CHOLINE ACETYLTRANSFERASE

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INTRODUCTION

The central role played by acetylcholine in the conduction of the nerve impulse is now generally accepted after decades of controversy. It is assumed that acetylcholine acts as a trigger, inducing changes in the cation permeability of electrically excitable membranes through attachment to a receptor biopolymer. While an enormous amount of scientific work has been centered on the receptor proteins to which acetylcholine is attached and on the enzyme (acetylcholinesterase) responsible for the hydrolysis of acetylcholine, much less effort has been centered on studying the synthesis and the regulation of the synthesis of this molecule.

The enzyme responsible for the synthesis of acetylcholine, then called choline acetylase (ChA), was discovered in rabbit brain by Nachmansohn and Machado[120] in 1943. They obtained a cell-free system in which the acetylation of choline took place in the presence of ATP. This was the first observation that the energy of ATP could be used for biosynthesis and the first demonstration of enzymic acetylation in a cell-free system, a finding so surprising that *Science, Journal of Biological Chemistry*, and *Proceedings of the Society of Experimental Biology and Medicine* all refused to publish it.[116] It was also noted that, on dialysis, the enzyme rapidly lost activity,[119,120] suggesting the presence of a coenzyme in this system. A purified preparation of this coenzyme was obtained by Nachmansohn and Berman,[118] in the presence of which ChA, inactivated by dialysis, regained its ability to acetylate choline.

It soon became apparent that the coenzyme initially discovered in the ChA system was identical to the cofactor which Lipmann[90] found to be essential for the acetylation of sulfanilamide in the pigeon liver system. The name coenzyme A (A for acetylation) was proposed by Lipmann for the cofactor.

As the generality of the involvement of coenzyme A in biological acylation and condensation reactions became appreciated, a great deal of effort was centered on the elucidation of the structure of this cofactor, particularly in the laboratories of Lipmann,[91] Lipmann et al.,[92] Snell et al.,[157] Baddiley and Thain,[5] and Baddiley et al.[6] The structure of coenzyme A is shown in Figure 1.

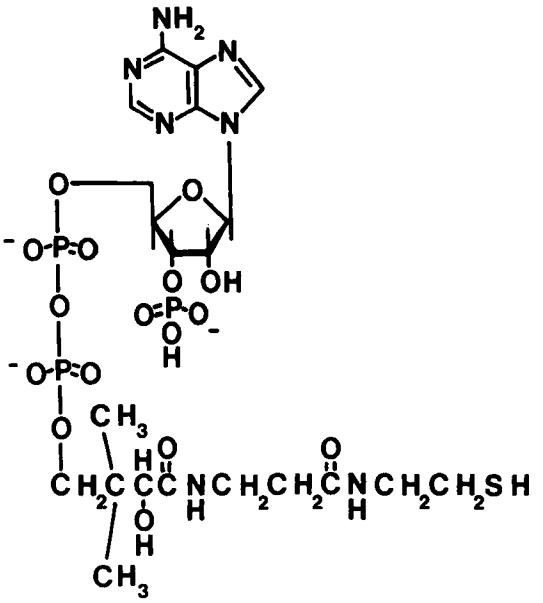


FIGURE 1. Coenzyme A.

While the mechanism of action of coenzyme A in acylation reactions was obscure initially, Lynen and his collaborators[94,95] showed that coenzyme A forms thiolesters capable of transferring acyl groups to suitable acceptors, as well as undergoing aldol condensations.

It is the function of ChA to catalyze the following reaction:

$$\text{CoA SC(=O)CH}_3 + (\text{CH}_3)_3\overset{+}{\text{N}}\text{CH}_2\text{CH}_2\text{OH} \rightleftharpoons \text{CoA SH} + (\text{CH}_3)_3\overset{+}{\text{N}}\text{CH}_2\text{CH}_2\text{OC(=O)CH}_3$$

Progress has been slow in elucidating the mechanism of action of this enzyme and the control mechanisms which regulate the formation and utilization of acetylcholine in nervous tissue; mainly because sources of the enzyme suitable for isolating it in large amounts are rare and because methods for relatively efficient purification have become available only very recently. Thus, more than 30 years after the discovery of ChA, it is still not clear whether it is a soluble or a membrane-bound enzyme. There are more theories than facts regarding the transport of the enzyme and its substrates and products, while the control mechanisms regulating the interaction of ChA, acetylcholine receptor, and acetylcholinesterase remain enigmatic.

SOURCES OF CHOLINE ACETYLTRANSFERASE

Since the initial discovery of ChA in rabbit brain,[120] this enzyme has been found in a large variety of nervous tissues ranging from sensory and motor nerves[117] to all portions of the human brain.[93] The claim that ChA and acetylcholinesterase are found in all nervous tissues led to the postulate that acetylcholine is essential for linking cationic permeability changes of axonal membranes to the propagation of the nerve impulse.[115] A general scheme for the role of an "acetylcholine cycle" in the regulation of the ion permeability of membranes has been proposed.[117,117a] This postulate has been attacked because of reports that the concentration of ChA in primary afferent fibers is extremely low[69,85] or that this enzyme is absent altogether. The low levels of ChA in sensory fibers have been cited as evidence for the noninvolvement of acetylcholine in axonal conduction.[69] It should be noted that inability to detect an enzyme or finding low levels of an enzyme in a given tissue does not prove that this enzyme might not play an essential role in the tissue from which it is – or is not – isolated. This is particularly true if the enzyme in question is rather unstable in the early stages of purification and has low specific activity, common problems

for investigators working with ChA. Contrariwise, presence of an enzyme at a given site does not prove its essentiality at that site; it does, however, raise the question of what it might be doing there.

While the ubiquitous distribution of ChA in nervous tissue is accepted fairly generally, even though its functions are still being argued about, the finding of ChA, often in large amounts, in nonnervous tissue remains baffling. This enzyme has been found in sources as diverse as placenta,[110] microorganisms,[158] and nettles.[11] The astonishingly high concentration of acetylcholine in the royal jelly of bees (1 mg/g)[74] suggests the presence of a ChA system in these insects. The extremely low levels of ChA in erythrocytes[69] are somewhat surprising, since erythrocytes contain relatively high levels of acetylcholinesterase as well as what appears to be acetylcholine receptor protein.[76]

The presence of ChA in some microorganisms and in the protozoa paramecium[13] and *Trypanosoma rhodesiense*[27] raises the interesting possibility that the phenomenon of chemotaxis[2,17] (in which, depending on the presence of an attractant or a repellent, the direction of rotation of flagellae and the overall direction of movement of the microorganism are reversed) may involve a cholinergic mechanism, particularly since there is evidence indicating that the coupling between sensory receptors and flagellae occurs through changes in the membrane potential.[52] ChA would have to be an essential component of such a mechanism.

Table 1 summarizes some of the sources of ChA

TABLE 1

Sources of Choline Acetyltransferase

Source	μmol AcCh/min/mg protein	Reference
Mammalian		
Human placenta	2.3	Morris[110] (1966)
	0.04	Roskoski[135] (1975)
	0.015	Banns[10] (1976)
	17.2	Hersh et al.[74,8] (1977)
Human brain	0.007	Bull et al.[29] (1970)
	0.15	White and Wu[168] (1973)
	0.143	Singh and McGeer[152] (1974)
	0.70	Roskoski[135] (1975)
Bovine brain	0.315	Glover and Potter[64] (1971)
	1.45	Chao and Wolfgram[36] (1973)
	2.5	Malthe-Sørenssen[97] (1976)
	48.0	Ryan and McClure[146] (1976)
Rat brain	0.62	Potter et al.[125] (1968)
	20.0	Rossier[138] (1976)
	40.2	Ryan[145,146] (1976)
Pigeon brain	[a]	Malthe-Sørenssen[100] (1972)
Invertebrates		
Squid head ganglia	0.1[b]	Nachmansohn and Weiss[121] (1948)
	1.8	Prince[126] (1967)
	66.7	Husain and Mautner[77] (1973)
Snail (*Helix aspersa*)	0.018	Emson and Malthe-Sørenssen[53] (1974)
Cockroach (*Periplaneta americana*)	0.318	Emson and Malthe-Sørenssen[53] (1974)
Horseshoe crab (*Limulus polyphemus*)	1.05	Emson and Malthe-Sørenssen[53] (1974)
Blowfly (*Lucilia sericata*)	0.01[b]	Smallman[154] (1956)
Housefly (*Musca domestica*)	0.017[b]	Mehrotra[109] (1961)
Microorganisms		
Schistosoma mansoni	1.25×10^{-5}[b]	Bueding[26] (1952)
Lactobacillus plantarum	[a]	Stephenson and Rowatt[158] (1947)
	0.58	Alpert et al.[4] (1966)

[a]Specific activity not given.
[b]μmol AcCh/min/mg acetone powder.

which have been investigated. No attempt will be made to refer to all of the studies that have been carried out. The search continues for ChA in a wide variety of nervous and nonnervous tissues. The recent observation of ChA activity in spermatozoa[23,66] is particularly interesting, again suggesting the possibility of a cholinergic system involved in chemotaxis. The observation that reversible sterility may be induced in rats by ChA inhibitors[66] indicates the functional importance of ChA.

Attempts are being made to correlate ChA activity to changes seen in human disease. For example, it has been claimed that ChA activity is reduced in dystrophic compared with normal muscle.[108] Reduced axoplasmic transport of ChA in sciatic nerves of dystrophic mice has also been reported.[79] In Parkinsonism, a reduction of ChA activity, particularly in the substantia nigra, has been reported to be taking place[93] even though degeneration of the nigrostriatal dopaminergic pathways is reported to be the primary lesion in this disease. While it is not clear whether alterations in ChA activity and ChA transport in dystrophy, Parkinsonism, or tardive dyskinesia are causes or symptoms of these diseases, these changes have accelerated work in determining ChA levels in increasingly localized areas of the nervous system. These studies, which have been greatly facilitated by improvements in the sensitivity and simplicity of ChA assay methods, have been carefully discussed in a review by Hebb.[70]

Initially, ChA activity was followed by applying the method of Hestrin.[75] In this procedure, the acetylcholine produced by the enzymic reaction is permitted to react with hydroxylamine to form a hydroxamic acid, which, in turn, produces a bright red color on reacting with ferric ion.

A more sensitive procedure was that of McCaman and Hunt,[107] who used ^{14}C-acetyl coenzyme A and determined the levels of ^{14}C-acetylcholine formed. Their procedure used precipitation of acetylcholine as a reineckate salt to separate the radioactive product from the radioactive substrate. A more effective separation method was introduced by Schrier and Shuster,[147] who used an anion-exchange resin which retains essentially all the applied labeled acetyl coenzyme A without retaining the labeled acetylcholine. At this time, the most sensitive method is probably that of Fonnum.[58] Labeled acetylcholine, produced by the action of ChA, is isolated by liquid cation exchange using sodium tetraphenyl boron in acetonitrile and a toluene scintillation mixture. Acetylcholine complex is extracted into the toluene phase and counted, while acetyl CoA remains in the aqueous phase.

It can readily be seen that ChA activity assays based on the formation of labeled acetylcholine from labeled acetyl coenzyme A are not suitable for studies in which rapid rate measurements are used to probe the details of intermediate steps in the catalytic process. A really sensitive assay, in which disappearance of reactants or formation of products could be followed directly without a separation step, would be extremely helpful to workers in this field.

As in all enzyme assays, the results must be treated with caution. It is difficult to be certain that extraction of the enzyme from the tissue from which it is isolated is complete. Conditions used by different workers may differ so that inhibition or activation might occur. ChA activity is very sensitive to salt concentration, thiol concentration, and other factors. Finally, the problem of allotopy,[128] the modification of the activity of an enzyme depending on whether it is studied in solution or membrane bound, cannot be ignored.

PURIFICATION OF CHOLINE ACETYLTRANSFERASE

As can be seen in Table 1, attempts to purify ChA progressed rather slowly. Only in the last few years has it been possible to obtain enzyme with activities exceeding 10 μM/min/mg protein. The slowness of this progress is due primarily to the relatively low levels of ChA in most tissues investigated and the lack of stability of the enzyme during the early stages of purification.

