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A RADIOISOTOPIC, CHROMATOGRAPHIC MICROASSAY FOR ACETYL-COENZYME A SYNTHETASE AND SOME PRELIMINARY STUDIES ON THE RAT BRAIN ENZYME

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SUMMARY

1. An isotopic, chromatographic assay for acetyl-CoA synthetase (acetate:CoA ligase (AMP), EC 6.2.1.1) has been developed which is both sensitive and rapid to perform.

2. The rat brain synthetase activity behaves the same as the synthetase activity from other sources when exposed to increasing concentrations of Na^+, K^+, or NH_4^+.

3. The subcellular location of the rat brain synthetase is primarily mitochondrial, but 13% of the activity was found in the cytoplasm.

4. Kinetic studies on the rat brain synthetase preparation indicated higher substrate affinities than previously reported and a marked substrate inhibition which occurred at moderate substrate concentrations.

5. In view of the differences in substrate affinities and substrate inhibition of the rat brain acetyl-CoA synthetase compared to the synthetases from other sources, it is suggested that the results of comparative studies be interpreted cautiously.

INTRODUCTION

Acetyl-CoA is an important metabolite in many cellular functions, including the tricarboxylic acid cycle, fatty acid synthesis, ketone body formation, pyruvate metabolism, and acetylcholine synthesis. However, consistent with the concept of intracellular pools of acetyl-CoA[1], the metabolic fate of the acetyl-CoA derived from different sources is distinct in certain systems[2–9]. To access the relative importance of acetate in the synthesis of acetyl-CoA in such structures as autonomic ganglia and peripheral nerves, it is apparent that a microassay for acetyl-CoA synthetase (acetate: CoA ligase (AMP), EC 6.2.1.1) will be useful. We report here on the development of such an assay which can easily measure the formation of 0.10 nmole of acetyl-CoA

from acetate. The radiometric, chromatographic assay is based on the coupling of two reactions:

$$\text{Acetate} + \text{CoASH} + \text{ATP} \leftrightarrows \text{acetyl-CoA} + \text{AMP} + \text{PPi} \quad (1)$$

$$\text{Acetyl-CoA} + \text{choline} \rightarrow \text{acetylcholine} + \text{CoA} \quad (2)$$

Reaction 1 is catalyzed by acetyl-CoA synthetase and Reaction 2 is catalyzed by choline acetyltransferase (EC 2.3.1.6). Under conditions of the assay, Reaction 1 is the rate-limiting step. Radioactive acetate is used as substrate and radioactive acetylcholine is recovered quantitatively by ion exchange chromatography and counted. This assay may be considered a modification of the choline acetyltransferase assays of Tuček[10] and Fonnum[11]. The assay has the desirable characteristics of being quick and simple to perform while it maintains a high sensitivity.

The new assay has been used to characterize acetyl-CoA synthetase from rat brain. Results show that the crude rat brain enzyme has many characteristics in common with the acetyl-CoA synthetase from pig and beef heart[12,13] but several important kinetic differences were found. The similarities and differences of this enzyme from different sources are examined and possible complications of comparative studies are discussed. A preliminary account of this work has been published in abstract form[14].

METHODS

Chemicals

[^{3}H]Acetic anhydride (50 Ci/mole), acetyl[1-^{14}C]choline iodide (2.43 Ci/mole), [^{3}H]acetyl-coenzyme A (980 Ci/mole), and aquasol universal L.S.C. cocktail (scintillation fluid) were products of New England Nuclear Corporation, Boston, Mass. Dowex ion exchange resins (CGC-240 and CGA-540) were purchased from J. T. Baker Chemical Company, Phillipsburg, N.J. Whatman No. 1 chromatography paper was obtained from Scientific Products, Santa Ana, Calif. Choline chloride was a product of Sigma Chemical Co., St. Louis, Mo. All other chemicals used were reagent grade.

