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A COMPARISON OF SOLUBILIZED AND MEMBRANE BOUND FORMS OF CHOLINE-O-ACETYLTRANSFERASE IN MOUSE BRAIN NERVE ENDINGS

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A COMPARISON OF SOLUBILIZED AND MEMBRANE BOUND FORMS OF CHOLINE-O-ACETYLTRANSFERASE IN MOUSE BRAIN NERVE ENDINGS

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ABSTRACT

Mouse brain choline-O-acetyltransferase (E.C. 2.3.1.6) (ChAT) activity was studied in vitro using a 100mM sodium phosphate buffer (pH 7.4) wash of a crude vesicular fraction containing solubilized ChAT and a washed crude vesicular fraction containing membrane bound ChAT. Both the solubilized and membrane bound forms of ChAT can acetylate choline linearly for 30 minutes. High concentrations of acetylcholine (Ach) can inhibit solubilized ChAT to a greater degree than the membrane bound enzyme form. Forty percent of the Ach synthesized by membrane bound ChAT survives hydrolysis in the presence of an excess of acetylcholinesterase (AchE), whereas none of the Ach synthesized by the solubilized enzyme form does. 4-(1-naphthylvinyl)-pyridine inhibits solubilized ChAT more than the membrane bound form, either in vivo or in vitro, and reduces mouse locomotor activity by 80-90% for 3 hours. Solubilized ChAT is totally sodium dependent whereas the membrane bound form is only partially sodium dependent. Membrane bound ChAT has both a high (Km 3.2 uM) and a low affinity (Km=0.48 mM) Michaelis constants as a function of added choline when velocity values are not corrected for acetylation of endogenous choline. Upon correction, only a low affinity Michaelis constant (Km=0.47 mM) is obtained for the membrane

bound enzyme form, similar to that of solubilized ChAT (Km=.17 mM). The choline analogues triethylcholine and homocholine are acetylated preferentially by membrane bound ChAT. The results suggest the existence of a membrane bound form of ChAT in mouse brain which is able to synthesize Ach.

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To my Mother, Father, Aunt, and Cousin

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INTRODUCTION

Acetylcholine is a putative transmitter in the central nervous system, and is beleived to be stored in two subcellular pools in the central cholinergic nerve ending, the cytoplasm and the synaptic vesicles (DeRobertis et al, 1963; Whittaker et al, 1964; see review by Hebb, 1972). The majoroty of the enzyme which synthesizes acetylcholine(Ach) from choline and acetyl CoA, choline-o-acetyltransferase (E.C.2.3.1.6.) (ChAT) is beleived to exist freely in the cytoplasm of the nerve ending under physiological conditions (Fonnum, 1966; 1967; 1968; 1973; 1975). The Ach stored in synaptic vesicles is beleived to originate in the cytoplasm (Fonnum, 1973; 1975). In the classical model of central cholinergic neurotransmission, Ach is synthesized in the cytoplasm and transferred to vesicles, where it is released during depolarization. However, the transfer of cytoplasmic Achinto vesicles (Marchbanks, 1968; Katz et al, 1973; Carroll and Nelson, 1978) or synaptic vesicle ghosts (Suszkiw, 1976) above diffusion levels has not been demonstrated. Recently, it has been reported that some ChAT is associated with synaptic vesicles (Hattori et al, 1976; Feigenson and Barrnett, 1977), although the relative physiological roles of the enzyme in this location and that present in the cytoplasm remain to be established.

Several investigators have suggested that a form of ChAT may be associated with the presynaptic neuronal membrane (Guyenet et al, 1973; Barker and Mittag, 1975; Haubrich and

Chippendale, 1977), and may be responsible for the efficient acetylation of choline accumulated by the high affinity choline transport system, which is beleived to be associated with cholinergic nerve endings, in contrast to the low affinity choline transport process (Kuhar et al, 1973; 1975). The high affinity transport process has a K_t value for choline in the micromolar range (Yamamura and Synder, 1972; 1973; Barker and Mittag, 1975; Carroll and Buterbaugh, 1975a; 1975b; Carroll and Gol berg, 1975) and consequently, ChAT may be associated with membranes and have a lower K_m for choline than solubilized, or cytoplasmic, ChAT, the most widely studied form of the enzyme, which has been reported to have K_m values for choline in the millimolar range (see Table 1).

Furthermore, evidence concerning the substrate specificity of ChAT indicates that a form of ChAT exists in nerve endings which is able to efficiently acetylate homocholine, a choline analogue which is not acetylated by the solubilized form of ChAT (Burgen et al, 1956; Dauterman and Mehrotra, 1963; Currier and Maunter, 1974; Collier et al, 1977), even at micromolar concentrations (Collier et al, 1977). One group of investigators has reported that 4-(1-naphthylvinyl)pyridine (4-NVP), a potent in vitro inhibitor of solubilized ChAT (Smith et al, 1967; Allen et al, 1970; Cavallito et al, 1970; Baker and Gibson, 1971; 1972; Goldberg et al, 1971; Krell and Goldberg, 1975)is only able to maximally inhibit nerve ending ChAT in vivo by 65% without affecting intracellular Ach levels (Krell and Goldberg, 1975).

One possible explanation for these results is that ChATiis an excess in the nerve ending (Schuberth et al, 1970; Carsson et al, 1972; Ross et al, 1971; Hebb, 1972; Glick et al, 1973; Saelens et al, 1973; Jenden et al, 1974; Krell and Goldberg, 1975). An Excess of ChAT in the nerve ending could account for choline acetylation from high affinity choline transport concentrations and a 65% nerve ending ChAT inhibition without an affect on Ach levels.It should be noted that nerve ending ChAT was inhibited by 65% at dosage levels of either 200 or 400mg/kg 4-NVP. Since complete inhibition of nerve ending ChAT by 4-NVP could not be reached, and the choline analogue homocholine could be acetylated by intact synaptosomes but not the solubilized preparation of nerve ending ChAT, other possible explanations may be that other forms of ChAT are present in vivo which are not inhibited by 4-NVP and are able to maintain brain Ach levels, and are also less substrate specific thansolubilized ChAT, as has been suggested by Collier et al (1977), or that compartments exist in the nerve ending which are not identically exposed to the inhibitor or choline analogues and enough ChAT is either protected from 4-NVP inhibition to maintain intracellular Ach levels, or enough ChAT is exposed to choline analogues to allow acetylation. The existence of such compartments has been suggested by studies of nerve endings and synaptosomes employing non-osmicating fixation techniques which have revealed a membranous presynaptic network (Bloom and Aghajanien, 1966; Jones, 1969; 1970) and a presynaptic vesicular grid (Jones, 1972).

The purpose of this research was to determine if a non-soluble or membrane bound form of ChAT exists in mouse brain nerve endings, and to compare some of its properti es with those of the solubilized enzyme form.

The enzyme forms used in this study were prepared from minces offforebrain of male (CF-1) albino mice, which were incubated in a 35mM K^+ Krebs bicarbonate buffer medium (pH7.4). This incubation procedure is beleived to simulate physiological stimulation (Salehmoghaddam and Collier, 1976; Carroll and Goldberg, 1976) and reduce intracellular Ach levels, which maybe inhibitory to the synthesis of Ach (Kaita and Goldberg, 1969; Glover and Potter, 1971; Harris et al, 1978). Subcellular fractions were prepared according to the method of Gray and Whittaker (1962), as modified by Collier et al (1972) and Salehmoghaddam and Collier (1976). The stnaptosomal fraction was hypo-osmotically shocked by an approximate 10 fold excess of glass distilled water (pH 4.0), and high ionic strength washes (100mM sodium phosphate buffer, pH 7.4, ionic strength 160mM) of the crude vesicular fraction, obtained after high speed centrifugation, solubilized that ChAT ionically bound to membranes (Fonnum,1966; 1967; 1968; 1975; Hebb, 1972). The washed crude vesicular fraction, containing nonionically membrane bound ChAT, was then suspended in a three fold excess of glass distilled water (pH 4.0). The enzyme activities were determined using the buffer substrate described by McCamenband Hunt (1965), containing 350uM acetyl (^{14}C) CoA. After incubation at 39 C (Hebb et al, 1975) in the prescence of added choline and an aliquot of the subcell

lular fraction being studied, (^{14}C) Ach is then separated from acetyl (^{14}C) CoA by liquid cation exchange with sodium tetraphenyl boron in a 3-heptanone (5mg/ml) at an approximate 7 fold excess, according to the method pf Fonnum (1969). Aliquots of the 3-heptanone layer were counted in a toluene based scintillation fluid, and the amount of (^{14}C) Ach produced was calculated from the known specific activity of acetyl (^{14}C) CoA. Protein determinations were done according to the method of Lowry et al (1951).

LITERATURE REVIEW

Recently, the involvment of the central cholinergic system in mental disorders has been discussed (Davies et al, 1978). Cholinergic mechanisms may play a role in Huntington's disease and tardive dyskinesia, since the use of choline chloride may improve the involuntary movements involved in these diseases (Davies et al, 1976). Choline treatment can also be associated with an improvement in the symptoms of schizophrenics (see Davies et al, 1978), and anticholinergic drugs can aggravate schizophrnia. Cholinergic underactivity may be involved in mania (Janowsky et al, 1972; Davies et al, 1975) an d euphoric manics less manic symptoms following cholinomimetic drugs, although irritable manics are not affected (Davies et al, 1978). The administration of acetylcholinesterases(AchE) inhibitors has been reported to develop depression, and/or impaired memory and concentrations (Rowntree et al, 1950; Gershon and Shaw, 1961; Bower et al, 1964; Medestein et al, 1973; Davies et al, 1977).

Choline-o-acetyltransferase (E.C.2.3.1.6.) (ChAT) catalyzes the synthesis of acetylcholine(Ach) in the following reversible reaction:

$$(CH_3)_3\overset{+}{N}CH_2CH_2OH + CoAS\text{-}\overset{O}{\overset{\|}{C}}\text{-}CH_3 = (CH_3)_3\overset{+}{N}CH_2CH_2O\text{-}\overset{O}{\overset{\|}{C}}\text{-}CH_3 + CoA$$

choline acetylCoA acetylcholine

ChAT was first discovered in tissue containing cholinergic nerve endings by Nachmansohn and Machado (1943), but it has been shown that the enzyme is present in nervous tissue rather than the tissue innervated (Bannister and Scrase, 1950; Collier and Katz, 1971; Hebb et al, 1964; Potter, 1970)..

ChAT is also found in the non-innervated placenta of higher primates (man and Rhesus monkey) although its functional role has not been determined (Hebb and Ratkovic, 1962; Morris 1966; Morris et al, 1971; Rama Sastry and Hendersen, 1972; White and Wu, 1973; Rama Sastry et al, 1976; Welch, 1976). ChAT is found in the bacterium, Lactobacillus Plantarum (Stevenson and Rowatt, 1947; White and Cavallito, 1970), and in the gill plates of Mytilus edulis (Bulbring et al, 1953).

In nervous tissue, the distribution of the enzyme and ChAT activity is similar to the distribution of Ach and cholinergic nerves (Feldberg, 1945; Hebb and Morris, 1969; Kasa, 1971). The concentration and total amount of enzyme in human brain increases during fetal development (Bull et al, 1970); however, the level of ChAT activity in adult brain is less than the ChAT activity in nine year old human brain (Bull et al, 1970).

ChAT is synthesized within the cell bodies of cholinergic neurons and transported to the nerve terminal (Hebb et al, 1964) by fast or intermediate axoplasmic transport (Fonnum, 1973) and its half life has been reported in the range of 12-21 days (Fonnum, 1973; Oesch, 1974). Neuronal activity may induce new ChAT synthesis (Oech, 1974). Only 10% of the total amount of ChAT present in ventral spinal neyrons is located in the cell body (Weil et al, 1977).

Under phsiological conditions, it is beleived that the majority of the enzyme is localized freely in the cytoplasm of the nerve ending (Fonnum, 1966; 1967; 1968; 1970; 1975) and not associated with synaptic vesicles or other membrane

organelles in the presynaptic neuron (Whittaker et al, 1964). Vesicles containing Ach have been discovered in the nerve ending (De Robertis et al, 1963; Gray and Whittaker, 1962; Whittaker et al, 1964; Whittaker and Sheridan, 1965) and release of Ach from these vesicles has been postulated in accordance with the quantal hypothesis (Fatt and Katz, 1952). This hypothesis states that Ach, and other neurotransmitters within the nerve terminal, are released in discrete units, and elicit miniature endplate potentials (m.e.p.p.'s) of equal magnitude in the abscence of depolarization and a nerve impulse stimulates the nerve terminal to release quanta simultaneously, causing an increase in the frequency of m.e.p.p.'s, resulting in an end plate potential (e.p.p.).

The subcellular distribution of the enzyme is known to be be dependent and influenced by the conditions of synaptosomal hypo-osmotic shock (Fonnum, 1966; 1967; McCamen et al, 1965; Tucek,1966). For all species, however, an increase in the ionic strength at physiological pH solubilizes ChAT from membranes, and a decrease in the ionic strength facilitates binding of the enzyme to membranes (Fonnum, 1966; 1967; 1968; Potter et al, 1968). The binding of ChAT to membranes is beleived to result from an ionic attraction between a positively charged enzyme and a negatively charged membrane (Fonnum, 1968). The isoelectric point and surface charge of ChAT differ in pigeon and guinea pig brain (Fonnum, 1968; Malthe-Sorensson and Fonnum, 1972), perhaps explaining the species variability observed in subcellular distribution studies.

Two different forms of partially purified ChAT have been identified in human neostriatal tissue and can be distinguished by elution patterns from phosphocellulose columns, stability, and antibody producing capacity (Singh et al, 1975). ChATmmayy also exist in at least two forms in rat and cat brain (Malthe-Sorensson, 1971; 1972) and in three forms in rat hippocampus, caudate nucleus, and sciatic nerve, indicating that in some species, closely related molecular forms of ChAT exist and these forms may differ in their membrane affinity.

The molecular weight of purified ChAT has been reported to be from 35,000 daltons (White and Cavallito, 1970) to 1,500,000 daltons (Chao and Wolfgram, 1974). The higher molecular weight forms of ChAT may have been produced by treatment of the initial extract with ammonium sulfate, since Chao and Wolfgram (1974) have reported that this treatment may cause aggregation of ChAT. It has been shown that storage for 2 to 3 weeks at -20 C causes disappearance of the higher molecular weight forms (Banns, 1976). The apparent ammonium sulfate effect may be partially responsible for the multiple forms of ChAT found in rat and cat brains by isoelectric focusing techniques (Malthe-Sorensson and Fonnum, 1972) and the multiple forms found in human brain and sciatic nerve (White and Wu, 1973), since both groups of investgators used ammonium sulfate in their purification procedures. Evidence for isozymes of the 65,000 dalton form of ChAT may be limited to the asymmetrical peak of enzyme activity obtained by Glover and Potter (1971). In contrast, the 65,000 dalton form of ChAT obtained by Chao and Wolfgram (1974) without the use of

ammonium sulfate appeared to be of only one molecular species o f ChAT. In a later publication, Chao (1976) purified ChAT and found its molecular wieght to be 87,000 to 89,000 daltons and to have 6 identical subunits, although these are resistant to separation by a variety of techniques (Weber and Osborn, 1969; Swank and Munkres, 1971; Peterson, 1972; Moss and Rosenblum, 1972; Davies and Stark, 1970; Chao and Wolfgram, 1973). The amino acid sequence of the enzyme is known (Chao, 1976).

