# Choline and Carnitine Acetyltransferases of Heart†

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ABSTRACT: Choline acetyltransferase (ChAc) and carnitine acetyltransferase (CarAc) were estimated in crude and partially purified extracts of rabbit and human heart and brain. Both enzymes were assayed by isotopic methods previously used for ChAc. Isoelectric focusing on acrylamide gel allowed a separation of the two enzymes and indicated that choline is an alternate substrate for CarAc of rabbit heart extracts. Further support for this conclusion was derived from kinetic studies and the finding that ChAc was inhibited approximately 90% by 50 μM (naphthylvinyl)pyridine, while CarAc, with either carnitine or choline as substrate, was not inhibited by this compound.

A number of workers have reported that significant levels of choline acetyltransferase (acetyl-CoA:choline *O*-acetyltransferase, ChAc,[1] EC 2.3.1.6) activity can be detected in crude extracts of mammalian heart tissue (Ekström, 1970; Mahoney *et al.*, 1971; Thoenen *et al.*, 1972). Therefore our interest in a suitable peripheral source of ChAc led us to attempt a purification of the enzyme from mammalian heart.

Although assays of crude homogenates of rabbit or human heart initially indicated the presence of ChAc levels similar to the high values reported by Mahoney *et al.* (1971), most of this activity appeared to be dependent only on the substrate, acetyl-CoA, and was not stimulated by added choline. In addition, more than 90% of this apparent activity disappeared during the initial steps of purification. Evidence is herein presented which demonstrates that most of the initially observed activity was actually due to the acetylation of endogenous carnitine by the enzyme carnitine acetyltransferase (acetyl-CoA:carnitine *O*-acetyltransferase, CarAc, EC 2.3.1.7). Isoelectrofocusing and kinetic experiments indicated moreover that a part of the low choline acetylating activity in heart extracts results from the ability of choline to serve as a relatively poor substrate for CarAc.

## Methods

*Extract Preparation.* New Zealand white rabbits were killed by cervical dislocation, and brains and hearts were quickly removed and rinsed at 0° in iced 0.9% KCl. Human heart (atrial) and brain (basal ganglia) tissue, obtained at autopsy less than 8 hr after death, was frozen immediately after dissection. All further purification steps were performed at 0–4°. Tissues were homogenized in 0.1 mM EDTA containing 0.5 mM *p*-tosyl fluoride as an inhibitor of proteolysis (Koshland, 1966), pH 7.0, at a 1 to 8 tissue to solvent ratio in either a Waring Blendor (hearts) or with a Teflon–glass motor-driven homogenizer (brains). Crude homogenates were dialyzed overnight at 4° against 1000 volumes of 0.05 M Tris-chloride–0.5 mM dithiothreitol–0.1 mM EDTA (pH 7.0). Dialysis membranes were pretreated by boiling in 0.1% $Na_2EDTA$ for 5 min in order to remove inhibitory substances.

Further purification was by a modification of the method of Potter *et al.* (1968) for ChAc. Homogenates were made 1% in 1-butanol (v/v) and were centrifuged at pH 8.0 (16,000*g* for 30 min). The supernatant was adjusted to pH 5.0 and recentrifuged to yield a pellet which was resuspended in 0.2 M KCl. Another centrifugation (16,000*g*, 30 min) produced a supernatant which was fractionated with ammonium sulfate. The fraction between 30 and 55% ammonium sulfate was resuspended, centrifuged at 100,000*g* for 1 hr, and then dialyzed against 0.05 M Tris–0.1 mM EDTA–0.1 mM dithiothreitol (pH 7.4). Protein was determined by the method of Lowry *et al.* (1951). Bovine albumin at 0.05% was added to these partially purified extracts as a stabilizing agent, and they were stored at −20°. Repeated thawing and freezing did not significantly affect enzyme activity over a period of several months. Extracts at this stage of purity were designated "$SP_{55}$ fractions."

