mM) compared with choline (1 mM) varied between 0 and 28 per cent depending on the procedure used for isolation of the acetylated product.

4. It is suggested that this variation arises out of differences in the relative percentage recoveries of acetyl HC-3 and acetylcholine by the different methods.

5. The possibility that HC-3 is acetylated in vivo has been suggested and the significance of this possibility discussed.

CHAPTER 5.

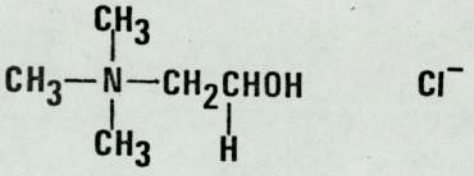
THE IN VITRO ACETYLATION OF HEMICHOLINIUM-3 AND TRIETHYLCHOLINE BY A RAT BRAIN HOMOGENATE.

INTRODUCTION.

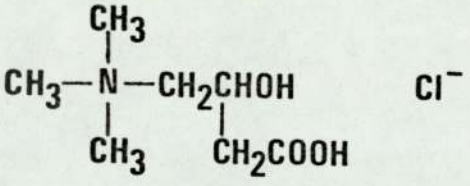
Earlier results presented in the previous section showed that hemicholinium-3 (HC-3) was acetylated by a rat brain homogenate and this acetylation was attributed to ChAc present within the homogenate. However, other acetylating enzymes may also be responsible for some or all of the observed acetylation and non-enzymic acetylation is also a possibility which must be considered.

White and Wu (1973c) indicated that the action of carnitine acetyltransferase (acetyl CoA: carnitine O-acetyltransferase, CarAc, E.C. 2.3.1.7.) can be wrongly attributed to ChAc in two ways. Firstly, acetylcarnitine (AcCar) formed by the action of CarAc may be indistinguishable from ACh formed by the action of ChAc, depending on the extraction method used to isolate the acetylated product. Secondly, since the structures of choline and carnitine are similar (Figure 15), the acetylation of choline to ACh may be catalysed by CarAc as well as by ChAc.

Fritz, Schultz and Srere (1963) reported that CarAc did not react with choline but White and Wu (1973c) have shown that choline can serve as substrate for CarAc although it was shown to be a poor substrate compared with carnitine. Roskoski, Mayer and Schmid (1974) confirmed this and reported that the percentage acetylation of choline (2mM) compared with carnitine (2 mM) was 0.004% for crystalline CarAc from pigeon breast muscle. In an attempt to determine acetylation due only to ChAc, Hamprecht and Amano (1974) devised a differential assay for



Choline



Carnitine

Fig. 15. Structural formulae of choline and carnitine.

ChAc which distinguished between AcCar and ACh by the specific hydrolysis of ACh with acetylcholinesterase. However, the method does not distinguish between ACh formed via ChAc and ACh formed via CarAc.

The results discussed above suggest the possibility that CarAc may be involved in the in vitro acetylation of HC-3 and also possibly in the acetylation of TEC, which has previously been shown to occur. (Hemsworth and Smith, 1970). The results of Barker and Mittag (1975) also suggest the involvement of an enzyme other than ChAc in the acetylation of TEC since the ChAc inhibitor N-hydroxyethyl-4-(1-naphthylvinyl) pyridinium bromide (NVPH) inhibited TEC acetylation by only 70% at a concentration of inhibitor which inhibited choline acetylation by 95%. The acetylation of HC-3 was inhibited by 93% by the same concentration of NVPH suggesting that ChAc might be solely responsible for the acetylation of HC-3. Preliminary experiments in the present work however showed a similar situation for HC-3 as that reported to exist for TEC by Barker and Mittag (1975) in that a ChAc inhibitor, in this case 4-(1-naphthylvinyl) pyridine, hydrochloride (NVP), inhibited HC-3 acetylation to a lesser extent than choline acetylation. This result prompted further work designed to explain the discrepancy between the present results and those of Barker and Mittag (1975) and also to further investigate the involvement of ChAc and other enzymes in the in vitro acetylation of HC-3 and TEC by a rat brain homogenate.

RESULTS.

RAT BRAIN HOMOGENATE.

1. Acetylation of choline carnitine, HC-3 and TEC.

Recovery of the acetylated products by precipitation with ammonium reineckate (see Methods) indicated that choline, carnitine,

HC-3 and TEC were all acetylated by a rat brain homogenate. The percentage acetylations of the various substrates compared with the acetylation of choline at the same concentration (1 mM) are shown in Table 17; the acetylation of HC-3 has been discussed more fully in a previous section.

Table 17 shows that the percentage acetylations of HC-3 and TEC compared with choline obtained using an enzyme preparation which had been partially purified by ammonium sulphate fractionation were greater than similar values obtained using a crude preparation. The percentage acetylation of carnitine compared with choline was however greater for the crude preparation.

During all experiments blank values were determined by using denatured enzyme in the incubation mixture and these were subtracted from the results obtained using active enzyme (see Methods). It was found that blanks containing HC-3 as substrate were higher than those containing choline, carnitine or TEC. This meant that the observed percentage acetylation of HC-3 compared with choline was much lower when a HC-3 - containing blank was used than when a choline-containing blank was used. (Table 17). This result suggested that when HC-3 was included in the incubation mixture there was either some non-enzymic acetylation of the HC-3 or possibly an increased 'trapping' of labelled acetyl CoA during precipitation with ammonium reineckate.

Electrophoresis of an incubation mixture containing HC-3 but no enzyme showed no evidence of non-enzymic acetylation of HC-3 since no acetylated product was detected. (Figure 16).

2. Inhibition of the acetylation of choline, carnitine, HC-3 and TEC by NVP and NVPH.

Solutions of the ChAc inhibitors NVP and NVPH were freshly prepared for each experiment since the inhibition produced by these

TABLE 17.

Acetylation of choline, carnitine, HC-3 and TEC by a rat brain homogenate.

SUBSTRATE	Percentage acetylation compared with choline		
	Original homogenate	Partially purified preparation	
	Choline blank	Choline blank	Separate blanks
Carnitine (1 mM)	69	20	20
HC-3 (1 mM)	26	82	38
TEC (1 mM)	4	14	14

The incubation mixture used was as described in Methods. Blank values were determined by using an enzyme preparation denatured with trichloroacetic acid (TCA) and including choline (choline blank) or carnitine, HC-3 or TEC (separate blank) in the incubation mixture. These blank values were subtracted from values obtained using active enzyme.

The enzyme preparation used was either the 20,000 g supernatant derived from an original homogenate of rat brain prepared in 0.1% Triton X-100/200 mM KCl or a preparation partially purified by ammonium sulphate fractionation. Both preparations were passed through Sephadex G-50 to remove endogenous substrate.

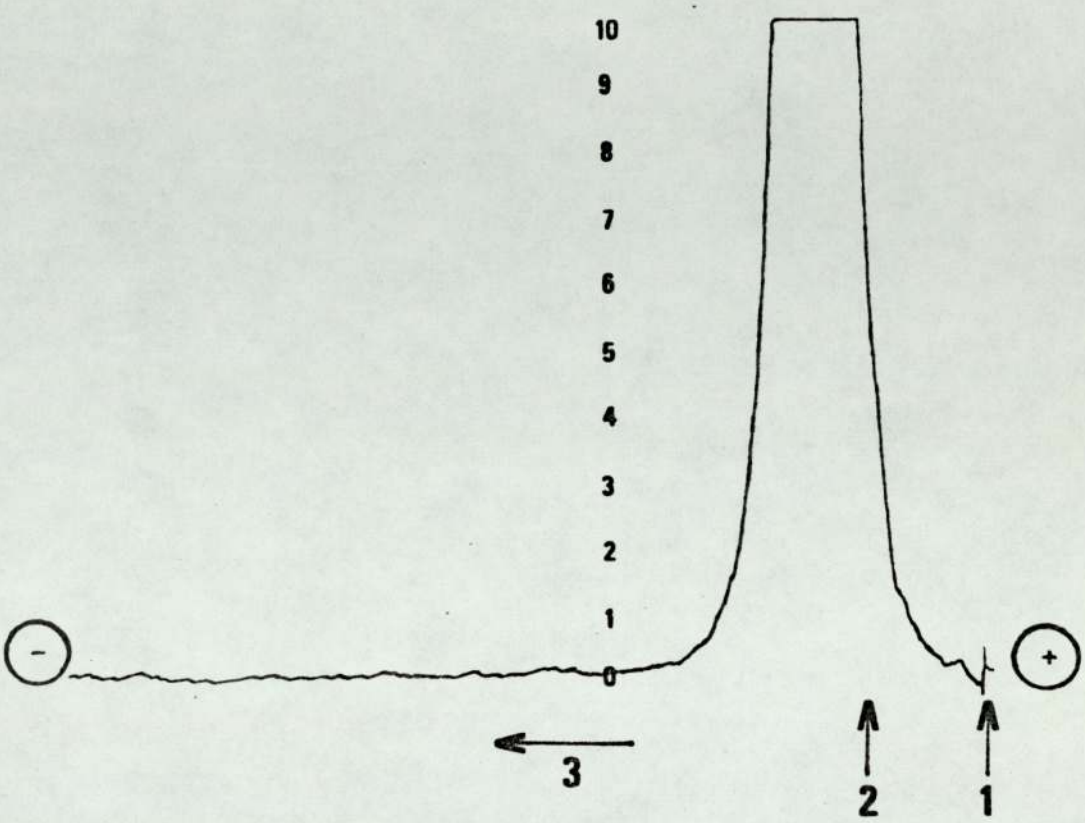


Fig. 16. Electrophoresis paper strip scanned for radioactive peaks. The incubation mixture contained HC-3 but no enzyme. The arrows represent the same conditions as those described in Fig. 11. Full scale deflection corresponds to 1,000 cpm. The peak represents unreacted 14-C acetyl CoA. The absence of any other peak suggests that no non-enzymic acetylation of HC-3 has occurred.

compounds was shown to decline with time, presumably due to their photosensitivity (White and Cavallito, 1970c).

The inhibition of TEC acetylation was initially studied using an enzyme preparation containing endogenous substrate and corrections of the results were made for this. The purpose of this procedure was to compare the present results with those of Barker and Mittag (1975) and Table 18 shows that the present results were similar to those of Barker and Mittag (1975) and that TEC acetylation was inhibited to a lesser extent than choline acetylation by both NVP and NVPH. The fact that Barker and Mittag (1975) used a lower concentration of NVPH than was used in the present study does not mean that the two sets of results are incomparable since the inhibitor concentrations were chosen in order to obtain a maximal inhibition of ChAc in both cases. In any case, the percentage inhibition of acetylation will depend on the enzyme concentration in addition to the inhibitor concentration. The results however suggest that a maximal inhibition of ChAc was achieved in both studies and the results are therefore comparable. It should be noted that neither set of results shows 100 per cent inhibition of choline acetylation and this suggests that a small percentage of choline acetylation by a rat brain homogenate may be non-enzymic or due to an enzyme other than ChAc.