It should be noted that 15-30% of the total nerve ending ChAT remains attached to membranes (Hebb, 1972) and is beleived to be nonionically membrane bound (Kucenski et al, 1975). The cytochemical studies of Hattori et al (1976) and Feigenson and Barrnett (1977) have suggested that at least some synaptosomal ChAT adheres to vesicles, and other investigations indicate that ChAT may be within vesicles (Hattori et al, 1978) and associated with the membrane of the presynaptic nerve ending (Barker and Mittag, 1975; Haubrich and Chippendale, 1977).

THE MECHANISM OF SOLUBILIZED ChAT

The generally accepted mechanism for solubilized ChAT is the Theorell-Chance mechanism (Cleland, 1963) based on kinetic studies (White and Cavallito, 1970; Morris et al, 1971; Hendersen and Rama Sastry, 1972; White and Wu, 1973; Emson et al, 1974), which predicts that the formation of an intermediate enzyme-acetyl CoA-choline complex is immediately followed by product formation, this permitting only a low concentration of intermediate to exist (Currier and Mautner, 1974; Malthe-Sorensson, 1976). Inhibition studies suggest the involvements of both a

thiol group (Potter et al, 1968; Mannervik and Sorbo, 1970; Roskowski, 1973) and a histidine residue (White and Cavallito, 1970; Currier and Mautner, 1974; Roskowski, 1974). Thiol reagents such as p-chloromercuribenzoate, N-ethyl maleimide, iodoacetate, and 5,5'dithiobis-(2-nitrobenzoate (DTNTB) appear to inhibit the enzyme, and acetyl CoA protects the enzyme from inhibition (Malthe-Sorensson, 1976; Berman-Reisburg, 1957; Potter et al, 1968; Mannervik and Sorbo, 1970; Roskowski, 1974). However, the possibility exists that the action of the thiol reagents may be more peripheral in nature, causing conformational c anges and denaturation (White and Cavallito, 1970; Mannervik and Sorbo, 1970). Other complications include species differences in the sensitivity of ChAT to such reagents (Glazer, 1970; White and Cavallito, 1970).

The essential involvement of a histidine residue has been implicated by Malthe-Sorensson (1976) by incubation of the purified enzyme with diethylpyrocarbonate, and the formation of N-carbethoxy histidine residues was measured. Imidazole residues have also been implied to be related to Ach synthesis by Hebb et al, (1975) and it can catalyze the formation of Ach nonenzymatically (Burt and Silver, 1973; Aquilonius, 1976). Imidazole has also been shown to activatew ChAT, sometimes by 80-100% (White and Cavallito, 1970; Hebb et al, 1975).

Active ChAT has been immobilized on mercurisepharose gels (Malthe-Sorensson, 1976). The validity of this technique to study an enzymatic reaction may be questioned since it has beenshown that the immobiliza ion of enzymes to different types of supports changes their affinities for substrates, their pH optimum (Golstein et al, 1964; Homby et al, 1968) and their

reaction kinetics (Kasche et al, 1971; Goldman et al, 1971). However, the number and type of different residues involved in the reaction meahanism are not beleived to change. The binding of ChAT to mercurisepharose gels is beleived to occurr at the thiol group in the active site, since preincubation of the enzyme in acetyl CoA inhibited binding to the gel. The bound enzyme is able to form Ach, indicating a nonessential thiol group (Malthe-Sorensson, 1976).

Further evidence implicating a functional histidine residue is provided by the fact that diethylcarbonate prevents the immobilized enzyme to form ACH (Malthe-Sorensson, 1976). In such a reaction meahanism, the rate limiting step would be the nucleophilic attack of histidine on acetylCoA with a fast reaction with choline (Bruice, 1959).

In a more recent study, it has been suggested that choline and acetyl CoA bind in a random, not ordered, mechanism, since dead end complexes of enzyme-acetyl CoA Ach and enzyme-choline-CoA were present, and the dossociation of CoA appeared rate limiting in the forward reaction (Hersh and Peet, 1978).

The effect of sodium chloride on the synthesis of Ach in rat striated synaptosomes has been investigated (Guyenet et al, 1973), and decreased sodium concentrations are associated with decreased synthesis of Ach from glucose, but this inhibition can be reversed by the addition of choline, indicating that the reduction of Ach synthesis is not due to impaired metabolic activity (Guyenet et al, 1973).

Rossier et al (1977) have found that the chloride anion

activates the V_{max} of unpurified solubilized ChAT from rat brain more effectively than several other anions, wheras the cation only affects the V_{max} slightly. These investigators and others (Glover and Potter, 1971) have reported that purification procedures can affect the response of the enzyme to salts, and the enzyme's K_m for choline and acetylCoA. Hersh et al, (1978) using a highly purified preparation of solubilized ChAT, has found that its activity is regulated by ionic strength, and divalent cations such as Mg^{+2} and Ca^{+2} inhibit the enzyme already activated by Na^{+}. In this study, the total anionic strength was able to influence ChAT's activity, and no selectivity for Cl^{-} was found.

Therefore, it appears that solubilized ChAT is able to catalyze the synthesis of Ach through an acetyl transfer by a histidine residue in the active site, and an apparently nonessential thiol is also in or near the active site (Currier and Mautner, 1974; Malthe-Sorensson, 1976). Furthermore, at physiological ionic strength, unpurified ChAT is activated by both sodium (Guyenet et al, 1973) and chloride inns (Rossier et al, 1977), suggesting that either or both may be necessary for the regulation and maintenance of ChAT activity(Hersh et al, 1978)

Kinetic studies, in addition to providing information about the mechanism of ChAT, also provide information concerning the concentrations of the substrates likely to be required for the synthesis of Ach in living tissue. Product inhibition studies can also reveal mechanisms by which ChAT may be regulated. K_m values for choline and acetyl CoA are

consistent across several species (see Table 1). It should be noted that Kucenski et al (1975) has described a membrane bound form of ChAT in rat brain, and found it to be kinetically similar to solubilized ChAT.

Although the mechanism of the reaction has not been elucidated with available kinetic evidence, it is generally agreed that a sequential mechanism exists and both substrates must interact with the enzyme before any products are released, (see review by Hebb, 1972). The products, Ach and CoA, have both been found to be inhibitory to Ach synthesis (Potter et al, 1968; Kaita and Goldberg, 1969; Morris and Hebb, 1970; Morris et al, 1971; Glover and Potter, 1971; White and Wu, 1973), and CoA has a K_i similar to the K_m of acetyl CoA. It should be noted that although high concentrations of Ach can be inhibitory to Ach production, the maximum ChAT inhibition attained by Ach is 50% (Potter et al, 1968; Kaita and Goldberg, 1969; Glover and Potter, 1971).

INHIBITORS OF ChAT

To date, no inhibitor of ChAT has been found that produces effects on cholinergic transmission due to a direct inhibition of ChAT. In vitro inhibitors of ChAT include styrylpyridine analogues such as 4-(1-naphthylvinyl)pyridine (4-NVP), which are able to inhibit AchE to varying degrees (Smith et al, 1967; Cavallito et al, 1970; Goldberg et al, 1971; Krell and Goldberg, 1975), halogenated analogues of Ach (Chaseaand Tubbs, 1966; Morris and Grewaal, 1969; 1971), acroyloyl choline (Malthe-Sorensson, 1974), acetylsecohemicholinium-3 (Domino et al, 1973)

and 3-bromoacetonyl-trimethylammonium bromide., or bromoketone (Persson et al, 1967), although this last inhibitor has been shown to inhibit an enzyme capable of hydrolyzing acetylCoA in Purkinje cells of the cerebellum and sensory root ganglion cells (Kasa et al, 1970).

Of the styrylpyridine analogues, Hebb (1972) has suggested that 4-NVP would inhibit ChAT specifically and not have significant action on cholinesterases. Goldberg et al (1971) has found that 4-NVP inhibits mouse brain ChAT in adose dependent manner in the range of 25 to 100mg/kg in vivo, but maximum inhibition (*#83%) occurred 4 hours after a 200mg/kg i.p. injection. Signs of depression and ataxia were observed at this dose. 24 hours after a 200mg/kg dose, no inhibition of ChAT was detectable. 4-NVP was able to inhibit the hexabarbitone-metabolizing enzyme system more effectively than ChAT in vitro, and the sleep time of mice was potentiated 3.5 fold by an i.p. dose of 25mg/kg of 4-NVP when given 30 minutes before barbituate treatment. The acute i.p. LD50 for 4-NVP at 24 hours was 337mg/kg.These investigations have also found that 4-NVP potentiated the behavoral effects of the AChE inhibitor physostigmine. Repeated attempt by several investigators to administer 4-NVP in vivo and reduce the levels of brain Ach levels have been unsuccessful (Carson, et al, 1972; Glick et al, 1973; Haubrich et al, 1974; Krell and Goldberg, 1975). One report indicates that i.p. administration of 200 or 400 mg/kg of 4-NVP to mice inhibits brain ChAT activity 65% without altering brain levels of Ach (Krell and Goldberg, 1975).

Furthermore, Feigenson and Barrnett (1977) have reported in vitro with a partially purified mouse brain ChAT preparation 65% inhibition was attained with either a 5or 10 uM concentration of 4-NVP. Also, Aquilonius et al (1970) was unable to demonstrate any effect of 4-NVP on neuromuscular transmission in the cat that would be related to ChAT inhibition. These results were interpreted to indicate that brain ChAT activuty is not rate limiying in the formation of Ach by the nerve ending. An alternative possibility is that the majority of brain Ach may be synthrsized by ChAT which is in a subcellular location that is inaccessible to the inhibitor.
The in vivo administration of bromoacetylcholine , an effective in vitro unhibitor of ChAT (Morris and Grewaal, 1969; 1971), has been unsuccessful in reducing rat brain Ach levels, even after 93% of the AchE had been inhibited, thus inhibiting the hydrolysis of the inhibitor, bromoacetylcholine ($peth et al, 1976). No change in brain Ach levels were found, suggesting that either 7% of the normal AchE activity was sufficient to hydrolyze the inbibitor, or that spontaneous hydrolysis of the inhibitor occurred, since Hebb (1972) had found that at neutral pH, these analogues are unstable.

CHOLINE

Free choline does not appear to be highly concentrated in cholinergic nerve endings (Mann and Hebb, 1977), its concentration in rat brain tissue being approximately 33uM (Stavinoha and Weintraub, 1974), about one tenth the K_m for choline of

solubilized ChAT. Therefore, changes in the intracellular choline concentration would be expected to affect Ach synthesis. The rate of release of Ach from the superior cervical ganglion of cat (Collier and Katz, 1974), rat (Sacchi et al, 1978), and mouse brain minces (Carroll and Goldberg, 1975) is enhanced when choline is present int perfusing or bathing medium. Choline induces an increase in the concentration of Ach in the hippocampus, a brain reqion rich in cholinergic nerve endings but not cell bodies, and denervation of rat adrenal gland abolishes this increase in Ach following choline administration (Haubrich and Chippenadale, 1977).

Sources of free choline in the brain include dee novo synthesis, an extremely slow process if present at all(Freeman and Jenden, 1976), phospholipid degradation, since choline phospholipids can be synthesized in the brain or transported to the brain via the blood, suggesting that these compounds can be catabolized to form free choline (Ansee and Spanner, 1968; 1971; 1975; Illingworth and Portman, 1972; Hoelzl and Franck, 1973; Orlando et al, 1975), a base excange reaction in which choline may enter or be released from phosphatidyl choline directly (Abdel-Latif and Smith, 1972; Brammer and Sheltawg, 1975; Dahlberg and Schuberth, 1977), and plasma choline which crosses the bl blood brain barrier, either by a saturatable uptake system (Sparf, 1973;Cornfed et al, 1977) or by diffusion (Freeman et al, 1975). Another source of choline in the brain is that choline produced by the hydrolysis of Ach b y AchE.

The concentration of choline in the synaptic cleft produced in this way has been estimated to be in the millimolar range (Whittaker, 1963).

In light of the several sources of choline present in brain, it should be noted that the majority of releaseable Ach is derved frm extracellular choline (Gardiner, 1961; Browning and Schulman, 1968; Carroll and Goldberg, 1975), and that newly synthesized Ach is preferentialy released over preformed Ach (see review by Hebb, 1972). However, it has been shown that brain tissue can synthesize relatively large amounts of Ach when incubated in the abscence of choline (Browning and Schulman, 1968; Collier et al, 1972) indicating that both endogenous and exogenous stores of choline can be used for Ach synthesis.

Extracellular choline is transported into synaptosomes (Yamamura and Synder, 1972; 1973; Barker an d Mittag, 1975; Carroll and Buterbaugh, 1975) and minces (Carroll and Goldberg, 1975) prepared from brain by two distinct uptake mechanisms, a high affinity system with a K_t of 0.5 to 5uM and a low affinity system with a K_T of 50-100uM. Most of the choline transported into synaptosomes by high affinity transport is beleived to be converted to Ach (Barker and Mittag, 1975).

The high affinity choline transport system is beleived to be localized specifically in cholinergic nerve terminals in brain (Kuhar et al, 1973; 1975) since denervation studies have shown that depletion of cholinergic nerve terminals is accompanied by a selective loss of high affinity choline transport [illegible] Sodium and chloride ions are beleived to be necessary for high affinity transport (Guyenet et al, 19

et al,1973; Haga and Noda, 1973; Simon and Kuhar, 1976; Kuhar 1978), as is a source of energy, since the metabolic inhibitor 2,4-dinitrophenol inhibits high affinity transport by 25% (Dowdall and Simon, 1973; Yamamura and Synder, 1973; Simon and Kuhar, 1976), and a sodium-potassium ATPase may be involved (chultz and Curran, 1970).

