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ACETYLATION OF THE CHOLINE
ANALOG HOMOCHOLINE BY MEMBRANE
BOUND
CHOLINE-O-ACETYLTRANSFERASE IN
MOUSE FOREBRAIN

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ACETYLATION OF THE CHOLINE ANALOG HOMOCHOLINE
BY MEMBRANE BOUND CHOLINE-O-ACETYLTRANSFERASE
IN MOUSE FOREBRAIN

BY

CHRISTINA G. BENISHIN

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OF

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ABSTRACT

The choline analog homocholine is not acetylated in vitro by choline-O-acetyltransferase (ChAT, EC 2.3.1.6) which is solubilized by 100 mM sodium phosphate buffer washes of a crude vesicular fraction of mouse forebrain. However, both homocholine and choline are acetylated by a form of ChAT which is non-ionically associated with a subcellular fraction of mouse forebrain containing membrane associated organelles and occluded ACh (P_4). Acetylation of homocholine by membrane associated ChAT is saturable. 4-(1-Naphthylvinyl)pyridine (NVP) inhibits the acetylation of both choline (60%) and homocholine (40%) by membrane associated ChAT but reduces the acetylation of only choline by soluble ChAT (76%). Choline and homocholine serve as competitive alternative substrates for the same membrane associated ChAT whereas homocholine only acts as a competitive inhibitor of choline acetylation by soluble ChAT. Acetylhomocholine competitively inhibits the acetylation of choline by both soluble and membrane associated ChAT more dramatically than does the natural end product ACh.

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TABLE OF CONTENTS

	PAGE
ABSTRACT.	ii
ACKNOWLEDGEMENTS.	iii
INTRODUCTION.	1
LITERATURE REVIEW	2
Historical.	2
Subcellular location of ChAT.	6
Substrates for ChAT	8
Kinetic mechanism of ChAT	10
Studies using homocholine	12
MATERIALS AND METHODS	14
Enzyme preparations	14
Enzyme assays	16
Chromatography.	17
Electron microscopy	17
RESULTS	19
Recovery of P ₃ ChAT activity following sucrose density gradient centrifugation	19
Kinetics of homocholine acetylation by the P ₄ fraction.	20
Ability of NVP to inhibit choline and homocholine acetylation by the P ₄ fraction.	20
Inability of NVP to reduce acetylation of homocholine by solubilized ChAT	21
Does homocholine serve as a competitive alternative substrate to choline for acetylation by P ₄ ChAT	21
Does homocholine serve as a competitive alternative substrate to choline for acetylation by soluble ChAT.	23
Ability of ACh and AHCh to inhibit acetylation of choline by solubilized and P ₄ ChAT.	24
DISCUSSION.	26
TABLES.	29,30
FIGURES	31-49
LITERATURE CITED.	50

INTRODUCTION

According to the classical model of a cholinergic nerve ending, acetylcholine (ACh) is synthesized by choline-O-acetyltransferase located in the cytoplasm of the nerve ending. ACh is then transferred into the synaptic vesicles where it is stored. Depolarization of the nerve ending stimulates the release of ACh from vesicles by a Ca^{++} dependent exocytotic process.

Presently not all studies support the classical model. In particular, the transfer of ACh from the cytoplasm to vesicles has not been demonstrated (Marchbanks and Israel, 1972; Suszkiw, 1976; Nelson et al., 1980). Also not all nerve ending ChAT is soluble. Some appears to be non-ionically associated with a crude vesicular fraction.

Recent studies using the choline analog homocholine suggest that ACh released upon depolarization may not necessarily be synthesized by soluble ChAT. Homocholine is similar to choline in most all aspects of cholinergic metabolism. However, homocholine differs from choline in one very important respect - it is not acetylated by soluble ChAT (see lit. review), although it is acetylated by slices of rat cerebral cortex (Boksa and Collier, 1980). Therefore the objective of this study was to determine if an alternative form of ChAT, which is non-ionically associated with a crude vesicular fraction, can acetylate homocholine.

LITERATURE REVIEW

Historical

Acetylcholine (ACh) is a neurotransmitter at autonomic ganglia, the neuromuscular junction, at parasympathetic and some sympathetic neuro-effector junctions, and a putative neurotransmitter in the central nervous system. Katz and his associates (Fatt and Katz, 1951, 1952, 1953; del Castillo and Katz, 1954) presented evidence that neurotransmitter released spontaneously from nerve endings of the neuromuscular junction (ACh) in multi-molecular quanta generates post-synaptic miniature end-plate potentials (m.e.p.p.s.). Nerve stimulation increases the frequency of m.e.p.p.s. Later, several investigators (e.g. Palade and Palay, 1954; deRobertis and Bennett, 1954, 1955) reported that electron microscopy revealed the presence of large numbers of spherical organelles ("synaptic vesicles") contained within nerve endings. These synaptic vesicles were considered to be the morphological correlates of the multi-molecular quanta reported earlier. The demonstration of quantal release of neurotransmitter, and the observation of synaptic vesicles in nerve endings led to the formulation of the vesicle hypothesis (first described by del Castillo and Katz, 1957). According to this hypothesis, ACh which is stored in synaptic vesicles is released into the synaptic cleft upon nerve stimulation, or depolarization, by a Ca^{++} dependent exocytotic process. Birks and MacIntosh (1961) demonstrated that the sympathetic ganglion contains several ←

pools of ACh. One pool, known as "stationary" ACh (approximately 15% of the total ACh content of the ganglion) was thought to be contained within nerve axons and cell bodies, and remains unaltered upon nerve stimulation. The other pool (remaining 85% of total ACh), termed "depot" ACh, was thought to be present in nerve endings, and was at least partially releasable upon depolarization. These authors noted that this latter pool seemed to be made up of two subfractions, one of which is smaller than the other and more readily releasable upon depolarization. In 1962, Gray and Whittaker isolated nerve ending particles with synaptic vesicles contained within them (synaptosomes) from homogenates of guinea-pig brain. Hebb and Whittaker (1958) also presented evidence suggesting that the synaptosomes contained "bound" ACh, which corresponds to the depot ACh of Birks and MacIntosh (1961). In 1964, Whittaker et al. reported that hypo-osmotic rupture of synaptosomes released approximately 50% of the bound ACh (S_3 fraction, cytoplasmic or labile-bound) while the other 50% remained associated with membrane fragments (P_3 fraction, vesicular or stable-bound). The stable-bound ACh was bimodally distributed in subcellular fractions of hypo-osmotically ruptured synaptosomes spun through a discontinuous sucrose gradient: one fraction (D) was enriched in synaptic vesicles and the other (H) contained large membrane fragments, then thought to be only nondisrupted synaptosomes. Several investigators using in vivo (Chakrin et al., 1972) or in vitro (Richter and Marchbanks, 1971a,b; Molenaar et al., 1973) techniques, reported that after labelling the subcellular pools of ACh to different specific activities using the precursor labelled choline, the specific activity of the ACh subsequently released was considerably higher than that con-

tained in either the cytoplasmic or vesicle-bound stores. These results indicate that newly synthesized ACh is preferentially released in response to nerve stimulation, and also suggest that a small subpool of vesicles may turn over its ACh content faster than the main vesicle population. Barker et al. (1972) then demonstrated that a tail vein injection of labelled choline into the guinea pig led to labelling of ACh in the different subcellular fractions of ruptured synaptosomes of the cortex to different specific activities, also suggesting that different pools of ACh turn over at different rates. Fraction H (containing the large neuronal membrane fragments and some membrane associated vesicles) had the highest specific activity, and fraction D (containing monodisperse synaptic vesicles) had the lowest specific activity of ACh. Soluble (cytoplasmic, or labile-bound) ACh had an intermediate specific activity.

In the early 1970s, most investigators agreed that most if not all of the enzyme which synthesizes ACh, choline-O-acetyltransferase (ChAT) is soluble within the nerve ending (see review on the subcellular location of ChAT). Therefore, based on the experimental evidence summarized thus far, and the evidence that most all ChAT is cytoplasmic, the classical model of the cholinergic nerve ending was developed. According to this model, all nerve ending ACh is synthesized by soluble ChAT. ACh is then transported into vesicles where it is stored, until depolarization stimulates the release of ACh by exocytosis. As mentioned earlier, not all studies support this model. Although attempted by several investigators, using vesicle fractions (Marchbanks and Israel, 1972; Nelson et al., 1980) or vesicle ghosts (Suszkiw,

1977) transfer of ACh from cytoplasm into vesicles above diffusional levels has never been demonstrated.

Another alternative hypothesis presented, from studies using Torpedo electroplaque (see review by Israel, Dunant, and Manaranche, 1979), was that ACh is synthesized by cytoplasmic ChAT, and may be released directly from the cytoplasm in response to depolarization. Ca^{++} regulates gates in the neuronal membrane through which ACh is thought to be released during depolarization. In this case the vesicles serve only as storage sites for ACh.

The development of freeze-fracture techniques has provided evidence that ACh is released from nerve ending vesicles by exocytosis. Heuser and Reese (1973) and Heuser et al. (1974) have observed clusters of "dimples" in the presynaptic membrane in a regular array around "active zones." Thin sections of similar tissues reveal the presence of numerous vesicles clustered near electron-dense regions in the neuronal membrane postulated to be the active-zones of release. These investigators also found that nerve stimulation increases the number of dimples adjacent to the active zones. The dimples are thought to be vesicles in the process of merging with and collapsing into the neuronal membrane during exocytosis.

Recently Katz and Miledi (1977) and Gorio et al. (1978) have presented studies suggesting that ACh may be released from nerve endings by more than one process, as follows: 1) Ca^{++} dependent depolarized release (generating electrophysiological end plate potentials); 2) Ca^{++} dependent spontaneous release (generating m.e.p.p.s.); and 3) Ca^{++} independent spontaneous release (generating background "noise," thought

to account for approximately 90% of spontaneous release, Katz and Miledi, 1977). Carroll and Aspry (1980) have also presented evidence that these different forms of ACh release may originate from different subcellular pools of ACh within nerve endings. The functional significance of these several forms of release remains to be elucidated.