*Enzyme Assays.* For determination of enzyme activity, assay components were as follows: [1-$^{14}$C]acetyl-CoA, 0.1 mM, 4 Ci/mol; NaCl, 150 mM; dithiothreitol, 0.05 mM; EDTA, 0.1 mM; potassium phosphate, 70 mM; 20 μl of enzyme extract; and either choline or carnitine (0.5–10 mM), in a total volume of 50 μl at pH 7.0. The second substrate was omitted in blanks. Eserine (0.1 mM) was added to crude extracts. Incubation was at 37° for 10 min, after which assay tubes were quickly chilled in an ice–water bath, and 20 μg of unlabeled ACh was added as a carrier. Separation of [$^{14}$C]ACh from the radioactive substrate was achieved by either of the following methods. (1) Disposable ion-exchange columns were prepared in 9 in. Pasteur pipets, plugged with a small wad of glass wool. Water-washed Bio-Rad Ag 1-X8 resin was added to each column to a height of 3 cm. The incubation mixture was pipetted on top of the resin and washed through the column directly into a scintillation vial with three 0.5-ml portions of distilled water. This is a modification of the method of Schrier and Shuster (1967). Any $^{14}$C-labeled compound which bears a positive or neutral charge is eluted into the scintillation vial, and therefore both acetylcarnitine and acetylcholine were eluted in this method. Fifteen milliliters of scintillation fluid (4 g of Omnifluor/l. of 2:1 toluene–Triton X-100) was added to each vial, and scintillation counting was carried out in a Packard TriCarb counter at 70% efficiency. (2) The $^{14}$C-labeled products were more accurately identified by a paper electrophoretic method which is described elsewhere (Potter and Murphy, 1967; White and Cavallito, 1970). Under the acidic conditions of this separation, the mobility of acetylcarnitine was 8 cm/hr and that of ACh was 9 cm/hr. Therefore the two products could be separated. The position of unlabeled ACh used as carrier was located on the paper strips by expos-

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[1] Abbreviations used are: ChAc, choline acetyltransferase; CarAc, carnitine acetyltransferase; ACh, acetylcholine.

ing them to iodine vapor. A transient complex between ACh and iodine appeared as a brown streak which could be marked with pencil before it faded. Paper strips after electrophoresis were scanned with a Nuclear-Chicago Actigraph III strip scanner. Activity in peak positions was determined by placing appropriate portions of the dried paper strips in scintillation vials, adding 15 ml of scintillation fluid, and counting at 63% efficiency.

*Isoelectric focusing* on acrylamide gel was performed using an Ortec Model 4200 electrophoresis chamber and pulsed power supply. The anodic solvent was 0.1 N HCl and the cathodic solvent was 0.15 M monoethanolamine, as recommended in Ortec Manual AN32. The gel slab contained 6% acrylamide, 0.25% *N,N'*-methylenebisacrylamide, 0.1 mM dithiothreitol, 0.1% Tween 80, and 2.4% ampholine (pH range 3–10). Extracts were dialyzed against 20% sucrose in order to increase their density, and aliquots containing less than 0.4 mg of protein were applied in small wells near the anodic end of the gel slab. A cap gel solution was then layered over the samples and allowed to polymerize. Electrofocusing was at 4° for 18 hr at 100 pulses/sec and 105 V. Sections of gel containing the enzyme were sliced horizontally in 2-mm sections, each of which was placed in a small tube and eluted with 0.10 ml of 0.07 M phosphate buffer (pH 7) for 1 hr at 4°. Substrates were added in a volume of 20 μl to give 60 μM [$^{14}$C]acetyl-CoA (4 Ci/mol), either 2 mM choline or 2 mM l-carnitine, and 100 mM NaCl. A gel strip containing no enzyme was assayed in a similar manner to determine background activities, which increased gradually from 30 cpm at the anodic (low pH) end of the gradient to 200 cpm at the pH 8.5 position in the gel. At pH 9.5, about 5 mm from the cathodic end of the gel, a peak of about 2000 cpm appeared in assays of blank gel strips. This peak was apparently caused by a basic impurity in the ampholine preparations, which reacted with acetyl-CoA, since its height varied with the particular batch of ampholine. It was not caused by acrylamide because it also appeared in fractions obtained after column electrofocusing on a sucrose gradient. Because of it, results in the pH region above 8.5 were discarded.

In every electrofocusing experiment standard proteins were electrofocused separately in adjacent wells of the same gel slab. These were bovine albumin, human hemoglobin, chymotrypsin, and chymotrypsinogen. At the end of the experiment, portions of the gel slab containing these standards were stained for protein by soaking the gel in 0.2% Bromophenol Blue in a 50:45:5 volume ratio of ethanol–water–glacial acetic acid (Awdeh, 1969). The pH gradient in the gel was then determined by the positions of the various standards, after a correction was made to allow for any change in length of the gel which occurred during staining (always less than 5% change). This method of determining the pH gradient was verified by eluting 5-mm sections of the gel slab with 2 ml of distilled water and then obtaining pH with a Beckman expandomatic pH meter. The gradient determined with both methods coincided, except in the region above pH 9, where the latter method gave somewhat lower pH values.