Several researchers claim to have obtained electrophoretically homogeneous protein after applying relatively simple purification methods. For instance, Chao and Wolfgram[36] claimed to obtain homogeneous ChA from the caudate nuclei of bovine brains by making an acetone-chloroform powder and then passing the aqueous buffer-soluble proteins through an organomercurial-Sepharose® column followed by DEAE – cellulose and hydroxyapatite columns. This enzyme preparation had a molecular weight of 69,000. In a subsequent paper,[35] Chao claimed to have evidence for ChA being a hexamer with six identical subunits with a molecular weight of 14,700 each.

His ChA preparation was used for the preparation of antibodies and the immunofluorescent localization of ChA in frozen sections of bovine spinal cord.[54] Unfortunately, the activity of the enzyme obtained by Chao and his collaborators was only a fraction of the activity obtained by Rossier[138] or Ryan and McClure,[146] yet the latter laboratories were careful to point out that they still had not achieved homogeneity. When Roskoski et al. reported obtaining human placental ChA purified to electrophoretic homogeneity,[135] the activity of his enzyme was minimal compared to that obtained by Morris[110] several years earlier. It should be noted that very impure ChA, may in some systems, move as a single band on electrophoresis.

The question of deciding when a "pure" enzyme is indeed pure is one that bedevils all enzyme chemists, and this problem seems to be accentuated in the case of ChA. Obviously, several of the studies reporting electrophoretically pure ChA deal either with proteins containing ChA only as an impurity or with denatured enzyme which is exhibiting only a fraction of its maximal activity. This problem is accentuated by several factors: the extreme instability of some species of highly purified ChA (particularly the brain enzyme),[145] the modification of enzyme activity and sensitivity to some inhibitors or activators after passage through some columns used during purification, and the considerable sensitivity of ChA activity to the ionic composition of the medium in which it is tested.

In spite of these problems, preparations of brain ChA[138,146] and squid ganglia ChA[77] of relatively high specific activity have been obtained during the last few years. This progress has been achieved primarily because of the introduction of improved column chromatographic techniques and because of new approaches to minimizing the lack of stability of the purified enzyme.

Dialysis against solid sucrose[77,124,124a,151] can greatly increase the stability of ChA. Similar stabilization can be provided by the presence of glycerol.[36,152] The usefulness of dithiothreitol for the stabilization of ChA seems to vary with the source from which this enzyme has been isolated. While generally believed to be essential for stabilizing the enzyme, purified ChA isolated from the head ganglia of squid seems more stable in its absence.[51]

Several attempts have been made to apply affinity chromatography[46] as a tool for the purification of ChA. Since mercurials are inhibitors of this enzyme with inhibition reversible in the presence of mercaptoethanol, it seemed reasonable to investigate the usefulness of organomercurial columns, which had already proved their usefulness for the separation of thiol proteins from nonthiol proteins.[153] Organomercurial-Sepharose columns have been utilized during the purification of ChA from squid ganglia[77] and the caudate nuclei of cow brains.[36] In the former case, mercaptoethanol was used in the elution buffer, and in the latter, cysteine was used. Ryan[145] reported quantitative binding of bovine brain ChA to an organomercurial-Sepharose column, but was able to recover only a small portion of enzymic activity. Since the mercurial columns used were all prepared by the procedure of Cuatrecasas[45] in which succinylated 3-aminopropyl-Sepharose is permitted to react with *p*-aminophenyl mercuriacetate, they must be considered "long-armed" columns. The difficulties frequently encountered in removing ChA from such columns are not surprising, but they raise the question whether "affinity chromatography" or "hydrophobic chromatography"[149] had been taking place. As can be seen in Figure 2, ChA isolated from squid ganglia is retained strongly on agarose columns to which simple alkyl chains have been attached, with the binding strengthened as the length of the alkyl chains is increased.[104b] Thus, ChA appears to be a hydrophobic protein, and the use of affinity columns with shorter arms should be investigated.

Similarly, while a styrylpyridinium Sepharose column[77] proved useful during the purification of ChA isolated from squid ganglia, it again was not clear whether affinity chromatography or hydrophobic chromatography had been taking place. It should be noted that styrylpyridinium derivatives (which will be discussed later), while potent inhibitors of ChA isolated from some species, are very poor inhibitors of ChA isolated from others. The tendency of these compounds to undergo light-catalyzed, *cis-trans* isomerizations which affect their inhibitory activity must also be considered.

Impressive purification of brain ChA was achieved by Ryan and McClure[146] using a CoA-Sepharose column, prepared by the procedure of Chibata et al.[39] Sepharose 4B activated with cyanogen bromide, and CoA are permitted to react with each other, the assumption being[39] that CoA is attached through the 6-amino group of adenine.

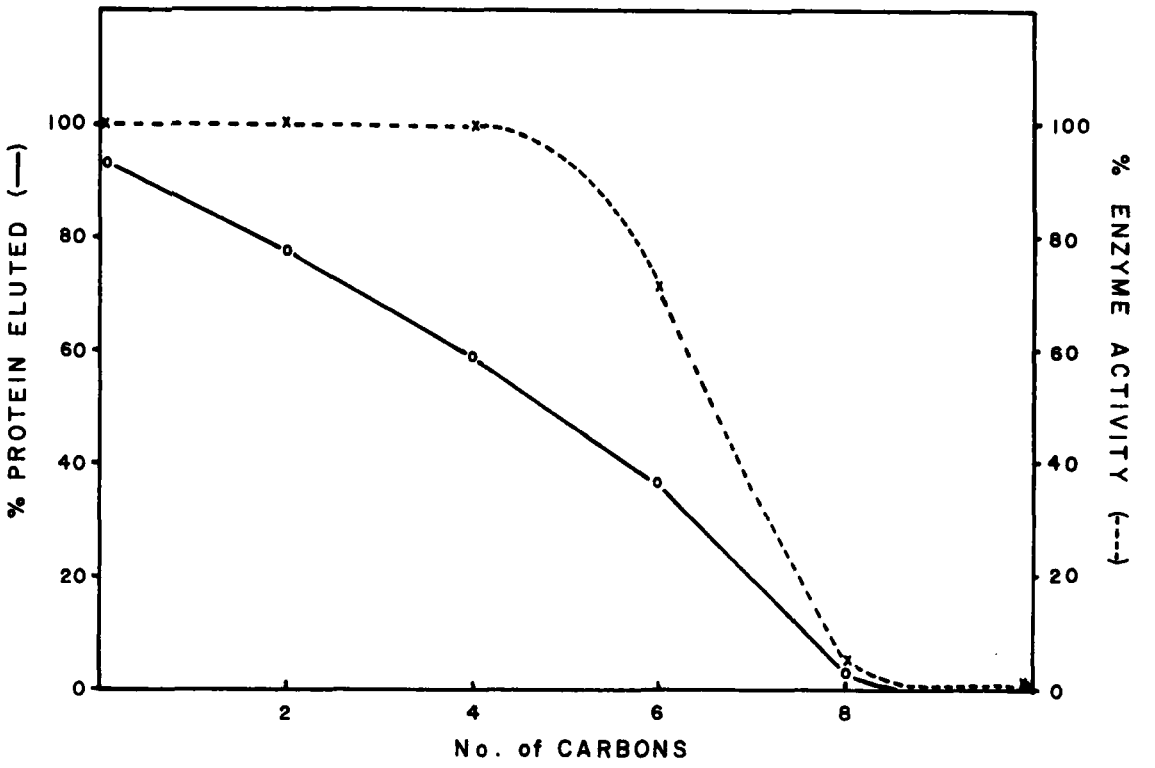


FIGURE 2. Chromatography of ChA from squid head ganglia on agarose, methyl-agarose, butyl-agarose, hexyl-agarose, octyl-agarose, and decyl-agarose.[104b]

Since it is rather easy to attach CoA to Sepharose 4B but impossible to attach CoA methyl disulfide by the identical procedure, it seems likely that the sulfur and not the 6-amino group of CoA is responsible for binding the coenzyme to the column.[51] Whatever the point of attachment, CoA columns should be very useful for the purification of those species of ChA in which CoA is bound tightly, but much less promising for those species of ChA in which only loose attachment of the coenzyme occurs.

Roskoski et al.,[135] Hersh et al.,[74a] and Weber[163] have had success in using blue dextran-Sepharose columns for the purification of ChA from brain and placenta[135] and from *Drosophila* heads.[163] This column was introduced by Thompson and his co-workers[160] as being useful for the purification of enzymes containing the dinucleotide fold to which binding of suitable coenzymes occurs. Thompson et al. claim that Reactive Blue 2, the chromophore of blue dextran, is structurally related to the adenosine diphosphoryl portion of coenzymes[160] such as NAD, FMN, ATP, ADP, or CoA. While the similarity of Reactive Blue (Figure 3) to ADP strikes this

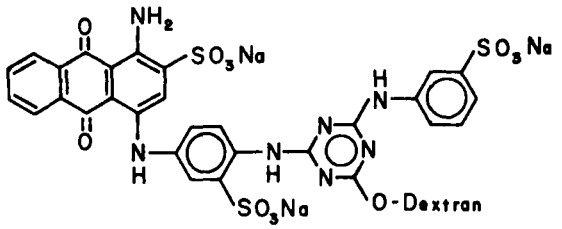


FIGURE 3. Reactive blue attached to dextran.

reviewer as elusive, it has been shown that Reactive Blue 2 is a competitive antagonist of acetyl CoA of brain and placental ChA.[135] However, a molecule as simple as 8-anilinonaphthalene sulfonate (Figure 4) is also a competitive antagonist of acetyl CoA in ChA isolated from squid ganglia[51a] and binds to the acetyl CoA site on the pyruvate dehydrogenase (E_1) enzyme in the pyruvate dehydrogenase complex.[150] This observation is of interest since it suggests the preparation of affinity columns carrying ligands much simpler than Reactive Blue 2. It also suggests the use of 8-anilino-1-naphthalene sulfonate and related fluorescent compounds as probes of the acetyl CoA binding site of ChA and as a tool for

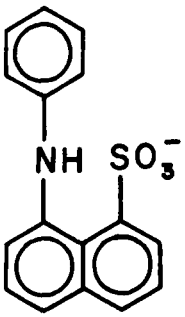


FIGURE 4. 1,8-Anilino-naphthalene sulfonic acid.

fluorescence resonance energy-transfer measurements. Such measurements have proved very useful for obtaining information about fluorophore environment and distances between fluorescence donor and fluorescence acceptor molecules.[25]

Immunoabsorbent chromatography was used in another approach to the purification of ChA. Several groups had reported obtaining antisera active against ChA from different sources.[54,99,139,141,151,152] In an unusual approach, Malthe-Sørenssen et al.[99] and Rossier and his coworkers[139,144] used antibodies to contaminant proteins in their ChA preparations and attached these to chromatographic columns. These retained immunogenic proteins without adsorbing ChA. These experiments emphasize the difficulties encountered in utilizing antibodies to ChA as a tool for the immunochemical localization of ChA. While such claims had been made[54] based on the use of enzyme claimed to be pure, but very low in activity, Rossier[136] was unable to substantiate them using a much more active enzyme preparation. At this time, antibodies monospecific against ChA do not seem to have been obtained.

As the methods of purification of ChA continue to be improved, the search for new and more plentiful sources of this enzyme is continuing as well. Even though, as already discussed, it has been possible to obtain very highly purified ChA from mammalian brain, Rossier[138] and Ryan and McClure[145,146] are rather pessimistic about obtaining sizeable amounts of homogeneous ChA from this source. Assuming a 10% overall yield, Ryan[145] points out that 850 g of tissue would be required to obtain 0.1 mg of pure ChA. This would correspond to 400 adult rat brains or 30 bovine brains. Rossier, who used the brains of 7000 rats,[137] during his studies also feels that other sources should be investigated in the search for homogeneous ChA, with immature human placenta and the electric organ of the *Torpedo* being particularly promising. Microorganisms seem a suitable source of ChA in relatively large amounts, while squid ganglia have been used as such a source for a long time.