A stock solution of [^{3}H]acetate was prepared from [^{3}H]acetic anhydride by incubating the anhydride with 1.0 ml of 100 mM KCl solution at pH 2 for 15 min at 0 °C. Subsequently, the solution was neutralized with KOH, and brought to 5.0 ml with a KCl–potassium phosphate buffer solution such that the final ion concentrations were: KCl, 100 mM; potassium phosphate buffer (pH 7.4), 12 mM; [^{3}H]-acetate, 10 mM. There were 55 500 dpm/nmole of acetate in this stock solution.

Preparation of rat brain homogenates

Male Sprague–Dawley Rats (approx. 250–300 g) were killed by cervical dislocation and the entire brain excised within 5 min. Surface blood vessels were carefully removed as the brain tissue was being rinsed in homogenizing buffer [1 mM EDTA, 50 mM potassium phosphate buffer (pH 7.4)]. The brain tissue was then blotted and weighed. All subsequent steps were performed at 4 °C. A 10% (w/v) homogenate was prepared in homogenizing buffer by 10 passes of a teflon plunger in a glass homogenizing vessel (0.10 mm–0.15 mm clearance) at 620 rev./min. The homogenate was then centrifuged at 30 000 × g in a Sorvall RC-2B centrifuge for 30 min and the supernatant (approx. 20 ml) was dialyzed for 8.5 h against 2 changes

of the same buffer (1500 ml each). Aliquots of the dialyzed supernatant were added to vials, frozen, and stored under liquid nitrogen until needed.

Partial purification of choline acetyltransferase

Rat brain choline acetyltransferase was partially purified by a modification of the procedure of Potter *et al.*[15]. A 10% homogenate was prepared as above in a 1 mM citrate–phosphate buffer (pH 5.0). The homogenate was spun at 100 000 × *g* for 30 min and the pellet was redissolved in a solution which was 100 mM KCl, 2.0 mM citrate–phosphate buffer (pH 7.2). This solution was then spun at 100 000 × *g* for 30 min and the pellet discarded. The supernatant was then fractionated with $(NH_4)_2SO_4$ and the 20–55% fraction dialyzed against a 10 mM citrate–phosphate buffer (pH 5.0) with 1 mM EDTA. The resulting solution was stored at −16 °C. It was completely devoid of acetyl-CoA synthetase activity and is referred to as partially purified choline acetyltransferase.

Chromatography of acetyl-CoA synthetase reaction products

Samples of the acetyl-CoA synthetase reaction were prepared for paper chromatography by freezing the reaction products in dry ice–ethanol and lyophilizing to dryness. The residue was then resuspended in a small volume of water (approx. 30 µl) and spotted on Whatman No. 1 chromatography paper. The chromatogram was run in an ascending solvent system of *n*-butanol–acetic acid–water (4:1:1, by vol.) for approx. 7 h. The dried chromatogram was subsequently cut into 2 inch strips and scanned for radioactivity on a Packard Model No. 7201 Radio-Chromatogram Scanner System.