Subcellular location of ChAT

The subcellular location of ChAT was the subject of controversy for a number of years. Hebb and her coworkers (Hebb and Smallman, 1956; Hebb and Whittaker, 1958) demonstrated that ChAT in brain homogenates is partially "soluble" and partially "particle bound." The soluble ChAT is that which originates from the disrupted nerve axons and cell bodies, and the particle bound ChAT (which represents the majority of ChAT in brain) is that which is present in synaptosomes. Hypo-osmotic rupture of synaptosomes releases their contents and different groups of investigators isolated ChAT in different subcellular fractions. Whittaker and his coworkers (Whittaker, Michaelson and Kirkland, 1964) recovered ChAT in a soluble cytoplasmic fraction of ruptured synaptosomes, which is removed from the occluded ACh stores (vesicle fractions). deRobertis et al. (1963) found that ChAT was associated with a subcellular fraction which did contain occluded ACh. Initially the difference was attributed simply to species variations (Tucek, 1966 a,b). However, the work of Fonnum (1967, 1968) appeared to resolve the situation. He found that the subcellular location from which ChAT was recovered depended on the pH and ionic strength of the suspending medium. Positively charged ChAT binds to the negatively charged membranes more readily at a low pH and low ionic

strength, and can be removed by washing the nerve ending fractions with a high ionic strength buffer. Also he found that rat and rabbit brain enzymes (which deRobertis studied) were much more resistant to these effects than guinea-pig (which Whittaker used) and pigeon enzymes. Fonnum concluded (1968) that at physiological pH and ionic strength "much if not all of the [ChAT] must be in a free form," and this concept has been generally accepted until recently.

Several investigators have also attempted to establish the subcellular location of ChAT using electron microscopic histochemical techniques. Kasa (1970, 1975), utilizing Pb capture of Coenzyme A, found the reaction product in the cytoplasm, and adsorbed to mitochondria and vesicles, but not within vesicles. However, Fiegenson and Barnett (1977) using different histochemical techniques (utilizing Mn^{++} and ferrocyanide) found reaction product within the vesicles, and the appearance of the product was sensitive to inhibition by the ChAT inhibitor 4(1-Naphthyl vinyl)pyridine (NVP). Therefore, based on cytochemical techniques, the subcellular location of ChAT was still somewhat controversial.

ChAT from mammalian tissue can be separated into several molecular forms on the basis of isoelectric point (e.g. White and Wu, 1973,c). It is not presently clear which form (or forms) is (are) functionally significant in neurotransmission. Krell and Goldberg (1975) reported that the ChAT inhibitor NVP, when injected into mouse or guinea-pig, inhibited soluble ChAT but there was no significant alteration in steady state brain ACh levels. Even at a neutral pH and high ionic strength, a certain amount (20-40%) of nerve ending ChAT remains insoluble. Hebb (1972) has suggested that "Even if reduced to 10%, the

enzyme remaining bound, if strategically located, could be responsible for the production of as much ACh as is present in the storage system, the 'depot' ACh of Birks and MacIntosh" (1961).

Recently Smith and Carroll (1980) have demonstrated that a crude vesicular fraction (P_3) of mouse forebrain, prepared from hypo-osmotically ruptured synaptosomes, retains approximately 15% of synaptosomal ChAT activity, after washing the fraction twice with 100 mM sodium phosphate buffer at a pH of 7.4. ChAT which is solubilized by the high ionic strength buffer washes appears to differ from the bound ChAT, in that it is more sensitive to inhibition by ACh and NVP, and is also more dependent on Na^+ . The fraction (P_3) which contains membrane bound ChAT also contains stable bound ACh (protected from hydrolysis by AChE in the absence of a cholinesterase inhibitor). Also ACh synthesized by membrane bound ChAT is partially protected from hydrolysis when excess AChE is added to the incubation medium, whereas ACh synthesized by soluble ChAT is not. This membrane bound form of ChAT may be functionally significant in neurotransmission.

Substrates for ChAT

ChAT catalyzes the synthesis of ACh from choline and acetyl CoA. An exogenous supply of choline is required to maintain ACh synthesis over a period of time since choline is not synthesized in the brain. The concentration of choline present in the extracellular space is maintained by choline from the plasma, and also choline resulting from the hydrolysis of ACh by acetylcholinesterase present in the synaptic cleft. Choline may also be partially supplied by the breakdown of membrane lipids. Uptake of choline into nerve terminals from the

synaptic cleft is thought to occur by one of two transport processes: low-affinity transport, with a K_t of 50-100 μM , and a high-affinity transport, with a K_t of 1-5 μM . The latter has been postulated to be specific for cholinergic nerve endings (Kuhar et al., 1973), and also to be coupled to ACh synthesis (e.g. Barker and Mittag, 1975) (see reviews by Kuhar and Murrin, 1978; and Jope, 1980).

The origin of acetyl CoA for ACh synthesis in nerve endings is presently unknown. It is synthesized within mitochondria; but whether acetyl CoA for ACh synthesis is generated at another location in the nerve ending, remains to be elucidated. An external source of acetate groups is required to maintain ACh synthesis. Several investigators have found glucose and pyruvate to be good precursors, both in vivo (e.g. Tucek and Cheng, 1974) and in vitro (e.g. Lefresne et al., 1973; Guyenet et al., 1973). Balfour and Hebb (1952) found that acetone extract powders of brain tissue are able to synthesize ACh from choline and either citrate or acetate. Tucek (1967 a,b,c) has reported that citrate-ATP lyase (EC 4.1.3.8) and acetyl CoA synthase (EC 6.2.1.1) (which synthesize acetyl CoA from citrate and acetate, respectively) are at least partially extramitochondrial enzymes. Alternatively, Sollenburg and Sorbo (1970), and Sterling and O'Neill (1978) have suggested that citrate may simply mediate the transport of the acetyl moiety from mitochondria to the site of synthesis of ACh. Gibson and Shimada (1980) report that citrate may not be the only intermediate in acetyl CoA metabolism in brain tissue, but that acetate and carnitine may also be involved. They also present evidence that acetyl CoA involved in ACh synthesis may be distinguished from acetyl CoA involved in lipid synthesis, by using (-) hydroxycitrate and n-butylmalonate,

both inhibitors of citrate metabolism. This agrees with a previous study by Tucek and Cheng (1974) which reports that when rats were injected with [1,5-¹⁴C] citrate, the lipid stores in the brain became labelled, whereas ACh did not. However this last study only examined whole brain ACh, and did not distinguish between the different sub-cellular pools of ACh.

Another alternative, proposed by Tucek (1967, c) is that acetyl CoA may itself be transported out of mitochondria during mitochondrial contractions.

Kinetic mechanism of ChAT

Several different mechanisms have been proposed by different groups of investigators on how ChAT catalyzes the synthesis of ACh. Kinetic studies have often led to different conclusions for several reasons. ChAT has not yet been isolated in pure form and often enzyme preparations may be contaminated with other proteins which interfere with the assay. Also ChAT decreases in specific activity the more it is purified (Hebb, 1972). Finally a variety of different species have been used in kinetic studies. In general, most investigators believe that ChAT functions by the Theorell-Chance mechanism (e.g. White and Wu, 1973, b; Morris et al., 1971). According to this mechanism of enzyme activity both the reactants (choline and acetyl CoA) bind and the products (ACh and CoA) dissociate from the enzyme in a particular order. However, Hersh and Peet (1977) have suggested that not all experimental evidence uniquely supports this type of mechanism. These authors employed dead-end inhibitors and alternate substates, and suggest that ChAT functions by a sequential random mechanism, in which the substrates bind and the products dissociate in random order.

They indicate that product inhibition studies (in which ACh competitively inhibits acetyl CoA but noncompetitively inhibits choline) can be also explained by the sequential random mechanism. Also, the deuterium isotope effect observed by Currier and Mautner (1974) (interpreted as general base catalysis) is not consistent with the Theorell-Chance mechanism.

Several investigators have attempted to classify ChAT as a sulfhydryl enzyme (i.e., the enzyme requires a free sulfhydryl group in its active site) utilizing various sulfhydryl agents (e.g. Reisburg, 1957; Mannervik and Sorbo, 1970). In general most investigators now agree that ChAT contains "nonessential" sulfhydryl groups, and that inhibition of ChAT by sulfhydryl reagents may be caused by conformational changes in the enzyme, and not by direct binding to the active site (e.g. White and Cavallito, 1970; see also review by Hebb, 1972).

Imidazole (or histidine) residues have also been implicated by Currier and Mautner (1974) among others (e.g. White and Cavallito, 1970), particularly in view of the previously mentioned general base catalysis observed by these authors. Imidazole groups have been shown to activate ChAT (White and Cavallito, 1970; Hebb et al., 1975; Davies, 1979) and may also synthesize ACh nonenzymatically (Burt and Silver, 1973; Aquilonius and Eckernas, 1976).

Ionic requirements of ChAT have also been studied by several groups of investigators. Rossier et al. (1977) demonstrated that only Cl^- activates ChAT solubilized from rat brain. However, Hersh (1980) found that all monovalent anions activate ChAT to the same extent.

Guyenet et al. (1973) and Smith and Carroll (1980) have found that ChAT is dependent on Na^+ for activity. Additionally, Hersh and his coworkers (1978, a,b,c) demonstrated that enzyme activity and mechanism may be regulated by ionic strength, and that divalent cations such as Ca^{++} and Mg^{++} inhibit the enzyme.

Studies using homocholine

Recent results obtained with the choline analog homocholine (3-trimethylamino propan-1-ol) support the possibility that the activity of soluble ChAT may not be essential to the formation of transmitter released during depolarization (Collier et al., 1977). The structure of homocholine is shown in Fig. 1. Homocholine is similar to choline in many aspects of cholinergic metabolism. It is transported into rat brain synaptosomes (Collier et al., 1977) and electroplaques of Torpedo (von Schwarzenfeld et al., 1979; Luqmani et al., 1980) and is acetylated and released as acetylhomocholine (AHCh) from the superior cervical ganglion (Collier et al., 1977), guinea-pig cortex (von Schwarzenfeld, 1979), Torpedo electroplaques (Luqmani et al., 1980) and minces of mouse forebrain (Carroll and Aspary, 1980) by a Ca^{++} dependent process during depolarization. A crude vesicular (P_3) fraction of mouse forebrain, depleted of its acetylcholine content independently of the cytoplasm (S_3) by incubation of mouse forebrain minces in a lithium-high potassium Krebs medium, can also be refilled with either newly synthesized AHCh or newly synthesized ACh formed from their respective precursors homocholine or choline. This refilling does not occur by transfer of the acetylated product from the S_3 fraction since the ratio of newly synthesized to preformed transmitter

in the P₃ fraction exceeds that of the S₃ fraction (Carroll and Nelson, 1978; Nelson et al., 1980).

Homocholine, however, differs from choline in one very important respect. Homocholine is not acetylated by soluble ChAT (Burgen et al., 1956; Dauterman and Mehrotra, 1963; Currier and Mautner, 1974; Barker and Mittag, 1975; Collier et al., 1977). Therefore the objective of this investigation was to determine if homocholine is acetylated by the membrane associated form of ChAT present in the nerve endings of mouse forebrain.