*Materials.* Acetyl-CoA, 96% pure, was purchased from P-L Biochemicals, Inc. (Milwaukee, Wis.); [1-$^{14}$C]acetyl-CoA, 99% radiopurity, 55 ± 5 Ci/mol, and Omnifluor from New England Nuclear Corp. (Boston, Mass.); choline chloride, >99% from Nutritional Biochemicals Corp. (Cleveland, Ohio); l-carnitine from General Biochemicals (Chagrin Falls, Ohio); pigeon muscle carnitine acetyltransferase, 80 U/mg, from Boehringer Mannheim Corp. (New York, N. Y.); Bio-Rad 1-X8 anionic exchange resin, 100–200 mesh, chloride form, from Bio-Rad Laboratories (Richmond, Cal.); *p*-tosyl fluoride from Aldrich Chemical Co. (Milwaukee, Wis.); Ampholine carrier ampholytes from LKB Instruments, Inc. (Rockville, Md.). Standards for electrofocusing were human hemoglobin and bovine albumin from Schwarz/Mann (Orangeburg, N. Y.); chymotrypsin and chymotrypsinogen from Worthington Biochemical Corp. (Freehold, N. J.). The ChAc inhibitor 4-(1-naphthylvinyl)pyridine was the generous gift of Dr. C. J. Cavallito. All solutions containing this compound were protected from light (White and Cavallito, 1970).

## Results

*ChAc and CarAc of Heart.* When crude eserine-treated homogenates of whole rabbit or human heart were assayed for choline acetylating activity, using the anion exchange procedure to separate the radioactive product from substrate, relatively high and variable apparent activities were obtained, ranging from 10 to 50 nmol of acetylated product per min per g of wet tissue. Exhaustive dialysis of these homogenates resulted in a loss of 95–97% of the initial activity, to ChAc levels of between 0.5 and 1.5 nmol per min per g of wet tissue. Since an optimal choline concentration was used in these assays, it was not likely that the high initial activities were due to the presence of endogenous choline. A paper electrophoretic separation of the incubation mixture indicated that the product in undialyzed homogenates was a positively charged substance with lower mobility than ACh. The results of such an experiment with a crude homogenate of human atrial tissue are given in Figure 1. For the upper scan in this figure, undialyzed human heart extract was incubated at 37° for 10 min with only [$^{14}$C]-acetyl-CoA and no added second substrate. The large peak is that of unchanged substrate, and the small cathodic peak represents the product. After this extract was exhaustively dialyzed atainst a buffer containing 70 mM potassium phosphate–0.1 mM EDTA–0.1 mM dithiothreitol (pH 7.0) to remove endogenous substrates, a variety of compounds was added in assays with acetyl-CoA. The second and third scans of Figure 1 show the effect of adding carnitine or choline, respectively, to this dialyzed extract. The large stimulation of product formation with added carnitine, with corresponding acetyl-CoA utilization, indicated that CarAc was present in the crude extract and that this enzyme in the presence of endogenous carnitine can produce the product, acetylcarnitine, which is known to migrate toward the cathode more slowly than ACh during electrophoresis at low pH (Potter and Murphy, 1967). The relatively small amount of ACh formed by this extract in the presence of choline was not clearly seen under the conditions of Figure 1 (scan 3) because the amount of product was more than 100-fold less than that obtained with carnitine. For the lowest scan of Figure 1, [$^{14}$C]ACh was added before electrophoresis so that its mobility could be determined.

Relative choline and carnitine acetylating properties of several extracts of heart and brain are given in Table I. Crude extracts were extensively dialyzed to remove endogenous carnitine prior to these assays. The last column in Table I indicates that the ratio of CarAc to ChAc in heart is much higher than in brain. The following experiments were designed to test the possibility that the relatively low level of choline acetylating activity in heart extracts might result from the action of CarAc, if choline were an alternate poor substrate for this enzyme.