Acetyl-CoA synthetase assay procedure

The acetyl-CoA synthetase assay was run in a total volume of 60 µl at the following final concentrations unless otherwise indicated: [^{3}H]acetate (potassium) $7.5 \cdot 10^{-4}$ M; ATP (dipotassium), $2.5 \cdot 10^{-3}$ M; CoA (reduced) $4.0 \cdot 10^{-5}$ M; dithiothreitol, $4.0 \cdot 10^{-5}$ M; L-cysteine, $2.5 \cdot 10^{-3}$ M; neostigmine methylsulfate, $5.0 \cdot 10^{-3}$ M; acetylcholine chloride, $3.3 \cdot 10^{-6}$ M; magnesium chloride, $2.5 \cdot 10^{-3}$ M; choline chloride, $12.5 \cdot 10^{-3}$ M; potassium chloride, $1.0 \cdot 10^{-1}$–$2.8 \cdot 10^{-1}$ M; potassium phosphate buffer (pH 7.4), $5.0 \cdot 10^{-2}$ M. In typical assays, a 10 min preincubation without choline was employed to preform some acetyl-CoA. Omission of this preincubation resulted in a non-linear time course. The choline solution was then added at 0 time and the complete reaction mixture incubated an additional 30 min at 37 °C. Blank tubes involved omitting choline, ATP or CoASH from the final reaction mixture. The reaction was stopped by cooling to 0 °C in an ice bath and adding 1.0 ml of a 10 mM KCl solution to each tube. Each reaction mixture was then put over a separate Dowex 50-Na^+ ion exchange column. Columns which measured 0.5 cm × 3.0 cm were prepared above a glass wool plug in a 9 inch pasteur pipette. Each column had been previously washed with at least 3 ml of 10 mM KCl solution. After the reaction mixture was put over a column, an additional 5 ml of 10 mM KCl solution and then 1 ml of 0.50 N choline chloride solution were added to wash the non-binding components from the column. The effluent was discarded. The bound acetylcholine was then eluted from the columns with 3 ml of 1.0 N choline chloride solution. The effluent was collected, 10 ml aquasol scintillation fluid added, and the solution counted in a Beckman LS-250

scintillation counter using an external standard. Tritium counting efficiencies were 30–33%.

Other procedures

Choline acetyltransferase was assayed by the radiochemical assay of Schrier and Shuster[16]. The final product was counted in 15 ml of Toluene–Triton X-100–PPO (1500 ml: 376 ml: 18.8 g) scintillation fluid for ^{14}C-labelled products or a modified Brays solution for ^{3}H-labelled products. Counting efficiences were about 30% for tritium and 91% for carbon. Succinate dehydrogenase (EC 1.3.99.1) was assayed as previously described[17]. Protein was determined by the procedure of Lowry *et al.*[18] with bovine serum albumin serving as a standard. Subcellular fractions of brain were prepared according to procedures of Whittaker[19] and Gray and Whittaker[20].

TABLE I

DOWEX-50 Na^+ COLUMN RETENTION CHARACTERISTICS

Solutions of labelled molecules with known specific activities were prepared. The total radioactivity of 10 μl aliquots of each sample was determined by adding 10 μl of the labelled solutions to 3.0 ml of 1.0 N choline chloride and counting in aquasol. The amount of recovered radioactivity was then determined by adding 10 μl aliquots of each sample to 1.0 ml of 10 mM KCl. These solutions were then put over Dowex-50 Na^+ columns, the columns treated as described in Methods, and the 3.0 ml of 1.0 N choline chloride elutant collected and counted in aquasol. The amount of labelled molecule present was in each case calculated from the known specific activity and the measured radioactivity.

	*Amount added** (*pmoles*)	*Amount recovered†* (*pmoles*)	*% Recovered*
[^{14}C]Acetylcholine (0.3 Ci/mole)	41 730	40 450	96.93
[^{3}H]Acetate (25 Ci/mole)	15 540	1.0	<0.01
[^{3}H]Acetyl-CoA (13.2 Ci/mole)	2 405	1.7	0.07

* Each value is the mean of 3 determinations.
† Each value is the mean of 6 determinations.

RESULTS

The isotopic assay depends on the complete separation of products from reactants since both are similarly labelled. Table I shows that with standard solutions, cationic acetylcholine can be quantitatively separated from the other labelled reaction components by a Dowex-50 column and the appropriate wash procedure. Blank tubes from the assay averaged about 400 cpm. Washing the columns with more concentrated salt solutions (to 50 mM KCl) was without effect in lowering the blank further. Although, the column procedure is efficient with respect to its separation of cationic and non-cationic molecules, it is not specific for acetylcholine. The recovered radioactivity could be due to a heterogeneous mixture of labelled cationic products. To rule this out, paper chromatography of lyophilized reaction mixtures and suitable standards was performed as described (see Methods). The only detectable radioactive product was found to co-chromatograph with the acetylcholine standard.