PHYSICAL PROPERTIES OF CHOLINE ACETYLTRANSFERASE

Numerous measurements of the molecular weight of choline acetyltransferase have been carried out. The first was that of Bull et al.[28] who, using sedimentation methods, reported rabbit brain ChA to have a molecular weight of 67,000. For bovine ChA, Glover and Potter,[64] using Sephadex G-200, found a molecular weight of 65,000. On the other hand, Chao and Wolfgram,[36] also using ChA isolated from bovine brain, claimed isolating two nonidentical subunits with molecular weights of 51,000 and 69,000. Using the same enzyme preparation, Chao[35] then claimed to be dealing with a hexamer, composed of identical subunits with a molecular weight of 14,700 each. These findings disagree with those of Ryan and McClure,[145,146] who, using a bovine enzyme of much higher specific activity than that of Chao, found it to have a molecular weight between 65,000 and 70,000. In good agreement are values of 68,000 reported by Rossier[140] for rat brain and 62,000 reported by White and Wu[168] for human brain ChA. Similarly, White and Cavallito,[165] using gel chromatography, reported that ChA isolated from *Lactobacillus plantarum* had a molecular weight of 70,000.

The formation of aggregates of ChA has been reported by White and Cavallito[165] with a molecular weight exceeding 100,000 seen in ChA from calf caudate nucleus after passage through a Sephadex G-100 column. Chao and Wolfgram[37] claimed that aggregation in this enzyme was the result of exposure to ammonium sulfate used during the early stages of purification, with molecular weights as high as 1,000,000 being observed. However, Ryan and McClure,[145,146] who purified bovine brain ChA by a procedure in which ammonium sulfate precipitation was used, did not find high molecular weight aggregates of this enzyme.

An interesting observation was made by Rossier,[140] who found that rat brain ChA, when subjected to sucrose gradient centrifugation, was recovered in a single symmetrical peak sedimenting at 4.6 S in the presence of dithiothreitol and phenanthroline. In the absence of dithiothreitol and phenanthroline, two peaks were observed sedimenting at 4.6 S and at 6.9 S. These peaks correspond to molecular weights of 68,000 and 136,000, respectively, and suggest the possibility that reversible reduction of a disulfide linking two identical subunits might occur. Treatment of the high molecular weight form with dithiothreitol results in the reformation of ChA with a molecular weight of 68,000. The turnover numbers of monomer and dimer were found to be identical. It is possible that the molecular weight of 120,000 to 125,000 reported for ChA isolated from squid ganglia[77] may also represent the dimer of this enzyme. Rossier believes that the aggregation of ChA might be the result of low protein concentration and the absence of a reducing agent.[140]

The possibility that ChA might be composed of identical subunits linked by disulfides could contribute to the complexity of the effects which thiols or thiol reagents may have on this enzyme. Disulfide formation and disulfide rearrangements might also be involved in the formation of isoenzymes of ChA which have been reported by several groups.

Malthe-Sørenssen for Fonnum[100] claimed that rat brain ChA could be separated by isoelectric focusing into three species with isoelectric points of 7.3 to 7.6, 7.7 to 7.9 and 8.3. Cat brain enzyme could be separated into three fractions as well. No such separation was seen with ChA from pigeon brain or guinea pig brain. The separate identities of the different forms were confirmed by refocusing. Similarly, White and Wu[168] reported obtaining multiple forms of human brain ChA as the result of isoelectric focusing and came to the conclusion that the different enzyme forms obtained by electrofocusing were all convertible to the same form.

Subsequently, Fonnum and Malthe-Sørenssen[60] reported that different forms of rat brain ChA had differential abilities to bind to synaptosomal membranes, with the 8.3 isoelectric point fraction binding most tightly. Polsky and Shuster[124,124a] reported the presence of two isoenzymes in squid ganglia ChA, each of which may exist in the form of different aggregates with different stabilities and different abilities to be activated by salt.

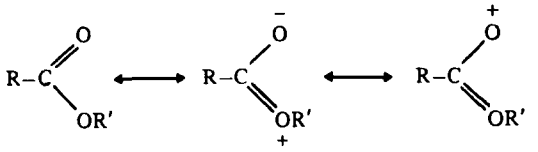
It seems possible that disulfide redox reactions might convert monomeric to dimeric ChA, perhaps altering its ability to be bound to membranes. This hypothesis remains to be proven.

Since analytically pure ChA is not as yet available, very little is known about its detailed structure. In the case of the rat brain enzyme, it is known to be a globular protein for which a Stokes radius of 3.39 nm and the relatively low density of 1.275 g/ml was reported.[140] Considering that this enzyme was discovered more than 30 years ago, this paucity of detailed information serves to emphasize the difficulties investigators have had in obtaining adequate amounts of pure material.

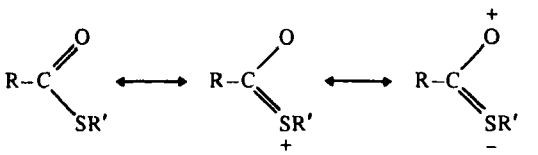
THE FUNCTIONS OF CHOLINE ACETYLTRANSFERASE

It is well known that esters of coenzyme A are "high-energy" compounds with the $\Delta G^{o\prime}$ for the hydrolysis of acetyl thiolesters ranging from -7.5 to -8.2 kcal, depending on the system investigated.[80] Similarly, Jencks and Carriuolo[83] found the $\Delta G^{o\prime}$ for the hydrolysis of *S*-acetylmercaptopropanol to be -7.7 kcal at pH 7. This value appears to be quite similar for thiolesters of quite different structures and exceeds the free energies of hydrolysis of normal oxygen esters which are normally about -5.1 kcal. In investigating the isomerization of 3-mercaptopropyl acetate, Jencks and Carriuolo[83] found that, at equilibrium, the amount of ester exceeded that of thiolester by a factor of 56.

This shift in equilibrium is generally attributed to the fact that ester resonance forms are energetically almost equivalent:



while the resonance forms of thiolesters:



are not. For this reason, resonance stabilization of esters exceeds that of thiolesters. Since sulfur, in contrast to oxygen, can accommodate electrons in its d orbitals, the charge-separated form with a partial negative charge on the sulfur atom is favored, as evidenced by spectroscopic and other data.[7,162,169] Calorimetric investigations of Wädsö[161] provide additional evidence for the relative lack of resonance stabilization of thiolesters compared to esters.

It must, of course, be remembered that $\Delta G^{o\prime}$ values are valid only for systems in which all reactants are present at unimolecular concentration. Recently, Pieklik and Guynn[123] investigated the equilibrium constant of the ChA reaction under physiological conditions. Equilibrium was approached from both directions and all the reaction components were assayed.

This work indicated that the equilibrium constant for the reaction catalyzed by ChA is considerably smaller than previous measurements had indicated.

$$K_{obs} = \frac{[\Sigma \text{ CoA}]\,[\Sigma \text{ Acetylcholine}]}{[\Sigma \text{ Acetyl CoA}]\,[\Sigma \text{ Choline}]} = 12.3 \pm 0.6$$

This value was determined at pH 7, and an ionic strength of 0.25 *M* and was found to be insensitive to alteration of ionic strength between 0.03 and 0.375 *M*.

The difference between the $\Delta G^{o\prime}$ for the hydrolysis of acetyl CoA and for the hydrolysis of acetylcholine is smaller than the free energy differences normally encountered when the hydrolysis of esters and thiolesters is compared. The reason for this is that acetylcholine may be considered a "high-energy compound". The free energy of hydrolysis of this ester, at pH 7, is between -6.47 and -6.99 kcal.[83,123] Presumably, the ester grouping of acetylcholine is labilized by the electron-attracting effect of the trimethylammonium group. X-Ray diffraction studies carried out on crystalline acetylcholine[31] and NMR studies carried out in solution[47,96] show the –OCCN– grouping of this molecule to be in the gauche conformation, with the trimethylammonium grouping and the acyloxy oxygen of the ester group quite close to each other (Figure 5). Their vicinity has been attributed to a "hard acid", "hard base" interaction.[106]

It can be seen that in the ChA-catalyzed reaction of acetyl CoA with choline, the formation of the products acetylcholine and CoA is favored, although less than would be expected if acetylcholine were not itself a "high-energy" compound. However, is must be remembered that thermodynamic information cannot be used to predict reaction rates. As "high-energy" compounds, thiolesters react much more rapidly than corresponding esters with amines[44,68] or with carbanions.[89] However, thiolesters do not undergo hydrolysis particularly rapidly. In fact, in many cases, thiolesters react more slowly than do analogous esters with hydroxide ion.[41,44] It seems likely that one of the primary reasons for the central metabolic role of thiolesters derived from CoA resides in the fact that these compounds undergo reaction with carbanions, amines, and other biologically relevant nucleophiles extremely rapidly, while being stable to hydrolysis.

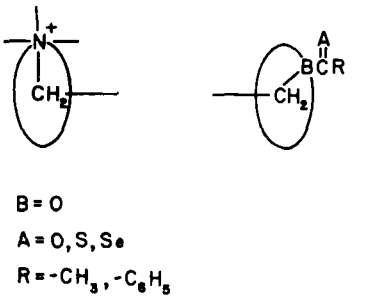


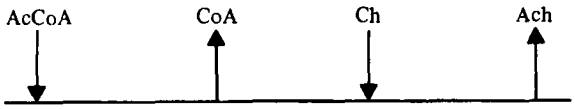
FIGURE 5. Gauche configuration of acetylcholine and related esters.

It is the function of ChA to catalyze the alcoholysis of a thiolester, a reaction favored slightly thermodynamically, but not kinetically, in the absence of this enzyme.

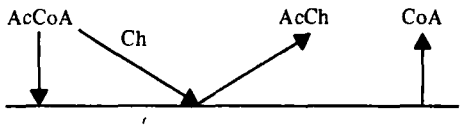
THE ORDERING OF THE CHOLINE ACETYLTRANSFERASE-CATALYZED FORMATION OF ACETYLCHOLINE

Since the reaction catalyzed by ChA involves two substrates, (choline and acetyl CoA) and two products (acetylcholine and CoA), much effort was expended to establish the ordering of the reaction. Studies of rat brain ChA by Potter and his co-workers[125] suggest that both choline and acetyl CoA combine with the enzyme before any products are released. These findings are in contrast to a report by Schuberth[148] suggesting that choline and acetyl CoA combine independently with placental ChA. By making double-reciprocal plots of initial velocity against fixed concentration of one substrate at different concentrations of the second

substrate, Schuberth obtained a set of parallel lines, one for each concentration of the fixed substrate. According to Cleland's rules[43] such a plot is diagnostic of a ping-pong mechanism in which binary but not ternary complexes are formed:



The ordering of the placental enzyme reaction was reexamined by Morris and his co-workers[113,114] and by Rama Sastry and Henderson.[129] In contrast to the report of Schuberth, these workers found acetylcholine to inhibit competitively with respect to choline, and acetyl CoA to inhibit competitively with respect to CoA. On the other hand, CoA inhibits noncompetitively with respect to choline, while acetylcholine is a noncompetitive antagonist of acetyl CoA. These findings exclude a ping-pong mechanism and suggest formation of a ternary rather than a binary complex to be required:



Convincing evidence against the formation of a binary complex was also obtained by Morris and Grewaal,[113] who found that ChA could catalyze the isotopic exchange between acetylcholine and ^{14}C-methyl-labeled choline only in the presence of CoA.

While it seemed clear that a sequential mechanism was observed, several questions remain. The competition experiments between the substrates and products of the reaction suggested a Theorell-Chance bi-bi mechanism in which the steady state concentration of the ternary complex is extremely low. It should be added that while it is generally assumed that acetyl CoA is the first substrate to be attached to the enzyme and CoA is the last product to depart, supporting evidence is not unequivocal since the order of substrate attachment and product release cannot be obtained from kinetic data alone. Very recent evidence obtained by Hersh and Peet[74b] supports a random binding mechanism in which the rate-determining step is at least partly dependent on the rate of interconversion of the ternary complex.

The observation that acetyl CoA binds considerably more tightly to ChA than does choline suggests that acetyl CoA is the leading substrate, while the tight binding of CoA compared to the binding of acetylcholine suggests CoA to be the last product to be released in brain and placental ChA. However, as will be seen, ChA isolated from different species can behave very differently and it cannot, at this time, be assumed that the ordering of all ChA reactions is identical. Apparent inhibition constants for the substrates and products of the ChA reaction can be obtained by the procedure of Cleland.[43]

It should be noted that an already complex situation is further complicated by the effects of changing salt concentration. This effect, which will be discussed later, has been noted by several groups. For example, Morris et al.[114] reported that increasing sodium chloride concentration results in looser binding of either substrate. Since the passage of the nerve impulse is obligatorily accompanied by considerable changes in ion concentration in the vicinity of the nerve membrane, the possibility cannot be ignored that such changes may affect the ordering of the ChA reaction.