Some attempts to couple high affinity choline transport and choline acetylation have suggested that choline must cross the high affinity transport system to be acetylated (Lefresne et al, 1975),while others (Barker and Mittag, 1975; Suszkiw and Pilar, 1976; Jope and Jenden, 1977) have fevored a kinetic coupling between transport and enzyme (controlled by the intracellular choline concentration available for Ach synthesis). Atterwill and Prince (1978) have suggested that one of the molecular forms of ChAT, which is present in adult rat brain but not in 7 day old brain, may be responsible for the couppling of high affinity choline uptake and choline acetylation. However, experiments with cat superior cervical ganglion suggest a lack of coupling (Collier and Ilson, 1977; Colli r et al, 1977) and in brain tissue (Barker, 1978).

In depolarized in vitro preparations, high affinity transpopt is reduced or lost, and choline taken up by the low affinity system is acetylated effeciently (Carroll and Goldberg, 1975; Murrin and Kuhar, 1976; Vaca and Beach, 1977). However, direct electrical stimulation of cholinergic tracts and administration of convulsant drugs in vivo have been shown to increase the Vm_{ax} of high affinity choline transport (Atweh, et al, 1975; Simon and Kuhar, 1975; Jenden et al, 1976; Simon et al, 1976).

In contrast to the high affinity choline transport system, low affinity transport is not beleived to be specific to cholinergic nerve endings (Diamond and Kennedy, 1969; Yamamura and Synder, 1973) and may supply choline for phospholipid synthesis.

Parental administration of choline may result in an increase in plasma and brain choline levels and barin Ach levels (Haubrich et al, 1974; 1975; Cohen and Wurtman, 1975). These results may suggest that the low affinity choline uptake is involved in Ach formation, and the elevation of plasma choline may promote Ach synthesis at sites remote from choli ergic terminals where only the low affinity transport system is operating. However, carnitine acetyltransferase, an enzyme present in brain tissue, may be able to acetylate chol ne (White and Wu, 1973; Schrier et al, 1974), although Bradshaw and Hemsworth (1976) have shown that carnitine is acewtylated 200 times as well as choline by carnitine acetyltransferase.

CHOLINE ANALOGUES

ChAT is not absolutely specific for its physiological substrate, choline (Hemsworth and Smith, 1970; Currier and Mautner, 1974; Barker and Mittag, 1975; Mann and Hebb, 1975; Collier et al, 1977; Ilson et al, 1977).

It has been assumed that the inhibition of Ach synthesis in synaptosomes by hemicholinium-3 (HC-3) is due to the inhibition of the choline uptake mechanism (Diamond and Kennedy, 1969; Fonnum, 1973). It has been argued, hoever, tthat the reversal of this inh bition by the addition of choline occurrs too slowly to be accounted for by a mechanism on the outside

of the presynaptic membrane (such as a transport carrier), and that this reversal is more likely to occurr within the nerve ending (MacIntosh, 1963). In this regard several investigators have reported that HC-3 can be acetylated by partially purified ChAT, although the identification of the acetylated product was incomplete (Arnaiz et al, 1970; Mann and Hebb, 1975; Barker and Mittag, 1975).

The choline analogue triethylcholine (TEC) is capable of prejunctional blocking action at neuromuscular junction (Bowman and Rand, 1961; Bowman and Hemsworth, 1965; Bowman et al, 1967), and Bull and Hemsworth (1965) and Potter (1968) have shown that TEC inhibits the transport of choline into nerve endings, suggesting that the pharmacological action of TEC is a reult of the depletion of available choline for Ach synthesis, leading to a decreased amount of releaseable Ach. In vitro, partially purified ChAT is capable of acetylating the mono-, di-, and triethyl analogues of choline, with monoehtylcholine (MEC) acetylated almost as readily as choline, and the di- and triethyl choline analogues being less acceptable as substrates, respectively (Hemsworth and Smith, 1970). TEC was also found to be acetylated in vitro with a K_m of 50mM (Mann and Hebb, 1975; Barker and Mittag, 1975). These studies were important since they suggest that TEC could be acetylated in vivo, and possibly form a false transmitter in cholinergic nerve endings. A false transmitter is a substance which is stored by a nerve terminal and released upon nerve stimulation under conditions which cause the release of the physiological transmitter.

Ilson and Collier (1975) have demonstrated that the superior cervical ganglion of the cat is capable of taking up TEC, acetylating TEC, and releasing acetyl-TEC in the prescence of Ca^{+2}. Preganglionic nerve stimulation increases both the uptake and acetylation of TEC, al hough the large proportion of transported unacetylated TEC (76% of normal) Suggests that the process of transport and acetylation are independent processes in ganglia (Ilson et al, 1977), at variance to the suggestion that choline uptake and acetylation are coupled (Barker and Mittag, 1975). In the rat cerebral cortex, 88% of the total amount of TEC taken up remained unacetylated, and only 3% was acetylated after the tissue had been depolarized for 48 minutes prior to exposure to nondepolarizing medium with 10uM TEC (Ilson et al, 1977).The characteristics of the release of acetylTEC from brain tissue was similar to the characteristics of Ach release in brain tissue (Ilsonb et al, 1977); release was stimulated by high K^+ and atropine (Polak and Meuws, 1966; Bertel-Meuws and Polak, 1968) and was Ca^{+2} dependent (Molenaar and Polak, 1970; Molenaar et al, 1973; Carroll and Goldberg, 1975).

The choline analogue homocholine (3-trimethylamino-propan-1-ol) has been shown to block choline uptake in synaptosomes (Simon et al, 1975) and to be taken up by both a high (K_t=3uM) and a low affinity (K_t=14.5uM) synaptosomal uptake system (Collier et al, 1977). Partially purified preparations of ChAT are unable to acetylate this analogue (Currier and Mautner, 1974; Collier et al, 1977), although synaptosomes are capable of forming acetyl-homocholine (Collier et al, 1977).

At 3uM homocholine, the percent acetylation of the total amount of homocholine transported by synaptosomes is 32%. The percent acetylation of total choline taken up by synaptosomes from a 3uM choline concentration 50.5%, indicating that under these conditions, homocholine is almost as acceptable a substrate as choline (Collier et al, 1977). Synaptosomes incybated in higher concentrations of homocholine acetylated a much lower percentage of the total taken up than synaptosomes incubated in higher concentrations of choline (Collier et al, 1977).

Preganglionic herve stimulation of sympathetic ganglion accelerates the acrtylation of homocholine in the prescence of physostigmine, and the amount is 70% of the amount of choline acetylated under comparable conditions (Collier et al, 1976), although homocholine acetylation cannot be demonstrated with less than 60 minute stimulation (Collier et al, 1977). The amount of ho ocholine acetylated by stimulated ganglia is not enough to replace all the Ach released by nerve stimulation, and Collier e al (1977) has suggested that the rate of acetylation is limiting. Stimulated non-eserinized ganglia acetylated less than 10% of the accumulated homocholine, and release of acetyl-homocholine is Ca^{+2} dependent (Collier et al, 1977).

ACETYL CoA AND ANALOGUES

The acetyl CoA analogues, propionyl CoA and butryl CoA, can be used for substrates for rat brain ChAT and have affinities equal to that of acetyl CoA, although the V_{max} is decreased as the acyl chain lengthens (Rossier, 1977).

Acetyl

Acetyl CoA for Ach synthesis can be produced inside the nerve ending from extracellular glucose, lactate, or pyruvate, but not exracellular succinate, citrate or acetate (Browning and Schulman, 1968; Jope et al, 1978). Acetyl CoA is formed from pyruvate, NAD^+, and CoA by pyruvate dehydogenaase within the mitochondria (Sandi, 1963; Tucek, 1967). Transport of acetyl CoA directly out of the mitochondria is unlikely, since the mitochondrial membrane is poorly permeable to acetyl CoA (Tucek, 1967; Lowenstein, 1968). Tucek (1970) has suggested that mitochondrial contraction may promote lleakage of acetyl CoA out of mitochondria. Other transfer mechanisms of possible acetyl CoA precursors out of mitochondria i clude transfer as citrate, acetate, and transfer as acetylcarnitine. However, the enzymes that catalyze the formation of acetyl CoA from acetate, acetyl CoA synthetase (E.C.6.2.1.1., acetate:CoA ligase), or from acetylcarnitine, carnitine acetyltransferase (E.C. 2.3.1.7. acetyl CoA:carnitine-o-acetyltransferase) are found within the mitochondria (Barker et al, 1968; Neidle et al, 1969). Acetyl CoA synthetase is not foynd in sufficient quantities in the cytoplasm to maintain Ach synthesis (Tucek, 1967).

Citrate is known to be transported into the cytoplasm of the cell from mitochondria for fatty acid synthesis (Barker et al, 1968). Citrate thus transported could then be converted to acetyl CoA by the cytoplasmic emzyne citrate ATP lyase (Tucek, 1967). It has been suggested that over 80% of the extramitochondrial acetyl CoA produced from pyruvate in rat liver mitochondria is supplied via citrate ATP lyase (

citrate ATP lyase (Daikuhara et al, 1968).-(-)Hydroxycitrate, an inhibitor of citrate ATP lyase, also inhibits incorporation of glucose into brain lipids, suggesting that citrate conversion to cytoplasmic acetyl CoA is involved in brain lipid synthesis (Watson et al, 1969; Patel and Owen, 1976). Work wth mixed label glucose has suggested that citrate is an intermediate in AcH synthesis (Sollenberg and Sorbo, 1970) and as the immediate precursor to the acetyl moiety of Ach (Sterling and O'Neill, 1978). The latter investigators have shown that the $^{3}H/^{14}C$ ratio of Ach produced is equivalent to the $^{3}H/^{14}C$ ratio of acetyl CoA produced by citrate ATP lyase, the citrate being produced intramitochondrially from 6(^{3}H,^{14}C)glucose. However, the $^{3}H/^{14}C$ ratio of Ach produced in the prescence of inhibitors of citrate cleavage, such as -(-)hydroxycitrate, and n-butyl malonate, an inhibitor of citrate transport (Robinson and Chappell, 1967) is significantly higher than the ratio obtained in the abscence of inhibitors (Sterling and O'Neill, 1978), suggesting that alternative sources of acetyl CoA for Ach synthesis exist. It is important to note, however, that other investigations (Tucek and Cheng, 1974; Lefresne et al, 1977) have found that labelled citrate, which had entered the tissue, is not significantly incorpoated into Ach either in vitro or in vivo.

Feigenson and Barrnett (1977) have suggested that endogenous acetyl CoA stores are capable of producing a significant amount of Ach. It should be noted, however, that the source of endogenous acetyl CoA is undetermined, and could be formed by alternate pathways to the citrate pathway. In brain tissue,

acetate is not a precursor for Ach formation (Scuberth, 1965; Tucek, 1967; Neidle et al, 1969; Aas, 1971), as it is in neuromuscular junction (Dreyfus, 1975). Recently, diebler and Morot-Gaudry (1977) have found that, in the electric organ of Torpedo marmaruta, acetyl CoA synthetase is 30 times less efficient in producing acetyl CoA than ChAT under optimum conditions, suggesting that this could be an alternative pathway of acetyl CoA production in brain tissue as well. Gibson and Blass (1976) have reported that Ach synthesis in mouse and rat brain is related to the NAD/NADH ratio in both the cytoplasm and across mitochondrial membranes, but may not be related to a decrease in ATP or adenylate energy charge. These results imply that several sources of acetyl CoA fo r Ach synthesis may exist.

REGULATION OF ChAT

Regulation of ChAT activity is beleived to exist peripherally, since Ach release promotes increased synthesis so that tissue levels are maintained at normal levels (Birks and MacIntosh, 1961; Br wning ang Schulman, 1968; Carroll and Goldberg, 1975; Sacchi et al, 1978). This regulation may occurr by product inhibition, but Ach has been found to be a weak inhibitor and is only capable of a 50% inhibition of enzyme activity at Ach concentrations of 100mM or more (Potter et al,1968; Kaita and Goldberg, 1969; Glover and Potter, 1971). Heterogeneity may exisrt, since Singh et al (1975) have reported that one molecular form of ChAT, a low activity form, is less resistant to Ach inhibition than a higher activity form. CoA is a much more effective inhibitor of Ach synthesis, but the accumulation

of CoA under physiological conditions is thought to be unlikely, since the ratio of CoA to acetyl CoA would be expected to change during Ach release, and these changes would affect the synthesis of other acetyl CoA requiring materials such as fatty acids (Hebb, 1972).

Another possible way ChAT may be regulated is by substrate availability. For acetyl CoA to limit the activity of ChAT, its availability would have to increase during Ach release. While no direct evidence exists that supports this possibility, it is beleived that Na^+, whose intracellular levels increase as a result of depolarization, increase the oxidation of glucose to CO_2 ((Diamond, 1971), suggesting that the amount of Krebs cycle intermediates produced may be enhanced. It should be noted that Ach synthesis is beleived to be somewhat Na^+ dependent (Mann and Hebb, 1975; Hersh et al, 1978; Jope and Jenden, 1978), as is high affinity choline uptake (see Kuhar and Murrin, 1978). Conditions of hypoxia or hypogylcemia inhibit the production of Ach (Gibson and Blass, 1977).

The rate of Ach synthesis in nerve terminals may be related to high affinity choline uptake (Barker and Mittag, 1975; Guyenet et al, 1973), and this uptake mechanism may be stimulated by in vivo treatment with drugs that lower levels of brain Ach (Jenden et al, 1976) or direct electrical stimulation that stimulates nervous activity (Simon and Kuhar, 1975; Atweh et al, 1975; Simon et al, 1976). High affinity choline transport is reduced or lost in depolarized in vitro preparations (Carroll and Goldberg, 1975; Murrin and Kuhar, 1976; Vaca and Beach, 1977), and choline uptake is accelerated (Roskowski, 1978),

probably mediated by the low affinity transport system, and acetylated efficiently, Experiments with sympathetic ganglia suggest that preganglionic nerve stimulation increases the total uptake of choline (Collier and MacIntosh, 1 969; Collier and Katz, 1976), and de-emphasizes the importance of high affinity choline uptake for Ach synthesis in ganglia. Furthermore, lesioning of the preganglionic nerve terminal has no influence on choline transport supplying the substrate for acetylation (Katz and Collier, 1974).

It has been suggested that neuronal activity may increase the production of ChAT in the cell body (Oech, 1974), but it is beleived that enzyme activity is not rate limiting for Ach synthesis, since Ach synthesis by brain in vivo is approximately 30 fold less than that found in vitro (Schuberth et al, 1970; Hebb, 1972; Saelens et al, 1973; Jenden et al, 1974) and inhibitors of ChAT do not appear to lower Ach levels (Glick et al, 1973; Krell and Goldberg, 1975). If neuronal activity enhances ChAT production and ChAT were rate limiting, the amount of Ach synthesized in the tissue during depolarization would be expected to increase. This is not beleived to occurr peripherally. It may be possible that the enzyme may be released during depolarization, since dopamine beta hydroxylase has been shown to be released (DePotter and Chubb, 1971).