When an enzyme preparation from which endogenous substrate had been removed was used, the acetylation of TEC still appeared to be less susceptible to inhibition by NVP and NVPH than did the acetylation of choline. (Table 19).

Initial experiments in which a blank containing no substrate was used as a control suggested that HC-3 acetylation was also inhibited to a lesser extent than was choline acetylation by NVP and NVPH. A comparison of the inhibition of HC-3 acetylation with the inhibition

TABLE 18.

Inhibition of TEC acetylation by NVP and NVPH.

	Percentage inhibition of acetylation.		
SUBSTRATE	NVP 2.5×10^{-3} M	NVPH 2.5×10^{-3} M	NVPH 0.2×10^{-3} M Barker and Mittag (1975)
Choline	81	99	95
TEC	48	69	70

The enzyme preparation used contained endogenous substrate and the results have been adjusted accordingly. This procedure was performed in order to obtain the comparison with the results of Barker and Mittag (1975) shown above.

TABLE 19.

Inhibition of the acetylation of choline, carnitine, HC-3 and TEC by NVP and NVPH.

SUBSTRATE	Percentage inhibition of acetylation	
	NVP 2.5×10^{-3} M	**NVPH** 2.5×10^{-3} M
Choline	90	96
Carnitine	100	0
HC-3	86	98
TEC	76	80

The enzyme preparation used had been partially purified by ammonium sulphate fractionation. Endogenous substrate was removed from the enzyme preparation immediately before each experiment by gel filtration.

Separate substrate blanks were used for all determinations.

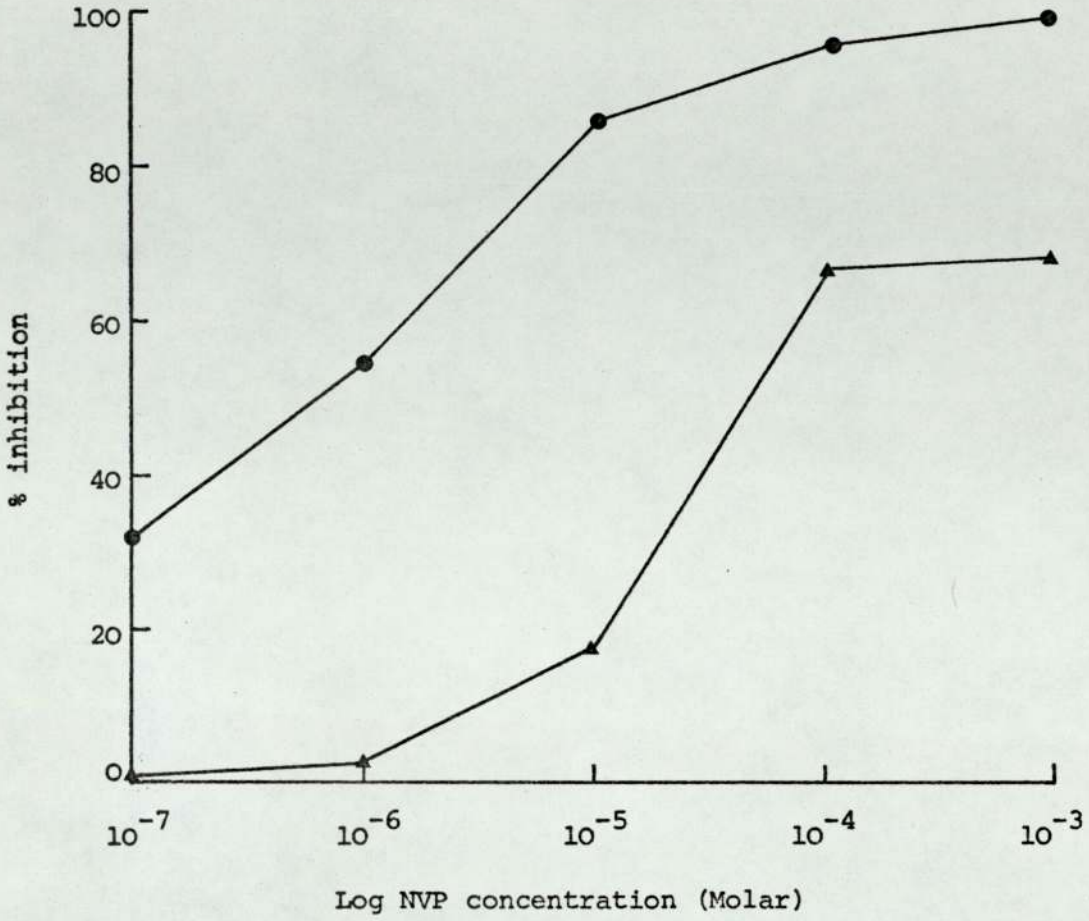


Fig. 17. Inhibition by NVP of the acetylation of HC-3 (▲) and choline (●). The enzyme preparation used was the supernatant from a rat brain homogenate prepared in 0.1% Triton X-100/200mM KCl and centrifuged at 20,000g for 20 minutes. Endogenous substrate was removed from the preparation by gel filtration using Sephadex G-50. Blank determinations were performed using an incubation mixture which contained no substrate.

of choline acetylation over a range of concentrations of NVP is shown in Figure 17.

When separate substrate blanks were used there was little difference between the inhibition of HC-3 acetylation and the inhibition of choline acetylation by NVP and NVPH (Table 19).

The acetylation of carnitine by a rat brain homogenate was inhibited completely by NVP but was unaffected by NVPH (Table 19).

CARNITINE ACETYLTRANSFERASE.

1. Acetylation of choline, carnitine, HC-3 and TEC.

The acetylation of the various substrates by CarAc was studied by substituting commercial CarAc from pigeon breast muscle (2×10^{-2} units/ml of incubation mixture) for ChAc in the incubation mixture (see Methods). Using carnitine (1 mM) as substrate the enzymatic reaction was linear with respect to time up to 17,500 cpm recovered and the enzyme concentration used was within this range (Figure 18). Separate substrate blanks were used for all the determinations involving CarAc.

As shown in Table 20, compared with carnitine, choline, HC-3 and TEC were very poor substrates of CarAc.

2. Inhibition of the acetylation of carnitine, choline, HC-3 and TEC by NVP and NVPH.

Neither NVP nor NVPH appeared to have any effect on the acetylation of choline, HC-3 and TEC by CarAc (Table 20). The acetylation of carnitine was inhibited by 30% by NVP but appeared to be unaffected by NVPH (Table 20). This indicates that NVP is not entirely specific for ChAc.

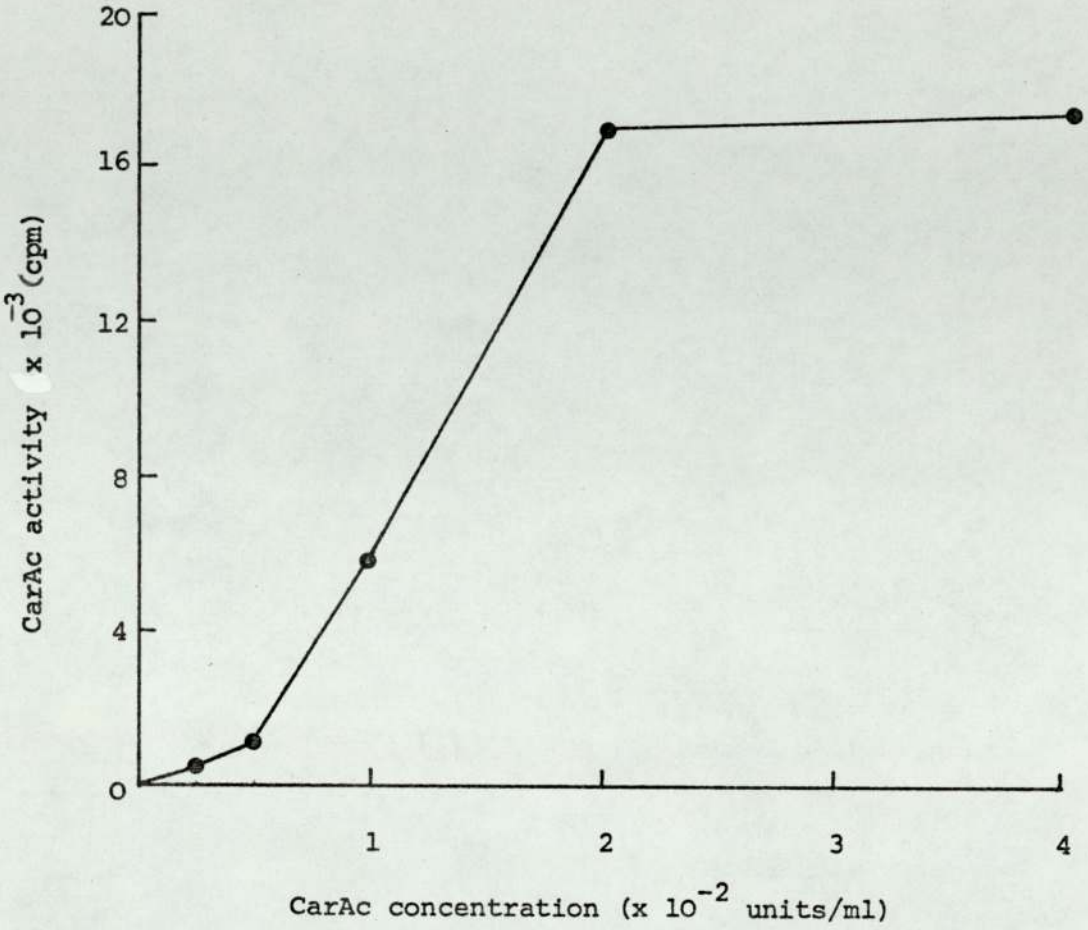


Fig. 18. Determination of CarAc activity. Activity v. enzyme concentration. The CarAc preparation used was the commercial enzyme obtained from pigeon breast muscle. Dilutions of the enzyme were prepared using 0.1% Triton X-100/200mM KCl.

TABLE 20.

The acetylation of carnitine, choline, HC-3 and TEC by carnitine acetyltransferase. Inhibition of the acetylation by NVP and NVPH.

SUBSTRATE	Percentage acetylation compared with carnitine	Percentage inhibition of acetylation	
		NVP 2.5×10^{-3} M	NVPH 2.5×10^{-3} M
Carnitine	100	30.0	0
Choline	0.5	0	0
HC-3	1.5	0	0
TEC	2.0	0	0

Final concentration of CarAc in the incubation mixture was 2×10^{-2} units /ml.

Separate substrate blanks were used for all determinations.

Assay performed as for ChAc (see Methods).

DISCUSSION.