TABLE II

DETERMINATION OF LIMITING ENZYME

Each value is the average of 4 determinations. (A) Enzymes assayed as described. (B) 10 μl of partially purified rat brain choline acetyltransferase was added to a normal reaction mixture. Control samples received 10 μl of boiled choline acetyltransferase solution.

	pmoles acetylcholine/μl per h
A. Maximum enzyme activities in brain extract	
Choline acetyltransferase	256
Acetyl-CoA synthetase	51
B. Effect of added choline acetyltransferase on synthetase activity	
Brain extract	37
Brain extract + choline acetyltransferase	37

Determination of limiting enzyme

Since our assay involves the coupling of two enzymes, the amount of accumulated product will reflect only the rate of the slowest step. It is therefore imperative that the choline acetyltransferase reaction be the faster step if we are to really measure synthetase activity. Table IIA shows the maximum activities of the two enzymes in one of the rat brain homogenates used. It is evident that the synthetase reaction has a slower rate under our conditions. The ratio of these two enzyme activities was routinely measured whenever different assay conditions were employed, as when looking at enzyme activities in subcellular fractions. In cases when the endogenous choline acetyltransferase activity was not sufficiently high, 10 μl of partially purified choline acetyltransferase was added to the reaction mixture.

The question of which enzyme was limiting was also examined in a separate experiment where excess choline acetyltransferase was added to a normal reaction (Table IIB). Since there was no increase in acetylcholine product, it again follows that the production of acetyl-CoA was the rate limiting step. Thus, the amount of

TABLE III

KINETIC DATA

K_m + V values for rat brain were calculated from double-reciprocal plots. The data from beef heart is that of the Campagnari and Webster[13]. The large discrepency between the calculated and observed V emphasizes the marked substrate inhibition of this enzyme.

	Apparent K_m values (M)	
	Rat brain	*Bovine heart*
Acetate	$2.2 \cdot 10^{-4}$	$7.9 \cdot 10^{-4}$
ATP	$2.7 \cdot 10^{-4}$	$1.8 \cdot 10^{-3}$
CoASH	$1.9 \cdot 10^{-5}$	$1.4 \cdot 10^{-3}$
Mg^{2+}	$2.2 \cdot 10^{-4}$*	$1.4 \cdot 10^{-3}$
	V values (rat brain homogenate)	
Calculated	55.6 nmoles acetyl-CoA/mg protein per h	
Observed	34.8 nmoles acetyl-CoA/mg protein per h	

* The K_m for Mg^{2+} could not be determined from a double-reciprocal plot due to an irregular saturation curve. The reported value is 1/2 the concentration at the maximum enzyme activity measurable.

acetylcholine formed seems to be a good indicator of the amount of acetyl-CoA formed.

Other assay characteristics

The rate of formation of acetylcholine after a 10 min preincubation without choline was constant for at least 90 min. A lag in the time curve was eliminated by the addition of 0.2 nmole of acetylcholine to the reaction mixture (Fig. 1). This lag,

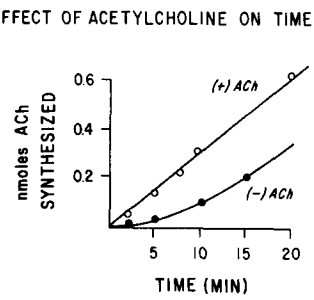

Fig. 1. Samples were pre-incubated for 10 min with or without acetylcholine (ACh) ($3.3\ 10^{-6}$ M). Choline was then added at 0 time and the reaction stopped at the indicated time (see Methods). Each point is the average of 2 determinations.

which has been noted on previous occasions[2], was insensitive to choline (12.5 mm) and endogenously generated acetyl-CoA. The possibility that this lag period and its elimination by acetylcholine were due to effects on choline acetyltransferase was ruled out by showing that when synthetic acetyl-CoA was used as substrate, no lag period resulted in the presence or absence of acetylcholine. The effect of acetylcholine on the synthetase reaction is not understood, but it is probably not physiological since most of the synthetase is intramitochondrial and acetylcholine is synthesized and package extramitochondrially. The most likely explanation seems to be a non-specific activation of the synthetase by acetylcholine, although it is clear that the acyl moiety alone or the choline moiety alone is not able to activate.