*Kinetics.* The partially purified $SP_{55}$ extract of rabbit heart was assayed with varying concentrations of choline at a constant saturating concentration of acetyl-CoA to give an appar-

TABLE I: Relative Carnitine and Choline Acetyltransferase Activities in Extracts of Heart and Brain.

| Extract | Acetyltransferase Activity[a] | | CarAc: ChAc |
|---|---|---|---|
| | Carnitine | Choline | |
| Rabbit heart, crude | 18.8 | 0.024 | 784 |
| Rabbit heart, $SP_{55}$ | 68.0 | 0.24 | 284 |
| Rabbit brain, $SP_{55}$ | 9.4 | 20.0 | 0.5 |
| Human heart,[b] crude | 0.94 | 0.0056 | 168 |
| Human brain, crude | 0.96 | 0.23 | 4.2 |
| Human brain, $SP_{55}$ | 2.7 | 14.4 | 0.19 |

[a] Enzyme activity is expressed as nmol of acetylated product formed per min per mg of protein at saturating concentrations of the substrates, acetyl-CoA and 1-carnitine or choline. All assays were in duplicate with a maximum variation of ±5%. [b] Human heart atrial tissue had been stored at −60° for 6 months prior to extract preparation.

ent $K_m$ value for choline. Extracts of rabbit brain and sciatic nerve were also assayed under the same conditions. Plots of reciprocal of enzyme activity as a function of reciprocal of choline concentration yielded straight lines for all three extracts. However, the apparent $K_m$ value obtained for the heart extract was approximately fivefold higher than the corresponding values for brain or sciatic nerve extracts (Table II).

Highly purified CarAc of pigeon muscle was assayed in a similar manner, using 0.1 mM acetyl-CoA, and a range of choline concentrations from 2.0 to 20 mM. A reciprocal plot of the data yielded a straight line with an apparent $K_m$ value of 8.7 mM. The $K_m$ for 1-carnitine with the same enzyme was found to be 0.4 mM. The $K_m$ of 5 mM for choline found with rabbit heart extract lies about midway between the values of 1.1 and 8.7 mM obtained with brain ChAc and pigeon muscle CarAc. Examples of paper strip scanning profiles obtained after electrophoresis of the products of pigeon muscle CarAc are shown in Figure 2. The appearance of [$^{14}$C]ACh was accompanied by a decrease in the substrate peak when choline was added to the incubation mixture.

If, as the above experiments indicate, choline is an alternate substrate for CarAc, one would predict that choline should inhibit the acetylation of carnitine. When the concentration of 1-carnitine was varied in experiments with pigeon muscle CarAc, choline competitively inhibited the formation of acetylcarnitine. ACh was a competitive inhibitor of the same system.

*Inhibition by (Naphthylvinyl)pyridine.* The ChAc inhibitor, (naphthylvinyl)pyridine, was dissolved in aqueous solution at pH 4.5 and then diluted into 0.07 M potassium phosphate buffer at pH 7.0. The inhibitor was always added to the enzyme prior to the addition of substrates. As indicated in Table III, the choline acetylating activity of rabbit heart was considerably less sensitive to inhibition by (naphthylvinyl)pyridine than was ChAc from a number of other sources, including rabbit brain and sciatic nerve. At 50 μM (naphthylvinyl)pyridine, the ChAc from human brain or sciatic nerve and from rabbit brain were inhibited by approximately 90%, while the formation of ACh by rabbit heart extract was inhibited by only 29%. CarAc, with either carnitine or choline as substrate, was not inhibited by concentrations of (naphthylvinyl)pyridine as high as 0.1 mM. Therefore, the partial inhibition of heart extract by (naphthylvinyl)pyridine is consistent with the