1. Involvement of carnitine acetyltransferase in the acetylation of substrates by a rat brain homogenate.

The lower inhibition by NVP of the acetylation of HC-3 compared with its inhibition of choline acetylation as initially observed in the present investigation, and the similar situation described by Barker and Mittag (1975) for the inhibition of TEC acetylation by NVPH, suggested that an enzyme or enzymes other than ChAc might be involved in the acetylation of these compounds. It seemed possible that CarAc might be involved in the acetylation of these substrates since electrophoresis of an incubation mixture containing rat brain homogenate and ^{14}C acetyl CoA indicated that its substrate, carnitine, had been present in the homogenate and had been acetylated to ^{14}C acetylcarnitine (see 'Acetylation of hemicholinium-3 by choline acetyltransferase' page 125). Also, White and Wu (1973c) have demonstrated that the acetylation of carnitine and to a lesser extent choline by CarAc from mammalian heart can interfere with studies on the acetylation of substrates by ChAc. The present results confirm that choline can act as a very poor substrate for CarAc as can HC-3 and TEC.

An interesting finding was that NVP inhibited the acetylation of carnitine by CarAc whereas NVPH had no inhibitory effect on the reaction. This observation allows some inferences to be drawn regarding the acetylation of carnitine by a rat brain homogenate since NVPH was also shown to exert no inhibitory action on the acetylation of carnitine by a rat brain homogenate at a concentration of NVPH which inhibited choline acetylation by 96%. This suggests therefore that ChAc does not acetylate carnitine since if it did some inhibition of carnitine acetylation by NVPH would be expected. Also, NVP totally inhibits carnitine acetylation by a rat brain homogenate yet inhibits carnitine

acetylation by only 30%. This suggests that there is only a small amount of CarAc in a rat brain homogenate though it should be noted that the concentration of commercial CarAc chosen was deliberately high in order to better observe the acetylation of the low affinity substrates.

It does however appear that rat brain homogenate contains sufficient CarAc to acetylate an amount of carnitine equivalent to 69% of the amount of choline acetylated by the homogenate. Partial purification of the rat brain preparation by ammonium sulphate fractionation decreases the percentage acetylation of carnitine compared with choline possibly by removing some of the CarAc from the preparation.

The present results suggest therefore that the acetylation by CarAc, present within a rat brain homogenate, of endogenous carnitine could result in the formation of acetylcarnitine which could be wrongly identified as ACh if adequate separation of acetylcarnitine and ACh is not achieved by the extraction procedure. Gel filtration easily overcomes this problem by removing endogenous carnitine (see 'Acetylation of hemicholinium-3 by choline acetyltransferase' page **125**). It therefore seems unlikely that CarAc plays a significant part in the actual acetylation of choline, HC-3 or TEC by a rat brain homogenate.

2. The acetylation of hemicholinium-3 by a rat brain homogenate.

The use of separate substrate blanks indicated that NVP and NVPH inhibited HC-3 acetylation and choline acetylation to a similar extent, thus confirming the result of Barker and Mittag (1975). The greater value of blanks containing HC-3 compared with those containing choline suggested that there may be some non-enzymic acetylation of HC-3 or an increased trapping of ^{14}C acetyl CoA during the extraction procedure rather than the participation of an enzyme other than ChAc. Electrophoresis experiments ruled out the possibility of non-enzymic acetylation

of HC-3 since no acetylated product was detected. It would therefore appear that trapping of acetyl CoA probably accounts for what was originally suspected to be HC-3 acetylation not susceptible to inhibition by NVP or NVPH. Barker and Mittag (1975) may have avoided the complication of acetyl CoA trapping by the use of an ion-exchange extraction procedure as opposed to reineckate precipitation.

Although HC-3 appears to act as a weak substrate for high concentrations of CarAc it is unlikely that CarAc is involved in the acetylation of HC-3 by a rat brain homogenate since NVPH, which does not appear to inhibit CarAc, is as effective an inhibitor of HC-3 acetylation as of choline acetylation. The present results suggest that ChAc is probably wholly responsible for the acetylation of HC-3 by a rat brain homogenate.

3. The acetylation of TEC by a rat brain homogenate.

Unlike HC-3, TEC did not appear to increase the recovery of ^{14}C acetyl CoA during reineckate extraction, as shown by the similar values for TEC- and choline-containing blanks. It appears therefore that the lower inhibition of TEC acetylation by NVP and NVPH, compared with their inhibition of choline acetylation, is due to the involvement of an enzyme other than ChAc.

TEC was shown to serve as a weak substrate for high concentrations of CarAc. However, it is unlikely that CarAc contributes significantly to the acetylation of TEC by a rat brain homogenate as NVP at a concentration which inhibits carnitine acetylation, and so presumably CarAc, by 100 per cent only inhibits TEC acetylation by 76%. The present results suggest therefore that ChAc is mainly responsible for the acetylation of TEC by a rat brain homogenate and that another acetylating enzyme, but not CarAc, is responsible for the remainder.

It is not as yet clear why the acetylation of HC-3 and TEC compared with that of choline should increase when an enzyme preparation is partially purified by $(NH_4)_2SO_4$ fractionation. It may however be due to an increased enzyme concentration if the activity vs. enzyme concentration curves for the different substrates are non-parallel, as described for choline and its mono-, di-, and tri- ethyl analogues by Hemsworth and Smith (1970).

SUMMARY.

1. The acetylation of HC-3 and TEC by a rat brain homogenate has been investigated.

2. Preliminary results suggested that an enzyme or enzymes other than ChAc may be involved in the acetylation of HC-3 and TEC. CarAc was presented as a possible candidate as a second acetylating enzyme.

3. Choline, HC-3 and TEC were found to be only weak substrates of CarAc compared with carnitine.

4. HC-3 appears to increase the recovery of ^{14}C acetyl CoA by reineckate extraction and this can be confused with HC-3 acetylation.

5. The use of the inhibitors NVP and NVPH indicates that ChAc is probably wholly responsible for HC-3 acetylation and mainly responsible for TEC acetylation by a rat brain homogenate. The acetylation of TEC not accounted for by ChAc is probably due to another acetylating enzyme, but not CarAc.

CHAPTER 6.

A. THE ACETYLATION OF TWO HEMICHOLINIUM ANALOGUES.

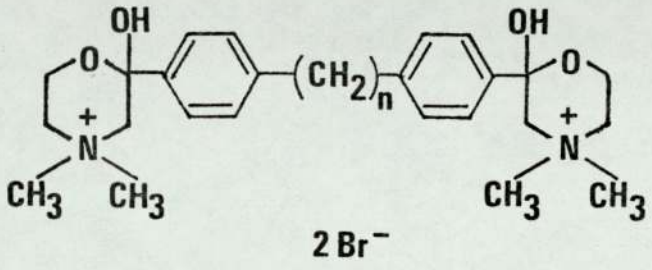
INTRODUCTION.

Two hemicholinium analogues were synthesised in the Department of Pharmacy, University of Aston by J.P. Boden with a view to studying their neuro-muscular blocking properties. The two analogues were structurally similar to hemicholinium-3 (HC-3) except that they contained one (HA-1) or two (HA-2) methylene groups linking the two phenyl rings (Figure 19).

The compounds were synthesised by the same procedure as that described for the synthesis of HC-3 (Long and Schueler, 1954; Schueler, 1955) except for the use of a different starting material. The starting material for the synthesis of HC-3 is diphenyl which consists of two linked phenyl rings whereas the compounds from which HA-1 and HA-2 were prepared contained one or two methylene groups respectively linking the two phenyl rings.

The neuromuscular blocking properties of HA-1 and HA-2 were compared with those of HC-3 on the rat phrenic nerve-diaphragm preparation and the frog rectus abdominis muscle preparation. (Boden, Bradshaw, Hemsworth and Veitch, 1975). The two hemicholinium analogues were shown to have a non-depolarising neuromuscular blocking activity and the results indicated that the analogues had an increasing post-junctional blocking activity as the distance between the quaternary nitrogen atoms was increased.

The present work involved a study of the acetylation of the compounds as an indication of their possible pre-synaptic effects and also as an indication of the effect of insertion of methylene groups between the



n = 1 or 2

Fig. 19. Structural formula of hemicholinium analogues.

phenyl rings on the ability of the hemicholinium molecule to serve as a substrate for ChAc.

RESULTS.

The acetylation of HA-1 (1 mM) and HA-2 (1 mM) was investigated by including the compounds with active enzyme and ^{14}C acetyl CoA in incubation mixtures which were subsequently extracted by reineckate precipitation (see Methods). The acetylation of the compounds was compared with the acetylation of HC-3 and choline at the same concentration.

The enzyme preparation used was the 20,000 g supernatant from a rat brain homogenate prepared in Triton X-100/200 mM KCl. Endogenous substrate was removed immediately before use by gel filtration using Sephadex G-50.

Blank determinations obtained using enzyme inactivated with trichloroacetic acid were greater for incubation mixtures containing HC-3, HA-1 or HA-2 than for those containing choline. This suggests that, like HC-3, HA-1 and HA-2 increase the recovery of ^{14}C acetyl CoA by the reineckate precipitation procedure.

Both HA-1 and HA-2 were found to be acetylated by the enzyme preparation and Table 21 indicates the percentage acetylation of the analogues compared with choline. These results show that the rate of acetylation of the analogues is reduced as the methylene chain length is increased.

DISCUSSION.

The two newly synthesised hemicholinium analogues showed a reduced acetylation with increasing methylene chain length and it is suggested that this may indicate a decrease in the pre-junctional activity which is

TABLE 21.

The acetylation of hemicholinium-3 and two of its analogues.

SUBSTRATE	PERCENTAGE ACETYLATION COMPARED WITH CHOLINE
HC-3	29
HA-1	14
HA-2	12

The incubation mixture contained active enzyme, ^{14}C acetyl CoA and either HC-3, HA-1 or HA-2. The acetylated products were recovered by reineckate precipitation.

Separate blank determinations were performed for each substrate.

a characteristic of hemicholinium-3 activity. Benz and Long (1969) suggested that HC-3 like activity depends on the distance between the cationic heads of the molecule and that an interquaternary distance of 14°A is suitable for this type of activity. The present results support this view in that an increase in the interquaternary distance from about 14°A for HC-3 to 15.3°A for HA-1 and 16.5°A for HA-2 increases the post-junctional activity of the compounds but decreases their acetylation. It should be noted that HC-3 has been reported to possess some degree of post-junctional activity in guinea-pig and frog preparations (Thies and Brooks, 1961; Martin and Orkand, 1961) so that the post-junctional activity of HA-1 and HA-2 can be considered as an increase in activity compared with HC-3, rather than a new type of activity.

Hemsworth (1971b, 1975) has reported that increasing the inter-quaternary distance in a series of polymethylene dicholine compounds increases the post-junctional activity of the compounds as was the case for the hemicholinium analogues used in the present work. This increased post-synaptic blocking activity with increasing methylene chain length may be due to the increased number of ACh receptor sites which can be sterically hindered by the larger molecules, whether or not the compounds bind to the receptors themselves. Alternatively, it could be due to an increased ability of the compounds to bind to adjacent receptor sites as the interquaternary distance is increased.