A lag in the time curve also resulted if the preincubation without choline was omitted. This lag occurred even in the presence of acetylcholine, but could be eliminated by synthetic acetyl-CoA if acetylcholine was also present. We interpret this in the following manner: Acetyl-CoA acts in our system as a reaction intermediate. As such, its concentration would be expected to increase initially until some steady-state level is reached. After this steady-state concentration is attained, the formation of acetylcholine will occur at the same rate as the formation of acetyl-CoA. This would result in a rectilinear time course. However, before this steady-state concentration is reached, the production of acetylcholine would occur at a slower rate than the production of acetyl-CoA. This would be expected to result in a lag in the time curve for acetylcholine formation which could be eliminated by synthetic acetyl-CoA or a preincubation to preform acetyl-CoA. This was found to be the case, so all reactions were run after a preincubation. The length of the preincubation is critical, however. If too much acetyl-CoA is formed, an initial burst of acetylcholine synthesis would occur when choline is added. If the preincubation time is too short, a lag in

acetylcholine synthesis would still result. 10 min was found to be the correct preincubation time to result in a rectilinear time course in our system.

To estimate the reproducibility of the assay, 12 samples, each containing 24 μl of rat brain homogenate, were incubated for varying lengths of time (15–90 min). The radioactivity of the recovered acetylcholine was then normalized to 1 h for the subsequent calculations. The mean value ($\pm$ S.E.) was 61.11 $\pm$ 0.75 pmoles of acetylcholine synthesized per μl of rat brain homogenate per h. Thus, the variability of the assay is less than 1.3%.

Studies on the rat brain acetyl-CoA synthetase

Subcellular fractionation studies on this enzyme have been performed previously[4,21,22]. The highest activities are always associated with the mitochondrial fraction, but some activity is always recovered in the soluble fraction. Application of our assay to rat brain subcellular fractions yielded results in general agreement with the data of Schuberth[21]. Although direct comparisons are difficult because we did not prepare our enzyme samples from acetone powders, one can see from Fig. 2

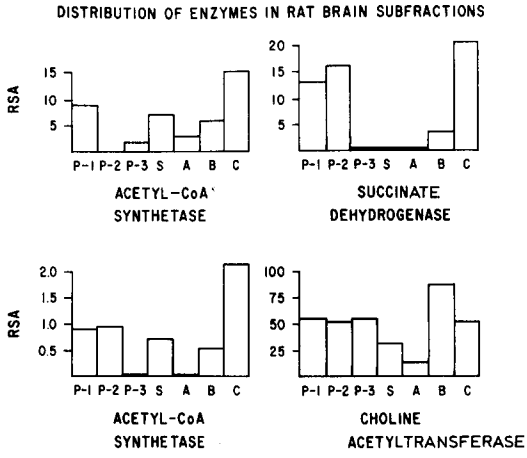

Fig. 2. Rat brains were fractionated by the methods of Whittaker[19] and Gray and Whittaker[2c]. P-1 = nuclei fraction; P-2 = crude mitochondrial fraction; P-3 = microsomes; S = soluble fraction. A, B and C refer to the subfractions of P-2: A = myelin; B = synaptosomes; C = mitochondria. Enzymes were assayed as described. *Data of Schuberth[21]. The acetyl-CoA synthetase activity of P-2 was not reported.

that the relative distribution of acetyl-CoA synthetase in our subcellular fractions is similar to the distribution for rat brain reported by Schuberth. The activities of the mitochondrial enzyme succinate dehydrogenase and the soluble cytoplasmic enzyme choline acetyltransferase are also shown. It seems clear that the rat brain acetyl-CoA synthetase is primarily mitochondrial in nature, but we recovered 13% of this enzyme in the soluble fraction. The recovery of this enzyme from initial subcellular fractionation procedures was 89%, whereas subsequent fractionation of the crude mitochondrial pellet (P-2) on sucrose gradients resulted in recovery of only 46% of the synthetase.