THE MECHANISM OF ACTION OF CHOLINE ACTEYLTRANSFERASE

Attempts to assign the mechanism by which an enzyme accelerates the reactions undergone by its substrates tend to follow a rather simple pattern. The pH optimum of the enzyme-catalyzed reaction is determined. Inferences as to the amino acid residues essential for catalysis are drawn from such studies. Since the acid dissociation constants of residues within the active site of enzymes may be entirely different from the dissociation constants seen in solution, such assignments can be quite misleading. Inhibition studies with group-specific reagents are carried out, leading to assignments of "essential groups" within the active site. Since many (probably most) group-specific reagents are not reliably group specific, such studies may also lead to misleading results.

Structure-action relationships of compounds related to the substrates or the products of the enzyme reaction are explored in the hope of finding antagonists of the enzyme with high structural specificity and stereospecificity. From these, inferences can be drawn about the topography of the active site of the enzyme, although

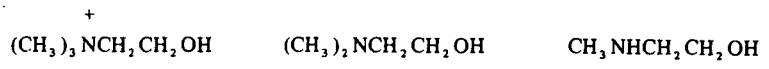
the possibility of conformational changes of small molecule, enzyme, or both within the small molecule-enzyme complex may complicate such interpretations.

While such studies strike most modern enzyme chemists as rather primitive compared to the information which can be provided by X-ray diffraction, NMR spectroscopy, or fluorescence resonance energy transfer methods, they can at least be carried out with impure enzyme.

Since (as already discussed) ChA is not available as a reliably homogeneous protein; is not available in amounts exceeding very few milligrams in purified form; and is subject to poorly defined activation or inactivation by salts, thiol reagents, or thiols, claims regarding the mechanism of action of this enzyme must be treated with extreme caution.

Studies With Molecules Related to Choline

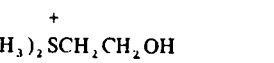
Since choline is a very simple molecule, it is not surprising that studies of its analogs were undertaken soon after the discovery of ChA. Berman et al.[18] compared the enzymic acetylation of choline with that of its dimethylamino and monomethylamino analogs. The trimethylammonium compound is acetylated 16 times more rapidly than its dimethylamino analog which, in turn, is acetylated four times more rapidly than the monomethyl derivative.



Choline | 2-Dimethylaminoethanol | 2-Methylaminoethanol

Since a similar requirement for a trimethylammonium group is encountered in the hydrolysis of acetylcholine by acetylcholinesterase and in the depolarizing action of acetylcholine in a variety of electrically excitable membranes,[117] it was postulated that the active sites of ChA, acetylcholinesterase, and the acetylcholine receptor of excitable membranes all possess an anionic site in the region to which the ligand is bound. The considerable difference between quaternary ammonium and tertiary amino compound suggested[18] that attachment of the trimethylammonium group to its binding site might result in a conformational change in the protein, enabling it to engulf the entire quaternary group.

Recently, a Swedish group[61] compared the acetylation by ChA of choline with that of the dimethylsulfonium analog of this compound. They found that while the maximal acetylation rates observed when choline or its dimethyl sulfonium analog were present at saturating concentrations were almost identical (4.1 and 3.6 μM/l/min, respectively), the binding of the dimethylsulfonium compound to ChA was considerably looser than that of the natural substrate. The K_m for choline was 0.99 $10^{-3}M$, and for 2-dimethylsulfonium ethanol it is 7.2 $10^{-3}M$.



Sulfonium Analog of Choline

In the case of acetylcholinesterase, maximal velocity is lowered and binding to the enzyme is reduced when the trimethylammonium group of acetylcholine is replaced by the dimethylsulfonium group. As noted by Ing et al.,[78] the dimethylsulfonium analog of acetylcholine is a relatively poor depolarizing agent when studied in several preparations.

It is interesting that the abilities of choline and its dimethyl sulfonium analog to be bound to ChA are different, while the maximal velocities of their enzymic acetylation are almost identical. Clearly, the presence of a cationic group is essential for effective acetylation by ChA. As shown by NMR spectroscopy, it is also essential for the maintenance of the gauche conformation of the –OCCN– (–OCCS–) grouping of these compounds in solution.[96,104a] However, while the presence of a third methyl group promotes tighter binding to ChA, this tighter binding does not significantly affect the rate-limiting step of the acetylation reaction.

Replacement of the methyl groups of choline with ethyl groups greatly lowers the ability of the alcohol to be bound to rat brain ChA.[72,101] A systematic study of this effect was undertaken by Hemsworth and Smith[72] who compared the ChA-catalyzed acetylation of choline, monoethylcholine, diethylcholine, and triethylcholine. The following results were found:

Substrate	K_m	V_{max}
Choline	1.9×10^{-4}	18.83
Monoethylcholine	5.1×10^{-4}	17.9
Diethylcholine	7.5×10^{-4}	13.2
Triethylcholine	26.0×10^{-4}	3.0

The authors proposed that of the three alkyl groups attached to the nitrogen only two are directed to the anionic site, while the third one is directed toward the acetyl CoA binding site. Only probability determines whether methyl or ethyl groups are involved in binding to the anionic site.[72] These findings are in good agreement with the original predictions of Nachmansohn.[117]

The distance between the cationic group and the hydroxy group of choline or its analogs is also important. It could be shown that the enzymic acetylation of homocholine, with three rather than two methylene carbons inserted between the trimethylammonium and the hydroxy group, is no faster than the nonenzymic acetylation by acetyl CoA.[48]

$(CH_3)_3\overset{+}{N}CH_2CH_2CH_2OH$

Homocholine

The observation by Hemsworth and Smith[73] of stereospecificity in the acetylation of α-methyl- and β-methylcholine emphasizes the importance of alignment of the cationic and hydroxy groups of the substrate for ChA to be an effective catalyst of the acetylation reaction.

$(CH_3)_3\overset{+}{N}CHCH_2OH$ (with CH_3 branch)

α-Methylcholine

$(CH_3)_3\overset{+}{N}CH_2CHOH$ (with CH_3 branch)

β-Methylcholine

$(CH_3)_3\overset{+}{N}CH_2CH_2SH$

Cholinethiol

$(CH_3)_3NCH_2CH_2NH_2 \underset{-H^+}{\overset{+H^+}{\rightleftharpoons}} (CH_3)_3\overset{+}{N}CH_2CH_2\overset{+}{N}H_3$

Aminocholine

Currier and Mautner[48] and Mautner et al.[105] noted that ChA was capable of catalyzing the aminolysis of acetyl CoA even at pHs at which the amino group of this compound (pK_a 6.9) is protonated. It was also observed that the ChA catalysis of the acetylation of choline and aminocholine showed a considerable D_2O isotope effect, which was greatest at the pH (pD) at which enzyme activity was optimal. Although such interpretations must be treated with caution,[82] such D_2O isotope effects are generally believed to indicate that general-base catalysis with rate-determining proton transfer is taking place.[14,15]

While stereospecificity in the enantiomers carrying the methyl group on the carbon next to the nitrogen is rather slight, it becomes very important in the β-methyl isomer. No acetylation is observed for L-β-methylcholine, as can be seen in the following summary[73] of work using ChA isolated from bovine caudate nuclei:

Substrate	Apparent K_m	Apparent V_{max}
Choline	1.7×10^{-4}	31.0
D-α-Methylcholine	89.0×10^{-4}	17.1
L-α-Methylcholine	390.0×10^{-4}	9.9
D-β-Methylcholine	460.0×10^{-4}	7.6
L-β-Methylcholine	No enzymic acetylation observed	

Clearly, steric hindrance in the vicinity of the hydroxy group to be acetylated is even more important than steric hindrance in the vicinity of the trimethylammonium group to be attached to the anionic site of the enzyme.

Studies of the acetylation of aminocholine and thiocholine have also been carried out.[48] These studies were complicated by the fact that while thiolesters undergo alcoholysis very slowly, they undergo aminolysis and thiolysis very rapidly. Thus, rapid transacetylation from acetyl CoA to cholinethiol will occur in the absence of ChA; the enzyme does not accelerate the rate of this reaction. Similarly, spontaneous transacetylation from acetyl CoA to aminocholine can take place; however, as expected, this reaction is pH dependent since only aminocholine, but not its protonated form, is an effective nucleophile.

Figures 6 and 7 show the pH (pD) dependence of the nonenzymic and ChA-catalyzed acetylation of choline and aminocholine by acetyl CoA.

Methylation of the oxygen and sulfur of choline or cholinethiol to form methoxycholine and methylthiocholine, respectively, did not yield inhibitors of the ChA reaction even when substrate and inhibitor were present at a concentration of 50 m*M*. Weak inhibition was exerted by choline disulfide and choline diselenide.[48]

Needless to say, numerous attempts were made to prepare analogs of choline designed to label ChA by forming covalent bonds within the active

site. The first of these, 3-bromoacetonyl trimethylammonium bromide,[122] proved to have rather low potency, with inhibition of only 50% being observed at a concentration of $5 \times 10^{-4} M$. Even at a concentration of $5 \times 10^{-3} M$, only 95% inhibition of placental ChA was observed. The compound appeared to be attached irreversibly to the enzyme.

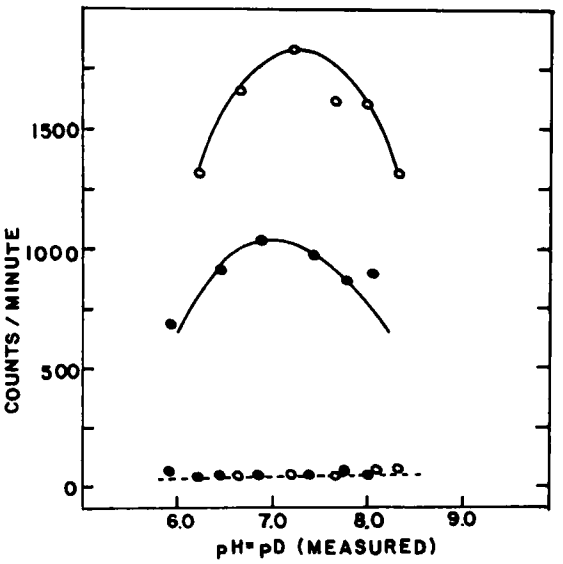
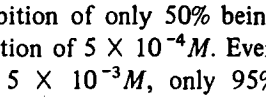


FIGURE 6. D_2O isotope effect in enzymic and nonenzymic acetylation of choline chloride. Enzymic reaction in H_2O (o–o), enzymic reaction in D_2O (•–•), and nonenzymic reaction in H_2O or D_2O (···). (From Currier, S. F. and Mautner, H. G., *Proc. Natl. Acad. Sci. U.S.A.*, 71, 3355, 1974, With permission.)



3-Bromoacetonyl Trimethylammonium Bromide

No such irreversible inactivation was observed when haloacetyl-cholines were tested as inhibitors of ChA.[40,112] While these compounds proved to be inhibitors of ChA, even the most potent derivative, iodoacetylcholine, inhibited human placental ChA by only 77% at a concentration of $1.2 \times 10^{-4} M$.[112] Alkylation did not occur, and dialysis restored full enzymatic activity. The action of these compounds is complicated by their ability to interact with acetylcholinesterase[40] as well as with ChA.