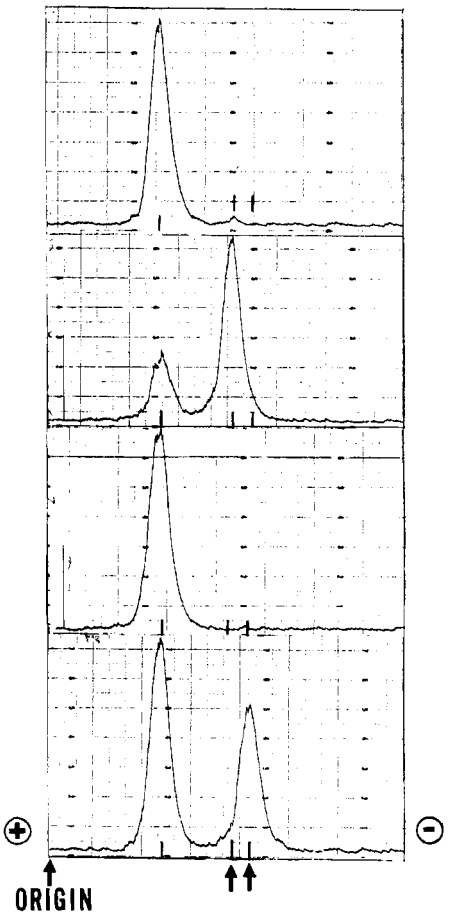


FIGURE 1. Photograph of paper strip scanning results after electrophoretic separation of reaction products of crude human heart extract. Incubation was at 37° for 10 min under the following conditions: upper scan—undialyzed extract, 0.1 mM acetyl-CoA, no second substrate added; second scan—dialyzed extract, 0.1 mM acetyl-CoA, 1.0 mM 1-carnitine; third scan–dialyzed extract, 0.1 mM acetyl-CoA, 5.0 mM choline; bottom—dialyzed extract, 0.1 mM acetyl-CoA, [$^{14}$C]ACh added after incubation and before electrophoresis. The expected peak positions of acetyl-CoA, acetylcarnitine, and ACh are indicated by vertical marks on each scan and by arrows at the bottom. These had mobilities of 5, 8, and 9 cm per hr, respectively. Full scale in the vertical direction represents 300 cpm with about 13% counting efficiency.

TABLE II: Kinetic Parameters for Choline Acetylation by Rabbit Extracts.[a]

| Tissue | $K_{m,app}$ (mM) | $V_{max}$ (nmol/min per mg of Protein) |
|---|---|---|
| Heart | 5.0 | 0.24 |
| Brain | 1.1 | 20 |
| Sciatic nerve | 1.0 | 18 |

[a] $P_{55}$ extracts were assayed with 0.10 mM acetyl-CoA and varying concentrations of choline (0.20–2.0 mM). Other assay conditions are described in Methods.

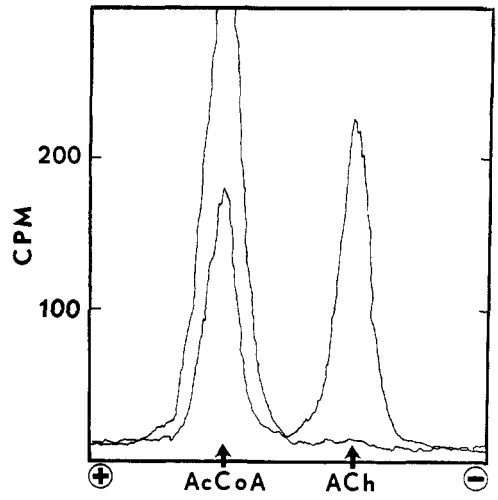


FIGURE 2: Tracings of paper strip scans after electrophoresis of incubation media. Highly purified pigeon muscle CarAc (25 $\mu$g) was incubated with 0.10 mM acetyl-CoA for 10 min at 37° in the absence and presence of 8 mM choline. The highest peak at the acetyl-CoA position was obtained in the absence of choline. This peak decreased as choline was added to the system, while a $^{14}$C-labeled product appeared in the expected position for ACh.

possibility that CarAc was responsible for the formation of part of the ACh.

*Isoelectric Focusing.* In order to more clearly distinguish between the choline and carnitine acetyltransferase activities of rabbit heart, the method of isoelectric focusing on acrylamide gel was used. Figure 3 is an electrofocusing profile obtained after two portions of $SP_{55}$ extract were simultaneously electrofocused in adjacent channels of the same gel slab. One gel column was assayed for ChAc activity and the other for CarAc. The main peak for CarAc occurred at pH 7.8. There were two small, but reproducible, peaks at pH 6.2 and 6.8. The major ChAc peak occurred at a pH value of 7.1. However, there was a significant amount of choline acetylating activity in the pH 6.2 and 7.8 peaks, corresponding to those which also exhibited CarAc activity.

When an $SP_{55}$ fraction of rabbit brain was electrofocused under the same conditions, a single large peak for ChAc was found at a pH value of 7.3 ± 0.2. This single ChAc form found in rabbit brain extract corresponds with the largest ChAc peak of rabbit heart in Figure 3.