The rate of acetylation of the dicholine compounds was also shown to increase with increasing methylene chain length (Hemsworth 1971b, 1975) in contrast to the reduced rate of acetylation observed on increasing the methylene chain length of the hemicholinium analogues. It may be that the interquaternary distance of the most rapidly acetylated dicholine compound, which contained 10 methylene groups between the nitrogen atoms, is similar to that of HC-3. In this case it is possible that the optimum

interquaternary distance of about 14°A is being approached from below in the dicholine series but is being exceeded in the hemicholinium series. Further information which could help to resolve this question could be obtained by studying the higher homologues of the dicholine series.

Although HC-3 is generally assumed to exist in a cyclic form in aqueous solution this has not been unequivocally proven and it is possible that it also exists in its straight-chain form (see 'Acetylation of hemicholinium-3 by choline acetyltransferase' page 117). N.M.R. spectroscopy indicates the possibility that, in D_2O at least, HA-1 and HA-2 have a greater ability to revert to the straight-chain form than does HC-3 and it may be that this is responsible for the observed differences in pharmacological activity of these hemicholinium analogues.

SUMMARY.

1. A study was made of the acetylation of two analogues of hemicholinium-3 which contained one or two methylene groups linking the phenyl rings. The compounds had previously been shown to have an increasing post-junctional blocking activity on neuromuscular preparations as the interquaternary distance was increased.

2. The rate of acetylation of the compounds was shown to decrease as the methylene chain length was increased.

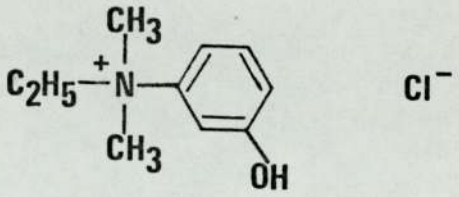
3. It has been suggested that the pharmacological activity of the compounds depends on their interquaternary distance and possibly also on the increased ability of these analogues to revert to their straight-chain form in water.

B. THE ACETYLATION OF EDROPHONIUM (TENSILON).

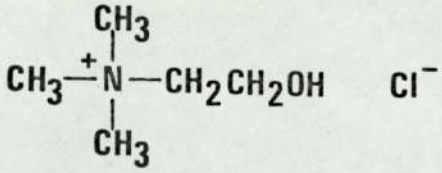
INTRODUCTION.

Edrophonium facilitates neuromuscular transmission (Randall, 1950) but as indicated by Barber, Calvey, Muir and Taylor (1976) the exact mode of action of edrophonium has not been fully elucidated, though its main action does appear to be of post-synaptic origin and probably involves the inhibition of acetylcholinesterase (AChE). However, Barber et al. showed that at high concentrations of edrophonium a biphasic facilitation of the twitch of a rat tibialis anterior muscle stimulated via its sciatic nerve was obtained, but the initial facilitation and its decline was not related to the plasma level of the drug or inhibition of red cell AChE. These workers postulated that 'receptor inactivation' might be responsible for the decline of the facilitation but they did not exclude other mechanisms of action. It is conceivable that these other mechanisms may include pre-synaptic effects of edrophonium which are possibly only apparent at high concentrations of the drug.

The structural (Figure 20) and pharmacological (Blaber and Bowman, 1959) similarities of edrophonium to choline suggest that edrophonium may be able to substitute for choline pre-synaptically at the neuro-muscular junction. In the present work, the possible acetylation of edrophonium by a rat brain homogenate in vitro has been investigated, mainly as a substrate specificity study for the enzyme choline acetyltransferase (ChAc) but also as an indication of a possible pre-synaptic effect of edrophonium which might account for the actions of edrophonium not adequately explained by its inhibition of AChE.



Edrophonium



Choline

Fig. 20. Structural formulae of edrophonium and choline.

RESULTS.

Edrophonium was included as a possible second substrate for ChAc, in addition to ^{14}C acetyl CoA, in the incubation mixture used for ChAc assays (see Methods). The enzyme preparation used was the 20,000 g supernatant from a rat brain homogenate prepared in Triton X - 100/200 mM KCl, from which endogenous substrate had been removed by gel filtration. Extraction of the incubation mixture by precipitation with ammonium reineckate (see Methods) indicated a greater recovery of radioactivity for incubation mixtures containing active enzyme and edrophonium in excess of 10^{-3}M than for blanks containing inactivated enzyme and 10^{-3}M edrophonium. The possibility that this was due to enzymic acetylation of edrophonium was further investigated.

For an enzyme preparation from which endogenous substrate was not removed the inclusion of edrophonium in the incubation mixture decreased rather than increased the amount of radioactivity recovered by reineckate precipitation. This suggests that edrophonium inhibits the acetylation of endogenous choline. Subtraction of blank values derived from incubation mixtures containing inactivated enzyme, quantifies this inhibition as approx. 97% and 88% for 10^{-1}M and 5×10^{-2}M edrophonium respectively. Table 22 summarises these results.

The recovered radioactivity from incubation mixtures containing enzyme inactivated with trichloroacetic acid was greater for incubation mixtures containing edrophonium as substrate than for those containing choline at the same concentration, and this was more marked at high concentrations of edrophonium. This results suggests non-enzymic acetylation of edrophonium or alternatively an increased recovery of ^{14}C acetyl CoA brought about through an increased trapping of the coenzyme by edrophonium.

TABLE 22.

Inhibition of endogenous choline acetylation by edrophonium.

SUBSTRATE	Recovered radioactivity (cpm)	Blank	cpm-Blank	Percentage inhibition of choline acetylation
Distilled water	2011	223	1788	
Edrophonium 10^{-1} M	1308	1247	61	96.6
Edrophonium 5×10^{-2} M	1080	869	211	88.2

The incubation mixture was as described in Methods and included distilled water or edrophonium as indicated above.

The enzyme preparation still contained endogenous choline.

Blank values were determined by including inactivated enzyme in the incubation mixture.

cpm-Bl represents the amount of recovered radioactivity due to acetylation of endogenous choline.

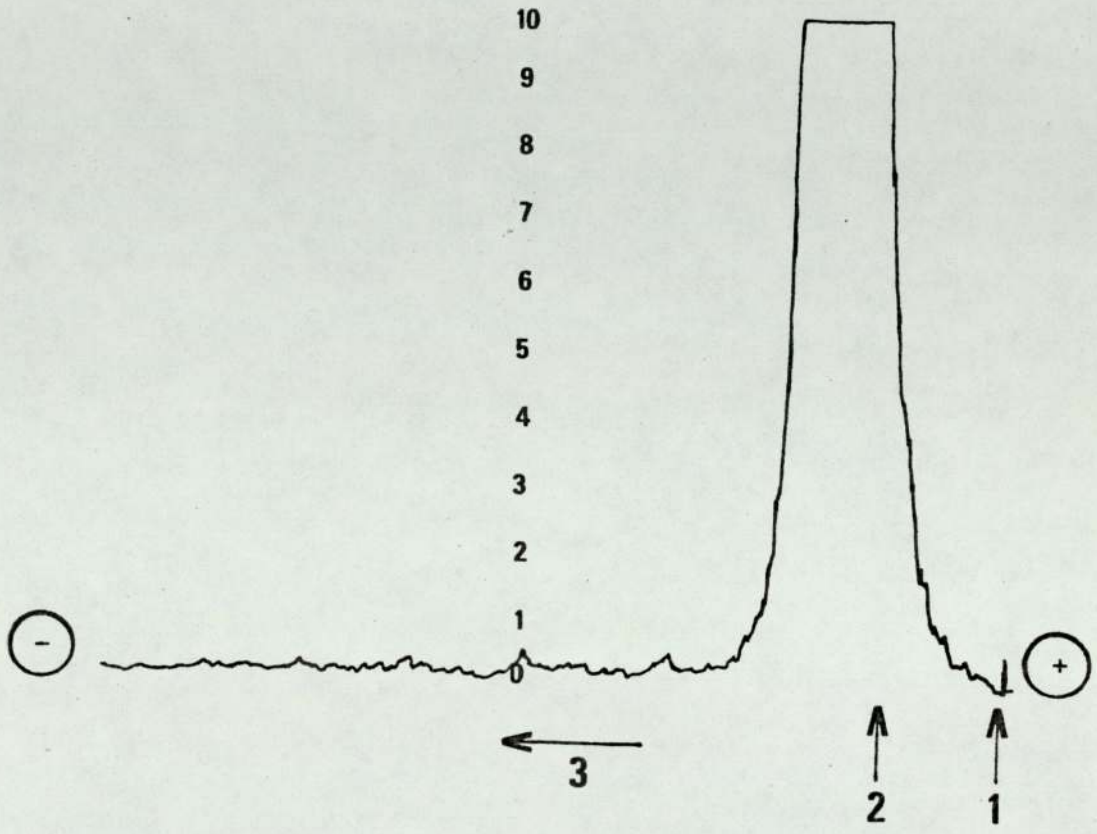


Fig. 21. Electrophoresis paper strip scanned for radioactive peaks. The incubation mixture contained edrophonium (0.1M). The arrows represent the same conditions as those described in Fig. 11. Full scale deflection corresponds to 1,000 cpm. The peak represents unreacted 14-C acetyl CoA. The absence of a second peak suggests that the edrophonium was not acetylated. Similar traces were obtained both in the presence and absence of an enzyme preparation.

Electrophoresis of incubation mixtures containing either active enzyme or no enzyme in addition to edrophonium at a concentration of 10^{-1}M showed no evidence of acetylation of the edrophonium (Figure 21).

DISCUSSION.

Initial experiments indicated that for incubation mixtures containing ^{14}C acetyl CoA and active enzyme the presence of edrophonium increased the amount of radioactivity recovered by reineckate precipitation. Three possible mechanisms by which this could occur are:-

1) the acetylation of edrophonium, either enzymically or non-enzymically,
2) the increased recovery of ^{14}C acetyl CoA due to 'trapping' by edrophonium,
3) the inhibition of non-specific esterases insensitive to the physostigmine present in the incubation mixture.

This third mechanism could only operate if the enzyme preparation contained endogenous choline from which ^{14}C ACh could be formed and if non-specific esterases were present in the enzyme preparation which could hydrolyse this ^{14}C ACh. The products of this hydrolysis would be ^{14}C acetate and choline and the ^{14}C acetate would not be detected by reineckate precipitation. The presence of edrophonium could reduce this non-specific ACh hydrolysis by inhibiting the non-specific esterases resulting in an increased recovery of radioactivity in the presence of edrophonium. However, edrophonium only increases the recovered radioactivity if an enzyme preparation containing no endogenous substrate is used and decreases rather than increases the amount of radioactivity recovered if an enzyme preparation is used

which still contains endogenous choline. Also, earlier experiments have shown a lack of hydrolysis of ^{14}C ACh added to incubation mixtures containing physostigmine suggesting that there are no non-specific esterases present in a rat brain homogenate which are capable of hydrolysing ACh (see Determination of ChAc activity page 99).