THE QUANTAL THEORY AND VESICLE HYPOTHESIS

The existence of minute transient fluctuations in voltage (m.e.p.p.'s) at neuromuscular junctions (Fatt and Katz, 1950; 1952) and at central synapses in spinal motoneurons (Katz and

Miledi, 1963) are beleiv d to represent the release of quantal units of transmitter, such as Ach, into the synaptic cleft. Furthermore, m.e.p.p.'s exhobit a regular size and time course, indicating that these changes in the end plate potential are not due to leakage of Ach, but from the simultaneous release of thousands of Ach molecules which are highly concentrated (Katz, 1966). The realease of these multimolecular packets of Ach in an all or none fashion from discrete points of the terminal axon menbrane is the basis of quantal spontaneous release (Katz, 1966). Eachquantum of Ach is thought to contain 5,000 to 10,000 molecules, and the release of large numbers of quanta is thought to cause the generation of an end plate potential or a postsynaptic potential (Katz and Miledi, 1963; Katz, 1966; 1969).

The significance of the quantal theory was enhanced by the electron-microscopic observation that synaptic vesicles (40-50nm in diameter) were concentrated at both neuromuscular junctions (Robertson, 1956) and central synapses (DeRobertis and Bennet, 1954; 1955; Fernandez-Moran, 1957). Subcellular fractionation studies found that pinched off nerve endings, or synaptosomes, contained Ach, ChAT, and AchE, and vesicles contain the highest concentration of Ach (Whittaker , 1959; Gray and Whittaker, 1962; DeRobertis et al, 1963; Whittaker et al, 1964; Whittaker and Sheridan, 1965). The relationship between the sy apyic vesicles and Ach led to the development of the vesicle hypothesis, which states that the transmitter substances are stored within synaptic vesicles which are then conveyed to the presynaptic membrane by some unkown process, and the

release of one vesicle's content corresponds to the release of one quantum (Jones, 1975), although most estimates of vesicle content indicate that a one-to-one relationship between quantum and vesicle is unlikely (Whittaker, 1970; Whittaker and Sheridan, 1965). It should be noted, however, that the quantal nature of Ach release in cerebral cortex has not been demonstrated. The response to Ach by cholinoceptive cells is slow and prolonged in cerebral cortex (Krnjevic et al, 1971), while a rapid and brief response to Ach is seen at the endplate region of skeletal muscle (Krnjevic and Miledi, 1958).

POOLS OF Ach

FREE Ach

20-30% of the total brain Ach content is released upon homogenization, is susceptible to AchE hydrolysis (Crossland and Slater, 1968; Tobias et al, 1946), and is thought to be outside of the nerve ending, probably that present in cholinergic axons, which are known to contain Ach along their whole length (MacIntosh, 1941). The source of the Ach in the axon maybe due to the fast axoplasmic transport of Ach storage particles or Ach bound to particles (Evans and Saunders, 1967; Schafer, 1973), or the simultaneous transport of ChAT, choline, and acetyl CoA (Fonnum et al, 1973; Hodgkin and Martin, 1965). The functional significance of this pool of Ach is unknown.

BOUND Ach: CYTOPLASMIC AND VESICULAR

The remaining Ach is bound within synaptosomes, and these particles can be disrupted by hypo-osmotic treatment (DeRobertis

et al, 1963; Whittaker, et al,1964) or pressure under iso-osmotic conditions (Takeno et al, 1969). After synaptosomal rupture, about half of the total bound Ach is released and is susceptible to AchE hydroylsis (cytoplasmic Ach); the remaining Ach (vesicular Ach) is protected from AchE and is found in the pellet of high speed centrifugation of lysed synaptosomes (DeRobertis et al, 1963; Whittaker and Sheridan, 1965). Exposing intact cerebral cortex to radioactive choline has been shown to labell these two nerve ending stores with specific activities (Aquilonius et al, 1973; Molenaar and Polak, 1973; Molenaar et al, 1973; Richter and Marchbanks, 1971), and the amounts of choline present in these stores can change independently (Collier et al, 1972), suggesting that cytoplasmic Ach is at least not entirely due to leakage from vesicular Ach.

LOCALIZATION OF ChAT

Fonnum (1975) has estimated that in the CNS, 5% of the total ChAT activity present is localized in the cell body, 15-25% in the axons and dendrites, and 70-80% in the nerve terminal. It was found that the location of ChAT within the nerveterminal, between the cytoplasm and membrane particles, including vesicles, was affected by the severity of the treatment used to hypo-osmotically shock synaptosomes, since DeRobertis et al (1963), who resuspended synaptosomes in 9-10 mls of water per gram of tissue, found that ChAT was localized with heavy membrane particles, including vesicles, and Wh ttaker et al, (1964), who resuspended synaptosomes in 2ml of water per gram of tissue, found that most of the ChAT was

activity appearred in the high speed supernate, suggesting cytoplasmic location. An explanation for these apparently divergent results was found by Fonnum (1967; 1968) who found that the proportion of solubilized to membranee bound ChAT in synaptosomes was largely a function of the ionic strength and pH of the suspending medium, maximum release of membrane bound enzyme occurring at an ionic strength of 150mM and a pH of 7.4. Furthermore, these studies suggested that species specificity is a factor, since rabbit and rat brain ChAT is more resistant to solubilization than ChAT from guinea pig and pigeon enzymes.

CLASSICAL HYPOTHESIS

Based on these findings, the classical hypothesis of Ach synthesis, storage and release was formulated by Fonnum (1975). According to this hypothesis, all Ach synthesis occurrs in the nerve ending cytoplasm, where the majority of ChAT is localized (Fonnum,1967; 1968). During nerve depolarization, Ach is released from the vesicular stores of the nerve terminal, and the Ach synthesized in the cytoplasm is then beleived to be transported by some mechanism into vesicles, and then released. A possible mechanism for the release of Ach from vesicles has been envisioned by Katz (1969), in which vesicles release their contents upon collision with reactive sites at the synaptosomal membrane. The choline uptake system, most probavly the high affinity system, is beleived to supply the cytoplasm with the choline necessary to maintain intracellular Ach levels (Fonnum,1975).

The major weakness of this hypothesis is that two pools

of Ach are found in synaptosomes, thecytoplasmic and vesicular pools, and only one site of synthesis, the cytoplasm (see Hebb, 1972; see Fonnum, 1975). The transport of cytoplasmic Ach into vesicles above diffusion levels has not yet been shown (Marchbanks, 1968; Katz et al, 1973; Suszkiw and Pilar. 1976; Carroll and Nelson, 1978), although binding of Ach to vesicles has been demonstrated (O'Brian 1976). The inability of vesicles to concentrate Ach supplied extravesicularly questions the supposed transfer of Ach from the Cytoplasm to the vesicles, since the vesicular concentration of Ach is estimated to be considerably higher than cytoplasmic Ach. Furthermore, it has been reported that the vesicular pool of Ach can empty and be refilled independently of the cytoplasmic pool (Carroll and Nelson, 1978), and that all of the vesicular Ach is replaced with newly synthesized Ach formed from extracellular choline rather tham extracellular Ach.

These investigations also suggest that synthesis of Ach destined to be incorporated in the vesicles occurrs at some other site than the cytoplasm. It should be noted that Kucenski et al (1975) described a membrane bound form of ChAT from rat brain,and two components of Ach synthesis have been observed in brain cells in culture ( ). Recent cytochemical studies have suggested that at least some ChAT is associated with vesicular membranes (Feigenson and Barrnett, 1977) and the number of vesicles present in the nerve ending may be related to the amount of active ChAT which can be found on vesicular membranes (Barrnett et al, 1978). Hattori et al (1976) has also reported that ChAT may be associated with both the outside of vesicular membranes and possibly intra-

vesicularly. The mean half life of vesicles, 21 days, (Arnaiz et al, 1970), agrees well with the estimated half life of ChAT, 12-20 days (Fonnum et al, 1973).

Arguments against the intravesicular location of ChAT state that acetyl CoA generating enzymes, which have not been found in vesicular fractions of brain (Tucek, 1967), must be present, along with substrate and ATP. However, Feigenson and Barrnett (1977) found that an endogenous pool of acetyl CoA in rat brain synaptosomes which is able to support Ach synthesis at 20% of normal amounts. The work of Lai et al (1977) may provide evidence that the vesicular fraction of acetyl CoA may be related to the apparent heterogeneous nature of brain mitochondria (Van der Berg, 1973). Two mitochondrial populations were found in rat brain, and both fractions were contaminated with membranes and Vesicles.

Another argument against the intravesicular production of Ach involves the generation of intravesicular CoA, a stong inhibitor of ChAT (Potter et al, 1968; Glover and Potter, 1971; Hebb, 1972), and would not allow the intravesicular Ach concentration to reach more than micromolar levels, assuming that CoA cannot be transported out of the vesicle.

FINE STRUCTURE OF THE PRESYNAPTIC NERVE TERMINAL

Several structures have been identified in the presynaptic nerve terminal (see Jones, 1975; 1978). These structurs may indicate that the activities of the cytoplasm do not occurr in a random fashion.

The orderliness of the area at the presynaptic terminal is emphasized by the prescence of a presynaptic vesicular grid, which appears to define the sites at which the vesicles can make contact with the presynaptic membrane (Jones, 1972). The observation that dense projections, exist as regularly arranged profiles projecting from the presynaptic membrane into the cytoplasm (Gray, 1963; 1966) may suggest that these projections are involved in drawing synaptic vesicles towards the presynaptic membrane prior to neurotransmitter release (Gray, 1966). Seversl investigations have suggested that dense projections are essential to presynaptic organization (Akert et al, 1969; Pfenninger et al, 1969; Akert, 1973), since they may be the nodal points of hexagonally arranged vesicles (Akert et al, 1969;1975; Akert and Pepu, 1975) and interconnected by filamentous strands.

Protuberances along the presynaptic neuronal membrane may represent temporary attachment sites for vesicles and constitute channels for the release of neurotransmitters from the vesicles trhough the presynaptic membrane (Del Castillo and Katz, 1957), or the protuberances may be the result of the release of neurotransmitter by an exocytotic mechanism (Streit et al, 1972; Jones, 1975).

Although vesicle loss upon stimulation has been observed by Barrnett et al (1978), the phenomenon is not well established (see Boyne, 1978). The long half life of vesicular membranes (Arnaiz et al, 1970) may be interpreted as evidence against exocytosis as a mechanism for transmitter release, since exocytosis implies single usage of the vesicle, and therefore a

high turnover rate (Cooper et al, 1977). Furthemore, Barker et al (1970) has suggested that the protein and lipid profiles of the vesicular and nerve terminal membrane are different, implying that fusion of vesicles with the nerve terminal membrane is unlikely. Another explanation for vesicle loss may be an alteration in vesicular shape and configuration. After stimulation, vesicles appear to become elliptical in frog neurmuscular junction (Heuser and Reese, 1973). In apparent support of exocytosis, vesicles appear to be released from electric organ of the electric ray (Boyne et al, 1975) and from cat superior cervical ganglion (Eysh and Wiley, 1974).

Coated vesicles, which have been assigned roles in the unfolding and fissioning of cell membranes (Roth and Porter, 1974), the cellular uptake and transport of proteins (Roth and Porter, 1974; Friend and Farquhar, 1967; Bowers, 1964) and enzymes (Broni et al, 1965; Holtzman et al, 1967), consist of a central vesicle surruonded by hexagonal and pentagonl shapes (Kanaski and Kadota, 1969; Kadota et al, 1976) composed of one protein, clathrin (Pearse, 1975). Coated vesicles have higher activities of certain enzymes than plain synaptic vesicles (Kadota and Kadota, 1973) and vesicular choline is preferntially associated with coated vesicls, while vesicular Ach is predominant in plain synaptic vesicles (Kamiya et al, 1974). Coated vesicles also appear to be preferntially located in the cytoplasm of the nerve ending (Gray, 1972; Jones and Bradford, 1971), and have been implicated in the transfer of membrane between the presynaptic and vesicular membranes (Marchbanks, 1976). The presynaptic network is beleived to be

of a microfilamentous nature of unknown components, extending throughout the presynaptic terminal and attaching at various points along the inner membrane of the presynaptic terminal, and may condense during fixation procedures to artifactually create coated vesicles (Gray, 1972; 1973; 1975).

It has been found by Gray (1975) that microtubules can be found in presynaptic nerve terminals, amd other investigations have implied a close association of vesicles and microtubules in axons of larval lamprey spinal cord (Smith et al, 1970; 1971). Complexes of microtubules and smooth endoplasmic reticulum have been found in CNS tissue, but rarely at presynaptic nerve terminals (Grahinger and James, 1969; Lieberman, 1971). Microtubules are beleived to be involved in rapid directional translocation of intracellular particles (Smith et al, 1975). In nerve cells, they may be involved in axoplasmic transport (Paulsen and McClure, 1975). The mitotic inhibitors viblastine and colchicine disrupt tubulin polymers and block rapid transport of protein (Karlsson and Sjostrand, 1969; 1971) and noradrenergic vesicles (Banks et al, 1971; 1972). The rate of tubulin production may be associated with functional stimulation, since eye opening causes a rise in tubulin synthesis in visual cortex (Roset et al, 1976; Cronly-Dillon and Perry, 1976).

Actomyosin proteins have also been found in presynaptic terminals (Fine and Brag, 1971; Pushkin and Berl, 1972; Bunge, 1973). It has been postulated that an interaction between membrane associated actin and cytoplasmic myosin may draw the synaptic vesicles towards the terminal membrane (Le Beux and

Willemot, 1975).

Synaptosomes have been shown to contain some of these structures (Jones etaal, 1975). Synaptic vesicles, coated vesicles, and dense core vesicles have been found using combined aldehyde-OsO_4 fixation and staining with uranyl and lead salts (Kuriyama et al, 1968; Ross et al, 1971; Dowdall and Whittaker, 1973), and dense projections and the presynaptic network have been revealed have been visualized using a non-osmicated fication technique (Bloom and Aghajanien, 1966; Jones, 1969; 1970).

MATERIALS AND METHODS

PREPARATION OF MOUSE BRAIN CHOLINE-O-ACETYLTRANSFERASE

Male (CD-1) albino mice were killed by cervical dislocation in a cold room (4 C), the brains quickly removed, and placed in several hundred millimeters of ice-cold 0.32M sucrose (isotonic sucrose). The brains were then blotted dry, the cerebellum, pons, medulla, and optic buttons excised, and sectioned through the medial saggital fissure. The brain halves were weighed, minced, and stored on a petri dish on ice until the onset of incubation.