Highly purified CarAc of pigeon muscle was also electrofocused and assayed in a similar manner for ChAc and CarAc activities. As seen in Figure 4, this preparation of CarAc exhibited two major peaks, one at pH 5.7, which was not seen in the rabbit heart, and the other at pH 7.8, similar to that in Figure 3. Of particular interest is the observation that choline acetylating activity was associated with the pH 7.8 fractions and not with the pH 5.7 fractions. Minor peaks were also seen at pH 6.1 and 6.7, similar to the minor peaks in Figure 3. The potent ChAc inhibitor (naphthylvinyl)pyridine, at 0.1 mM did not inhibit any of the activity found with CarAc.

Values of the isoelectric points of main peaks obtained after electrofocusing are summarized in Table IV. Rabbit heart and brain $SP_{55}$ fractions contained both ChAc and CarAc. However, the brain fraction did not contain sufficient CarAc to produce a measurable peak in the CarAc region when choline was the substrate.

TABLE III: Inhibition of ChAc by (Naphthylvinyl)pyridine (50 $\mu$M).[a]

| Extract Source | % Inhibn |
|---|---|
| Rabbit heart | 29 |
| Rabbit brain | 89 |
| Human caudate nucleus | 89 |
| Human sciatic nerve | 92 |

[a] Extracts were dialyzed high-speed supernatants after ammonium sulfate fractionation. Substrate concentrations were 0.1 mM acetyl-CoA and 5 mM choline. Above results are averages of at least two determinations with maximum variation of ±1% inhibition.

TABLE IV: p*I* Values[a] of Main Peaks after Electrofocusing.

| Extract | Substrate | |
|---|---|---|
| | Carnitine | Choline |
| Rabbit heart, $SP_{55}$ | 7.8 | 7.8 |
| | | 7.1 |
| Rabbit brain, $SP_{55}$ | 7.9 | 7.3 |
| Pigeon muscle (highly purified CarAc) | 5.7 | |
| | 7.8 | 7.8 |

[a] Variation of these p*I* values (isoelectric points) in repeated experiments was ±0.2 pH unit. Fractions after electrofocusing were assayed with 60 $\mu$M [$^{14}$C]acetyl-CoA and either 2 mM choline or 2 mM 1-carnitine, as described in Methods.

## Discussion

In the present study the choline acetylating activity in crude dialyzed homogenates of rabbit heart was found to be 1.5 ± 0.15 nmol of ACh per min per g of wet tissue. The three- to fourfold lower value for human heart may have been due to the fact that the human tissue had been stored at −60° for 6 months. Another consideration is that only human atrial tissue was used, whereas whole rabbit hearts were assayed. Furthermore, since CarAc was capable of acetylating choline in these preparations, the measured AcCh levels would reflect the activities of both enzymes, especially when the ratio of CarAc to ChAc was high. The levels of true ChAc in heart would therefore be lower than the values in Table I. Judging from the distribution of enzyme activity after electrofocusing (Figure 3) and the extent of inhibition by (naphthylvinyl)pyridine, between one-third and one-half of the choline acetylation might have been caused by CarAc in the $SP_{55}$ rabbit heart extracts. The lower level of CarAc in the human heart preparation would have had a smaller influence on the apparent ChAc activity of this tissue.

Several workers have reported activities for ChAc of crude undialyzed heart homogenates that are much higher than those found here. Mahoney *et al.* (1971), using a modification of the method of Schrier and Shuster (1967), reported average values of ChAc in rat hearts of 18 nmol/min per g of wet tissue, similar to brain levels. Their values for human heart were even higher than those for human brain tissue. As shown in the present study, the method of Schrier and Shuster may, because of the

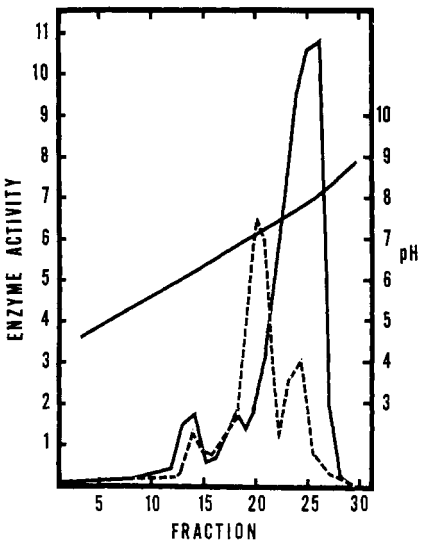


FIGURE 3: Electrofocusing profile of rabbit heart $SP_{55}$ extract. Adjacent portions of an acrylamide gel slab were sliced into 2-mm portions and assayed for enzyme activity, as described in Methods, with [$^{14}$C]acetyl-CoA and carnitine (———) or choline (------) as the second substrate. Amounts of protein initially placed in the gel columns were 400 $\mu$g for ChAc assays and 10 $\mu$g for CarAc assays. Enzyme activity is expressed as cpm $\times$ $10^{-3}$. The pH gradient in the gel is represented by the solid diagonal line and the right-hand ordinate. The anodic end of the gradient was on the left.