Edrophonium was shown to inhibit the acetylation of endogenous choline. The fact that for an enzyme preparation containing endogenous choline the amount of radioactivity recovered in the presence of a high concentration of edrophonium was less than in its absence suggests that if this inhibition is due to acetylation of edrophonium, then the acetylated form of edrophonium is probably not recovered by reineckate precipitation.

Enzymic acetylation of edrophonium was ruled out by the fact that the increased recovery of radioactivity in the presence of edrophonium was not dependent on active enzyme and occurred in incubation mixtures containing inactivated enzyme or water in place of enzyme.

No evidence was found for either the enzymic or non-enzymic acetylation of edrophonium when electrophoresis of incubation mixtures containing edrophonium was performed. The detection of only one radioactive peak by electrophoresis, that due to ^{14}C acetyl CoA, suggests that edrophonium does increase the 'trapping' of acetyl CoA when extraction is performed by reineckate precipitation.

The results suggest therefore that edrophonium is not acetylated. At high concentrations however, it does appear to inhibit the acetylation of choline but it is unlikely that this will contribute to any in vivo effect of the drug since it is unlikely that such high concentrations of edrophonium will occur at the sites of choline acetylation.

The inability of edrophonium to serve as a substrate for ChAc may be attributed to the differences in its structure from that of

choline. In this respect the presence of an ethyl group rather than a methyl group on the nitrogen atom may affect the binding of the molecule to the enzyme but it is unlikely that the binding is totally prevented since monoethylcholine has previously been shown to be acetylated (Hemsworth and Smith, 1970a). However, the aromatic ring may totally prevent attachment of the molecule to the enzyme binding site by steric hindrance.

Another factor which may be of importance is that the distance between the nitrogen atom and the hydroxyl group is probably different for edrophonium than for choline so that even if the edrophonium molecule was able to bind to the enzyme its hydroxyl group may not exactly align with the enzyme active site.

SUMMARY.

1. The structural similarity between edrophonium and choline suggested that edrophonium might be acetylated by ChAc and this was investigated using enzyme from a rat brain homogenate.

2. Initial experiments produced results which indicated that edrophonium might be acetylated but other possible explanations of the observed results were also sought.

3. Further investigation suggested that edrophonium was not acetylated and this was confirmed by electrophoresis of an incubation mixture containing the drug.

4. The initial results appear to have arisen from an increased recovery of ^{14}C acetyl CoA by reineckate precipitation in the presence of edrophonium.

5. High concentrations of edrophonium appear to inhibit the acetylation of endogenous choline.

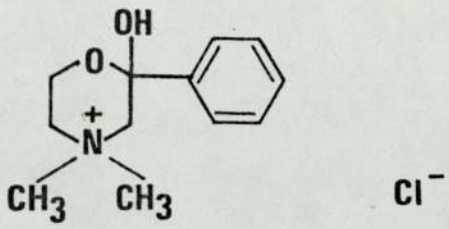
C. THE ACETYLATION OF TWO PIPERIDINOL COMPOUNDS.

INTRODUCTION.

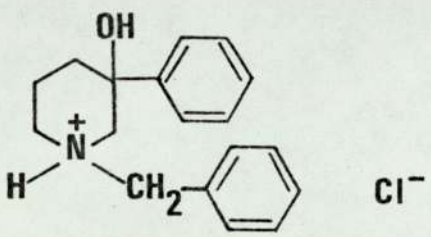
Two piperidinol compounds were synthesised by A. C. Wiggins, Department of Pharmacy, University of Aston. These compounds were 1-benzyl-3-phenyl-3-piperidinol hydrochloride (I), first synthesised by Iselin and Hoffmann (1954) and 1-benzyl-1-isobutyl - 3-phenyl-3-piperidinol bromide (II). The structure of the compounds was based on that of hemicholinium-15 (HC-15) (Figure 22), which has been shown to possess both a non-depolarising post-junctional action (Bowman, Hemsworth and Rand, 1967) and, in lower doses, a pre-junctional blocking action (Hemsworth, 1971a). The work was performed as a step towards obtaining neuromuscular blocking drugs of short duration of action and low toxicity which would be useful clinically.

Compounds I and II were tested on isolated neuromuscular preparations and were shown to possess a depolarising neuromuscular blocking action in contrast to the non-depolarising block caused by HC-15 (Hemsworth, Veitch and Wiggins, 1975). The compounds were also shown to possess a long duration of action and it was assumed that this, in addition to the difficulty found in reversing the block, was due to either a pre-synaptic effect of the compounds or the depolarising nature of their neuromuscular blocking activity.

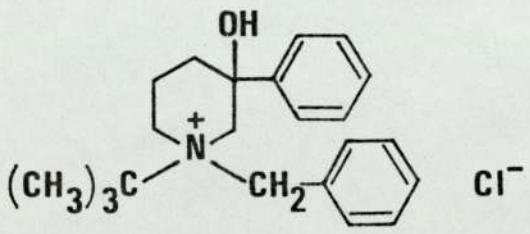
HC-15 has been shown to be acetylated by ChAc (Hemsworth, 1971a) and the present work is concerned with an investigation into the possible acetylation of compounds I and II, which might be expected on the basis of their similarity in structure to HC-15. The work was performed in order to obtain further information on the structure-activity relationships of substrates for ChAc and also to determine



Hemicholinium-15



Compound I



Compound II

Fig. 22. Structural formulae of HC-15 and two analogues.

whether there was any likelihood that compounds I and II could act pre-synaptically by substituting for choline.

RESULTS.

The acetylation of compounds I and II was studied by including them in an incubation mixture containing ^{14}C acetyl CoA and an enzyme preparation which was prepared in Triton X-100/200 mM KCl and partially purified by ammonium sulphate fractionation.

Blank values obtained from incubation mixtures containing compound I or compound II and inactivated enzyme were similar to those for choline-containing blanks, suggesting that there was no non-enzymic acetylation or increased 'trapping' of acetyl CoA (see 'Acetylation of hemicholinium-3 and 'Acetylation of edrophonium' pages 151 & 166).

When incubation mixtures containing compound I or compound II were extracted by precipitation with ammonium reineckate the amount of radioactivity recovered was similar to blank values. A similar determination for an incubation mixture containing choline showed a high recovery of radioactivity (21,305 cpm). Acetylation of compounds I and II therefore seemed unlikely but as it was possible that the acetylated forms of I and II were formed but not recovered by reineckate precipitation, electrophoresis of the incubation mixture was performed in order to detect any labelled acetylated product.

Electrophoresis of incubation mixtures containing compounds I or II showed no evidence of acetylation of the two compounds (Figure 23) compared with a control of an incubation mixture containing choline which showed a marked acetylation to ACh (Figure 24). It would appear therefore that compounds I and II are not acetylated.

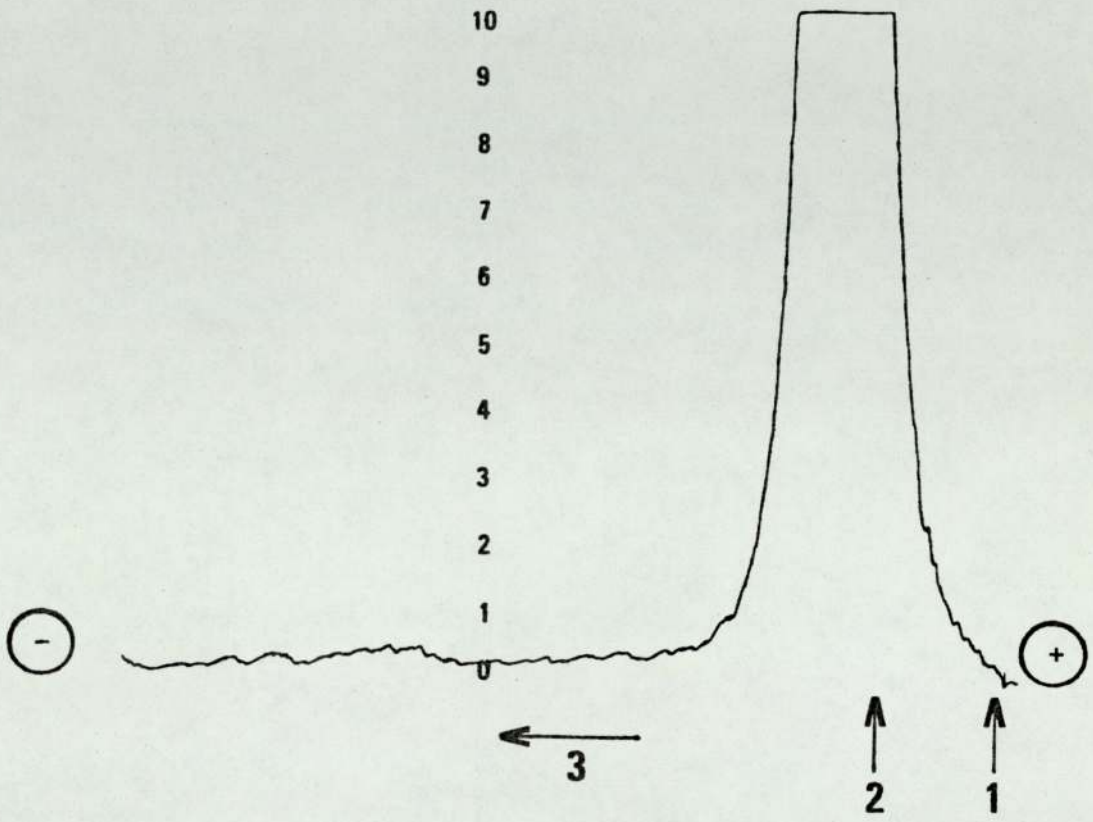


Fig. 23. Electrophoresis paper strip scanned for radioactive peaks. ChAc which had been passed through Sephadex G-25 was used in the incubation with Compound I (See Fig. 22) as substrate. The arrows represent the same conditions as those described in Fig. 11. Full scale deflection corresponds to 1,000 cpm. The peak represents unreacted 14-C acetyl CoA. The absence of any other peak suggests that Compound I was not acetylated. A similar trace was obtained when Compound II (See Fig. 22) was included in the incubation mixture.