Haloacetyl Cholines X = F, Cl, Br, I

The claim of Roskoski,[132] that the reversible inhibition of ChA by bromoacetylcholine and bromoacetyl CoA provides evidence for the presence of a thiol group in the active site of ChA, is unlikely. It seems surprising a bromoacetyl derivative should react with a thiol group, not by forming a covalent bond with elimination of

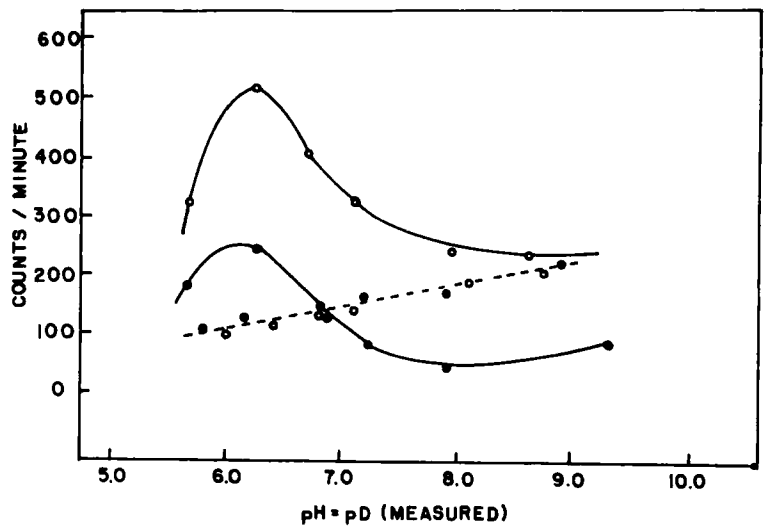
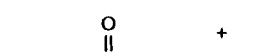


FIGURE 7. D_2O isotope effect in enzymic and nonenzymic acetylation of amino-choline bromide. Enzymic reaction in H_2O (o–o), enzymic reaction in D_2O (•–•), and nonenzymic reaction in H_2O or D_2O (···). (From Currier, S. F. and Mautner, H. G., *Proc. Natl. Acad. Sci. U.S.A.*, 71, 3355, 1974, With permission.)

bromine but as postulated by forming a bromoacetylthiolester. In fact, the weak potency and reversibility of the haloacetylcholines as inhibitors of ChA argues strongly against the presence of a thiol group near the site of attachment.

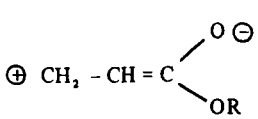
Recently, acryloylcholine was reported to be an inhibitor of ChA.[98] Malthe-Sørenssen et al.[98] reported this compound to inhibit rat and pigeon brain ChA at concentrations of 5×10^{-5} and $2.5 \times 10^{-5} M$, respectively. Preincubation with choline, acetyl CoA, or sodium chloride did not protect the enzyme. Double-reciprocal plots of the data obtained in experiments in which the concentrations of acryloylcholine, acetyl CoA, and choline were varied showed that the inhibition of ChA was uncompetitive with respect to both substrates, with the inhibitor changing both the K_m and the V_{max} of the enzyme. While the inhibition of pigeon brain ChA could be largely reversed by diluting the enzyme-inhibitor mixture with buffer, no reversal of the inhibition of rat brain ChA could be brought about. The observation that the inhibitory activity of acryloylcholine is additive with that of CoA implies attachment of these compounds at different enzyme sites.

Methylating either the α- or the β-carbon of acryloylcholine greatly lowered inhibitory potency, as did saturation of the double bond, with propionylcholine having only one thousandth the inhibitory activity of the acryloyl analog.



Acryloylcholine

Malthe-Sørensson and his co-workers[98] postulated that the resonance form of acryloylcholine:



would be favored rendering the β-carbon of the acyl group an effective electrophile. There is ample precedent for this postulate, and it seems reasonable to expect acryloylcholine to be able to react with suitably activated mercapto, hydroxy, and amino groups.

Recently, Rowell and Chiou[144] investigated the inhibitory activity of the dimethylamino analog of acryloylcholine. This compound, designed to increase ability to penetrate biological membranes, proved (as would be expected) to be a poorer inhibitor of ChA than acryloylcholine. In accordance with the prediction[98] that the β-carbon of the acryloyl group would be a good electrophile, this compound was found to react rather rapidly in aqueous solution to form *N,N*-dimethylaminoethyl-β-hydroxypropionate.

It seems likely that the inhibitory activities of acryloylcholine and its dimethylamino analog are due less to their analogy to acetylcholine than to the presence of a reactive double bond.

Molecules Related to Arylvinyl Pyridines

In 1967 it was reported[156] that several styrylpyridine analogs were rather potent inhibitors of ChA. Some, but not all, of these derivatives inhibited acetylcholinesterase as well. This finding led to the synthesis and study of large numbers of related compounds.[3,8,9,32,34,63,166]

The synthesis of these compounds seems to have been stimulated by the observation that some bisquaternary ammonium compounds such as decamethonium or hexamethonium had moderate ability to inhibit ChA. Among the arylvinyl pyridines and their naphthyl analogs synthesized initially,[32] the most potent proved to be compounds of the type shown in Figure 8.

It soon became apparent that the presence of the vinyl group linking the aromatic rings was essential for the inhibitory action of these compounds. Saturation of the double bond destroyed activity almost completely. Somewhat surprisingly, it was not essential for the pyridine nitrogen to be quaternized to provide an effective inhibitor of ChA. The structures and inhibitory potencies of some members of this series of compounds[32,166] are shown in Table 2.

In addition to being inhibitors of ChA, these compounds also tend to be inhibitors of acetylcholinesterase. In general, specificity for ChA as compared to acetylcholinesterase seems to be lower among the more potent quaternary pyridinium salts than among the analogous pyridines.

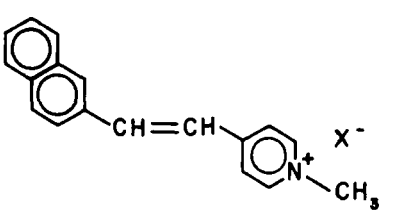
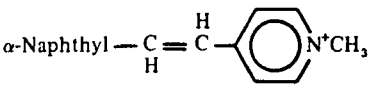
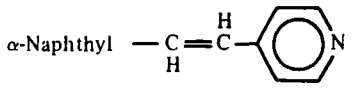
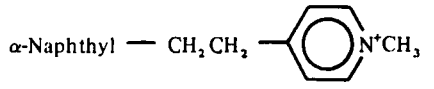
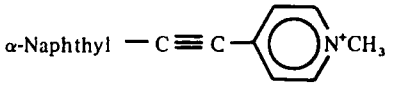
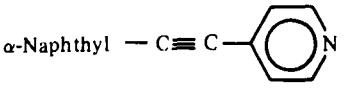


FIGURE 8. 4-(1-Naphthylvinyl) pyridinium methiodide.

TABLE 2

Inhibitory Activities of Arylvinyl Pyridines and Related Compounds

	I_{50} ChA	I_{50} AchE[a]
	3.3×10^{-6} (*M*)	4×10^{-5}
	2.5×10^{-5}	4% at 10^{-3}
	6% at 10^{-3}	10% at 10^{-4}
	1×10^{-6}	9×10^{-4}
	2×10^{-4}	2×10^{-4} no inhibition

[a] Acetylcholinesterase.

Further studies of structure action relationships of these compounds by Cavallito's group[3,32,34] and Baker's group,[8,9,63] showed that substitution of cyano or nitro groups in the 3- or 4-position of a phenyl substituent increases ability to inhibit ChA, while introduction of chloro, bromo, iodo, or alkyl groups lowers it. Introduction of these groups affected inhibition of acetylcholinesterase in the opposite fashion; this led to the generalization[8] that acetylcholinesterase binding is increased by the introduction, on the aryl substituent, of polar, electron-withdrawing groups, while inhibition of ChA is favored by introducing nonpolar, electron-releasing groups, most effectively in the meta position. Therefore, it is not surprising that the following 3,4-dichloro derivative (Figure 9) is one of the most potent, most specific inhibitors of ChA among this group of compounds.[8] This dichloro derivative causes 50% inhibition of rabbit brain ChA at a concentration of $1.1 \times 10^{-6} M$, while a concentration of $910 \times 10^{-6} M$ is required for equivalent inhibition of acetylcholinesterase.

From the studies cited above the following structure-action relationships could be deduced:

1. The presence of a vinyl or acetylenic bridge between the aryl and pyridine rings is essential for activity in inhibiting ChA. Saturation of this bridge or introduction of alkyl groups on either carbon lowers activity drastically.
2. Only *trans* but not *cis* vinyl compounds are active inhibitors. The linear acetylenic derivatives have high activity.
3. Quaternization of the ring nitrogen increases ability to inhibit ChA but increases ability to inhibit acetylcholinesterase more, thus lowering specificity.
4. As already noted, introduction of nonpolar, electron-releasing groups on the aryl ring increases ability to inhibit ChA while lowering ability to inhibit acetylcholinesterase.

While the initial studies of arylvinyl pyridinium compounds were undertaken with the assumption that these derivatives and choline would be competing for the anionic site of ChA, it soon became apparent that this was not the case. White and Cavallito[167] studied the action of *N*-methyl-4-(1-naphthylvinyl)pyridinium iodide as an inhibitor of

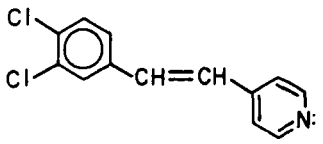


FIGURE 9. 3',4'-Dichloro-4-stilbazole.

calf caudate nucleus enzyme and showed that inhibition by this compound was noncompetitive with both choline and acetyl CoA. Inhibition was reversible initially; however, incubation of ChA with the inhibitor at 37° before addition of the substrate led to enzyme inhibition which was only partly reversible. Irreversibility was not detected during the first 10 min of incubation of ChA with inhibitor.

From these and other experiments, White and Cavallito[167] concluded that arylvinyl pyridines might:

1. Prevent removal of a proton from choline
2. Interfere with acetyl transfer from acetyl CoA to choline
3. Prevent release of acetylcholine from its site of formation

White and Cavallito also reported that in the presence of ^{14}C acetyl CoA, an apparent ^{14}C-acetyl CoA ChA-complex, could be detected by gel filtration. Formation of this intermediate was not inhibited by the presence of choline or by the presence of the arylvinyl pyridinium inhibitor. However, the inhibitor stabilized the acetyl CoA-ChA complex in the presence of choline.

Although capable of protecting ChA against inhibition by mercuric ion or the thiol reagent allicin (allyl-2-propene-1-thiolsulfinate), dithiothreitol does not protect it against the action of the arylvinyl pyridinium compound. However, some protection against this derivative is provided by cupric ion, which, at low concentrations, has been claimed[12] to interact preferentially with imidazole.

These findings led White and Cavallito[167] to suggest that arylvinyl pyridines might inhibit ChA by interfering with the catalytic action of the imidazole residue of histidine. As will be seen later, the role of histidine in the catalytic function of ChA seems well established, and it seems likely that the suggestions of White and Cavallito regarding the inhibitory action of their derivatives are substantially correct. Malthe-Sørenssen et al.[48] suggest that acryloylcholine inhibits ChA by a similar mechanism. It appears that arylvinyl pyridines interfere with the transfer of the acetyl group from acetyl CoA to choline, without directly competing with the attachment of either substrate, however, how this is accomplished remains unanswered.

Allen et al.[3] and Cavallito et al.[32,34] postulated that the most important features of the binding of arylvinyl pyridines were the electron-donating benzene ring, the electron-accepting pyridine ring, and the polarizable double or triple bond linking them. They suggested that binding to ChA involved π-donor and hydrophobic contributions by the aryl ring and π-acceptor interactions by the pyridine ring.

This interpretation was criticized by Baker and Gibson,[8,9] who proposed that binding of these derivatives directly involved the vinyl or acetylenic bridge. This could occur either through a direct interaction of the double bond with the enzyme or by polarization of the double bond by resonance interaction with the rings attached to it (Figure 10). Such interactions would place a partial negative charge on the pyridine ring and a partial positive charge on the carbon adjacent to the aryl group, making the latter susceptible to nucleophilic attack by suitable enzymic groups.

Of the two possibilities charge-transfer complex formation or direct interaction with the double or triple bond, the latter seems more likely. The rapid hydration of the dimethylamino analog of acryloylcholine has already been mentioned,[144] as has the partly irreversible character of inhibition by either arylvinyl pyridinium compounds[167] and acryloylcholine.[98]

Work with this group of compounds is complicated by their considerable photosensitivity. In the presence of a sunlamp, the *trans* compounds are rapidly converted to the much less inhibitory *cis* isomers.[166] This reaction can be followed by observing the spectral changes which are similar to those seen when *trans* stilbene is photoisomerized.[81]

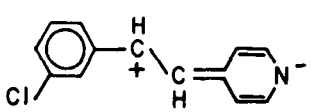


FIGURE 10. Possible resonance form for 3'-chloro-4-stilbazole.

In analogy to the observation of Lewis et al.[88] that *trans*, but not *cis*, stilbene is fluorescent, it is possible to follow the light-induced *trans* to *cis* isomerization of arylvinyl pyridines by following the decrease in fluorescence intensity.