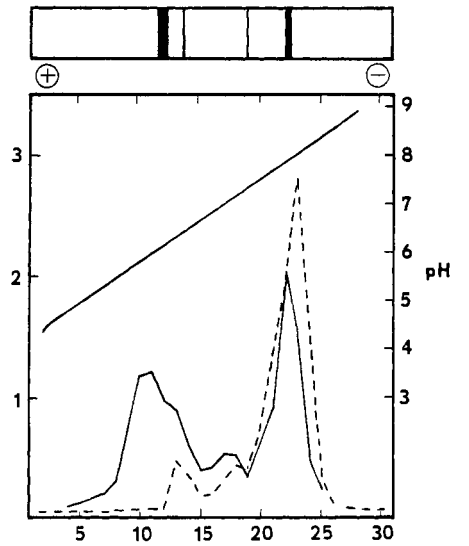


FIGURE 4: Electrofocusing profile of highly purified pigeon muscle CarAc. Adjacent portions of an acrylamide gel slab were sliced horizontally into 2-mm portions and assayed for enzyme activity with [$^{14}$C]acetyl-CoA and carnitine (——) or choline (-------). The abscissa records the fraction number, and the ordinate represents enzyme activity in cpm $\times$ $10^{-3}$. Amounts of protein electrofocused were 30 $\mu$g (for ChAc) and 0.3 $\mu$g (for CarAc). The pH gradient is indicated by the solid diagonal line and the right-hand ordinate. A diagram of protein bands obtained after staining an adjacent gel strip which was electrofocused at the same time is shown at the top, where the width of each band is meant to be roughly proportional to its intensity in the gel.

presence of carnitine in crude homogenates, give false values for ChAc approximately 20- to 30-fold higher than those obtained after extensive dialysis. A level of approximately 9 nmol/min per g of wet tissue was determined for ChAc in crude extracts of rat heart by Thoenen *et al.* (1972), who employed the assay method of Fonnum (1966), which involves precipitation of ACh by sodium tetraphenylborate. The effect of endogenous carnitine in this method has not been investigated. ChAc activities in rabbit heart were determined by Ekström (1970), who used the frog rectus bioassay method. His value of 80 $\mu$g of ACh/hr for a whole rabbit heart weighing 6 g can be converted to 1.2 nmol/min per g of tissue, which is similar to our value of 1.5.

High levels of CarAc and endogenous carnitine have been found in hearts of a number of species (Marquis and Fritz, 1965; McCaman *et al.*, 1966). Marquis and Fritz (1965) determined that the free carnitine level in peripheral tissues ranged from 800 $\mu$g/g dry weight of heart tissue down to 200 $\mu$g/g in kidney. The value for brain was lowest (100 $\mu$g/g). CarAc levels were also higher in peripheral tissues than in brain. Therefore, it is important that any determination of ChAc, especially in homogenates of peripheral tissues, be preceded by extensive dialysis in order to remove endogenous carnitine.

An earlier report (Fritz *et al.*, 1963) that choline was not a substrate for CarAc was based on a single low choline concentration which, in the spectrophotometric assay used, could not be expected to give a measurable effect. Choline was, however, found to be an inhibitor of CarAc (Fritz and Schultz, 1965). Three lines of evidence now support the conclusion that choline is, indeed, a substrate for CarAc, although a very poor one in comparison with l-carnitine. (1) Choline was acetylated by enzyme forms of rabbit heart which are indistinguishable from CarAc on electrofocusing. There is the possibility that the choline acetylating activity seen it p*I* values of 6.2 and 7.8 with rabbit heart could be due to peripheral forms of ChAc which do not exist in the brain. However, the appearance of higher CarAc activities in the same fractions strongly suggests that these CarAc forms are capable of using choline as an alternate poorer substrate. Highly purified CarAc of pigeon muscle also gave multiple peaks on electrofocusing when either choline or carnitine was used as substrate (Figure 4). None of these peaks corresponded with the known isoelectric point of pigeon ChAc (p*I* = 6.6) as determined in the electrofocusing experiments of Malthe-Sorensen and Fonnum (1972). (2) Kinetic experiments indicated that the formation of ACh by CarAc of either pigeon muscle or rabbit heart was hyperbolically dependent on choline concentration so that reciprocal plots were linear. In addition, choline appeared to be a competitive inhibitor of carnitine acetylation, consistent with its behavior as an alternate poor substrate. (3) (Naphthylvinyl)pyridine, a potent ChAc inhibitor, did not inhibit the formation of ACh by CarAc.