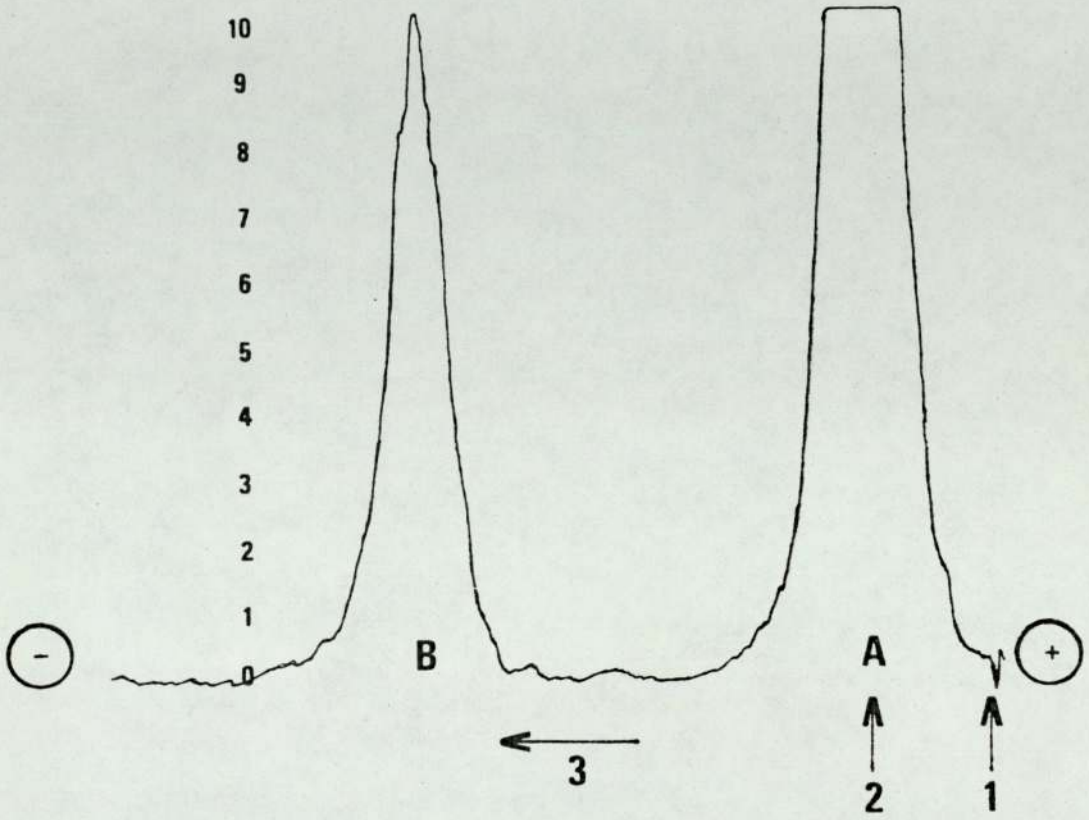


Fig. 24. Electrophoresis paper strip scanned for radioactive peaks. ChAc which had been passed through Sephadex G-25 was used in the incubation with choline as substrate. The arrows represent the same conditions as those described in Fig. 11. Full scale deflection corresponds to 1,000 cpm. Peak A represents unreacted 14-C acetyl CoA. Peak B represents 14-C ACh formed by the acetylation of choline.

DISCUSSION.

The two piperidinol compounds tested do not appear to be acetylated by the enzymes present within a rat brain homogenate. This can probably be attributed to the molecular structure of the compounds since they differ in structure from HC-15, which is acetylated (Hemsworth, 1971a), in their N-substitution and also in their lack of an oxygen atom within the aliphatic ring.

N-alkyl substitution has been shown to affect the acetylation of choline analogues so that acetylation decreases with the successive replacement of methyl groups with ethyl groups on the nitrogen atom (Hemsworth and Smith, 1970a). This reduced acetylation probably arises from steric hindrance by the larger chemical groups which prevents attachment of the molecules to the enzyme binding site. It might reasonably be expected therefore that N-substitution of even larger chemical groups, as is the case for compounds I and II, could result in further reductions in the ability of these choline-like compounds to serve as substrates for ChAc.

The absence of an oxygen atom within the aliphatic ring may also affect the ability of compounds I and II to serve as substrates for ChAc and in the previous section it was shown that edrophonium, which also lacks an oxygen atom within the aromatic ring, was similarly not acetylated. However, since other factors probably account at least partly for the lack of enzymic acetylation of these compounds the influence of this oxygen atom cannot be fully determined from these compounds. In order to obtain more information concerning the role of the aliphatic ring oxygen atom in the acetylation of HC-15 it would be of interest to investigate the susceptibility to acetylation of the compound lacking this oxygen atom but otherwise structurally the same as HC-15.

The inability of compounds I and II to serve as substrates for ChAc suggests that a pre-synaptic action of these compounds by substitution for choline is unlikely.

SUMMARY.

1. The acetylation of two piperidinol compounds by a rat brain homogenate was investigated.
2. Extraction, by reineckate precipitation or electrophoresis, of incubation mixtures containing these compounds showed no evidence to suggest that these compounds were acetylated.
3. The lack of acetylation of these compounds has been discussed in relation to their differences in structure from hemicholinium-15, which has previously been shown to be acetylated by ChAc.

CHAPTER 7.

INHIBITION OF CHOLINESTERASE ACTIVITY BY TWO METHYLACRIDINIUM COMPOUNDS.

INTRODUCTION.

9-azido-10-methylacridinium methosulphate and 9-chloro-10-methylacridinium methosulphate are both monoquaternary ammonium compounds (Figure 25) and were synthesised in the Department of Pharmacy, University of Aston, by Dr. G. A. M. Butchart. The compounds are hydrolysed in either aqueous or alcohol solvents (Mair and Stevens, 1972) and Figure 25 indicates the progress of this hydrolysis in water. This hydrolysis proceeds via the formation of an unstable intermediate containing a hydroxyl group in the nine-position of the acridinium ring, which immediately dissociates to form N-methylacridone. N-methylacridone is formed from hydrolysis of both the azido- and chloro-methylacridinium compounds. Butchart (1975) reported that the half-lives in water at 37°C, as determined by electronic absorption spectroscopy, were 8 hours for the azide and 1.5 hours for the chloro-compound.

The two methylacridinium compounds were synthesised for their possible anti-tumour activities but were found by the Cancer Chemotherapy National Service Centre, Bethesda, U.S.A. to be inactive. This was attributed to their rapid de-activation by hydrolysis and also to the fact that the salts, as permanent cations, were unable to penetrate cell walls (Butchart, 1975).

The compounds were however shown to be extremely toxic in mice and it was suggested that inhibition of cholinesterase enzymes might be involved. As a result of the quaternary nature of the two compounds it was suspected that there might be an interaction between the organic molecules and the anionic site on the cholinesterase. This possible action

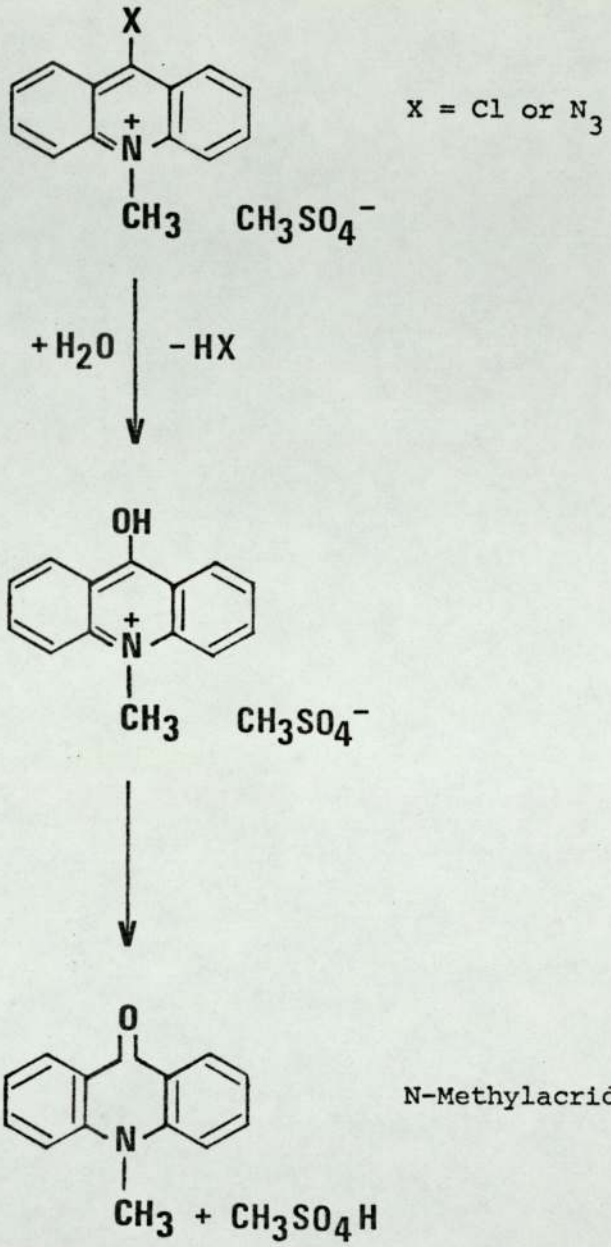


Fig. 25. Hydrolysis of methylacridinium compounds.
$t_{\frac{1}{2}}$ in water: X = Cl, 90 mins., X = N_3, 480 mins.

suggested that the compounds might be useful as insecticides or rodenticides and in this respect it was thought that their hydrolysis may be particularly useful in rendering them harmless after they had achieved their desired effect. It was assumed that the product of hydrolysis, N-methylacridone, which lacks the quaternary nitrogen of the original compounds, would be inactive as an anti-cholinesterase agent.

The present work was performed in order to determine the anti-cholinesterase activity of the two methylacridiniums and their hydrolysis product, N-methylacridone, with a view to assessing the potential use of the methylacridiniums as possible pesticides.

RESULTS.

1. Inhibition of cholinesterase activity by the two methylacridinium compounds.

The effect of the methylacridinium compounds on cholinesterase activity was determined by including the compounds in incubation mixtures containing enzyme, buffer and radioactive substrate (see Methods page 43). The degree of hydrolysis of the substrate was then compared with that obtained in controls containing distilled water in place of the compounds.

In order to determine the effects of the two compounds on acetylcholinesterase (AChE) and pseudo-cholinesterase (ChE) the compounds were tested against commercial preparations of these two enzymes from bovine erythrocytes and horse serum respectively. ^{14}C ACh was used as substrate. Also, a 20,000 *g* supernatant from a rat brain homogenate was used as a source of both AChE and ChE. In this case, ^{14}C ACh was used as substrate for AChE and ChE combined, ^{14}C acetyl β methyl choline was used as a specific substrate for AChE and ^{14}C butyryl choline was used as a specific substrate for ChE.

The azido-compound was readily soluble in water and all determinations were carried out using an aqueous solution of this compound. The chloro-compound was not so soluble in water and concentrations greater than 10^{-5}M did not dissolve completely in water. Solutions of the chloro-compound were therefore prepared in methanol and all determinations were carried out utilising this organic solvent. Control determinations for the chloro-compound were performed in the presence of methanol so that any effect of the methanol itself on the cholinesterase enzymes would not be misinterpreted as being due to the methylacridinium. Methanol was shown to have some inhibitory effect on the hydrolysis of acetyl β methyl choline by a rat brain homogenate preparation but did not affect the hydrolysis of butyryl choline.

The inhibition produced by the two compounds was compared on the different enzyme preparations using the different substrates by incubating them with a number of different concentrations of the methylacridiniums and estimating the I_{50} values (Table 23).