To reduce the tissue levels of Ach which may be inhibitory to the enzyme (Potter et al, 1968; Kaita and Goldberg, 1969; Glover and Potter, 1971), the brain minces, wieghing approximatelt 150-200mgs, were incubated in 8 mls of 35mM K^+ Krebs bicarbonate buffer medium(1012 mM NaCl; 3.5 mM KCl; 2.5 mM $CaCl_2$; 1.2 KH_2PO_4; 1.2 mM $MgSO_4/7H_2O$; 25.0 mM $NaHCO_3$; 11.11 mM glucose) at 37 C for 4 minutes under 95% O_2- 5% CO_2 in a Dubnoff metabolic shaker, set a 90 cycles/minute (Salemoghaddam and Collier, 1976; Carroll and Goldberg, 1976). The incubated minces were then washed twice with ice-cold isotonic sucrose, and subcellular fractions prepared according to the method of Gray and Whittaker (1962) as modified by Collier et al (1972) and Salemoghaddam and Collier (1976) (see Fig. 1). The washed minces were homogenized in 5 ml of isotonic sucrose at 840 RPM using 8 up and down strokes in a Teflon to glass homogenizer, with a clearance of 0.0025cm. This initial homogenate was centrifuged at 4 C at 1000g for 10 minutes and the pellet, P_1, which is composed primarily of nuclear debris and myelin, was discarded. The

supernate, S_1, which includes nerve ending synaptosomes, was centrifuged at 17,000g for 15 minutes. Following this centrifugation, the supernate (S_2), which contains microsmes, was discarded. The synaptosomal fraction (P_2), which is rich in synaptosomes (De Robertis et al, 1963; Gray and Whittaker, 1962; Whittaker et al, 1964), was the hypo-osmotically shocked in 2 mls of ice cold glass distilled water, pH 4.0, and homogenized at 400 RPM with a Teflon to glass homogenizer using 8 up and down strokes, in a effort to maximize synaptosomal rupture. This homogenate was then centrifuged at 100,000g for 60minutes to yield a pellet (P_3) and a supernate (S_3). The P_3fraction, or the crude vesicular fraction, consists of synaptic vesicles, ruptured synaptosomal menbranes, mitochondria, and most of the synaptosomal ChAT activity, since ChAT has been shown to bind to membranes under conditions of low ionic strength (Fonnum, 1967; 1968). The S_3 fraction contains cytoplasmic Ach and other cytoplasmic constituents. The P_3 fraction was then suspended twice in one ml of 100mM sodium phosphate buffer, pH 7.4, ionic strength 160mM, and centrifuged for 60 minutes at 100,000g. The supernates from these two centrifugations were then combined, and contain that fraction of synaptosomal ChAT which is ionically bound to membranes after hypo-osmotic synaptosomal rupture, and beleived to exist freely in the cytoplasm of the cholinergic nerve ending in vivo (Fonnum, 1967; 1968; 1975). The <u>washed</u> P_3 fraction was then surface washed twice with 10mls of glass distilled water, pH 4.0, in an effort to reduce endogenous choline. The enzyme activity in the <u>washed</u> crude vesicular fraction is beleived to be due to a form of ChAT which is non-ionically bound to membranes or

membrane organelles.

The recivery of ChAT and protein of the synaptosomal fraction (P_2) is shown in Fig. 1.

ASSAY FOR ChAT ACTIVITY

ChAT activity in the mouse brain sucellular fractions was determined by the procedure of McCamen and Hunt (1965) as modifoed by Spyker et al (1972). In this procedure, a saturating acetyl(^{14}C)CoA concentration and various concentrations of unlabelled choline are incubated in a 200mM sodium phosphate buffer, pH 7.4, in the prescence of an aliquot of the subcellular fraction in question. Aliquot volume was 2ul, and the buffer substrate mixture, containing acetyl(^{14}C)CoA, choline, sdium phosphate buffer, NaCl, $MgCl_2$, and 70mg/ml protien, had an aliquot volume of 20ul. The acetylated product was extracted from the incubation mixture with 5mg/ml sodium tetraphenyl boron in 3-heptanone via cation exchange (Hebb et al, 1975). Tissue-free samples were run as blanks.

Imidazole groups have been implicated as activators of the non-enzymatic production of Ach from choline and acetyl-CoA (Hebb et al, 1975; Aquilonius, 1976). Therefore, control (tissue-free) and enzyme containing incubation mixtures were boiled for 5 minutes, and found to be not to be significantly different from values obtained for the nonboiled control control (tissue-free) incubabation mixture.

Protein was determined by the method of Lowry et al, 1951.

DECSENDING PAPER CHROMATOGRAPHY

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Ach (and other acetylated products) extracted into sodium tetraphenyl boron in 3-heptanone was extracted into 0.4N HCl with a recovery of 85% (Nelson, personal communication), and lyophilized and concentrated several fold. The concentrated samples were straked on Whatman No. 5 chromatogrm paper and developed, in a descinding fashion, in a solvent system of n-butanol-methanol-acetic acid-distilled water (8:2:1:3, v/v) at room temperature for 8 hours. An Ach standard (unlabelled) was co-chromatographed and ita position detected by iodine vapor (Brante, 1949). The developed chromatograms were cut into 1 cm strips, eluted with 1 ml of hyamine-methanol (1:2) v/v), and counted by liquid scintillation spectrometry, using a tolune based fluor.

In some experiments, the incubation mixture contained no added choline, and endogenous stores of substrates for mouse brain ChAT, including choline, were acetylated with acetyl(^{14}C)CoA. The prescence of Ach and at one other acetylated compound with a quaternary ammonium group has been determined under these conditions.

In other experiments, a vast excess of AchE was present in the incubation mixture, based on the amount of Ach formed by the fraction under study, and the prescence of non-hydrolyzed Ach was confirmed by descending paper chromatography.

INHIBITION STUDIES

Solutions of 4-(1-naphthylvinyl)pyridine (4-NVP) were prepared in amber bottles, since 4-NVP is light-sensitive

(White and Cavallito, 1970). The increase in volume of the incubation mixture was 1 ul, and the increase in total volume did not effectively dilute tha Ach produced.

When the effect of 4-NVP was measured in vivo, mice were pretreated with either an i.p. injection of 4-NVP in saline or an equal volume of saline, and were killed by cervical dislocation 30 minutes later. Subcellular fractions were prepared as descibed as above, and ChAT activity was determined.

Inhibition of mouse brain ChAT in vitro by Ach was investigated by adding 1ul of a highly concentrated Ach solution to the incubation mixture immediately before the incubation of the sample, in an attempt to decrease the amount of choline produced by the reverse reaction of ChAT from the added Ach. The forward reaction is hoghly favored and has an equilibrium constant of approximatel 500 (Potter and Glover, 1971).

LOCOMOTOR ACTIVITY MEASUREMENTS

In these experiments, 4 mice were injected i.p. with 200mg/kg 4-NVP, an equal volume of saline, or not injected, the latter acting as a control for injection. Locomotor activities were determined for gruop of adult mice in activity chambers equipped with a Selective Activity Meter - Model S and activity measurements were recorded at 15 minute intervals. Two different chambers were used, and the activity obtained at the initial 15 minutes for the non-injected control group for each activity chamber was arbitrarily set at 100%.

Choline Analogue Synthesis

Homocholine and triethylcholine iodide salts were prepared by reacting the corresponding tertiary amino alcohols, 3-dimethylamino-1-propanol and 2-diethylaminoethanol, with methyl or ethyl iodides, respectively. The reactions were spontaneous at room temperature or upon gentle warming, respectively. Homogeneity of both of these analogues was ascertained by descending paper chromatographys and found to agree with other investigators (Barker and Mittag, 1975; Collier et al, 1977; Olson et al, 1977).

ANALYSIS OF ENZYME KINETICS

The steady state kinetics of the great majority of enzyme-catalyzed reactions can be described in terms of a hyperbolic relationship between the velocity of the reaction, defined in this study as the activity of ChAT at a known choline concentration, and the concentration of choline involved (Roberts, 1971). The doble reciprocal plot, in which 1/v is plotted against 1/s, is by far the most widely used transformation to study enzyme kinetics (Lineweaver and Burke, 19). This transformation was used in this study.

STATISTICS

The statistics used in this investigation include the paired and independent t-tests, as described by Lentner (1975).

LIMITATIONS OF THIS STUDY

The major assumptions of this approach include the use of high potassium concentrations to induce vesicular Ach release as a valid technique (Liley, 1956; Katz, 1962; Gaye, 1967), and that ChAT is specific for cholinergic neurons (Fonnum, 1979). The major weakness of this approach is that central Ach metabolism is studied in an in vitro preparation which has been estimated to beonly 15% cholinergic (MacIntosh,), and the degree of interaction with noncholinergic neurons has not been determined.

MATERIALS

Acetylcholinesterase(AchE) and 4-NVP were purchased from Sigma and Calbiochem, repectively. 2-Diethylaminoethanol and ethyl iodide, the corresponding amino alcolhols and alkyl iodide for the synthesis of triethylcholine (TEC), were supplied by Eastman and Fischer Scientific, respectively. 3-Dimethyl amino-1-propanol and methyl iodide, the corresponding amino alcohol and alkyl iodide for the synthesis of homocholine, were supplied by Aldrich Chemical. Acetyl(^{14}C)CoA was purchased from New England Nuclear, with a specific activity of 10.1 mCi/mole. Toluene and Liquifuor were purchased from Fischer Scientific and New England Nuclear, respectively.

Results

The subcellular fractionation procedure used in this study is desci
is described in Fig. 1. The recovery of synaptosomal ChAT and and protien is shown in Table 1, and these results are similar to those obtained by Fonnum (1968).

The results shown in Fig. 2. indicate that both the solubilized and membrane bound forms of mouse brain nerve ending ChAT can linbarly acetylate choline for 30 minutes, when saturated with both choline and acetyl CoA at 39 C. The membrane bound form of the enzyme appears to acetylate choline linearly for almost an hour under these conditions. The nonenzymatic conversion of choline and acwtyl CoA to Ach has been shown to exist and be enhanced by imidazole groups in extracts of rat brain (Hebb et al, 1975) and human cerebrospinal fluid (Aquilonius and Eckernas, 1976). To test the possibility that nonenzymatic production of Ach was occurring, both the solubilized enzyme form,present in the combined sodium phosphate wash fraction, and the membrane bound form of ChAT, present in the washed crude vesicular pellet, was boiled for 5 minutes and their respective abilities to form Ach from saturaring levels of choline and acetyl CoA determined. Boiling reduced the activity of both enzyme forms to the level of tissue free blanks, suggesting the abscence of nonenzymatic Ach production in these subcellular fractions.

Some reports suggest that Ach may regulate the activity of brain ChAT by mass action (Potter et al, 1968; Glover and Potter, 1971). Solubilized rat brain ChAT is also capable of producing acetyl CoA and choline as products, but the Ach synthesizing direction is favored, with an equilibrium constant of 450 (Glover and Potter, 1971). The physiological importance of Ach inhibition of ChAT has been investigated by Harris et al (1978). These inves-

tigators have suggested that synthesis of new Ach in vivo is inhibited by acetylcholinesterase inhibitors, which preserve released Ach, and atropine, which is beleived to bind yo presynaptic receptors, and acts to reduce the amount of Ach released. Several investigators have reported that total synaptosomal ChAT activity is not inhibited more than 50% at Ach concentrations of 100-150mM (Potter et al, 1968; Kaitia and Goldberg, 1969; Glover and Potter, 1971). Recently, Singh et al (1975) have reported the isolation of a low activity form of ChAT which is resistant to inhibition by high concentrations of Ach, which are capable of inhibiting a higher activity form to a greater degree. Therefore, the inhibition of both the solubilized and membrane bound enzyme forms by Ach was investigated in this study. The results presented in Table 3 indicate that the membrane bound enzyme form is less resistant to Ach inhibition at Ach concentrations greater than 150mM than the solubilized form of ChAT.

It should be noted that exogenous Ach concentrations referred to in this experiment are extravesicular. This exogenous Ach may be separated from a fraction of the membrane bound ChAT, thus accounting for the incomplete inhibition of total membrane bound ChAT activity.

Since vesicular Ach but not cytoplasmic Ach is protected from AcgE hydrolysis, (Whittaker et al, 1964; Whittaker and Sheridan, 1965), the ability of the subcellular fractions to produce Ach at saturating levels of choline and acetyl CoA in the prescence of a 100,000 fold excess of AChE was tested. These results (see Table 4) reveal that approximately 40% of the control level of Ach was maintained in the washed crude vesicular fraction, but 97% of the Ach produced by the solubilized enzyme form was hydrolyzed. The identity

of the product resistant to hydrolysis present in the crude vesicular fraction was found to be Ach with descending paper chromatography.

While these results may indicate that a portion of the enzyme present in the washed crude vesicular fraction is protected from the effect of an excess of AchE, it is possible that enclosed vesicles are formed during the initial homogenization of the brain minces, which may represent entrapped cytoplasmic components, including cytoplasmic ChAT. If this were occurring, the addition of an excess of AchE to the brain minces during the initial homogenization would case the incorporation of AchE into these artifactually produced vesicles, thus hydrolyzing the Ach produced by ChAT in this location. However, when this experiment was done no reduction in Ach production was found in either the combined washes of the crude vesicular fraction, representing cytoplasmic ChAT of the nerve ending, or the washed crude vesicular fraction, representing membrane and perhaps vesicular bound ChAT. However, 90% of the ChAT activity not bound to membranes following hypo-osmotic shock of the synaptosomal subcellular fraction was inhibited.

The ions Na^+ and Cl^- have been implicated as activators of brain ChAT activity (Potter etyal, 1968; Hebb et al, 1975; Rossier, 1907; Hersh et al, 1978) and uptake of choline (see Kuhar and Murrin, 1977). To determinr if the membrane bound and solubilized forms of ChAT have different degrees of dependency on the prescence of Na^+, both enzyme forms were incubated in lowered Na^+ concentrations (see Table 5). It is apparent that both forms of ChAT are dependent on Na^+, although the membrane bound form was less inhibted (62%) than the solubilized form, which was inhibited to nondetectable levels.

Styrylpyridine analogues, such as 4-(1-nahthylvinyl)pyridine (4-NVP) are potent in vitro inhibitors of solubilized ChAT (Smith

et al, 1967; Allen et al, 1970; Cavallito et al, 1969; Goldbeerg et al, 1971; Baker and Gibson, 1971; 1972; Krell and Goldberg, 1975). Repeated attempts by several investigators to administer 4-NVP in vivo and reduce the level of brain Ach have been unsuccessful (Carson et al, 1972; Glick et al, 1973; Haubrich et al, 1974; Krell and Goldberg, 1975). Nerve ending ChAT activity can be inhibited approximately 65% without affecting brain levels of Ach after an i.p. dose of 200 or 400mg/kg 4-NVP (Krell and Goldberg, 1975), indicating that brain ChAT activity is not rate limiting. An alternative possibility is that the majority of brain Ach maybe synthesized by ChAT in a subcellular location inaccessible to the inhibitor. TO test this possibility, the solubilized and membrane bound forms of the enzyme were incubated in the prescence of varying concentrations of 4-NVP and their activities determined. The results shown in Table 6 indicate that the soluble form of ChAT is more sensitive to inhibition by 4-NVP than the membrane bound form of the enzyme. 4-NVP, at a concentration of 1.2uM, inhibits the activity of the solubilized enzyme by 65% without significantly reducing the activity of the membrane bound form. In agreement with Krell and Goldberg (1975), the K_i of 4-NVP for the solubilized enzyme was found to be 1.0uM.