The differential inhibition by (naphthylvinyl)pyridine appears to be a useful method for distinguishing true ChAc activity from choline acetylation due to CarAc. Furthermore, if our results obtained *in vitro* are applicable to the *in vivo* situation, one would expect that a specific inhibition of ChAc would not completely inhibit ACh synthesis in the heart and other peripheral tissues. On the other hand, a compartmentation of enzymes might exist *in vivo*, so that most of the CarAc may not be available to endogenous choline.

The CarAc peak found at pH 5.7 with pigeon muscle enzyme may have been due to a species difference between rabbit and pigeon. It was not caused by a tissue difference because $SP_{55}$ fractions of rabbit muscle gave only a single peak at p*I* = 7.9. Nor was it caused by an inner mitochondrial form of CarAc which was not extracted in our $SP_{55}$ preparations, since inner and outer mitochondrial preparations of rabbit heart

gave the same CarAc pattern on electrofocusing (H. L. White and J. C. Wu, unpublished).

CarAc has been proposed as one of several possible enzyme systems which facilitate transport of the acetyl moiety from within mitochondria to the cytosol of nerve cells where ACh synthesis occurs (Tučcek, 1970). According to this theory, acetylcarnitine formed by intramitochondrial CarAc passes through mitochondrial membranes and is converted to acetyl-CoA by extramitochondrial CarAc. Acetyl-CoA then is able to stimulate ACh formation by cytoplasmic ChAc. The *in vitro* interactions of choline and ACh with CarAc may therefore have relevance with respect to *in vivo* regulation of ACh synthesis.

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# The Isolation and Reactions of a 1,4-Dihydropyridine General Intermediate for Vitamin B-6 Catalysis†

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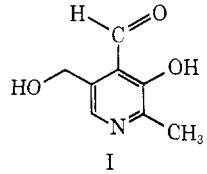
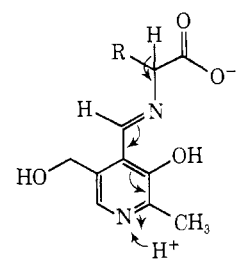
ABSTRACT: Evidence is presented that in cold concentrated aqueous solution, the Schiff base formed from pyridoxal (vitamin B-6) and diethyl aminomalonate exists principally in the form of its 1,4-dihydropyridine tautomer. The dihydropyridine may be precipitated from solution, representing the first isolation of what is postulated to be the general intermediate for vitamin B-6 catalysis. The corresponding methyl-substituted amine, diethyl aminomethylmalonate forms only normal Schiff bases with pyridoxal. Nuclear magnetic resonance (nmr) data show that in these systems pyridoxal catalyzes the rapid cleavage of the carboxyethyl group to form ethyl alcohol and ethyl alanate. The mechanism of this reaction is suggested to be analogous to vitamin B-6 catalyzed decarboxylations but to proceed *via* prior saponification. These data are used to discuss the factors controlling various products which are produced in vitamin B-6 catalyzed reactions.

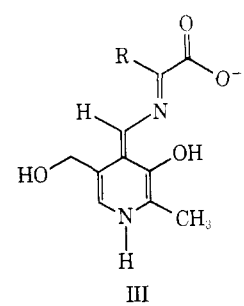
The heterocyclic aldehyde pyridoxal, I, and its 5-phosphate ester are active forms of vitamin B-6. This vitamin is an essential cofactor to a host of enzymes which catalyze amino acid reactions. Of particular interest to chemists is the fact that many of these reactions proceed slowly by pyridoxal catalysis even in the absence of the enzymes. This initially gave rise to the suggestion that the reactions go through Schiff base formation and subsequent tautomerization to the dihydropyridine II → III (Metzler *et al.*, 1954).




I




II

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