MATERIALS AND METHODS

Choline and 3-heptanone (practical grade) were obtained from Eastman Company (Rochester, NY) and the 3-heptanone was redistilled (collected at 146-147°C). Eserine sulfate and carnitine were purchased from Sigma Chemical Company (St. Louis, MO) and 4-(1-naphthylvinyl) pyridine (NVP) and acetyl Coenzyme A from Calbiochem (La Jolla, CA). Sodium tetraphenylboron and toluene (spectanalyzed) were purchased from Fisher Scientific Company (Fairlawn, NJ). Aquasol II, Hyamine hydroxide, Liquifluor, [acetyl-1-¹⁴C] acetyl Coenzyme A (spec. act. 48.3 mCi/mmol), [¹⁴C] methyl iodide (spec. act. 31.0 mCi/mmol) and [¹⁴C] acetylcholine (spec. act. 1.2 mCi/mmol) were purchased from New England Nuclear (Boston, MA). Homocholine, acetylhomocholine, [¹⁴C] homocholine and [¹⁴C] acetylhomocholine, were synthesized as described by Nelson et al. (1980).

Enzyme preparations

Male CD-1 albino mice were killed by cervical dislocation, the forebrains quickly removed and placed into ice-cold Normal Krebs (composition in mM; NaCl, 117; KCl, 3.5; MgSO₄, 1.2; NaHCO₃, 28.0; KH₂PO₄, 1.2; CaCl₂, 2.5; Glucose, 11.0). They were blotted dry, weighed, minced, and incubated for 15 min in Normal Krebs at 38°C. Minces were then washed twice with ice-cold 0.32 M sucrose, and subcellular fractions prepared according to the method of Gray and Whittaker (1962), as modified by Collier et al. (1972) and Salehmoghaddam and Collier (1976). Minces were homogenized in 5.0 ml of 0.32 M sucrose at 840

rpm with 8 up and down strokes. This homogenate was spun at 1000 g for 10 min, and the supernatant spun at 17,000 g for 15 min, to yield a P_2 (synaptosomal) pellet. Synaptosomes were hypo-osmotically ruptured by homogenizing the P_2 pellet in 2.0 ml of water at 400 rpm using 8 up and down strokes. This homogenate was spun at 100,000 g for 60 min to yield a crude vesicular fraction (P_3) and an S_3 fraction believed to contain soluble cytoplasmic contents. The S_3 fraction was discarded. The P_3 fraction, which contains occluded ACh, was then washed twice with 1.0 ml of 100 mM sodium phosphate buffer (pH 7.4) in order to remove ionically bound ChAT (Fonnum, 1968). Approximately 70% of the ChAT activity recovered from the hypo-osmotically ruptured P_2 fraction was present in the 100 mM sodium phosphate buffer wash of the P_3 fraction whereas 15% remained in the washed P_3 (Smith and Carroll, 1980). The washed P_3 fraction was then homogenized in 1.0 ml of water and layered on top of a discontinuous sucrose gradient containing 2 ml each of 0.4 M and 0.6 M sucrose and spun at 192,000 g for 30 min. The pellet was homogenized in 500 μ l of water and stored at -20°C until determination of ChAT activity. The 0.4 M sucrose layer was pelleted at 100,000 g for 60 min. This fraction was suspended in 200 μ l of water for enzyme assay. The 0.6 M fraction was also pelleted at 100,000 g for 60 min and suspended in 500 μ l of water for enzyme assay. P_4 , 0.4 M and 0.6 M fractions were never kept for more than 7 days, and the solubilized enzyme never more than 2 days, since it was noted that enzyme activity declined after these times even though stored at -20°C . In one set of experiments, the P_4 fraction was washed with 1.0 ml of 100 mM sodium phosphate buffer,

centrifuged at 100,000 g for 60 min and the ChAT activity determined in the wash fraction as well as the washed P₄ fraction.

Enzyme assays

ChAT activity in the various fraction was determined in duplicate or triplicate by the method of McCamen and Hunt (1965) as modified by Fonnum (1969). Aliquots of 2 ul of the tissue fractions or the pooled sodium phosphate buffer washes of the P₃ fraction were incubated for varying time periods (activity was linear for at least 30 min for all substrate concentrations used) at 39°C, a temperature which activates brain ChAT activity (Hebb, 1975), with 20 ul of a buffer substrate consisting of the following components: NaCl (234 mM), Bovine Plasma Albumin (0.0375%), MgCl₂ (17mM), sodium phosphate buffer, pH 7.4 (84 mM), Eserine (150 uM), [¹⁴C] acetyl Coenzyme A (343 uM, spec. act. 2 mCi/mmol) and one or several of the following substrates in the concentrations indicated: choline, homocholine, carnitine, or no added substrate. Acetyl CoA: Carnitine-O-acetyltransferase (carnitine acetyltransferase, EC 2.3.1.7) activity was determined by replacing choline with carnitine in the McCamen and Hunt assay. In some experiments the following compounds were tested as inhibitors of ChAT: ACh, AHCh, and NVP. Stock solutions were prepared fresh in water (pH 4.0 for ACh and AHCh) and 1 ul aliquots were added to tissue samples just prior to addition of the buffer substrates. One ul of water (pH 4.0) did not affect soluble or membrane bound ChAT activity. NVP solutions were always prepared in amber bottles and the assay conducted in a darkened room, since NVP is light sensitive.

Following incubation, samples were chilled and the acetylated products were extracted with 150 ul of sodium tetraphenylboron/

3-heptanone (TPB/3-hept., 75 mg/ml). One ml of Hyamine hydroxide was added to 100 μ l of the TPB/3-hept. extract and [14 C] acetylated products were counted in 15 ml of toluene containing Liquifluor. Acetylation was calculated from the specific activity of the buffer substrate, which was counted in the presence of TPB/3-hept. (75 mg/ml) (extraction of acetylated products into 75 mg/ml TPB/3-hept. was essentially complete). Net acetylation was corrected for acetylation of endogenous substrates. Because of variability in the tissue preparation, all experiments were conducted on forebrains from at least 6 experimental animals except where indicated.

Chromatography

Tissue aliquots were incubated as previously described except that acetylated products were extracted with 5 mg/ml TPB/3-hept. 100 μ l of the organic phase was mixed with 50 μ l 1N HCl and aliquots of the HCl pooled and dried. Two μ l of water was added to the dried extract and the sample was streaked on a strip of Whatman #3 paper. Acetylated products were separated by descending paper chromatography in a solvent system of n-butanol: ethanol:1N acetic acid: water (8:2:1:3) and identified by comparison with [14 C] ACh and [14 C] AHCh standards run simultaneously. Labelled products were dissolved from 1 cm strips in 1 ml of water and counted in Aquasol II.

Electron microscopy

Pellets obtained from the subcellular fractionation were fixed in 1% formaldehyde in 50mM cacodylate buffer, and post-fixed in 1% OsO₄ in Millonig's phosphate buffer. They were then dehydrated in ethanol and embedded in Spurr's resin according to standard procedures

(Spurr, 1969). Ultra-thin sections were stained with lead citrate and uranyl acetate, and examined under an RCA EMU-3F electron microscope.

RESULTS

Recovery of P₃ ChAT activity following sucrose density gradient centrifugation.

Aliquots of the subcellular fractions of mouse forebrain were assayed for ChAT activity using 10 mM choline. ChAT which is nonionically bound to the P₃ fraction, i.e., ChAT which remains associated with that fraction after two washes with 100 mM sodium phosphate buffer (pH 7.4), formed 429.7 ± 71.4 nmol/g wet wt/hr of acetylated product (n=4). Approximately 81% of the ChAT activity associated with the washed P₃ fraction was recovered from the 3 fractions of the sucrose density gradient: the 0.4 M sucrose fraction formed 9.0 ± 2.5 nmol/g wet wt/hr (n=24) and electron microscopy revealed that this fraction is enriched with spherical organelles resembling vesicles; the 0.6 M sucrose fraction formed 48.5 ± 14.9 nmol/g wet wt/hr (n=6); and the P₄ fraction formed 289 ± 61 nmol/g wet wt/hr (n=18). Electron micrographs of this fraction revealed that it contains large membrane fragments, which have spherical organelles associated with some fragments, similar to those shown in a recent report (von Schwarzenfeld, 1978). In the experiment in which the P₄ fraction was homogenized in 1.0 ml of 100 mM sodium phosphate buffer (pH 7.4), centrifuged, and its ChAT activity compared with an unwashed P₄ fraction, the ChAT activity was identical (353.4 ± 37.4 vs. 362.4 ± 14.9 nmol/g wet wt/hr, n=3). ChAT activity in the 100 mM sodium phosphate buffer wash of the P₄ fraction was not detectable. The P₄ fraction, which also contains occluded

ACh (Carroll and Benishin, 1979) most closely reflects the metabolic activity of the P₃, a conclusion supported by a recent publication in which Torpedo electroplaques were studied (Luqmani et al., 1980). Therefore, this fraction was selected for the subsequent kinetic analyses.

Kinetics of homocholine acetylation by the P₄ fraction.

The kinetic parameters for P₄ ChAT activity as a function of added homocholine were determined using substrate concentrations ranging from 10⁻⁵ to 10⁻² M. Each point represents the average velocity, corrected for acetylation of endogenous substrates, for 15 forebrains. Results in Figure 2 indicate that the apparent K_m value for homocholine acetylation is 49 uM, and the apparent V_{max} for homocholine acetylation is 70 nmol/g wet wt/hr. Boiling tissue prior to incubation with homocholine (10 mM, n=6) reduced the formation of acetylated products to the level of tissue-free blanks.

Ability of NVP to inhibit choline and homocholine acetylation by the P₄ fraction.

Several reports have suggested that carnitine acetyltransferase, which is present in cholinergic preparations, may acetylate cholinergic neurotransmitter precursors (White and Wu, 1973a; Tucek et al., 1978). To determine if choline and/or homocholine are primarily acetylated by P₄ ChAT rather than carnitine acetyltransferase, aliquots of the P₄ fraction were incubated either in the presence or absence of NVP, which inhibits ChAT but not carnitine acetyltransferase (White and Wu, 1973a). Results shown in Fig. 3 indicate that NVP

(4.6 μM) significantly reduces acetylation of choline (60%) and homocholine (40%), but stimulates acetylation of carnitine (18%) by the P_4 fraction.

Inability of NVP to reduce acetylation of homocholine by solubilized ChAT.

Results presented in Table 1 indicate that NVP (4.6 μM) significantly reduces choline acetylation by ChAT solubilized from the P_3 fraction 76%. This fraction acetylates carnitine and homocholine very poorly, and NVP does not alter acetylation of either substrate.

Does homocholine serve as a competitive alternative substrate to choline for acetylation by P_4 ChAT?