To determine if one or both enzyme forms were inhibited in vivo, mice were pretreated with either an i.p. injections of 4-NVP (200 mg/kg) or saline in equal volumes and then killed alternatively 30 minutes later. Both solubilized and membrane bound enzyme forms were prepared from both treated and control groups, and their respective enzyme activities determined in the prescence of a saturating choline concentration. The results shown in Table 7 indicate that i.p. administration of 200 mg/kg 4-NVP inhibits the activity of the soluble, cytoplasmic form of ChAT by 77% while ~~not significantly~~

inhibiting the membrane bound form by only 29%.

In the study of Krell and Goldberg (1975), it was reported that mice injected with 4-NVP appeared ataxic and depressed. This behavioral effect was observed in the present study. The effect was correlated to the locomotor activity of the mice in Fig. 3. In this experiment, 4 mice were injected with 4-NVP (200 mg/kg) or saline in equal volumes, or not injected, the latter group serving as an injection control. It was found that locomotor activities were rduced by approximately 80-90% with respect to either the injected or noninjected control group, and this decrease in locomotor activity was present 3 hours after a single injection.

Extracellular choline is transported into synaptosomes (Yamamura and Synder, 1972; 1973; Kuhar et al, 1973; Barker and Mittag, 1975; Carroll and Buterbaugh, 1975) and brain tissue minces (Carroll and Goldberg, 1975) by two distinct uptake mechanisms, a high affinity uptake system with a K_t of 0.5 to 5.0uM and a low affinity system with a K_t of 50 to 100uM. Most of the choline transported into synaptosomes by high affinity transport is converted to Ach, when an AchE inhibitor is present in the incubation (Yamamura and Synder, 1972; 1973; Guyenet et al, 1973; Kuhar et al, 1973; Barker and Mittag, 1975).

The K_m values reported for solubilized ChAT with respect to choline range from 0.1 to 0.7mM (see Table 8). All reported K_m values exceed the high affinity choline transport K_t by at least 100 fold. To determine if the K_m for membrane bound ChAT for choline might approximate that of high affinity choline transport, the choline kinetics of both solubilized and membrane bound enzyme forms were determined over a choline concentration of $4{,}25 \times 10^{-3}$M to 4.25×10^{-7}M. The results shown in Fig. 4 suggest that membrane bound ChAT appears to have both a high affinity (K_m=3.2uM and a low

affinity (K_m=0.478mM) Michaelis constant, whereas the solubilized form shows only a low affinity K_m (K_m=0.166mM) for choline (see Fig. 5). Hoever, the production of acetylated endogenous stores in the washed crude vesicular fraction has been shown to be approximately 14% of the amount of Ach produced when saturating choline levels are present (see Table 9). This level of production is similar to that determined when the membrane bound enzyme form is incubated in the prescence of $4.25x10^{-6}$M or $4.25x10^{-5}$M choline. The results shown in Table 10 indicate that the acetylated product present after incubation of the membrane bound enzyme form in the abscence of added choline is actually two moieties, one with an R_f value similar to that found for acetylcarnitine. However, it is believed that carnitine is a poor substrate for brainChAT (Bradshaw and Hemsworth, 1972).

The substrate specificity of ChAT has been studied by several investigators and recently, in cat superior cervical ganglion and rat brain synaptosomes, the choline analogues triethylcholine (TEC) and homocholine have been shown to be acetyla ed (Ilson and Collier, 1975; Collier et al, 1977; Ilson et al, 1977). Therefore, the ability of the membrane bound form of ChAT to acetylate these analogues was tested (see Figs. 6 and 7). In this study, both solubilized and membrane bound forms of ChAT were incubated at varying time intervals in the prescence of $4.25x10^{-2}$M TEC and $1.0x10^{-2}$M homocholine. Under these conditions, it was suggested that TEC was acetylated by the membrane bound form of ChAT at 5,15, 30, and 60 minutes of incubation linearly, whereas homocholine was also apparently acetylated by the membrane bound form at 5,15, 30, and 60 minutes of incubation. In contrast, the solubilized enzyme form was less able to acetylate TEC than the membrane bound form, and homocholine was not acetylated by the solu-

bilized enzyme at 30 minutes of incubation, even in the prescence of a 5 fold excess of the solubilized enzyme form (data not shown).

The identification of these acetylate d choline analogues was performe on descending paper chromatography, and R_f values were obtained (see Table 10). These results imply that the acetylated product(s) formed from both TEC and homocholine incubations are different from the value reported for Ach, but their identities with respect to the acetylated product(s) obtained during incubations performed in the abscence of choline are less clear. In fact, the R_f values reported for acetylated TEC and homocholine, which agree with those reported by Ilson et al (1977) and Collier et al, (1977), are similar to a R_f value obtained after incubation in the abscence of added choline. Therefore, the identification of the acetylated choline analogues TEC and homocholine by mouse brain membrane bound ChAT remains unconfirmed.

DISCUSSION

Theenzyme that synthesizes Ach, ChAT, is localized in cholinergic nerve terminals, and the majority of the enzyme is beleived to be soluble, and Ach synthesis is beleived to occur in the cytoplasm of the nerve ending (Fonnum, 1967; 1968; 1975). Following cytoplasmic synthesis, Ach is then beleived to be transferred into synaptic vesicles (Fonnum, 1975). However, the transfer of Ach into synaptic vesicles above diffusion levels has not been demonstrated (Katz et al, 1973; Suszkiw, 1976). Additionally, the vesicular emptying of Ach appears to facilitate the transport of extracellular choline but not Ach into vesicles independently of the cytoplasm, suggesting that maintenance of vesicular stores may not be dependent on cytoplasmic synthesis and transfer of Ach into vesicles (Carroll and Nelson, 1978).

Enzyme forms with different surface charges (Fonnum and Malthe-Sorensson, 1972), types of antibodies produced (Singh et al, 1975) and Ach inhibition characteristics (Singh et al, 1975) from the same enzyme source. Furthermore, the cytochemical studies of Feigenson and Barrnett (1977), Bene and Barrnett (1978) and Hattori et al (1976) have indicated that at least some of the nerve ending ChAT is associated with vesicular membranes.

Results obtained in this investigatio indicate that a membrane bound form of ChAT can synthesize Ach, and has properties beleived to be characteristic of enzymes on membranes, including altered enzyme kinetics, inhibition susceptibilities, and substrate specificities, than the enzyme off the membrane (Hochstadt and Quinlan, 1975). Other results obtainedinthis study suggest that some of the membrane bound ChAT is located in a position which allows some Ach to be synthesized and protected from AchE hydrolysis and allows some ChAT to be active in the prescence of high exogenous Ach concentrations.

4-NVP is a reversible, specific inhibitor of solubilized ChAT (Smith et al, 1967; Cavallito et al, 1969; Goldberg et al, 1971; Krell and Goldberg, 1975). In the present experiments, 4-NVP inhibited the solubilized form of ChAT to a greater extent than membrane bound ChAT in both invitro and in vivo studies.

It has beensuggested that brain ChAT is present in excess and is not rate limiting, since a 65% inhibition of synaptosomal ChAT can be inhibited by 4-NVP with no effect on brain Ach levels (Krell and Goldberg, 1975) and 4-NVP has no effect on cholinergic transmission (Aquilonius et al, 1970). Another interpretation is that solubilized ChAT may be present in a location susceptible to4-NVP, whereas membrane bound ChAT is relatively inaccessible to 4-NVP inhibtion, and may maintain vesicular Ach stores. Furthermore,

Barker (1973) has shown that 4-NVP treatment inhibits the low affinity uptake of choline, which is not beleived to be responsible for providing choline for acetylation and Ach production, and has only a neglible effect on high affinity choline uptake, which is beleived to provide choline almost exclusively for Ach synthesis (see Freeman and Jenden, 1976).

The regulation of ChAT by Ach product inhibition has been suggested by several investigators (Potter et al, 1968; Kaita and Goldberg, 1969; Glover and Potter, 1971), but it is only able to inhibit synaptosomal ChAT by 50% at Ach concentrations of 100mM or more. Therefore, for Ach to act as ac physiological inhibitor of ChAT, the enzyme must be exposed to higher concentrations than are beleived to be present in the nerve terminal cytoplasm. On the other hand, the intravesicular concentration of Ach has been estimated to be at least 200mM (Barker et al, 1970). It has been reported that one form of ChAT is able to be inhibited by phsyiological concentrations of Ach, whereas a low affinity activity form of ChAT (analogous to the membrane bound form of ChAT) is not profoundly inhibited by those levels of Ach. (Singh et al, 1975). This investigation indicates that the membrane bound form of ChAT may reach maximal inhibition at an extravesicular concentration of 150mM, while the solubilized form of ChAT can be completely inhibited by Ach.

It has been proposed that high affinity choline uptake regulates Ach synthesis (MacIntosh and Collier, 1976; Haubrich and Chippendale, 1977). Barker and Mittag (1975) and Lefresne et al (1973) have propsed that high affinity choline transport and Ach synthesis are coupled processes. However, Barker (1978) has found that a Compound (TMPYA : N,N,N-trimethyl-N-propylnyl-ammonium) can be taken up by the high affinity transport system and not be acetylated, and

Hope and Jenden (1977) have found that conditions which compromise the availability of acetyl CoA lead to a drop in Ach synthesis without impairing high affinity uptake. In this investigation, it was found that the degree of inhibition of the membrane bound form of ChAT by a lowered sodium concentration corresponds well with the degree of sodium dependency ascribed to the high affinity choline transport system in synaptosomes (Simon and Kuhar, 1976), suggesting that the sodium requirement of the membrane bound form of ChAT [illegible] in the prescence of a saturating acetyl CoA concentration may be related to the sodium reqiurement of the high affinity choline transport system.

The choline kinetics determinations on the enzyme forms, done without correction for endogenous acetylation, indicate that the membrane bound enzyme might have a high affinity component for choline (K_m=[illegible] 3.2uM) similar to the K_t values reported for high affinity choline transport. However, after correction for endogenous choline acetylation is performed, the choline Michaelis constants obtained for both solubilized and membrane bound ChAT are similar (K_m=0.167mM and 0.478mM, respectively), a result which agrees with that reported by Kucenski et al (1975).

Recently, it has been discovered that triethylcholine and homocholine can be acetylated by the cat superior cervical ganglion during electrical stimulation to a greater extent than by in vitro tests, which use solubilized ChAT preparations (Hemsworth and Smith, 1970; Currie and Mautner, 1974; Ilson and Collier, 1975; Collier et al, 1977; Ilson et al, 1977). Furthermore, it has been found that homocholine can be acetylated by a synaptosomal preparation of rat brain ChAT after a 5 minute incubation (Collier et al, 1977), and that, while homocholine can be transported by the high affinity uptake system, not all the homocholine transported during (Collier

et al, 1977) or subsequent to stimulation (Carroll and Nelson, 1979) is acetylated. These latter ressults may suggest that acetylation rather than high affinity uptake is rate limiting.

In this investigation, it was found that triethylcholine could be acetylated by both the solubilized and membrane bound ChAT at a concentration of 4.25×10^{-2}M, incubated for 30 minutes at 39 C. The R_f value obtained for the acetylated product on descending paper chromatography correspond to that value obtained by Ilson et al (1977). However, the apparent acetylation of homocholine by membrane bound ChAT appears to be similar to endogenous acetylation at 60 minutes of incubation, but not at 5 or 15 minutes. Furthermore, the acetylated compound(s) present after incubation in the abscence of added choline for 60 minutes have identical R_f values, agreeing with those reported by Collier et al (1977) for acetylated homocholine.

In conclusion, it appears that some nerve ending ChAT present in hypo-osmotically shocked synaptosomes is not solubilized by high ionic strength washes, and may be nonionically associated with synaptosomal membranes, including vesicular membranes. This membrane bound form of ChAT possess a similar affinity for its substrate choline as the solubilized enzyme, but only after the former is corrected for endogenous acetylation. Furthermore, membrane bound ChAT is less sensitive to inhibition caused by 4-NVP, Ach, reduced sodium levels, and hydrolysis of Ach formed than the solubilized form of mouse brain ChAT.

TABLE 1

Reported K_m values for brain choline-o-acetyl-transferase (ChAT)

Tissue source	K_m (choline)	K_m (acetylCoA)	
rat brain	184uM	13.0uM	Kucenski et al (!(&% (1975)
human putamen	510uM	11.0uM	White and Wu, (1973)
human caudate	600uM	6.6uM	"
rat brain	410uM	18.0uM	"
rabbit brain	640uM	11.0uM	"
bovine striatum	750uM	10.0uM	Glover and Potter, (1971)
bovine caudate	800uM	16.0uM	White and Cavallito, (1970)
mouse brain	700uM	17,0uM	Kaita and Goldberg, (1969)

TABLE 2

The Recovery and Distribution of Synaptosomal Choline-O-Acetyltransferase (ChAT) and Protein

Subcellular Fraction	ChAT Activity*	Protein**
S_3 (cytoplasmic Ach and ChAT not ionically bound to membranes after hypo-osmotic shock)	1.01% (2)	35% (4)
100mM sodium phosphate buffer wash (solubilized ChAT)	67.81% (2)	11.5 (4)
Washed P_3 (membrane bound ChAT)	15.6% (2)	53.4 (4)
P_2 (total synaptosomal ChAT recovered, and protein)	84.42% (2)	99.9% (4)

* ChAT activities were determined as pm Ach/ mg wet wt./ 15', and the fractions were incubated in the presence of a saturating choline concentration (4.25mM).

** Protein was determined by the method of Lowery et al, (1951).

( ) indicates number of brains used.

TABLE 3

Inhibition of Solubilized and Membrane Bound Choline-O-Acetyltransferase (ChAT) by Acetylcholine (Ach) (n=6).

Ach(mM)	Enzyme Activity (pm Ach/ug prot./15 min) (% of control for 15 min. incubation) Solubilized ChAT	Membrane Bound ChAT
0 (control)	100±4.4	100±6.5
10	94.5 ±17.1	116.2±9.8
50	112.1±13.7	77.7±8.7*
100	69.7±7.3*	52.8±4.3*
150	39.3±6.1*	31.0± 2.1 *
200	25.1± 2.6*	30.4±3.9*
383	nondectable	26.7±3.6*

Enzyme forms were prepared from mouse brain minces incubated in 35mMK$^+$Krebs medium for 4 minutes at 37 C.