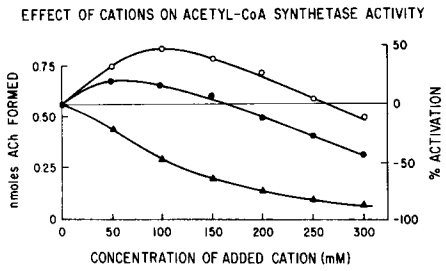

Fig. 3. Enzyme assays were performed at normal substrate concentrations but without added KCl. Zero salt concentrations refer to no added salts, but some K^+ is present because K^+ substrates are used throughout. ○, KCl; ●, NH_4Cl; ▲, NaCl. (ACh, acetylcholine).

The effects of Na^+, K^+, and NH_4^+ on acetyl-CoA synthetase activity were also examined (Fig. 3) and found to be similar to those reported for pig[12] and beef heart[13] acetyl-CoA synthetase. This enzyme characteristically exhibits a marked inhibition in the presence of Na^+ and an activation in the presence of K^+. The complete inhibition of acetyl-CoA by high [Na^+] means that we could stop the production of acetyl-CoA by adding Na^+ to the reaction mixture. When this was done by adding 10 μl of concentrated NaCl to reaction mixtures after the normal 30 min incubation periods (final [Na^+], 350 mM), the synthesis of acetylcholine stopped abruptly, even though the samples were incubated an additional 5 min. Since Na^+ at these concentrations does not inhibit choline acetyltransferase activity[15], any accumulation of acetyl-CoA (beyond its steady-state concentration) would have resulted in continued acetylcholine synthesis. We would have found an increase of up to 15% in the total amount of acetylcholine synthesized if acetyl-CoA had built-up during the normal 30 min incubation period. Since there was no increase in the amount of acetylcholine synthesized, this experiment confirms that the synthetase reaction was rate-limiting in our assay system.

Kinetic studies on acetyl-CoA synthetase from bovine heart have been performed by Campagnari and Webster[13]. Using our assay, we repeated these studies on the crude rat brain preparation with two significant results. First, the apparent K_m values for the rat brain enzyme are lower, indicating higher substrate affinities. This effect is most clearly seen with CoASH, which has a 100-fold lower K_m with the rat brain enzyme (Table III). All other substrates have 2 to 10-fold lower K_m values. It may be that the much lower K_m for CoASH results partly from the presence of dithiothreitol in the incubation media. This molecule has been shown to keep CoASH in its biologically active form in solution, whereas other reducing agents are less efficient in this respect (Arnold, Jr, L. J., personal communication). Thus, the decreased K_m may partly reflect an apparent increase in the biologically active concentration of CoASH. If so, then the 100-fold difference in K_m values for CoASH between rat brain and bovine heart synthetase will be at least partially artifactual, since dithiothreitol would also be expected to lower the apparent K_m of the bovine heart enzyme. The second significant result was that the rat brain enzyme is strongly inhibited by all its substrates except acetate. At optimal substrate concentrations, the observed V for this enzyme is only 63% of the calculated value from double-reciprocal plots (Table III). At higher concentrations of CoASH or Mg^{2+}, the enzyme

inhibition becomes very pronounced, and at the concentrations normally used to assay acetyl-CoA synthetase activity, (10–20 mM ATP, 10–20 mM acetate, 0.5–2.0 mM CoASH, 1.0–6.0 mM Mg^{2+}), the rat brain enzyme examined here would be inactive. Although greater than optimal concentrations of ATP do not cause a marked decrease in enzyme activity, there does seem to be some inhibition at lower concentrations. This can be inferred from the double-reciprocal plot for ATP, which is skewed upward near the ordinate. The same is true of the double reciprocal plot for CoASH. The inhibition by these two substratrs then probably accounts for the 37% inhibition of acetyl-CoA synthetase seen in Table IV.