Not surprisingly, Cavallito's group[34] observed photohydration when an aqueous solution of *N*-methyl-4-(1-naphthylvinyl) pyridinium iodide was placed in "direct, spring afternoon sunlight" for 2 hr. Although these studies have not been carried out, one would expect arylvinyl pyridines and their quaternized derivatives to undergo facile photochemical cycloaddition with the formation of substituted cyclobutane dimers. The *cis* compounds should also be able to form photocyclization products analogous to the formation of dihydrophenanthrenes from *cis* stilbenes.[159]

Azomethine analogs, in which either one or the other of the carbons in the ethylene bridge linking the aryl and pyridine rings, were replaced with nitrogen, were synthesized, and studied by Cavallito's group.[33] The results of these studies are summarized in Table 3.

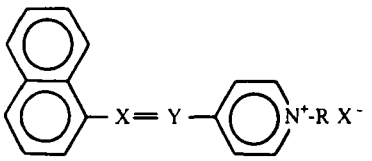
The relative inactivity of the azomethines compared to their styryl analogs was attributed to the fact that azomethines, are nonplanar even in the *trans* conformation.[67] However, Baker and Gibson[9] have pointed out that introduction of fairly bulky groups in the ortho position of either the pyridine or the aryl substituent, which also disrupt planarity, does not greatly alter the ability of these compounds to bind to ChA. They came to the conclusion that the difference in the ability of stilbazoles and their anil analogs to inhibit ChA is due to differences in the electronic nature of the double bond linking the rings. Replacement of carbon with nitrogen has a relatively small effect when carried out in the position vicinal to the naphthalene group, but abolishes activity almost completely when carried out in the position vicinal to the pyridine ring.

It is interesting to note that azo analogs of arylvinyl pyridines also have some ability to inhibit bacterial ChA, as does *p*-phenylazotrimethylammonium chloride,[1] a compound previously studied as an inhibitor of acetylcholinesterase.[21,62] In contrast to the arylvinyl pyridines in which the *trans* compound can be photoisomerized easily to the *cis* compound but the reverse reaction cannot be achieved conveniently, *cis* azo compounds may revert to the *trans* isomer in the dark or by exposure to light of a wavelength coincident with the maximal absorption by the *cis* compound (Figure 11).

Because of this property, azo compounds may be used as photochromic reagents,[21,55,62] that is, compounds capable of undergoing a reversible conformational change while attached to the active site of a biopolymer. It seems likely that photochromic azo analogs of arylvinyl pyridines capable of inhibiting ChA should be able to provide valuable information regarding the mechanism of action of this enzyme.

TABLE 3

Bridge-modified Analogs of Arylvinyl Pyridines[33]



X	Y	R	I_{50} ChA (*M*)
CH	CH	CH_3	4.7×10^{-7}
CH	CH	H	2.5×10^{-5}
N	CH	CH_3	1.5×10^{-6}
N	CH	H	7.5×10^{-5}
CH	N	CH_3	1×10^{-4} (15%)
CH	N	H	1×10^{-4} (15%)

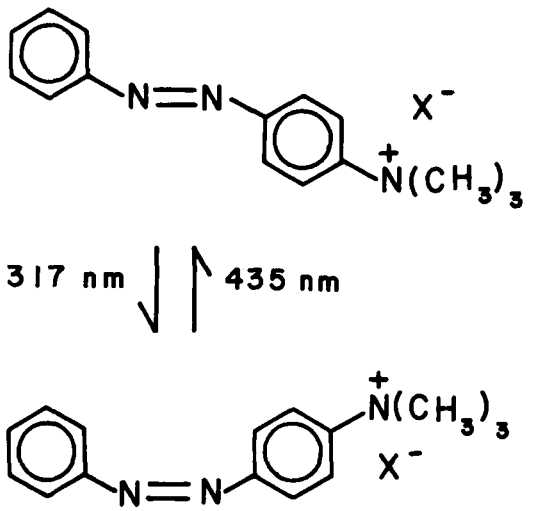


FIGURE 11. Interconversion of *cis* and *trans* *p*-phenyl azophenyltrimethylammonium halide.[62] (From Galley, K. T., de Sorgo, M., and Prins, W., *Biochem. Biophys. Res. Commun.*, 50, 300, 1973. With permission.)

It should be emphasized that the susceptibility to inhibition of ChA by arylvinyl pyridines and related compounds shows considerable species specificity. While brain and placental ChA are easily inhibited by these compounds, ChA isolated from snail, cockroach, and horseshoe crab is highly resistant.[53] Similarly, Yu and Booth[170] have reported fly head enzyme to be resistant to inhibition by the above compounds. It may be significant to note that ChA isolated from snail, horseshoe crab, and cockroach[53] has in common the property of binding CoA weakly in comparison to the binding of acetyl CoA. It seems that only those species of ChA which bind CoA tightly are inhibited effectively by arylvinyl pyridines.

Acryloylcholine is an uncompetitive inhibitor of ChA with respect to choline and acetyl CoA,[98] while a recent reexamination of the inhibition of ChA by arylvinyl pyridines showed these to be uncompetitive inhibitors as well,[145,146] in contrast to the earlier report that inhibition was noncompetitive.[167]

If one follows Cleland's rules,[42] this inhibition pattern suggests that acryloylcholine and arylvinyl pyridines interact with a different form of ChA than do the substrates of this enzyme and that an irreversible reaction step takes place between substrate attachment and inhibitor attachment. This reasoning also leads to the suggestion[145] that this group of inhibitors interacts with the ChA-CoA complex.

Thiol Reagents

The ability of thiol reagents to inhibit ChA was reported soon after the discovery of the enzyme. Berman-Reisberg showed inhibition of ChA by iodoacetate, *p*-chloromercuribenzoate, iodosobenzoate, and cupric ion,[19,20] the latter ion here being considered thiol rather than imidazole specific. She noted that incubation was necessary for maximal inhibition by thiol reagents, indicating that a relatively slow interaction was taking place. The possibility was raised that the acyl group of acetyl CoA might be transferred to an enzymic thiol group yielding an acyl–S–ChA intermediate. Alternatively, it was suggested that the nucleophilic attack of the hydroxy group of choline on the carbonyl carbon of acetyl CoA might be aided by a thiol group of ChA with a thioketal tetrahedral intermediate being formed in which this carbon would interact both with the sulfur of CoA and with an enzymic thiol group. Numerous other workers were also able to inhibit ChA with thiol reagents;[102,111,125,133,148] however, attempts to protect ChA from inhibition by the addition of substrates or products led to conflicting results.

Mannervik and Sörbo[102] reported that choline, acetylcholine, and CoA did not protect ChA isolated from bovine caudate nuclei against the action of thiol reagents, but noted slight protection by acetyl CoA. On the other hand, Roskoski,[133] using enzyme from the same source, claimed complete protection by acetyl CoA and substantial protection by acetylcholine and, using human brain and placental enzyme, complete protection by acetylthiolcholine.[135]

Roskoski[131] also claimed that, using bovine brain ChA, it was possible to obtain ^{14}C-labeled enzyme by incubating the enzyme with ^{14}C-acetyl CoA or ^{14}C-acetyl ^{3}H-choline, followed by passage through a Sephadex G-50 column. In contrast to White and Cavallito,[167] who presented some evidence for formation of a ChA-acetyl CoA complex, Roskoski believed that he was isolating a ^{14}C-acetylthio derivative of ChA which he claimed to be able to acetylate either choline or CoA. He concluded that the ^{14}C-acetyl group was attached to an essential enzymic thiol group forming a thiolester resistant to cleavage by trichloroacetic acid or guanidinium chloride, but susceptible to cleavage by hydroxylamine. He believed that the rate-limiting step in the transacetylation reaction was deacylation of the acetylthio-ChA intermediate, with CoA dissociation being the last step in the reaction.[131,135]

Roskoski[132] believed that the fact that bromoacetylcholine and bromoacetyl CoA were reversible inhibitors of ChA and that these compounds protected against inactivation by *N*-ethylmaleimide suggested formation of a bromoacetylthio-ChA intermediate. However, as already noted, these experiments rather than proving the presence of an active-site thiol group are more likely to indicate its absence.

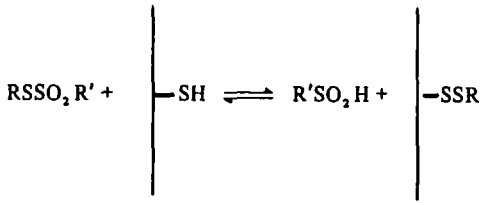
In view of the discrepancies between the experiments of Roskoski,[131-133,135] and the earlier work of Mannervik and Sörbo,[102] our laboratory investigated this problem using ChA isolated from squid head ganglia. In agreement with the latter authors, we found that acetyl CoA, but not acetylcholine or choline, provided protec-

tion against the inhibitory action of thiol reagents. Furthermore, using ^{14}C-acetyl CoA, we found[48,105] that incubation of the substrates with very highly purified ChA,[77] followed by passage through a Sephadex column by the procedure of Roskoski,[131] cleanly separated ChA from ^{14}C-acetyl CoA, with no radioactivity associated with the enzyme (Figure 12).

While our results were obtained with ChA from squid ganglia, which in some respects is rather different from the brain enzyme used by Roskoski, Malthe-Sørenssen[97] was unable to obtain an acetyl-enzyme intermediate following incubation of bovine brain ChA with ^{14}C-acetyl CoA, when enzyme with an activity of 2.5 μM/min/mg protein was used, however, such an intermediate could be observed using enzyme with substantially lower purity. It should be remembered that Roskoski recently reported[135] that only 10% of his protein forms a thiolester, and suggested that it is possible that "the acetyl thioprotein intermediate is adventitious." This seems, indeed, to be the case.

In investigating the action of thiol reagents as inhibitors of ChA, we considered it desirable to use small molecules, which when attached to a thiol group would minimize steric interactions with other portions of the target enzyme. It seemed possible that a thiol reagent attached to a sulfur distal from the active site might still be able to cause non-competitive inhibition by interfering with attachment of the substrate either through steric hindrance or, indirectly by inducing or interfering with a conformational change.

Alkyl alkanethiolsulfonates provide convenient reagents for the attachment of small groups to thiols. Smith et al.[155] reported them to be easy to synthesize, label, and remove. They are attached to their target by the following reaction:



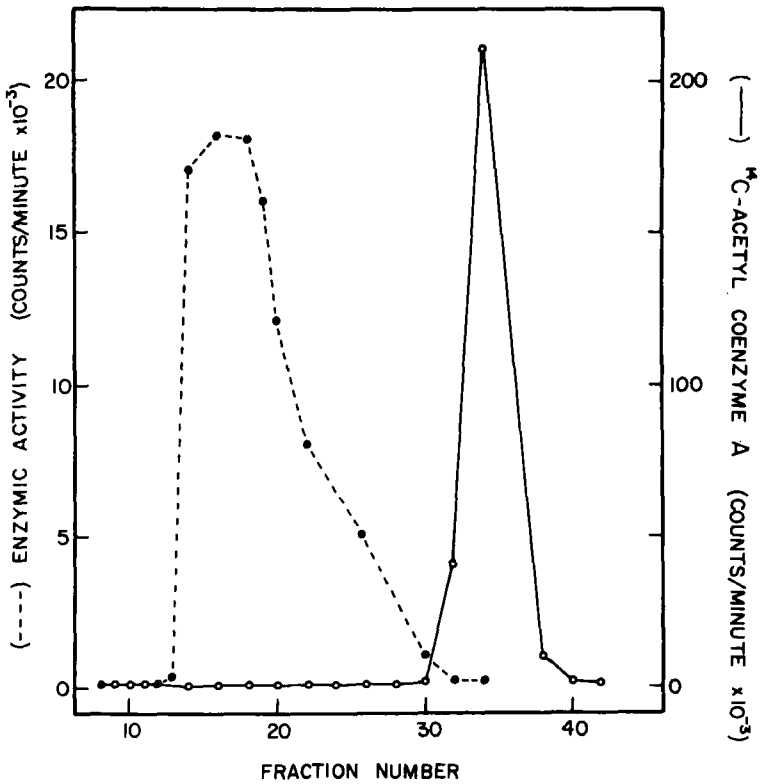


FIGURE 12. Separation of choline acetyltransferase from (^{14}C)-acetyl coenzyme A following the procedure of Roskoski[131] using highly purified enzyme from squid head ganglia.[48,105] (From Currier, S. F. and Mautner, H. G., *Proc. Natl. Acad. Sci. U.S.A.*, 71, 3355, 1974. With permission.)