Both compounds were effective as inhibitors of both AChE and ChE. For all enzyme preparations and for all the substrates used the azide was a more potent inhibitor than was the chloro-compound as shown by the lower I_{50} values for the azide. It is not possible from these results to determine positively whether inhibition was greater for AChE or ChE as the commercial enzyme preparations were of a different concentration. Also, for the rat brain homogenate, the ratio of the amounts of AChE and ChE is not known and a valid comparison of their degrees of inhibition can only be obtained if they are present in equal concentrations. However, the results suggest that the two compounds may be more active against ChE than against AChE.

TABLE 23.

I_{50} values for the inhibition of cholinesterase activity by azido- and chloro- methylacridiniums.

Enzyme	Methylacridinium		Substrate
	Azido	Chloro	
Bovine erythocytes, (AChE)	2.9×10^{-5}	5.5×10^{-3}	ACh
Horse serum, (ChE)	1.9×10^{-6}	3.3×10^{-4}	ACh
Rat brain homogenate	4.2×10^{-6}	1.2×10^{-3}	ACh
	2.3×10^{-6}	1.0×10^{-3}	Ac-β-MeCh
	1.5×10^{-6}	4.4×10^{-4}	BuCh

Incubation mixtures contained enzyme, ^{14}C - labelled substrates as indicated above and a range of concentrations of each inhibitor.

I_{50} values represent the concentrations of inhibitor which inhibited the hydrolysis of the various substrates by 50%.

2. Reversibility of the cholinesterase inhibition produced by the methylacridiniums.

In order to determine whether the cholinesterase inhibition produced by the compounds was reversible or irreversible, the azide was pre-incubated with rat brain enzyme for varying lengths of time before the addition of ^{14}C ACh as substrate. A control containing no inhibitor was similarly pre-incubated so that any decline in enzyme activity which occurred independently of the inhibitor, such as that caused by enzyme denaturation, could be taken into account.

It was found that pre-incubation of the inhibitor with the enzyme had no effect on the degree of inhibition produced on subsequent incubation with substrate. This suggests that the inhibitor was acting reversibly.

3. Effect of hydrolysis of the methylacridinium compounds on their anticholinesterase activity.

The cholinesterase inhibition produced by the two methylacridiniums was tested against a rat brain homogenate using ^{14}C ACh as substrate. The effect of hydrolysis of the compounds on their anticholinesterase activity was assessed by re-determining the inhibitory activity of solutions of the compounds at varying intervals after their preparation.

The inhibitory activity of the azido-compound showed no decline for solutions of the compound kept in the light or dark at room temperature over a period of 16 days.

The inhibitory activity of both methylacridiniums at a concentration of $10^{-5}M$ was compared with that of physostigmine at the same concentration (Table 24). The inhibitor solutions were then left

at 37°C and their inhibitory activities re-determined at intervals up to 5 days. The solutions were then left at room temperature for a further 9 days and their anticholinesterase activities re-determined.

For the azide, an increase in its inhibitory activity was obtained up to 5 days after preparation of the solution and this was followed by a decrease in activity over the next 9 days. It is possible that this initial increased activity was due to evaporation of water from the solution making it effectively more concentrated. A control experiment could not be performed to test this possibility as the supplies of the azide had at this stage been exhausted.

The chloro-compound showed a steady decline in inhibitory activity so that after 5 days little inhibitory activity remained. Physostigmine also showed a steady, though slower, decline in its anticholinesterase activity over the 14 days.

Table 24 summarises the above results and also indicates the lesser potency of the methylacridiniums compared with physostigmine.

It would appear from the above results that the inhibitory potency of the methylacridiniums does not parallel their hydrolysis to N-methylacridone.

4. Anticholinesterase activity of N-methylacridone.

A possible explanation of the above results is that N-methylacridone is itself active against cholinesterase. To test this N-methylacridone, prepared in methanol, was included in incubation mixtures containing a rat brain enzyme preparation and ^{14}C ACh as substrate.

High concentrations of the compound were found to inhibit the hydrolysis of ACh and a 37.5% inhibition of hydrolysis was achieved at a concentration of N-methylacridone of 10^{-3}M. The compound is therefore

TABLE 24.

Reduction in anticholinesterase activity of the methylacridiniums and physostigmine.

INHIBITOR	Days after preparation of the inhibitor solutions				
	0	1	2	5	14
	Percentage inhibition of cholinesterase activity				
Azido-compound	67.8	74.0	76.0	80.1	69.5
Chloro-compound	21.3	22.1	12.9	0.1	0
Physostigmine	98.2	98.0	97.8	96.0	95.5

All inhibitors were prepared at an initial concentration of 10^{-5}M. The inhibitor solutions were kept at 37°C for the first 5 days after their preparation and were subsequently stored at room temperature.

less potent an anticholinesterase than either of the two methylacridinium compounds. However, the inhibitory capacity it does possess may explain to some extent why the decline in inhibitory activity of the methylacridiniums does not parallel their hydrolysis.

5. Determination of K_i values for the methylacridiniums.

Different concentrations of each inhibitor were incubated with enzyme from a rat brain homogenate and two different concentrations of ^{14}C ACh substrate (10^{-3}M and 3×10^{-3}M). The rate of hydrolysis of ACh(V) was measured as before. The reaction was found to proceed at a constant velocity throughout the incubation even in the presence of a high concentration of inhibitor (Figure 26).

An attempt was made to determine the K_i values of the two methylacridinium compounds by the use of Dixon plots (i.e., graphs of $\frac{1}{V}$ against inhibitor concentration) (Dixon, 1953).

The Dixon plot for the azide is shown in Figure 27 and appears to be linear for both substrate concentrations and the K_i can be determined as 3.5×10^{-6}M. It is possible that the graph is not linear for the higher substrate concentration at high inhibitor concentrations but it was however unfortunately not possible to repeat the experiment due to insufficient supplies of the compound.

The Dixon plot for the chloro-compound was found to be curved at both substrate concentrations (Figure 28) and the K_i could not therefore be determined.

DISCUSSION.

As suggested by the quaternary nature of the two methylacridinium compounds, both were found to be inhibitors of cholinesterase though their potency was less than that of physostigmine. The azido-compound was shown

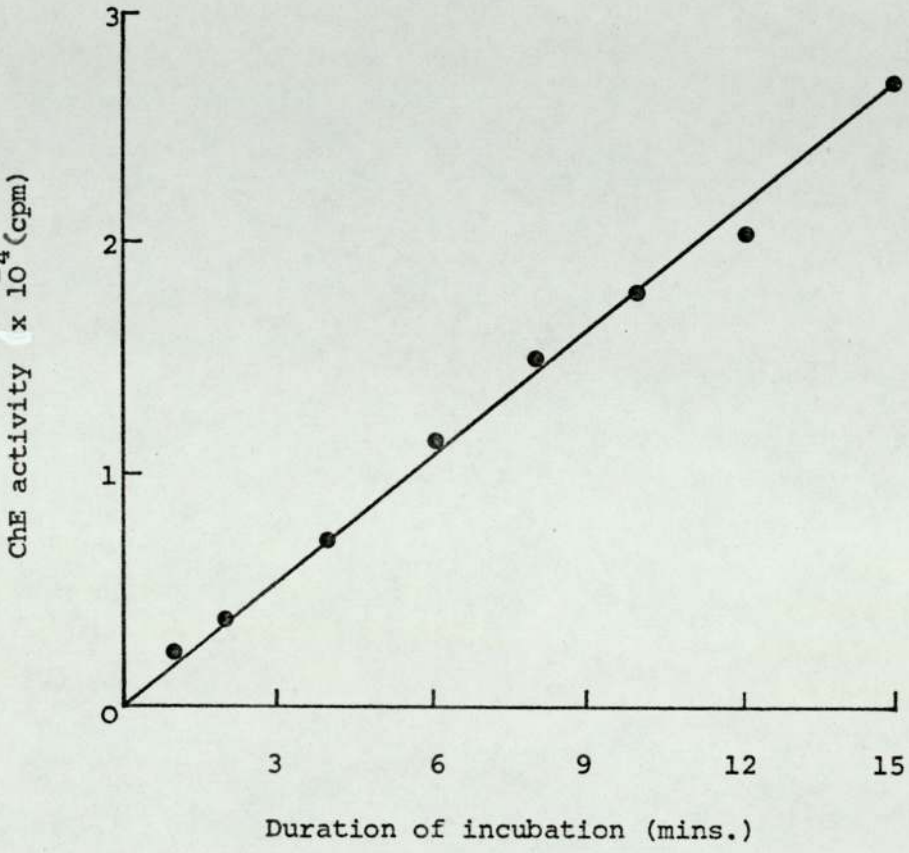


Fig. 26. Determination of cholinesterase activity. Activity v. duration of incubation. The enzyme preparation used was the supernatant from a rat brain homogenate prepared in 0.1% Triton X-100/200mM KCl and centrifuged at 20,000g for 20 minutes. The incubation mixture contained ChE, 14-C ACh ($3x10^{-3}$M) and buffer/salt /detergent mixture (See Methods). A linear relationship between activity and duration of incubation was also obtained when an incubation mixture containing 14-C ACh (10^{-3}M) and 9-chloro 10-methylacridinium methosulphate ($3x10^{-3}$M) was used.