Results obtained in this study with the ChAT inhibitor NVP suggest that both choline and homocholine are acetylated by ChAT in the P_4 fraction. To determine if both substrates are acetylated by the same or different forms of ChAT in the P_4 fraction, the kinetic parameters of choline acetylation were determined in the absence or presence of several fixed homocholine concentrations (0.5, 1.0, 5.0, or 10.0 mM) using 3 forebrains, according to the procedure of Cha (1968). In this method, two potential substrates are simultaneously incubated with the enzyme and the concentration of one substrate, A, is varied while the other, B, is held constant, and the combined velocity of products formed is determined. In the present experiments, the combined velocity equals the total amount of acetylated products formed, i.e., [^{14}C] ACh and [^{14}C] AHCh. Both of these products behave identically in the assay used and are extracted equally by TPB/3-hept.

When both substrates are metabolized by the same enzyme and substrate A is better than B (i.e., the V_{\max} for A exceeds the V'_{\max} for B), then a plot of $1/v$ (combined velocity) vs. $1/[A]$ will be hyperbolic. When a series of kinetic plots are generated using different fixed levels of substrate B or in the absence of B, and V_{\max} exceeds V'_{\max} , then the curves generated will intersect at a common point. A theoretical common point of intersection can be predicted from the following relationship: $S = V'_{\max} \cdot K_m / (V_{\max} - V'_{\max})$ where S represents the concentration of substrate A at the common point of intersection, and K_m represents the apparent Michaelis constant obtained for substrate A.

When the V_{\max} equals V'_{\max} the curves will merge at the Y axis and not be straight lines. If substrate B is not metabolized by the same enzyme as A but competes with A for binding to the same enzyme, then a series of straight lines is generated which yield similar $V_{\max} S$. If two enzymes are involved in the metabolism of the two substrates, the curves obtained will not intersect at one point.

Figure 4 depicts the pooled results obtained for two different experiments where $1/v$ (combined velocity) vs. $1/S$ (corrected for endogenous choline concentration) for each fixed homocholine concentration (0, 0.5, 1.0, 5.0, or 10.0 mM) is plotted. Values were normalized by multiplying $1/v$ by V_{\max} for choline and $1/S$ (corrected) by the K_m for choline for each of the two experiments. The correlation coefficients obtained for the least squares regression lines of the data points (dotted lines) decrease as the concentration of homocholine is increased. Polynomial regression analysis shows that a second degree polynomial fits the data points obtained better than a straight line when homocholine is present.

The combined plot (Figure 5) shows that the curves do in fact intersect at one common point as predicted when one enzyme metabolizes both substrates. When this experiment was repeated a third time with another forebrain, kinetic plots yielded a similar pattern.

Table 2 summarizes the kinetic parameters obtained for choline and homocholine for all three alternative substrate binding experiments. The V_{\max} for choline acetylation (295 ± 86 nmol/g wet wt/hr) exceeds the V'_{\max} for homocholine acetylation (79 ± 23 nmol/g wet wt/hr) by approximately 3.7 fold. The theoretical point of intersection (47 μ M choline) is similar to the actual point of intersection obtained for the three experiments (52 ± 5 choline). The mean V_{\max} value for homocholine acetylation by P_4 ChAT obtained using the Cha procedure (79 ± 23 nmol/g wet wt/hr, n=3) closely corresponds to the value obtained for homocholine acetylation by the method of Lineweaver and Burk (1934) (70 nmol/g wet wt/hr, n=15).

Does homocholine serve as a competitive alternative substrate to choline for acetylation by soluble ChAT?

Aliquots of the sodium phosphate buffer wash of the P_3 fraction were incubated with varying concentrations of choline in the absence or presence of fixed homocholine concentrations (2.0, 10.0, 20.0 mM) as described in the previous section for P_4 ChAT. Again values were normalized. The plot of V_{\max}/v vs. K_m/S for the sodium phosphate buffer was different (Figure 6) from that obtained for the P_4 fraction. For example, the correlation coefficients obtained for the least squares regression lines do not change with increasing homocholine concentration and polynomial regression analysis indicates that the

points are best fit by straight lines rather than second degree polynomials. Regression coefficients increase with increasing homocholine concentration and the V_{\max} s for choline acetylation do not change with increasing homocholine concentration suggesting that homocholine competitively inhibits the binding of choline to soluble ChAT but is not being acetylated.

Ability of ACh and AHCh to inhibit acetylation of choline by solubilized and P_4 ChAT

ACh competitively inhibits choline acetylation by ChAT (Kaita and Goldberg, 1969; White and Wu, 1973 b,c; Smith and Carroll, 1980). To determine if AHCh also inhibits acetylation of choline by soluble and/or P_4 ChAT, aliquots of the sodium phosphate buffer wash or P_4 fraction were incubated in the presence or absence of varying concentrations of AHCh and compared with similar concentrations of ACh. To insure that the added inhibitor did not interfere with the extraction of labelled acetylated product into the TPB/3-hept., ACh or AHCh was added to some samples following incubation with the buffer substrate. Their respective effects on the amounts of acetylated products extracted into TPB/3-hept. were determined to be negligible at the inhibitor concentration used.

ACh, as expected, inhibits acetylation of choline by ChAT in the sodium phosphate buffer wash and the P_4 (Figure 7). AHCh also inhibits both soluble and P_4 ChAT activity in a dose dependent manner, and is a more potent inhibitor of both enzyme forms than is ACh. Dixon plots of ACh inhibition of P_4 ChAT and AHCh inhibition of P_4 and soluble ChAT indicate that inhibition is competitive in all 3 cases, as

illustrated in Figures 8, 9, and 10. Plots of $1/v$ of AHCh or ACh concentration for the different concentrations of choline (1.1 and 4.3 mM) intersect in the fourth quadrant, which is consistent with competitive inhibition, and yield K_i s of 63mM, 10mM, and 200mM, respectively.

DISCUSSION

In agreement with the results of others (Burgen et al., 1956; Dauterman and Mehrotra, 1963; Currier and Mautner, 1974; Collier et al., 1977), the choline analog homocholine does not appear to be acetylated by soluble ChAT. The ChAT inhibitor NVP (Krell and Goldberg, 1975) does not reduce its acetylation at a concentration which reduces choline acetylation by approximately 75%. It does, however, compete with choline for binding to soluble ChAT since its presence alters the kinetic plots obtained for choline acetylation in a manner suggestive of competitive binding (Cha, 1968).

Homocholine, like choline, is acetylated by a form of ChAT which is nonionically associated with a fraction of mouse forebrain nerve endings that contains both occluded ACh and subcellular organelles associated with neuronal membranes (P_4). Acetylation of homocholine by this fraction is saturable and inhibited by the ChAT inhibitor NVP. Homocholine alters the kinetic plots obtained for P_4 ChAT as a function of choline in a manner which suggests that both it and choline are acetylated by the same ChAT in the P_4 fraction. Choline is a superior substrate to homocholine for acetylation by ChAT in the P_4 since the V_{\max} value obtained for choline by the method of Lineweaver and Burk (1934) (295 ± 86 nmol/g wet wt/hr, $n=3$) exceeds the V'_{\max} values obtained for homocholine using either the method of Lineweaver and Burk (70 nmol/g wet wt/hr, $n=15$) or that of Cha (79 ± 23 nmol/g wet wt/hr, $n=3$) by almost 4-fold.

Several results obtained in the present study and a previous one (Smith and Carroll, 1980) indicate that membrane associated ChAT does not represent soluble ChAT which has become artificially associated with membranes during tissue preparation or soluble ChAT contained in nondisrupted synaptosomes. When the P_4 fraction is washed with 100 mM sodium phosphate buffer following sucrose density gradient centrifugation rather than suspended in distilled water, the ChAT activity determined in the washed and unwashed P_4 fractions is identical. This result indicates that suspension of the P_3 fraction in distilled water prior to sucrose density gradient centrifugation does not cause soluble ChAT to bind ionically to membranes recovered in the P_4 fraction. Addition of excess acetylcholinesterase during the initial brain homogenization hydrolyzes at least 90% of the ACh formed by S_3 without hydrolyzing any ACh formed by the P_3 fraction washed twice with 100 mM sodium phosphate buffer (pH 7.4) indicating that tissue homogenization does not form subcellular organelles which trap cytoplasmic ChAT. NVP inhibits the acetylation of homocholine by membrane bound but not soluble ChAT, whereas it reduces the acetylation of choline by soluble ChAT to a greater extent than it reduces the acetylation of choline by membrane bound ChAT. If homocholine were acetylated by soluble ChAT contained in nondisrupted synaptosomes associated with the P_4 fraction, then NVP should inhibit the acetylation of homocholine by soluble ChAT to a greater extent than it reduces acetylation of homocholine by the P_4 fraction and it clearly does not. Homocholine competitively inhibits the binding of choline to soluble ChAT but is not itself acetylated by this form of the enzyme. Conversely, it competitively

inhibits the binding of choline to membrane bound ChAT and is acetylated.

Results presented here appear to differ from those of Boksa and Collier (1980) who concluded that homocholine is probably a substrate for ChAT based on in situ evidence, but were unable to demonstrate appreciable acetylation of homocholine in vitro relative to choline. Their investigation, in contrast to this experimental protocol, encouraged the association of soluble nerve ending ChAT to membranes by suspending ruptured synaptosomes in a low osmotic strength medium. Then they compared the ability of membrane associated ChAT to acetylate choline and homocholine and were unable to detect appreciable acetylation of homocholine relative to choline. The ability to detect acetylation of homocholine by membrane bound ChAT may be due to the fact that nerve ending ChAT was separated into soluble (acetylates choline but not homocholine, and represents at least 70% of nerve ending ChAT) and membrane bound ChAT (acetylates choline better than homocholine and only represents 15% of nerve ending ChAT).

Both AHCh and ACh compete with choline for binding to ChAT, as illustrated by the Dixon plots. The product AHCh is more potent than ACh in inhibiting ChAT solubilized from the P₃ fraction and ChAT non-ionically associated with the P₄ fraction. As Cha et al. (1975) have suggested, the larger chain analog may become "wedged" in the catalytic site and act as a more potent inhibitor than the natural end product.

TABLE 1. ABILITY OF NVP TO INHIBIT ACETYLATION OF CHOLINE, HOMOCHOLINE AND CARNITINE BY ChAT SOLUBILIZED FROM A CRUDE VESICULAR FRACTION^a

	CHOLINE	HOMOCHOLINE	CARNITINE
CONTROL	908 ± 114 (12)	78 ± 22 (12)	136 ± 16 (6)
NVP (4.6 μM)	217 ± 46 (12) ^b	65 ± 24 (12)	123 ± 8 (6)

^a Aliquots of subcellular fractions were incubated with either 10 mM choline or 10 mM homocholine, or 1.2 mM carnitine for 30 min at 39°C, and amounts of acetylated products determined following correction for acetylation of endogenous substrates. Results are expressed as nmol of acetylated product formed/g wet wt/hr.