The mixed disulfides thus formed can react with dithiothreitol or β-mercaptoethanol with regeneration of the original thiol. Using rabbit muscle creatine kinase, Kenyon and his co-workers[155] showed that inhibitor action of substituted thiolsulfonates increased with the size of the group introduced. Methyl methanethiolsulfonate did not completely inhibit this enzyme, but protected it against inhibition by thiolsulfonates carrying bulkier alkyl groups. The authors, therefore, concluded that the thiol group being blocked was not an essential one.

Methyl methanethiolsulfonate was found to be an excellent inhibitor of ChA isolated from squid ganglia[49] as well as of rat brain enzyme,[142] with inhibition being reversed competitively by acetyl CoA. A short lag period preceded inhibition (Figure 13).

These findings raised the possibility that methyl methanethiolsulfonate might inhibit ChA, not by reacting with an active-site thiol of the enzyme, but rather by reacting with the thiol group of CoA. The inhibition lag could then be due to the need for an adequate amount of CoA to be produced by the enzyme. To test this hypothesis, we synthesized the mixed methyl disulfide of CoA.[49] This compound inhibited ChA without a lag period, its K_i being $2 \times 10^{-6} M$ (Figure 14). On the other hand, dethia CoA,[38] the analog lacking the thiol residue, proved to be a weak inhibitor of ChA.

The binding constants of CoA, dethia CoA, acetyl CoA, CoA methyl disulfide, ethyl disulfide, propyl disulfide and the 3-carboxy-4-nitrophenyl (DTNB) disulfide,[50,51a] as well as those of choline and acetylcholine are summarized in Tables 4 and 5.

Alkyl disulfides of CoA appear to be the most potent, competitive antagonists of ChA available. The recently synthesized,[50] fluorescent derivative $1,N^6$-etheno CoA methyl disulfide (Figure 15) also proved to be a competitive antagonist of acetyl CoA, with a binding constant of $4.0 \times 10^{-6} M$. It should be useful for obtaining detailed information about the binding sites of this enzyme.

The differences in the abilities of acetyl CoA and disulfides of CoA to be bound to ChA from squid ganglia raise the question of whether these differences are due to increased hydrophobic bonding mediated through groups attached to the coenzyme's sulfur. If so, the region to which hydrophobic binding occurs must be quite small,

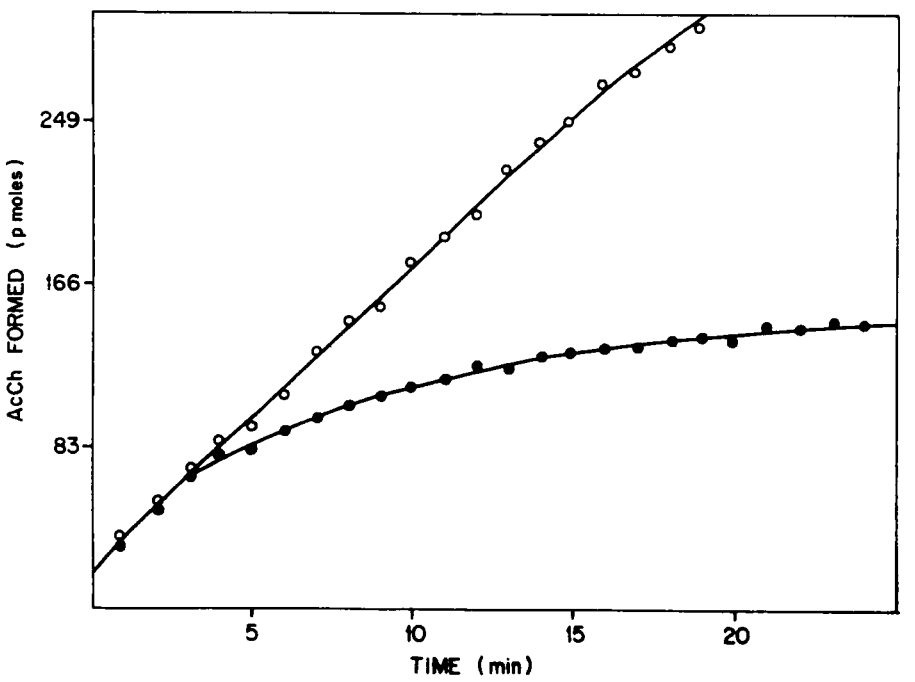


FIGURE 13. Inactivation of choline acetyltransferase. (o–o) Control; (• - •) 2.5×10^{-6} M methyl methanethiolsulfonate.[49] (From Currier, S. F. and Mautner, H. G., *Biochem. Biophys. Res. Commun.*, 69, 431, 1976. With permission.)

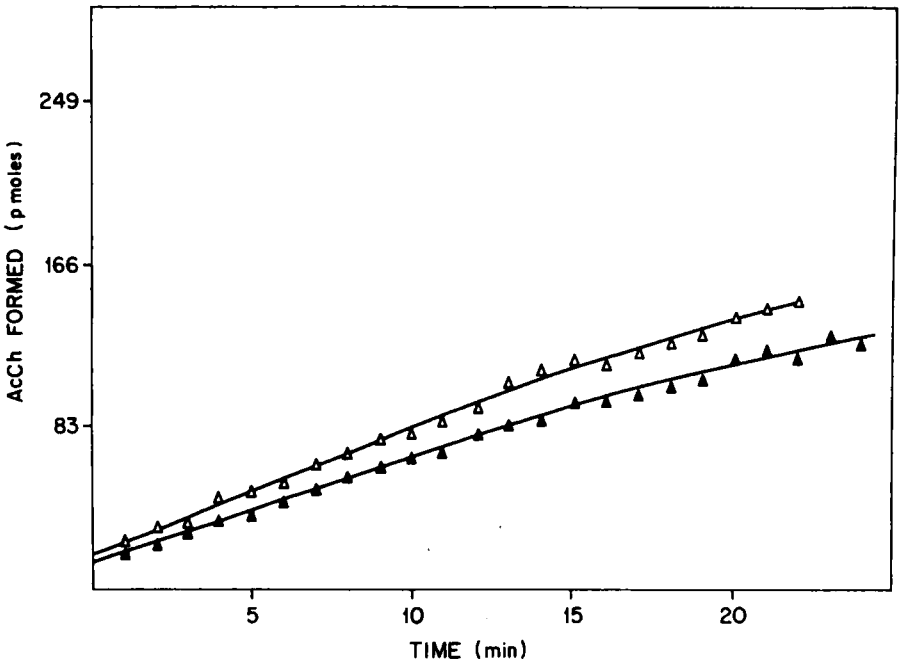
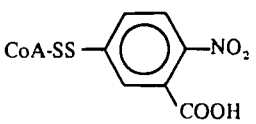


FIGURE 14. Inactivation of choline acetyltransferase. (▲-▲) 6.4 × 10^{-6} *M* synthetic CoA methyl disulfide at t = 0; (△-△) 2.5 × 10^{-6} *M* CoA and 2.5 × 10^{-6} *M* methyl methanethiolsulfonate.[49] (From Currier, S. F. and Mautner, H. G., *Biochem. Biophys. Res. Commun.*, 69, 431, 1976. With permission.)

TABLE 4

The Binding Constants of Acetylcholine and of CoA Analogs[51a]

Compound	K_I (*μM*)
Acetylcholine	37,000
CoA	75
Dethia CoA	78
1,N^6-Etheno CoA	400
3′-Dephosph. CoA	7,000
CoA-$SSCH_3$	2
CoA-$SSCH_2CH_3$	2
CoA-$SSCH_2CH_2CH_3$	5
	4
1,N^6-Etheno CoA-$SSCH_3$	4

since the abilities of methyl, ethyl, and propyl disulfides of CoA to be bound to this enzyme are almost identical. Therefore, only the methylene group vicinal to the sulfur can be engaged in binding.

It is not surprising, then, as noted by Nachmansohn's group,[18] that the abilities of ChA to bind acetyl, propionyl, and butyryl CoA are very similar, as are the maximal rates at which acetylcholine or propionylcholine are formed in the enzyme-catalyzed reaction. On the other hand, butyryl CoA and benzoyl CoA are inhibitors of ChA. The abilities of different acyl derivatives of CoA to interact with ChA are summarized in Table

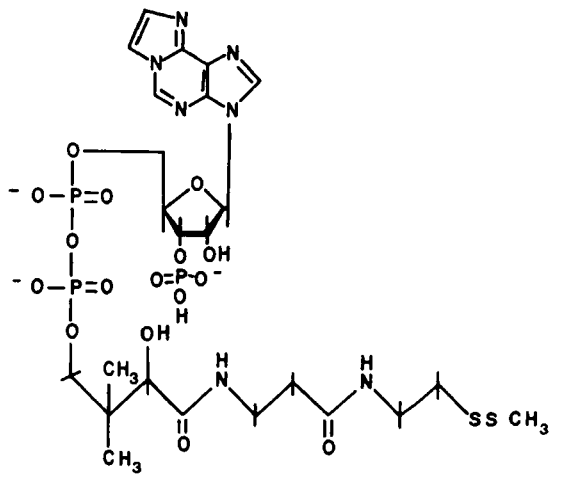


FIGURE 15. 1,N^6-Etheno coenzyme A methyl disulfide.

5. The possibility that substitution of the sulfur of CoA might result in a conformational alteration of the coenzyme, thus altering the abilities of other portions of this molecule to interact with its apoenzyme, cannot be excluded.[104]

It should be emphasized that the large differences between the abilities of acetyl CoA and disulfides of CoA to be bound to ChA are not seen in all species of this enzyme. ChA isolated from a group of invertebrates[53] shows rather large differences in the relative ability to bind CoA and acetyl CoA. On the other hand, in bovine brain enzyme, CoA is bound tightly with K_i values of 38 $\times 10^{-6}M$ for CoA and of 16.5 $\times 10^{-6}M$ for acetyl CoA reported by Ryan.[145] Similarly, in rat brain ChA, the ability to bind CoA and its acetyl derivative is very similar. CoA methyl disulfide is an excellent inhibitor of the rat brain enzyme, with a binding constant of $1 \times 10^{-7}M$.[142]

Relatively little work has been carried out to study the specificity of binding of CoA to ChA. The similarity in the binding abilities of etheno derivatives of CoA, and CoA methyl disulfide with those of the nonfluorescent analogs suggests that the contribution of the 6-amino group of the purine ring must be relatively unimportant. The contribution of the 3′-phosphate groups of CoA analogs to the binding of the cofactor to ChA is important, the K_m of acetyl dephospho CoA being $820 \times 10^{-6}M$,[51,51a] while the K_i of dephospho CoA is $7000 \times 10^{-6}M$.

The Mechanism of Action of Choline Acetyltransferase

In summarizing the information regarding inhibition studies with this enzyme, the following conclusions can be drawn. The evidence is unconvincing that ChA possesses a thiol group in the active site and that, as suggested by Roskoski,[131-133] and Roskoski et al.,[135] transacetylation from acetyl CoA to this mercapto group with formation of acetylthio enzyme takes place. However, the possibility exists that ChA possesses one or several thiol groups outside the active site. After all, mercurial columns can be used to separate ChA from other proteins.[36,77] The data of Rossier[140] suggest that, in the presence of dithiothreitol, reduction of a ChA disulfide with formation of two identical subunits might be taking place. Recently, several examples of nonallosteric enzymes have appeared in which flip-flop mechanisms of two subunits within a functional dimer are an integral part of the catalytic mechanism.[87] Such enzymes follow Michaelis-Menten kinetics and show relatively low substrate specificity, as does ChA with respect to acetyl CoA derivatives. It seems possible that ChA is a member of this class of enzymes.

Evidence has already been presented that catalysis by ChA shows a sizeable D_2O isotope effect, whether choline or aminocholine is acetylated.[48,105] In many hydrolytic enzymes, imidazole, through general base removal of the proton of the hydroxy group of serine, facilitates nucleophilic attack by the oxygen atom of serine;[24] thus, it seemed possible that imidazole might play a similar role here too.