The inhibitory concentrations of Ach are exclusively extravesicular.

*Results significantly differ from control ($p < 0.05$)

TABLE 4

Determination of Choline-O-Acetyltransferase (ChAT) Activity in Subcellular Fractions of Mouse Forebrain in the Prescence and Abscence of an Excess of Acetylcholinesterase (AchE) (n=7).

	Subcellular Fractions	
	100mM Sodium Phosphate Buffer Wash of P_3	Washed P_3
Control*	129.8 ± 20.6	3.9 ± 0.4
AchE**	3.9 ± 2.6	1.6 ± 0.2

*ChAT activity expressed as pm Ach/ug prot./5 min.

**Enough Eel AchE was added to the incubation medium to hydrolyze 2.84umoles of Ach to choline per min. This amount represents an excess of at least 100,000 fold.

TABLE 5

The Effect of Lowered Sodium ion concentration on Choline-O-Acetyltransferase (ChAT) in Subcellular Fractions of Mouse Brain

	ChAT Activity (pm Ach/ mg wet wt/5 min	
Subcellular Fraction	318mM Na^+ (Control)	236.3mM Na^+ (Lowered Na^+)
washed P_3	89.8 ± 8.7 (5)	34.35 ± 4.9 (5)*
pooled 100mM sodium phosphate buffer washes	180.1 ± 25.5 (5)	not detectable (5)*

*Results significantly differ from control (p 0.05)

TABLE 6

In vitro Inhibition of Solubilized and Membrane Bound Forms of Choline-O-Acetyltransferase (ChAT) by 4-(1-naphthylvinyl)pyridine (4-NVP) (n=7).

	Enzyme Activity (pm Ach/ug prot./ 5 min)	
4-NVP (uM)	Solubilized ChAT	Membrane Bound ChAT
0 (control)	88.0±16.8	7.56±0.43
0.072	74.2± 12.08	7.69±0.41
0.290	70.3±12.0	7.34±0.46
1.16	32.2±7.8*	6.94±0.34
4.63	12.4±6.1*	5.29± 0.33*

*Results significantly different from control ($p < 0.05$).

TABLE 7

In vivo Inhibition of solubilized and Membrane Bound Forms of Choline-O-Acetyltransferase (ChAT) by 4-(1-naphthylvinyl)pyridine (4-NVP)

	Enzyme Activity (pm Ach/ug prot./ 5 min.) (% of control for 5 min incubation)	
Treatment*	Solubilized ChAT	Membrane Bound ChAT
Control	100±10 (7)	100±19 (7)
4-NVP	23± 10** (8)	71±7** (8)

*Mice were injected with 200mg/kg 4-NVP i.p. or an equal volume of saline 30 minutes prior to cervical dislocation. Enzyme forms were prepared from mouse brain minces incubated in 35mM K^+ Krebs medium. ( ) indicates the number of brains used.

**Results significantly different from control (p 0.05)

TABLE 8

Comparison of Membrane Bound Enzyme Activities in the Prescence of Various Choline Concentrations (n=8).

choline (Molar)	Membrane Bound ChAT Activity (pm Ach/ ug prot./15 min.)
$4,25x10^{-3}$	68.3±9.3
$4.25x10^{-7}$	7.84±0.4*
0 (no added choline)	9.35±0.5

*Result significantly lower than value obtained in the abscence of added choline. (p 0.05)

TABLE 9

Apparent R_f values of the acetylated products obtained by paper chromatography with various substrates from mouse brain minces

Substrate	R_f value of acetylated compound synthesized by the subcellular fraction in question	
	100mM sodium phosphate buffer wash	washed P_3
choline (4.25mM)	0.30	0.30
triethylcholine (42.5mM)	0.92*	0.90*
homocholine (10.0mM)	0.74**	0.80**
no added substrate	0.30& 0.74	0.30 & 0.80

Descending paper chromatography was performed on Whatman No. 1 paper in a solvent system of n-butanol-acetic acid- water (8:2:3:1). Incubations were done at room temperature for 8 hours.

*R_f values agree with those of Ilson et al (1977).

**R_f values agree with those of Collier et al (1977).

Fig. 1

Subcellular Fractionation and Enzyme Preparation

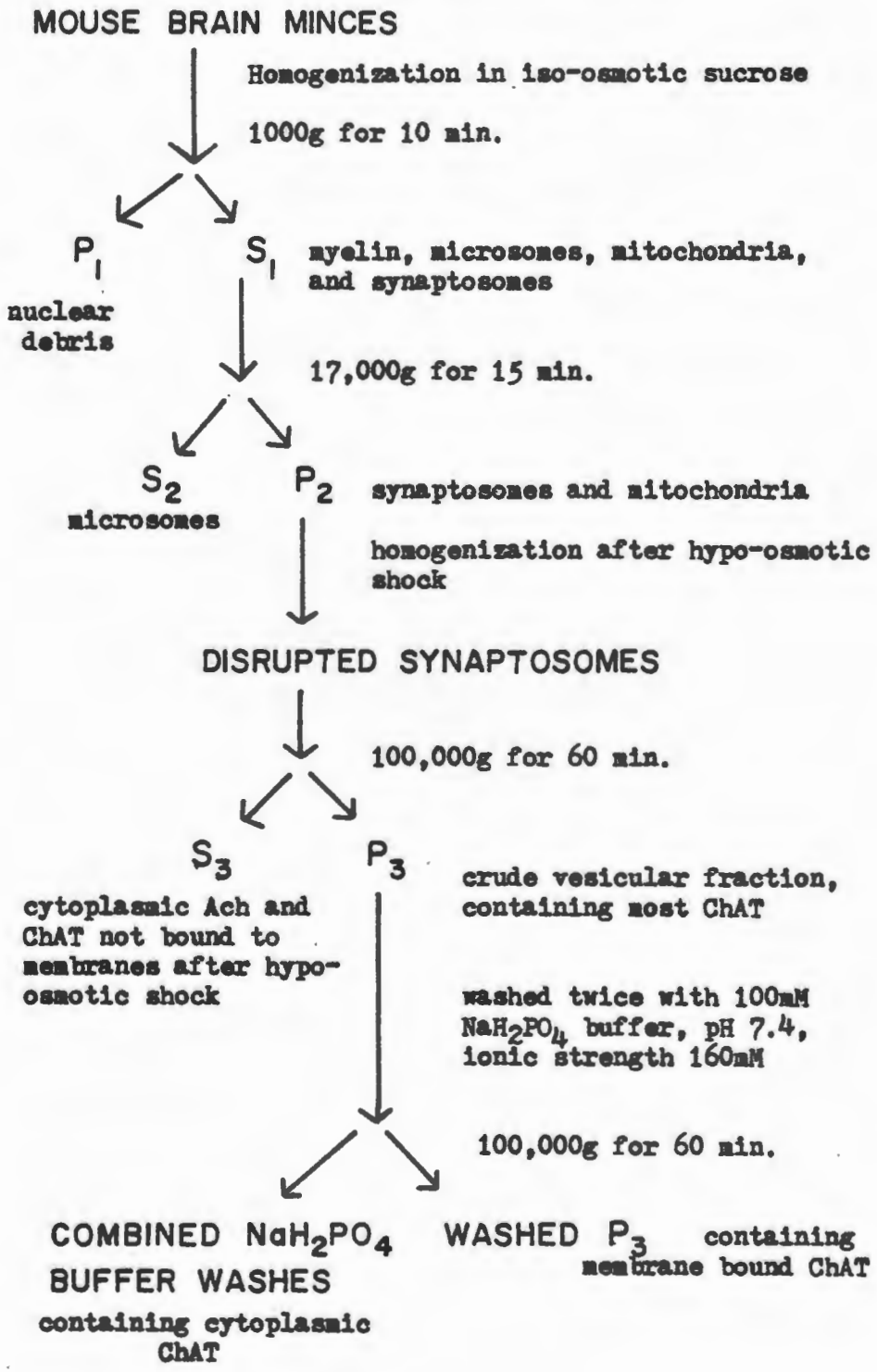


Fig. 2 Acetylation of Choline by Membrane Bound and Solubilized Forms of ChAT (□ and ▨ , respectively) over time. Enzyme activities expressed as pm Ach/ug prot./time (n=9).

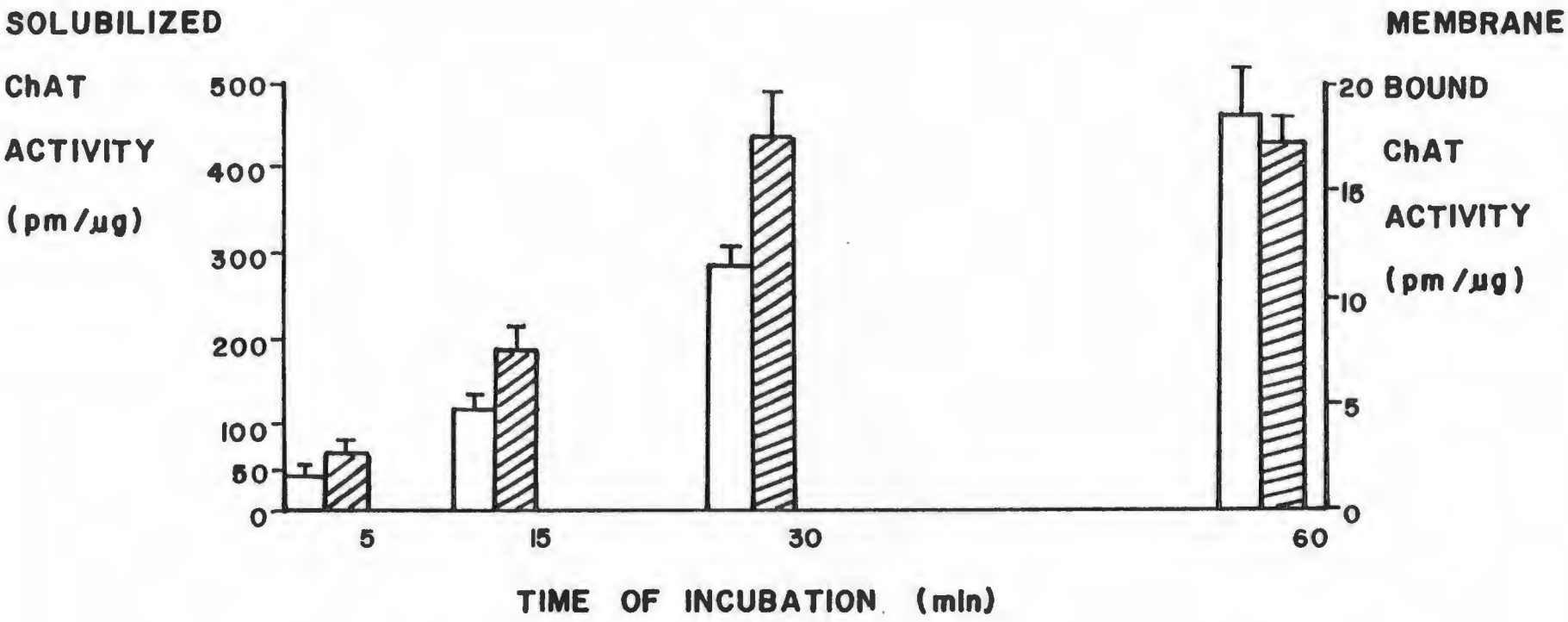
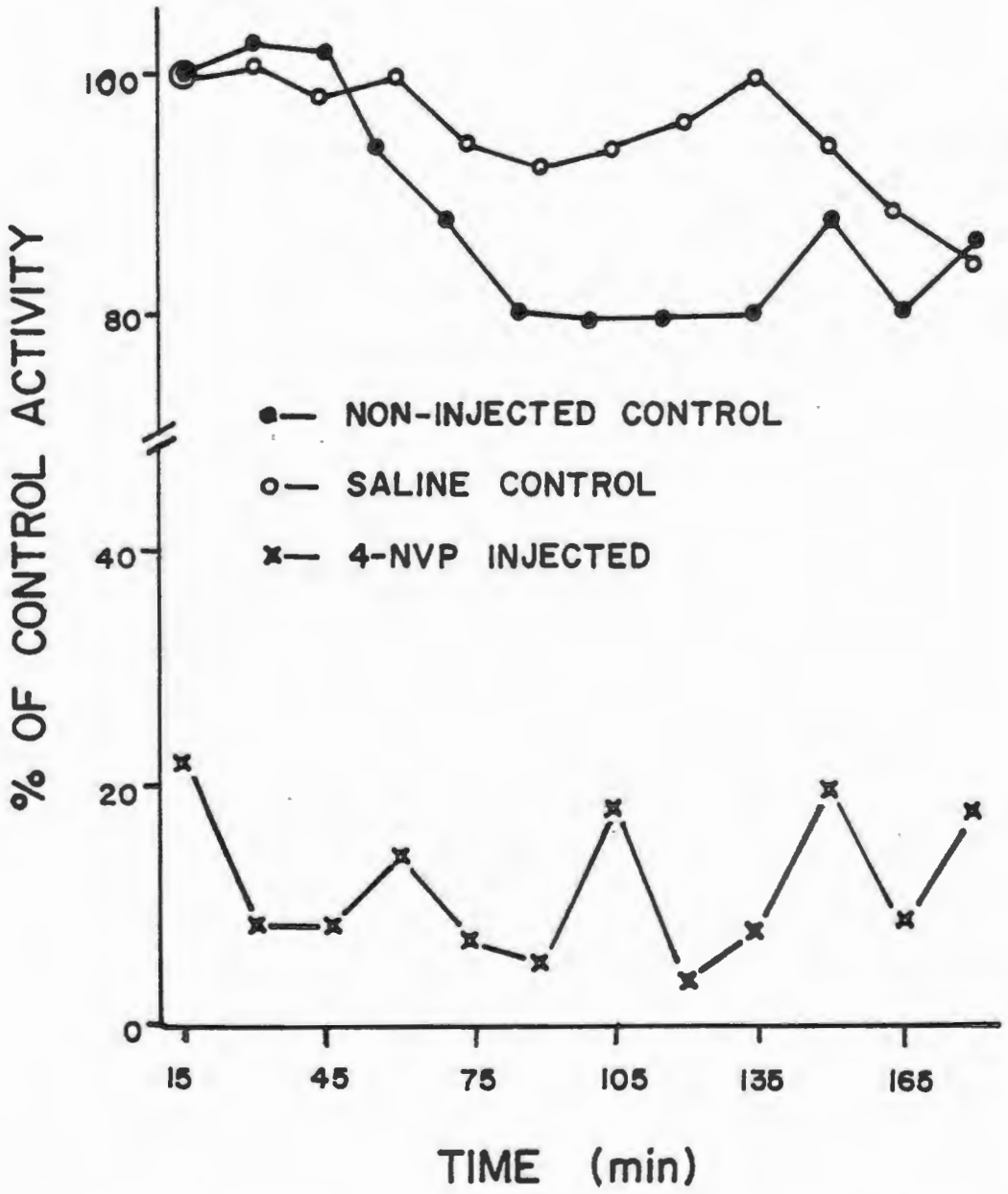