^b Results significantly different from control at P < 0.05 (t-test, unpaired).

TABLE 2. KINETIC VALUES FOR THE COMPETITIVE ALTERNATIVE SUBSTRATES OF P₄ ChAT, CHOLINE AND HOMOCHOLINE^a

	<u>K_m (choline)</u> <u>(uM)</u>	<u>V_{max} (choline)</u> <u>(nmol/g wet wt/hr)</u>	<u>V'_{max} (homocholine)</u> <u>(nmol/g wet wt/hr)</u>	<u>S</u> <u>(uM)</u>
EXPT. 1	123	465	125	45
EXPT. 2	144	187	50	52
EXPT. 3	172	233	61	61
Mean ± S.E.M.	146 ± 14	295 ± 86	79 ± 23	52 ± 5
THEORETICAL				47

^aNormalized values from Expt. 1 and Expt. 2 are illustrated in Fig. 3 and 4. In all three experiments, the K_m and V_{max} for choline were determined from line A (0 Homocholine). V'_{max} for homocholine was calculated from the Y-coordinate of the point of intersection (V_{max}/v = V'_{max}, Cha, 1968). S, the choline concentration at which homocholine appears to have no effect, was determined from the X-coordinate of the point of intersection (K_m/S). The theoretical value for S was determined from a plot of the theoretical equation

$$v = \frac{\frac{V_{\max}(S)}{K_m} + \frac{V'_{\max}(S')}{K'_m}}{1 + \frac{(S)}{K_m} + \frac{(S')}{K'_m}}$$

where K_m and V_{max} were the means from these three experiments (146 uM and 295 nmol/g wet wt/hr, respectively), and K'_m and V'_{max} obtained from Fig. 1 (49 uM and 70 umol/g wet wt/hr, respectively).

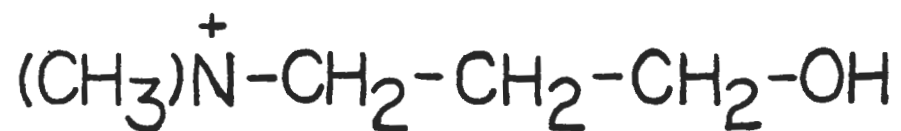
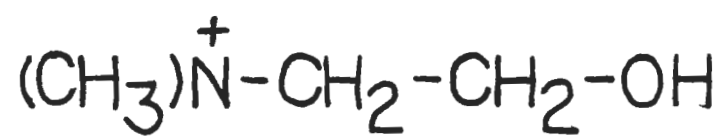


Figure 1. The structure of choline and the analog homocholine.

Figure 2. Lineweaver-Burk plot of homocholine acetylation of P₄ ChAT. 2 μ l aliquots of the P₄ fraction were incubated at 39° for 15 min in the presence of varying concentrations of homocholine ranging from 1×10^{-5} M to 1×10^{-2} M. Each point represents the average velocity for 15 forebrains (each forebrain assayed in duplicate), after correction for acetylation of endogenous substrates. The apparent K_m and V_{max} values for homocholine acetylation are 49 μ M and 70 nmol/g wet wt/hr, respectively, as calculated from the least squares regression line ($Y = 0.00072 x + 0.0147$; $r = .997$).

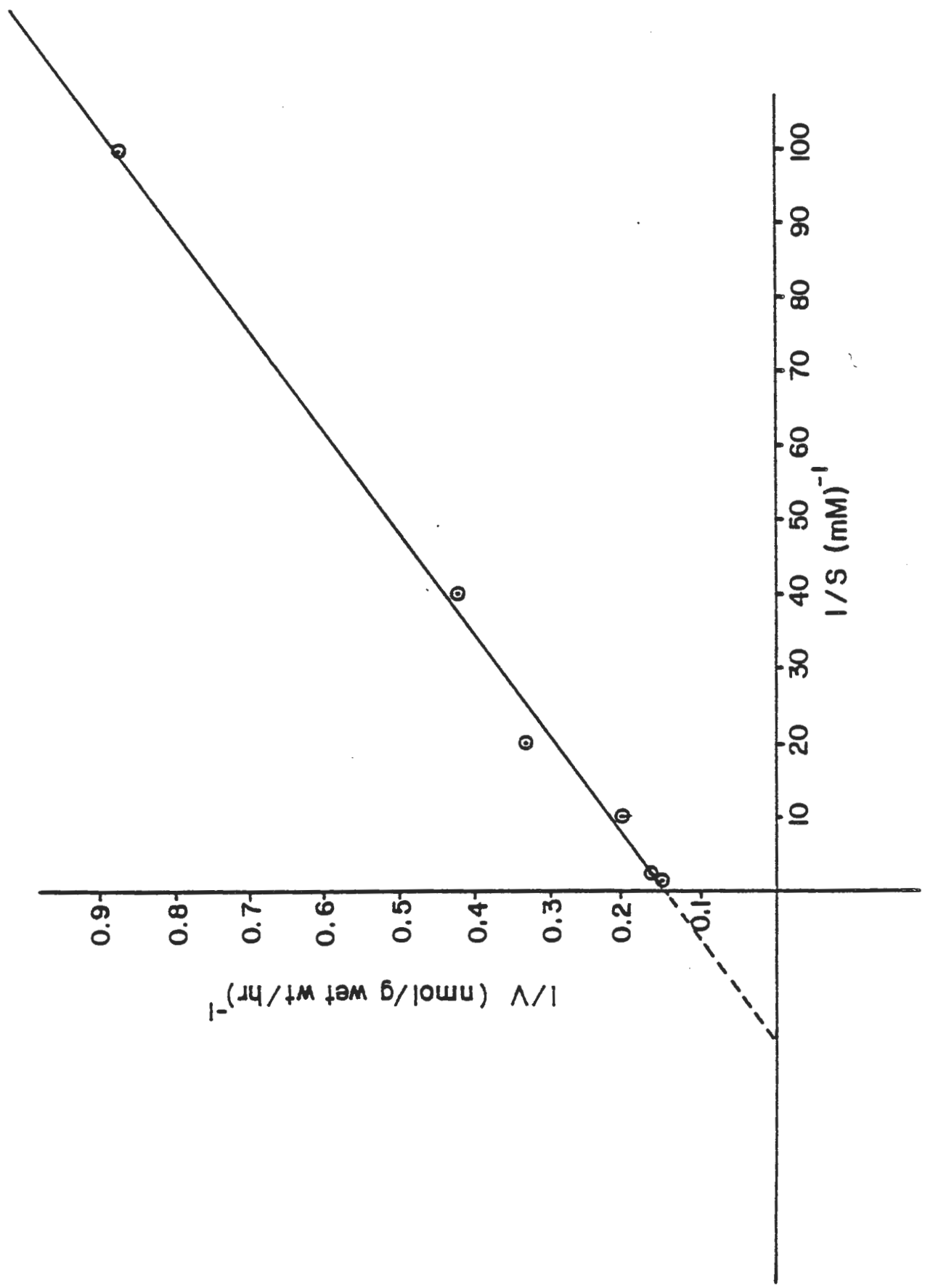


Figure 3. Ability of the ChAT inhibitor NVP to inhibit the acetylation of choline and homocholine but not carnitine by the P₄ fraction. 2 ul aliquots of the P₄ fraction were incubated at 39° for 30 min in the presence of the substrates indicated and the effect of NVP (4.6 uM) determined on the formation of acetylated products. Amounts of acetylated products formed were corrected for acetylation of endogenous substrates. () = number of experimental animals. An asterisk indicates that NVP treatment differs from control at P < 0.05 (t-test, unpaired).

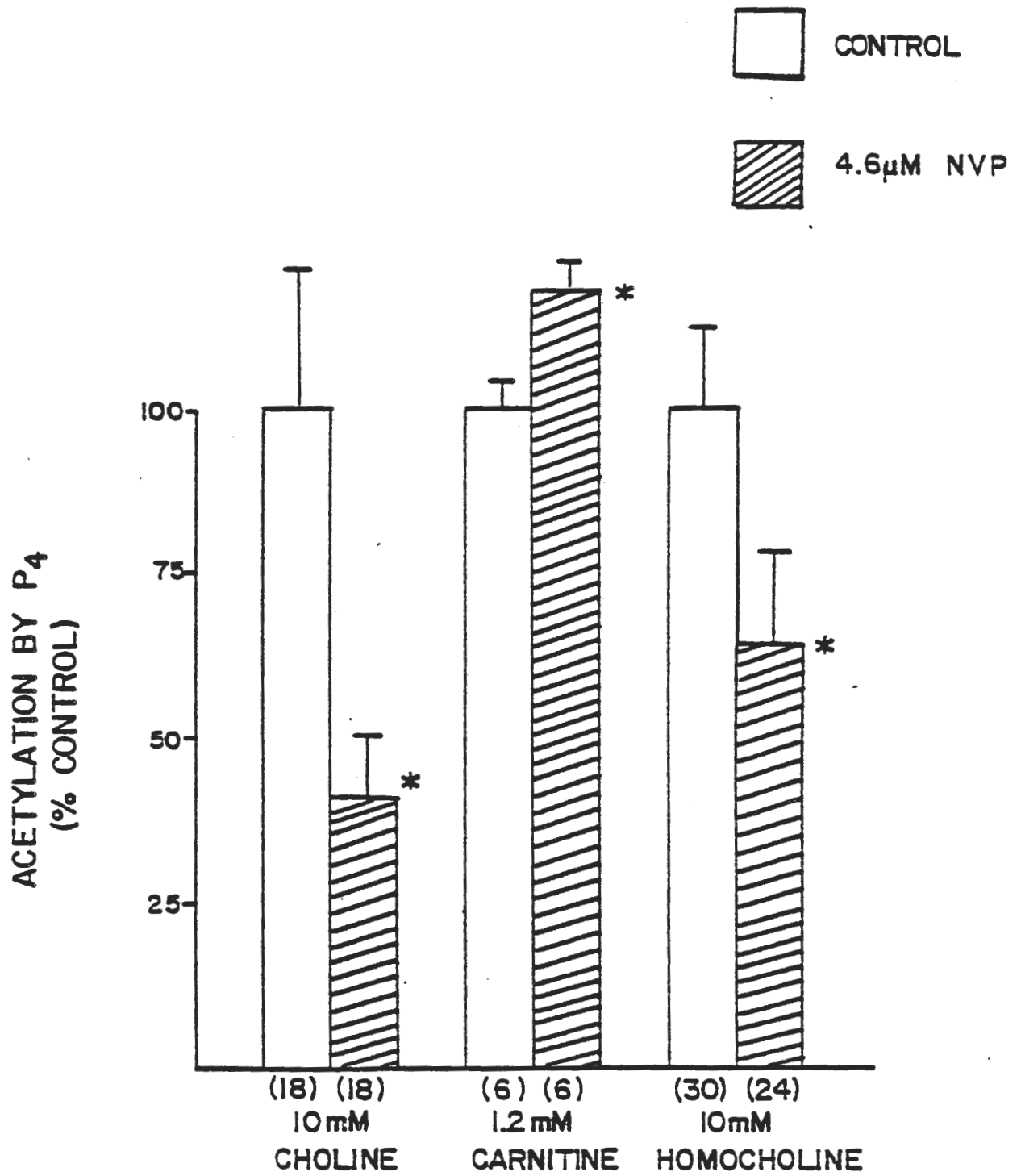


Figure 4. Ability of homocholine to serve as an alternative substrate to choline for acetylation by P₄ ChAT. 2 ul aliquots of the P₄ fraction were incubated at 39° for 15 min with added choline concentrations ranging from 20 to 1000 uM and either no added homocholine or with one fixed homocholine concentration (0.5, 1.0, 5.0, or 10.0 mM). Results from two separate experiments (two forebrains) are included in each plot.