A possible role for imidazole in the catalytic functions of ChA has been proposed by White and Cavallito,[167] on the basis of the observation that cupric ion partially protected ChA against inhibition by arylvinyl pyridinium compounds. Burt and Silver[30] also reported that imidazole catalyzed the nonenzymic transfer of an acetyl group from acetyl CoA to choline. It is not surprising that the hydrolysis of thiolesters is subject to catalysis by imidazole, a fact reported by Bender and Turnquest[16] almost 20 years ago. While Burt and Silver suggested formation of *N*-acetylimidazole as a catalytic intermediate in the nonenzymic transacetylation reaction, and Malthe-Sørenssen proposed an analagous intermediate in the enzymic reaction,[97] the D_2O isotope effect seen in the ChA-catalyzed transacetylation[48,105]

TABLE 5

Kinetic Data For Acyl CoA Derivatives[51a]

Derivative	K_m (μM)	V_{max}	K_I (μM)	K_m for choline (μM)
Acetyl CoA	47	16		1900
Propionyl CoA	31	15		2600
Butyryl CoA.	20	0.94	55	6700
Acetyl-3′-dephospho CoA	820	5.30		753

suggests that general-base catalysis takes place, at least in the enzymic reaction. In the imidazole-catalyzed hydrolysis of *p*nitrophenyl acetate, for which formation of *N*-acetylimidazole as intermediate is well documented, a D_2O/H_2O isotope effect of 1.0 is seen.[15] In the ChA-catalyzed reaction, the isotope effect is about 2.0, being greatest at the pH (pD) at which enzymic activity is maximal. As already noted, caution must be used in interpreting isotope effects;[82] however, the data available make general-base catalysis more likely than nucleophilic catalysis.

It remained to establish that imidazole was indeed essential for the catalytic functioning of ChA. It had been reported that methylene blue and, more specifically, rose bengal, on photooxidation, were capable of inactivating histidine residues.[103,164] It can be seen in Figure 16 that photooxidation in the presence of these dyes indeed results in rapid inactivation of ChA activity. This finding was confirmed by Roskoski,[134] who used ethoxyformic anhydride to inhibit brain ChA. Activity could be regenerated by treatment with hydroxylamine. Some protection against inhibition by ethoxyformic anhydride by CoA and acetyl CoA was reported. In view of the data now available, we proposed the mechanism shown in Figure 17 for ChA.[48]

It is suggested that general-base catalysis by an essential enzymic imidazole group enhances the ability of the hydroxy group of choline or the protonated form of aminocholine to react with the thiolester group of acetyl CoA bound to the enzyme. In other words, the nucleophilicity of the hydroxy group of choline bound to an active site of ChA is enhanced by interaction with an imidazole group in a fashion analogous to the imidazole-serine interaction seen in the "charge relay system" of chymotrypsin and related enzymes.[24]

It will, of course, be essential to carry out a careful study of the enzyme modified by photooxidation to prove that an essential imidazole group is indeed inactivated; however, all the data now available are compatible with that postulate.

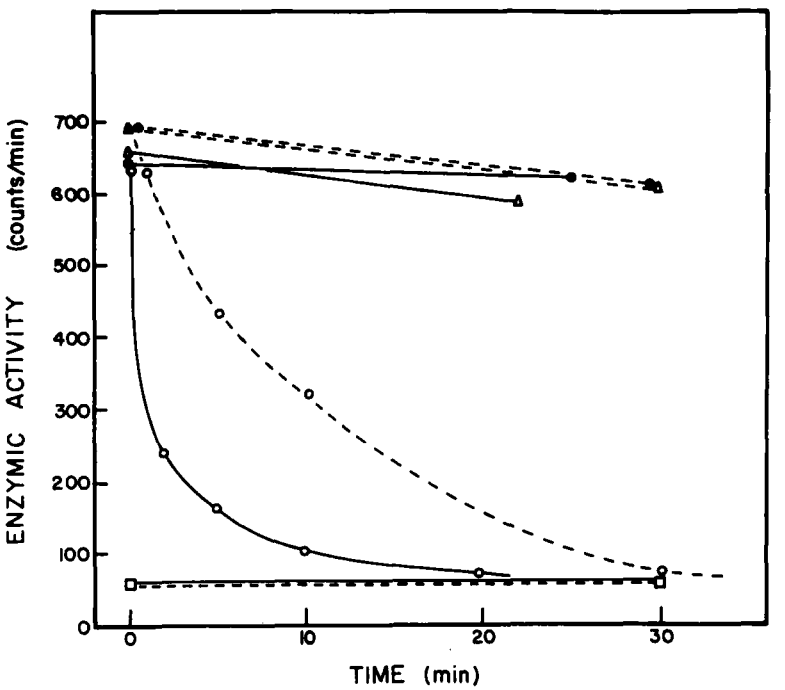


FIGURE 16. Photooxidation time profile using methylene blue (broken line) and rose bengal (solid line); irradiated ChA without dye (●); irradiated ChA with dye (○); ChA and dye not irradiated (△); and control, i.e., substrates, dye, no ChA (□).[48,105] (From Currier, S. F. and Mautner, H. G., *Proc. Natl. Acad. Sci. U.S.A.*, 71, 3355, 1974. With permission.)

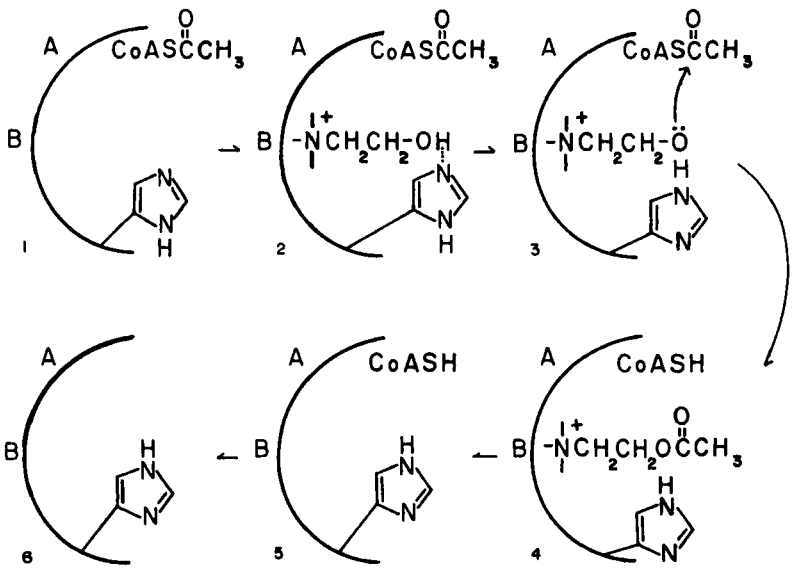


FIGURE 17. Proposed mechanism of action for choline acetyltransferase.[48] (A) Acetyl coenzyme A binding site; (B) choline binding site (anionic). (From Currier, S. F. and Mautner, H. G., *Proc. Natl. Acad. Sci. U.S.A.*, 71, 3355, 1974. With permission.)

THE CONTROL OF CHOLINE ACETYLTRANSFERASE ACTION

It was noted long ago that the levels of acetylcholine in nervous tissue remain relatively constant. As early as 1949 Richter and Crossland[130] reported that electrical stimulation in the cerebral cortex of the rat produced a transient fall in brain acetylcholine levels followed by increased acetylcholine synthesis. The rate of resynthesis rose within 1 min to 7 μg/g, more than five times the normal brain content.

In 1969, Kaita and Goldberg[84] suggested that since acetylcholine in concentrations from 10 to 100 m*M* progressively inhibited its own synthesis by ChA, direct inhibition of this enzyme by acetylcholine is involved in the regulation of its biosynthesis. Following nerve stimulation, there is an increase in the concentration of free choline. Since choline and acetylcholine are competitive inhibitors of the binding of each other, their relative levels would be expected to control ChA activity. Similarly, Potter and his co-workers[125] suggested that acetylcholine controlled its biosynthesis by mass action.

Birks[22] and McCaman and Hunt[107] observed that the activity of ChA is increased by sodium ion, the intracellular concentration of which, in turn, is increased by nerve depolarization. Birks suggested that the salt effect may serve "as a means of coupling cellular activity to metabolism."

Schuberth[148] claimed that chlorides specifically activate ChA. He reported that the initial velocity of the ChA-catalyzed reaction is increased in the presence of chloride, with a shift of the pH optimum to higher levels and a lowering in the temperature optimum of the reaction. Potter et al.[125] found that activation increased in passing from fluoride to chloride to bromide to iodide, although the activation observed was rather slight. While numerous papers have been written dealing with the effect of salts on the activity of ChA, there is considerable variability in the results observed. For example, Korey et al.[86] reported that sodium chloride in concentrations up to 100 μ*M* had no effect in the squid enzyme, while inhibition was observed at higher concentrations. Berman-Reisberg,[20] using the same enzyme, found no salt effects, while Prince,[126,127] also using squid ChA, reported salt activation. In contrast to Schuberth,[148] who found more activation with chloride and bromide than with iodide in the human placental enzyme, Prince found activation to be maximal with iodide as compared to other halide ions. He also noted that the binding of choline became looser as the ionic strength of the medium was increased, as did the binding of acetyl

CoA.[127] Recently, Rossier and his collaborators,[143,157a] using rat brain enzyme, also reported salt activation by several anions, the effect being maximal with chloride and bromide. Rossier et al.[157a] showed that the maximal velocity of the enzyme reaction is increased by the presence of chloride ion while, as noted previously, the K_m for choline and for acetyl CoA is increased. It was suggested that the functioning of this enzyme might be controlled through changes of chloride concentration in the vicinity of ChA, brought about by nerve stimulation. However, Hersh and Peet[74b] convincingly demonstrated salt effects to be related to ionic strength and not to be ion specific.

It should be noted that salt effects may differ greatly depending on the purity of the ChA being studied. Interestingly, Potter's group[125] noted that dialysis removes the ability of ChA to be affected by salt. Careful studies with highly purified enzyme will be required before a final assessment of the importance of salt in the control of ChA function can be made.

An important discovery was made by Fonnum[56] who observed that the binding of ChA to membranes is a reversible process dependent on the pH and the ionic strength of the medium. The enzyme was active both in the particulate and in the soluble form, suggesting that aggregation involved neither the active site of the enzyme nor induced a conformational change altering catalytic activity. This effect showed little ion specificity. Acetylcholine and choline solubilized ChA; thus, the enzyme would be expected to be soluble if compartmented in the presence of relatively high concentrations of these compounds.[56] Later, Fonnum[57] showed that the binding of ChA to membranes involved coulombic interactions between the positively charged ChA molecule and the negatively charged membranes.

Since the discovery by Hebb and Silver[71] that ChA accummulates proximally to a nerve ligature, several studies have dealt with the axonal transport of ChA. This work has recently been reviewed by Fonnum.[59] Considerable attention has also been paid to the high- or low-affinity uptake of ChA into synaptosomes[59] which, for the last 20 years, have been widely believed to be involved in transmitter release, a theory for which experimental proof remains elusive.[65] Both for studies of axonal ChA transport and for studies of the physiological significance of synaptic vesicles, it would be important to know whether ChA should be considered a soluble or a membrane-bound enzyme. Evidence suggests that ChA is a cytoplasmic enzyme which can be bound by coulombic interactions to vesicular and other membranes. Since this binding is affected by pH and salt concentration, both of which may be changed during the propagation of the nerve impulse, it is possible that the binding and release of ChA from membranes may be of importance to the regulation of acetylcholine synthesis. Changes in pH and salt concentration may also affect the abilities of the substrates and products of the ChA reaction to bind to the enzyme and, potentially, the ability of subunits to dissociate, thus, it is clear that parameters likely to be changed during the flow of the nerve impulse will have a great effect on the activity of ChA and on the uptake of its substrates and the release of its products.

Studies of the functional relationships between ChA, acetylcholine receptor protein, acetylcholinesterase, the storage and transport mechanisms of the substrates, products and macromolecules involved, as well as of the ions which may affect them, are of fundamental importance. Unfortunately, for the time being, even the much humbler aims of obtaining analytically pure ChA, studying its detailed mechanism of action, and obtaining reproducible methods for localizing it in cells have not been achieved.

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