DISCUSSION

We have described a radioisotopic microassay for the measurement of acetyl-CoA synthetase (acetate:CoA (AMP) ligase) activity. The assay is based on a coupling of this enzyme with choline acetyltransferase. The amount of radioactive acetylcholine formed is a good measure of the amount of acetyl-CoA formed from radioactive acetate as long as the choline acetyltransferase activity of the sample is not rate-limiting. In samples where this requirement is not initially met, one can add aliquots of partially purified choline acetyltransferase. The specificity of the reaction can also be checked by adding Na^+. High concentrations of this ion result in complete inhibition of the synthetase reaction. The principle advantages of this assay over other methods of estimating acetyl-CoA formation are speed, simplicity, and higher sensitivity. As little as 50–100 pmoles of acetyl-CoA synthesis from acetate can be detected, and 10 to 100-fold higher sensitivities seem reasonable if higher specific activity acetate is used.

Using this new assay, some properties of the acetyl-CoA synthetase from rat brain were examined. In subcellular fractions, we recovered 13% of the enzyme in the soluble fraction although the majority of the enzyme is clearly in the mitochondria. This is consistant with reports from other laboratories which found from 6–40% of the enzyme in the soluble fraction[4,22,23]. Although these figures are affected by the lysis of some mitochondria during the homogenization procedure, it seems clear from this and other studies that some of the enzyme is indeed soluble in the intact cell. The function of this cytoplasmic enzyme is unknown, but it may be important in generating cytoplasmic acetyl-CoA from acetate which is released from the mitochondria after β-oxidation of fatty acids[24]. The poor recoveries of this enzyme on density gradients is also well documented[4,22,23]. We were able to recover only 46% of this enzyme from our fractionation of the crude mitochondrial pellet (P-2) on sucrose gradients which is in keeping with the findings of others. The reason for these low recoveries could be due to the separation of an unknown activator or cofactor from the enzyme within the gradient, as suggested by Whittaker[25]. Alternatively, a denaturation of the enzyme may be occurring. We currently favor this latter possibility since there seems to be a loss of some enzyme activity by other biochemical treatments, *e.g.* freezing and thawing and dialysis or dilution after an $(NH_4)_2SO_4$ fractionation. Also, preliminary enzyme mixing experiments and additions of heat denatured enzyme solutions to active enzyme solutions have not given results consistent with the missing cofactor hypothesis.

The results of kinetic studies on the crude acetyl-CoA synthetase preparation

revealed very high substrate affinities. In general, the K_m values reported are about 10-fold lower than the K_m values previously reported for acetyl-CoA synthetase from beef heart mitochondria. There was also a 37% enzyme inhibition at optimal substrate concentrations, and a much greater inhibition at higher concentrations of CoASH or Mg^{2+}. This effect was also reported with the purified beef heart enzyme[13], although it was not as great. It is interesting that at the normal concentrations used to assay this enzyme from other sources, the rat brain enzyme would be completely inhibited. Conversely, mouse brain homogenates, rat heart muscle homogenates, and rat skeletal muscle homogenates are all without acetyl-CoA synthetase activity when treated identically and assayed at the substrate concentrations which were found to be optimal for the rat brain synthetase activity. These observations make difficult any type of comparative studies in which enzyme activities from different sources are being measured. In these studies, optimal substrate concentrations will have to be determined for the enzyme from each source before any meaningful comparisons can be made.

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