corresponds to Expt 1 and corresponds to Expt 2; see Table 2. Choline concentrations were corrected for endogenous choline. Values were normalized by multiplying $1/v$ by V_{\max} for choline and $1/S$ (corrected) by K_m for choline for each experiment. Least squares regression lines (---) for each homocholine concentration are included along with the correlation coefficients (r). Curved lines(—) through the data points of different kinetic plots were drawn independently. In all cases where homocholine was present, polynomial regression analysis indicated that a second degree polynomial fit the data point better than a straight line.

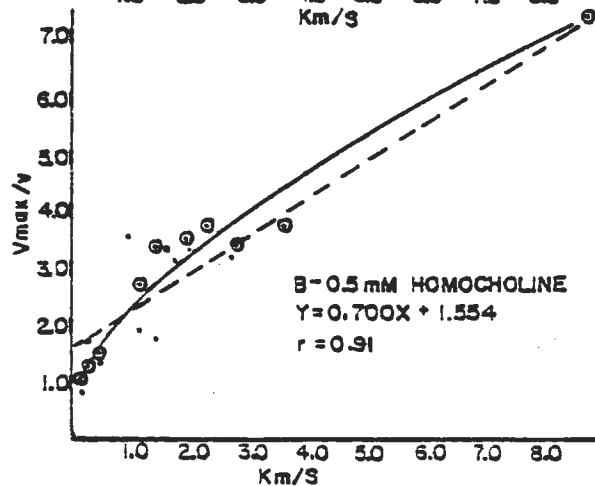
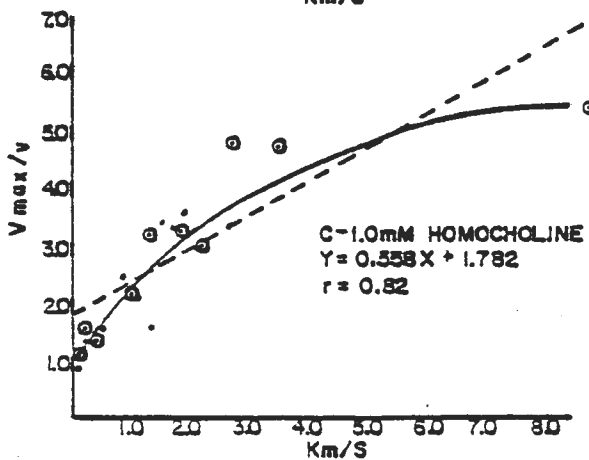
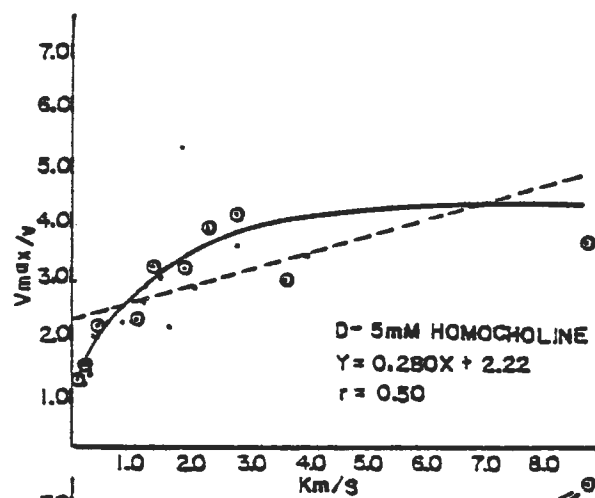
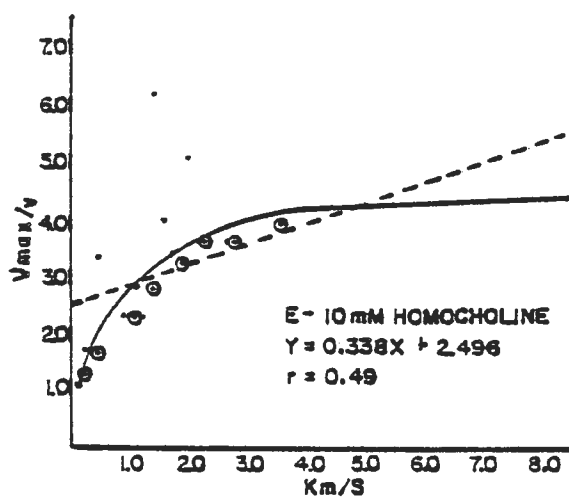
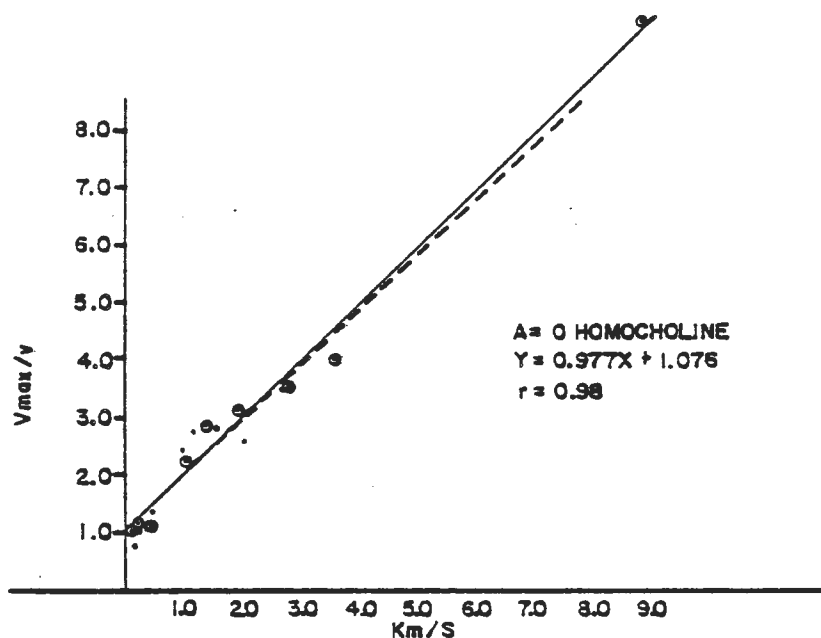


Figure 5. Combined kinetic determinations of P_4 ChAT activity as a function of added choline in the absence and presence of fixed homocholine. Superimposing the individual curves shown in Fig. 4 results in the curves intersecting at one common point, a result interpreted by Cha (1968) to indicate that both substrates are metabolized by the same enzyme. The Y coordinate of the point of intersection is 3.7, which indicates that the V_{\max} for P_4 ChAT as a function of choline exceeds the V'_{\max} for P_4 ChAT as a function of homocholine by 3.7 fold. The X coordinate (2.75) represents the concentration of choline (48.5 μM) at which homocholine, irrespective of its concentration, has no effect on the velocity of the total acetylated products being formed ($K_m = 133.5 \text{ } \mu\text{M choline} / 2.75 = S (48.5 \text{ } \mu\text{M choline})$).