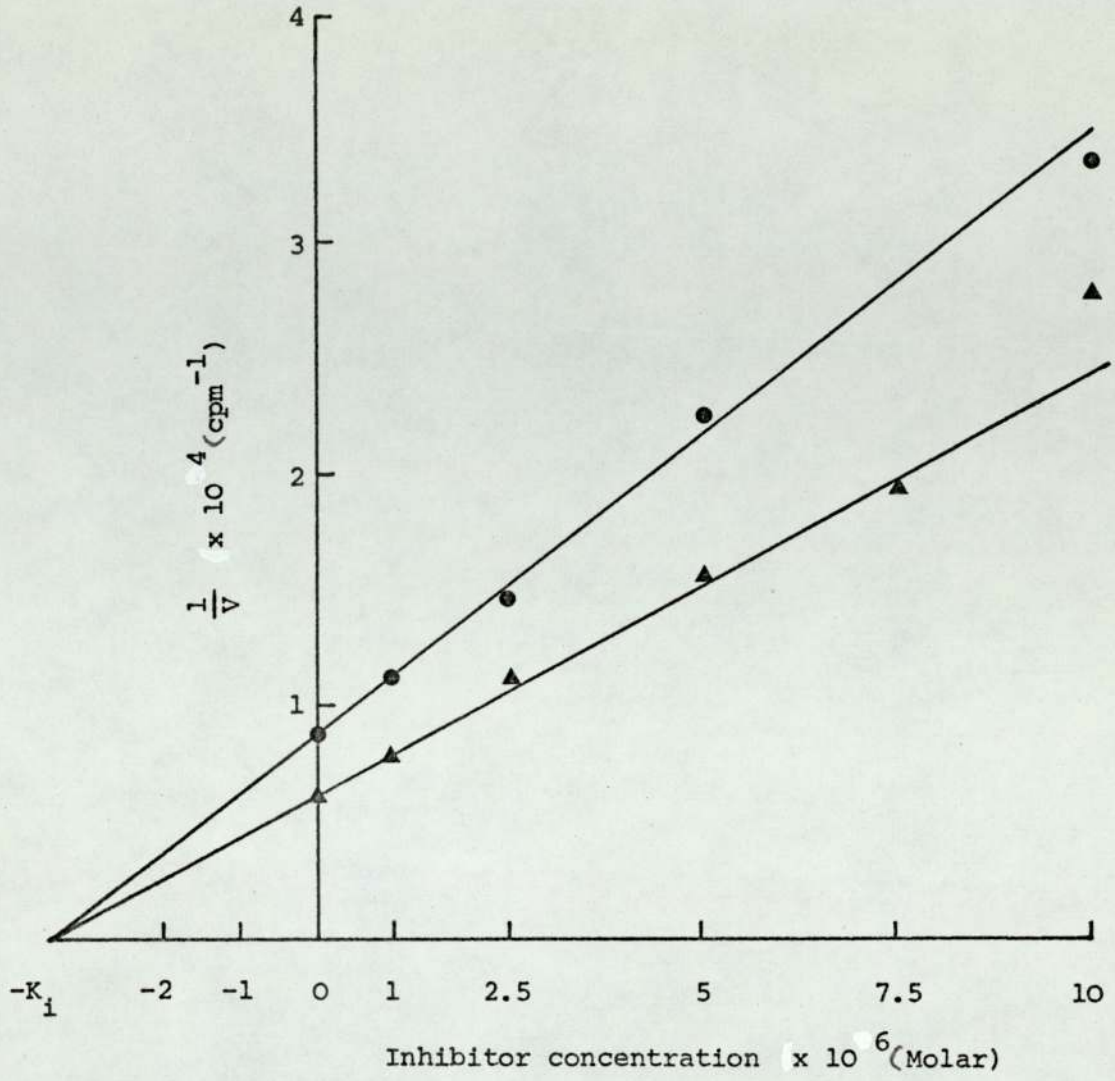


Fig. 27. Dixon plot for the inhibition of cholinesterase activity by 9-azido 10-methylacridinium methosulphate. The incubation mixture contained ChE, 14-C ACh (10^{-3}M, ● or $3x10^{-3}$M, ▲), inhibitor at the concentrations indicated above and buffer/salt/detergent mixture (See Methods). The K_i for the inhibition of ChE by the azide can be determined from the graph as $3.5x10^{-6}$M.

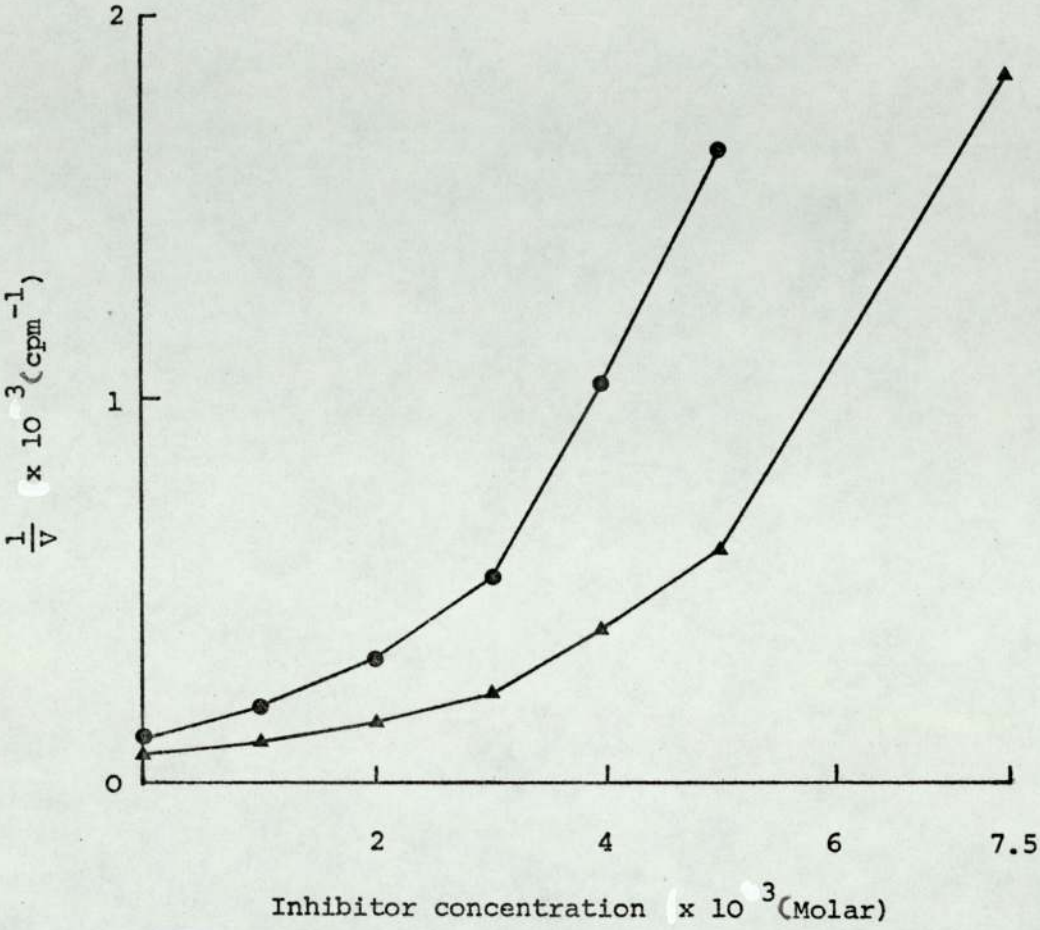


Fig. 28. Dixon plot for the inhibition of cholinesterase activity by 9-chloro 10-methylacridinium methosulphate. The incubation mixture contained ChE, 14-C ACh (10^{-3}M, ● or $3x10^{-3}$M, ▲), inhibitor at the concentrations indicated above and buffer/salt/detergent mixture (See Methods).

to be more potent than the chloro-compound in inhibiting cholinesterase and this correlates with the reported toxicity of the compounds observed in mice, thus supporting the view that the in vivo toxicity of the compounds occurs as a result of cholinesterase inhibition.

A kinetic study of the cholinesterase inhibition produced by the two compounds revealed a non-linear Dixon plot for the chloro-compound. This can arise if two or more molecules of inhibitor bind to the enzyme to inhibit access of one molecule of substrate to the enzyme active site. (Cleland, 1970; Houslay and Tipton, 1975). The kinetics of the azide suggest that only one molecule of this compound binds to the enzyme per enzyme active site. This may explain to some extent the greater anti-cholinesterase activity of the azide compared with the chloro-compound.

The greater potency of the azide could also be as a result of a stronger binding of this compound to the enzyme. This could occur if the azide was able to bind to the enzyme at both the quaternary and azide portions of the molecule, and the chloro-compound was able to bind at only its quaternary nitrogen site. This, if it occurs, may or may not be related to the number of molecules of inhibitor required to inhibit access of one substrate molecule to each enzyme active site.

The Dixon plots for the azide at the different substrate concentrations coincide on the abscissa and this suggests that the inhibitor is acting non-competitively. (Dixon, 1953). The reversible, non-competitive nature of the cholinesterase inhibition produced by the azide suggests that it binds to the enzyme at a different site from the substrate.

The anticholinesterase actions of the two methylacridiniums suggests that they may have potential use as pesticides. However, their lack of reduction of anticholinesterase activity on hydrolysis, which was first expected on the basis that their hydrolysis product N-methylacridone lacks the quaternary nitrogen, may prove to limit their value.

SUMMARY.

1. Two monoquaternary ammonium compounds, 9 azido- and 9-chloro methylacridinium methosulphate and their hydrolysis product, N-methylacridone, were examined for possible anticholinesterase activity.

2. The two methylacridiniums were shown to inhibit cholinesterase from bovine erythrocytes, horse serum, and rat brain. The azido-compound was shown to be more potent than the chloro-compound which correlates with the in vivo toxicity of the compounds in mice.

3. N-methylacridone was shown to possess a weak anticholinesterase activity which may limit the value of the methylacridiniums as pesticides since they cannot therefore be rendered harmless by hydrolysis.

4. A kinetic study of the methylacridiniums suggests that the greater anticholinesterase activity of the azide may result from the fewer molecules of this compound, compared with the chloro-compound, required to inhibit access of one substrate molecule to the enzyme active site.

CONCLUSIONS

CONCLUSIONS.

This thesis has been mainly concerned with the preparation of ChAc and the acetylation of various choline analogues by preparations of this enzyme. The results, obtained in in vitro experiments, have provided information not only on the specificity of ChAc but also on the possible mode of action of some of the analogues in the intact animal, although the limitations of extrapolating in vitro results to the in vivo situation have been recognised. The results have however indicated that the several precautions detailed below are necessary in order to ensure that the studies are concerned specifically with ChAc.

These precautions are necessary because the unsuitability of conventional purification techniques in the purification of ChAc and the lack of suitable specific inhibitors of the enzyme has meant that as yet pure preparations of ChAc cannot be obtained. Care must therefore be taken in attributing the actions of an enzyme preparation to ChAc and the present work has indicated that CarAc is probably responsible for the acetylation of carnitine by rat brain homogenates and that other acetylating enzymes present within the homogenates may be involved in the acetylation of TEC.

Distinction between ChAc and other acetylating enzymes can to some extent be made with the use of inhibitors directed against ChAc. However these inhibitors are not altogether specific for ChAc. NVP for example was shown to inhibit CarAc. The effect of ChAc inhibitors on other acetylating enzymes present within rat brain homogenates cannot be determined from the present results.

The presence of endogenous substrates within ChAc preparations can also cause some confusion in the determination of ChAc activity and in studies on the acetylation of various substrates. Although this problem can be overcome by removing endogenous substrates from the preparation by

gel filtration it has been shown that choline can be formed very rapidly in homogenates and the gel filtration must necessarily be performed immediately before the enzyme preparation is used.

Another precaution to ensure the specificity of the ChAc radiochemical assays used in the present work is to identify the product of acetylation. This not only distinguishes this product from products formed by the acetylation of substrates not under consideration but also ensures that the recovered radioactivity is not due to recovery of the initial second-substrate ^{14}C acetyl CoA or of ^{14}C acetate derived from this second-substrate. Electrophoresis has proved to be useful in identifying the products of acetylation by ChAc and results obtained with this method are of particular value when used in conjunction with quantitative determinations obtained by one of the other ChAc assay methods.

Until pure preparations of ChAc are available therefore, removal of endogenous substrates coupled with identification of the acetylated products and the lack of appearance of these products on treatment of the enzyme preparation with specific ChAc inhibitors, remain necessary precautions to ensure the specificity of the ChAc assay procedures. If these precautions are followed studies on ChAc can provide valuable information on cholinergic mechanisms and the involvement of these mechanisms in the action of drugs and possibly in disease states.

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