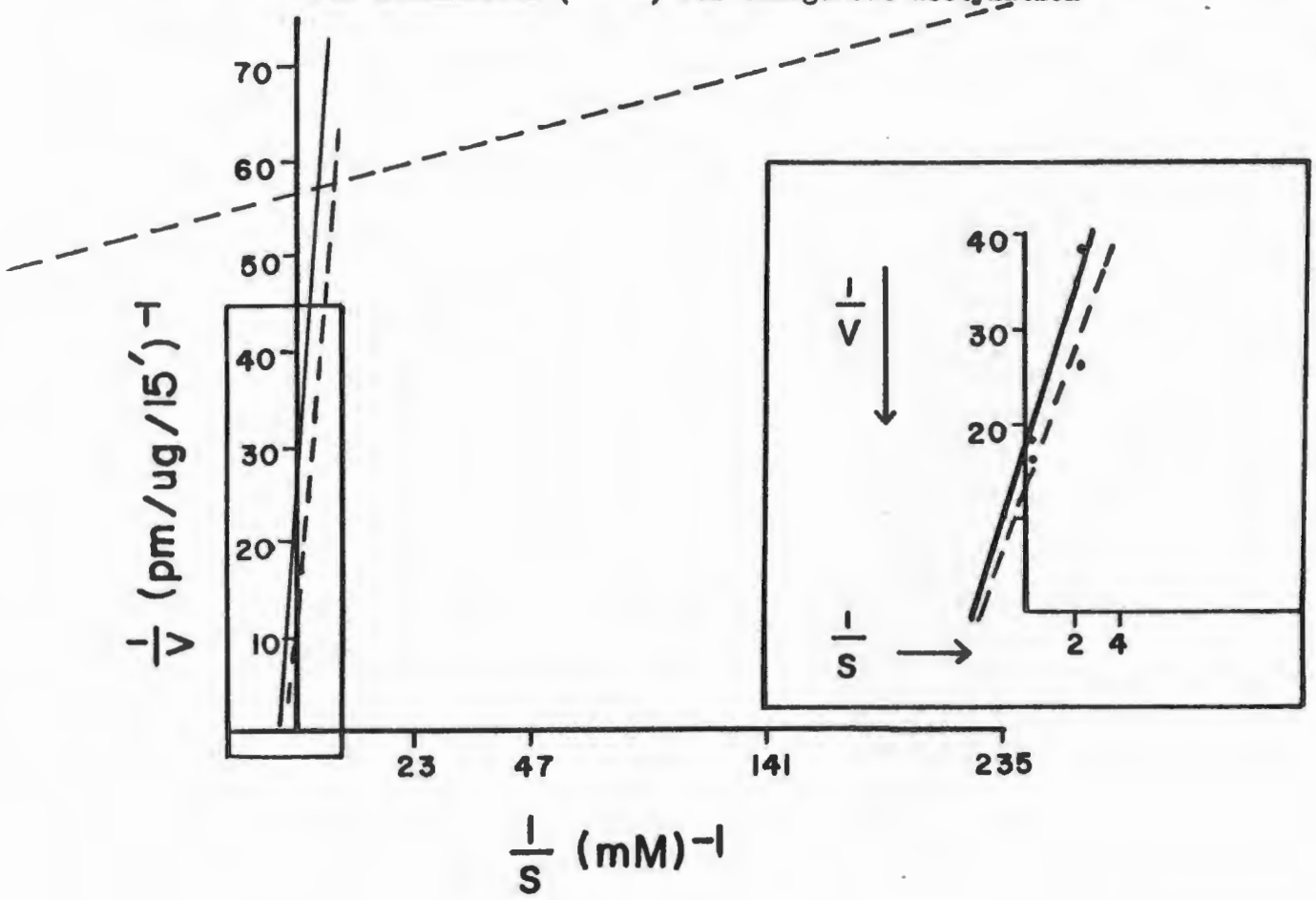
Fig. 3

Effect of 4-(1-naphthylvinyl)pyridine (4-NVP) on Mouse Locomotor Activity



4 mice were injected i.p. with 200mg/kg 4-NVP or an equal volume of saline, or not injected. Locomotor activities were determined for a group of 4 mice in activity chambers equipped with Selective Activity Meter - Model S at 15 minute intervals. Each point represents the average of 2 determinations.

Fig. 4 Choline Kinetics of the Membrane Bound Enzyme Form, corrected (--) and uncorrected (- - -) for endogenous acetylation



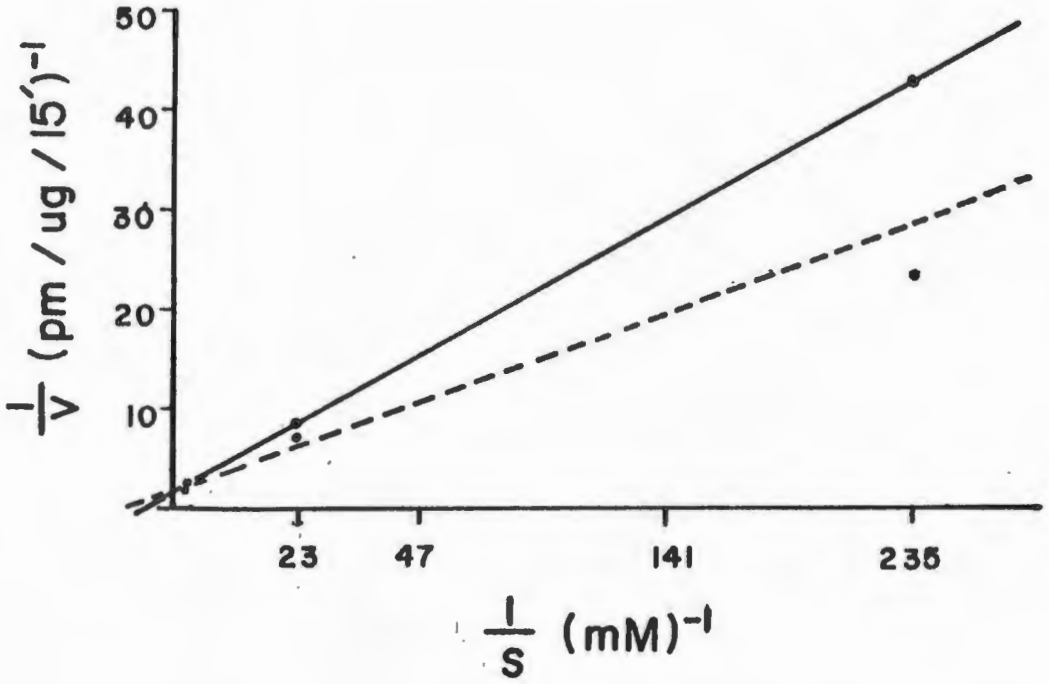
corrected for endogenous acetylation

K_m=0.273mM
V_{max}=49.41pm/ug/15'

uncorrected
low affinity component
K_m=0.478mM
V_{max}=75.9pm/ug/15'

high affinity component
K_m=3.2uM
V_{max}=17.4pm/ug/15'

Fig. 5 Choline Kinetics of the Solubilized Enzyme Form, corrected (--) and uncorrected (- - -), for endogenous acetylation.



corrected for endogenous acetylation

K_m=0.189mM
V_{max}=794.9pm/ug/15'

uncorrected

K_m=0.166mM
V_{max}=802.6pm/ug/15'

Fig. 6 Acetylation of Exogenous Triethylcholine (TEC) (▨), Homocholine (■), and Endogenous Substrate(s) (□) over time by Membrane Bound ChAT

( ) indicates number of brains used.

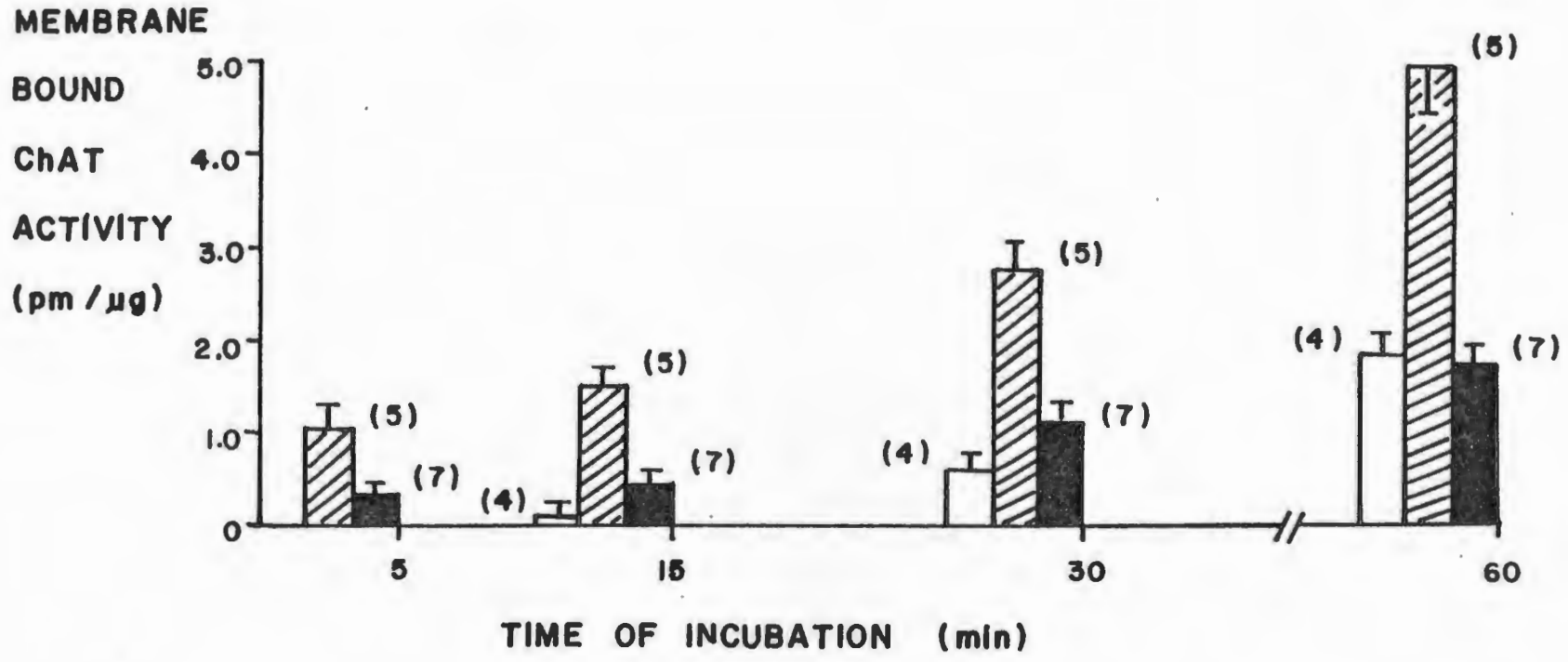
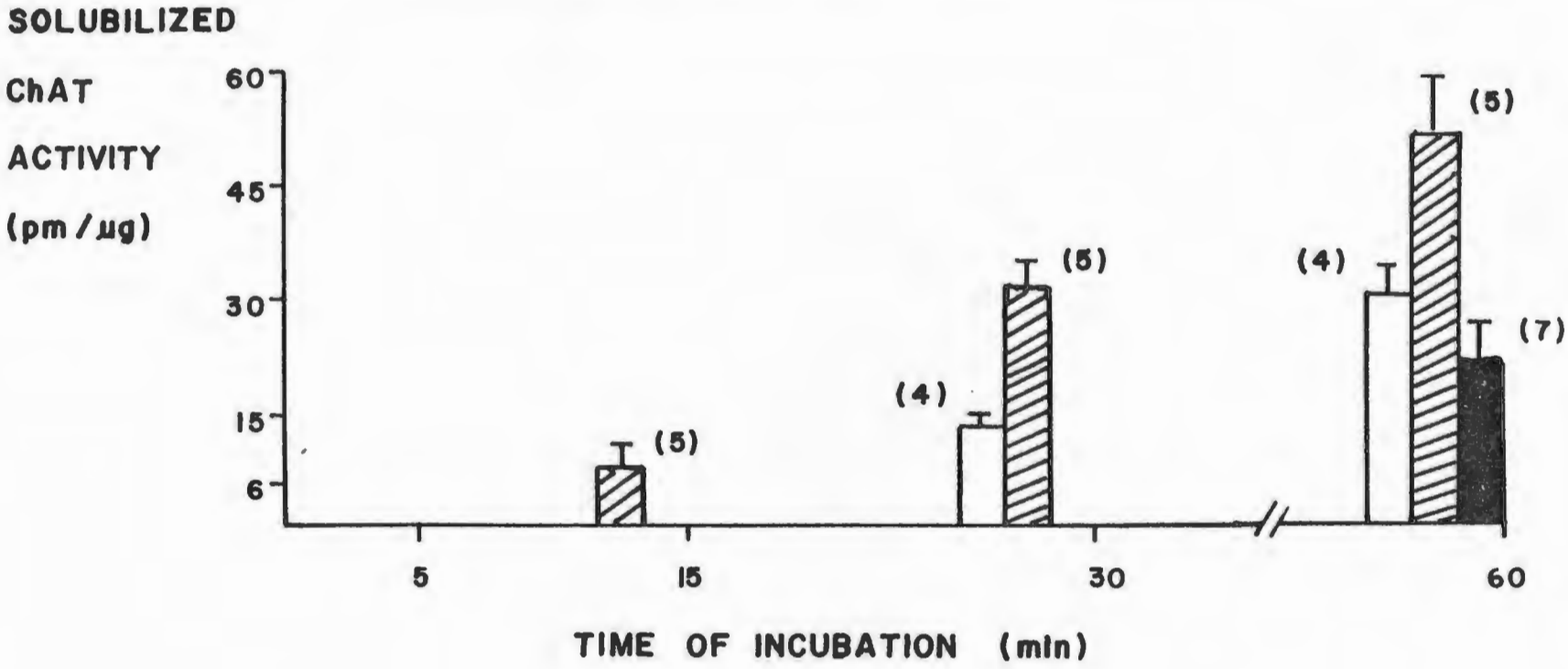


Fig. 7 Acetylation of Exogenous Triethylcholine (TEC) (▨), Homocholine (■), and Endogenous Substrate(s) (□) over time by Solubilised ChAT

( ) indicates number of brains used.



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