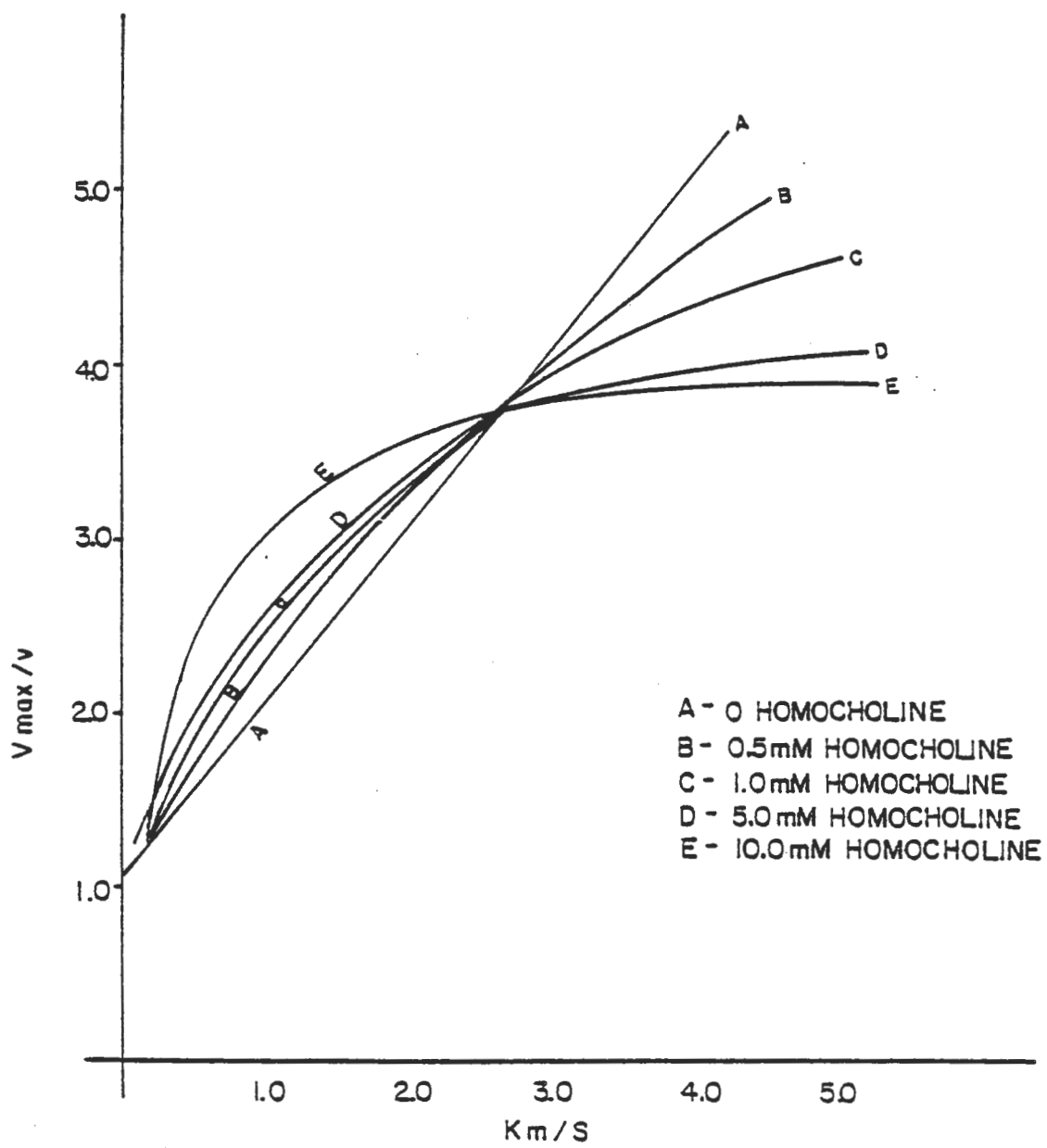


Figure 6. Ability of homocholine to competitively inhibit choline acetylation by soluble ChAT but not serve as a substrate. 2 μ l aliquots of the sodium phosphate buffer wash of the P_3 fraction were incubated for 15 min at 39° with varying concentrations of added choline ranging from 50 to 1200 μ M, and in the absence or presence of fixed levels of homocholine (2.0, 10.0, 20.0 mM). The least squares regression lines and correlation coefficients are as follows:

A (0 homocholine) $Y = 1.0007 x + 0.995$ $r = 0.94$

B (2 mM homocholine) $Y = 1.350 x + 0.814$ $r = 0.99$

C (10 mM homocholine) $Y = 1.470 x + 0.916$ $r = 0.98$

D (20 mM homocholine) $Y = 2.024 x + 0.754$ $r = 0.99$

Polynomial regression analysis indicated that in all cases a straight line fit the data better than a second degree polynomial.

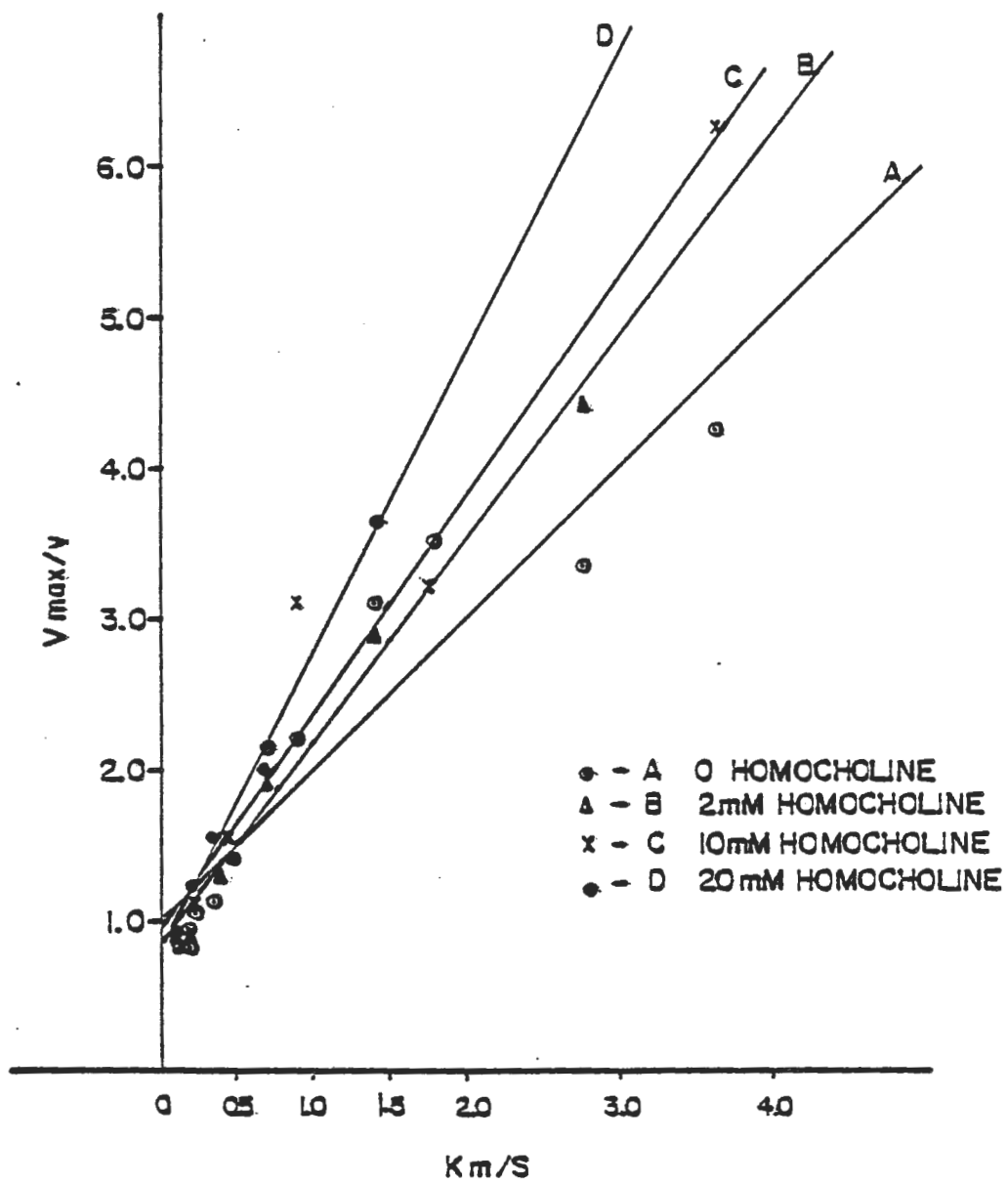


Figure 7. Ability of ACh and AHCh to inhibit acetylation of choline by soluble and P₄ ChAT. 2 ul aliquots of sodium phosphate buffer wash or the P₄ fraction were incubated for 15 min at 39° with 1.1 mM choline and varying concentrations of either ACh or AHCh. An asterisk indicates that ACh or AHCh treatment differs from control at P < 0.05.

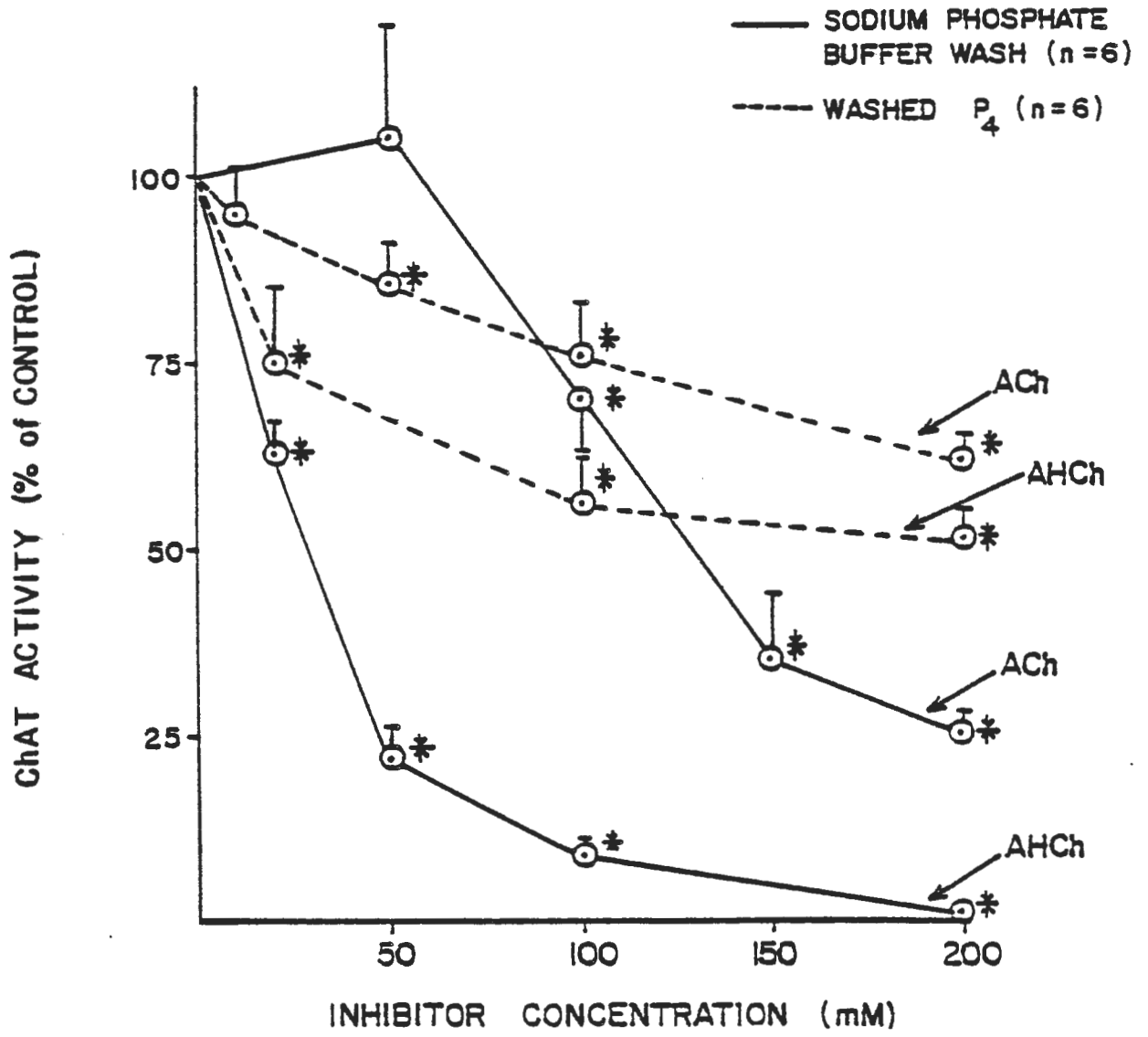


Figure 8. Dixon plot of acetylhomocholine inhibition of choline acetylation by P₄ ChAT. 2 μ l aliquots of the P₄ fraction were incubated for 15 min at 39° in the presence of choline (1.1 or 4.3 mM) and varying concentrations of acetylhomocholine. 1/v (choline acetylation) is plotted as a function of acetylhomocholine concentration. Acetylhomocholine did not interfere with the extraction of [¹⁴C] ACh into TPB/3-hept. at the concentrations used here. Intersection in the fourth quadrant indicates competitive inhibition; K_i = 63 mM.

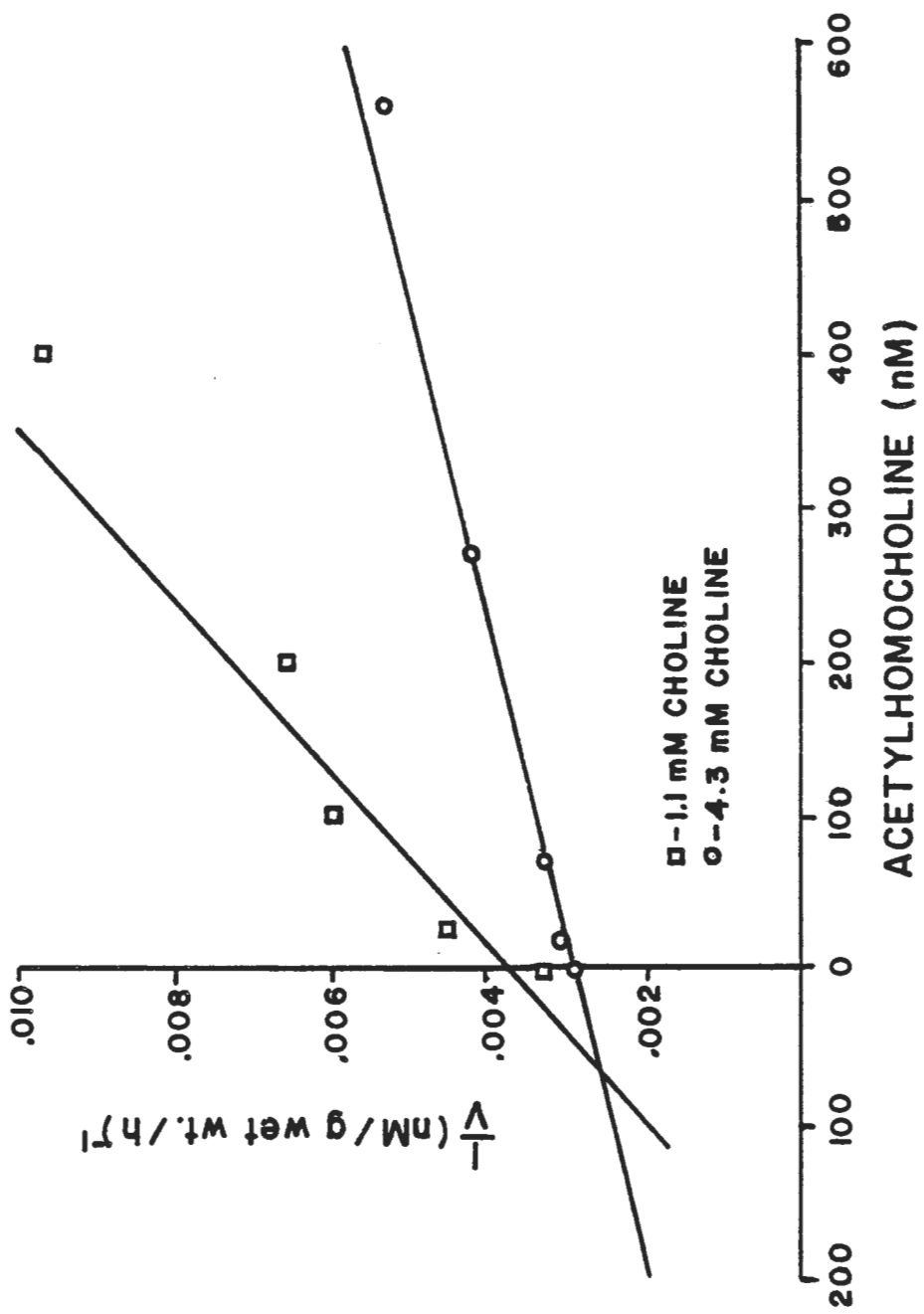


Figure 9. Dixon plot of acetylhomocholine inhibition of choline acetylation by soluble ChAT. 2 μ l aliquots of the sodium phosphate buffer wash were incubated for 15 min at 39° in the presence of choline (1.1 or 4.3 mM) and varying concentrations of acetylhomocholine, $1/v$ (choline acetylation) is plotted as a function of acetylhomocholine concentration. Acetylhomocholine did not interfere with the extraction of [14 C] ACh into TPB/3-hept. at the concentrations used here. Intersection in the fourth quadrant indicates competitive inhibition; $K_i = 10$ mM.

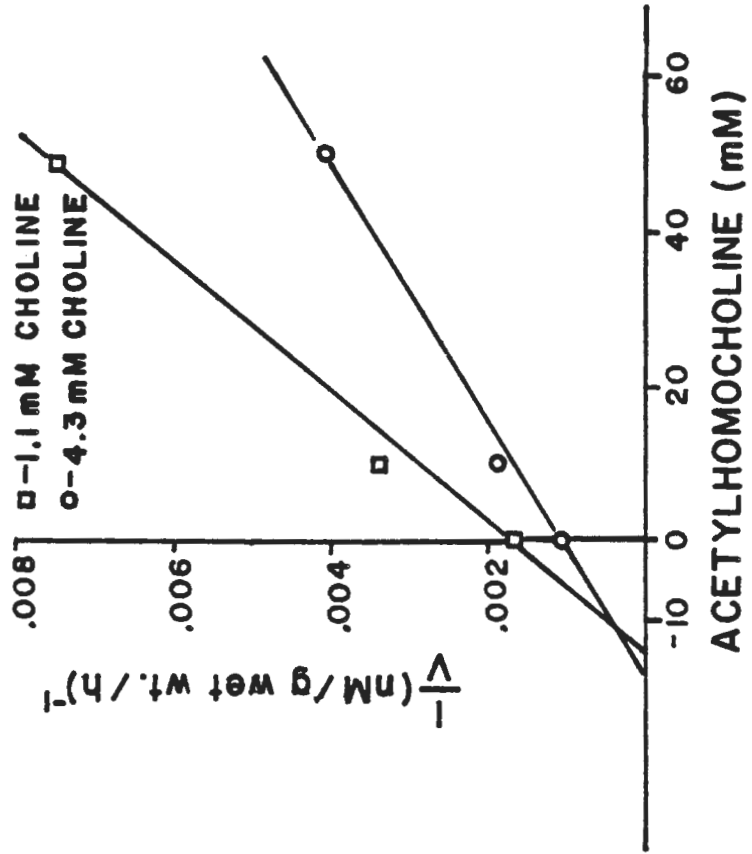
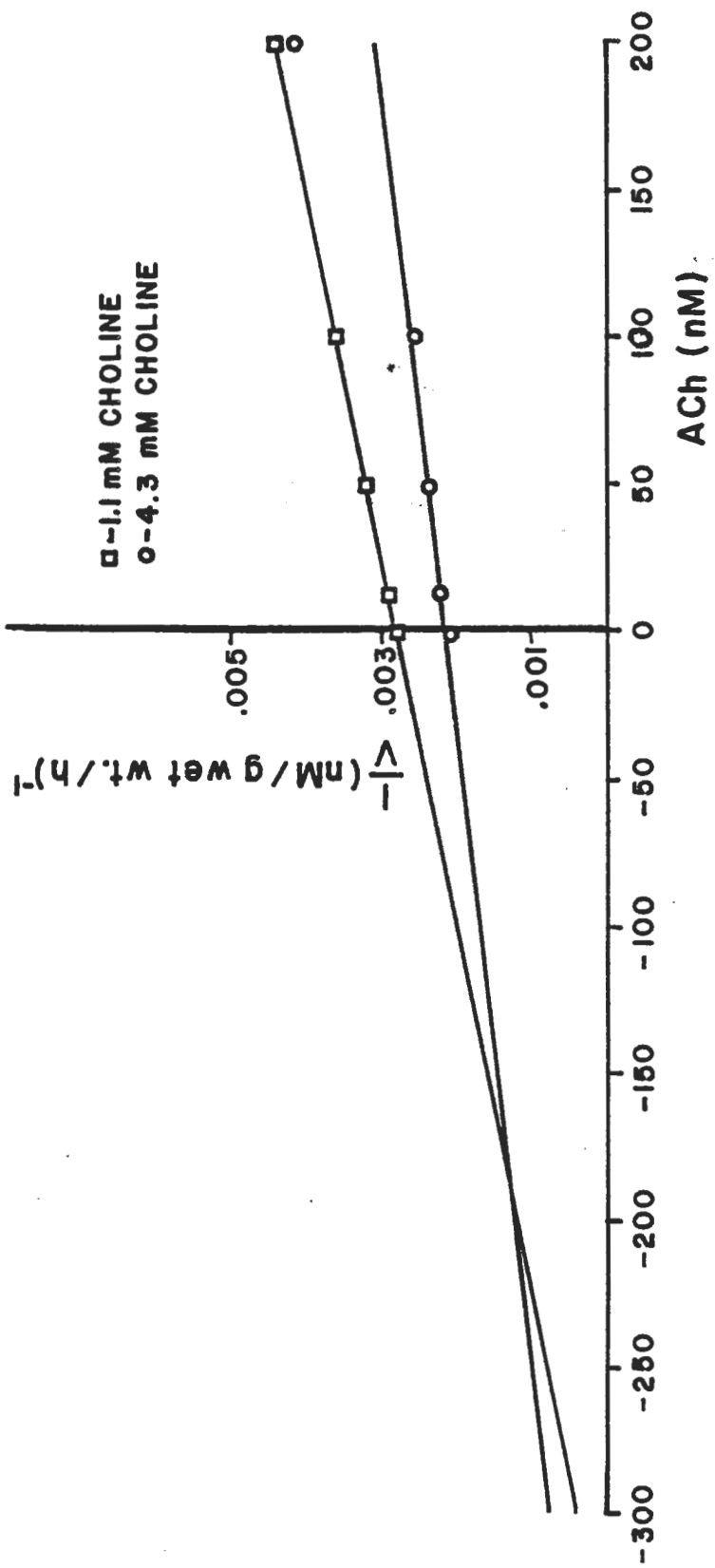


Figure 10. Dixon plot of acetylcholine inhibition of choline acetylation by P_4 ChAT. 2 μ l aliquots of the P_4 fraction were incubated for 15 min at 39° in the presence of choline (1.1 or 4.3 mM) and varying concentrations of acetylcholine. $1/v$ (choline acetylation) is plotted as a function of acetylcholine concentration. Acetylcholine did not interfere with extraction of [14 C] ACh into TPB/3-hept. at the concentrations used here. Intersection in the fourth quadrant indicates competitive inhibition; $K_i = 200$ mM.



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