I. SUBCELLULAR DISTRIBUTION OF ACETATE AND ACETOACETATE ACTIVATING ENZYMES OF RAT BRAIN

II. STUDIES ON RAT BRAIN ACYL COENZYME A HYDROLASE (SHORT CHAIN)

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LIST OF SYMBOLS AND ABBREVIATIONS

CoA	- Coenzyme A
Tris	- tris(hydroxymethyl)aminomethane
HA	- hydroxyapatite
DTNB	- 5,5'-dithio-bis-2-nitrobenzoic acid
AcCoA	- Acetyl Coenzyme A
nd	- not determined

PART ONE

SUBCELLULAR DISTRIBUTION OF ACETATE AND ACETOACETATE ACTIVATING ENZYMES OF RAT BRAIN

CHAPTER 1

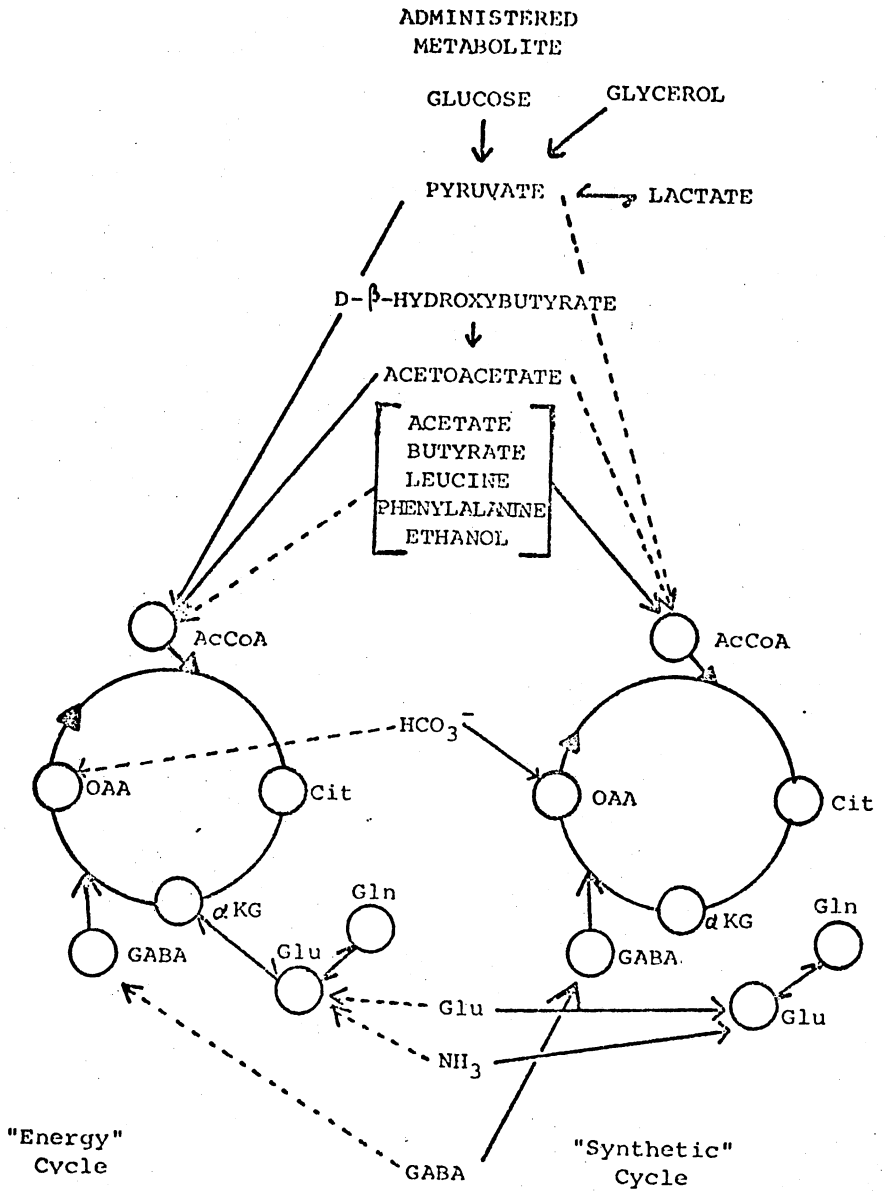
INTRODUCTION

Metabolic compartmentation in brain was first suggested by the experiments of Waelsch *et al.* (1), who showed that upon administration of ^{14}C-glutamate, the specific radioactivity of brain glutamine rose to five times that of glutamate, thus implying departure from a simple precursor-product relationship.

Three years later, in 1964, Cremer (2) showed that with ^{14}C-glucose the opposite effect was obtained, i.e., the specific radioactivity of glutamate was greater than that of glutamine. Then in 1966 Koeppe and O'Neal (3, 4) showed that labelled acetate and butyrate also gave brain glutamine with a higher specific radioactivity than glutamate, and on the basis of these and other results postulated that all experiments described so far could be explained by postulating two pools of tricarboxylic acid cycle intermediates which did not freely interchange. These two pools have historically been known as the "large", or glucose-utilizing pool, and the "small" tricarboxylic acid cycle, which is labelled by acetate and glutamate. Further studies on isotope experiments have been reviewed by Berl and Clark (5). They summarized the results schematically, as is reproduced in Figure 1.

Woven into the idea of two sets of Krebs cycles in brain is the idea of mitochondrial heterogeneity, first demonstrated in 1965 in the work of Salganicoff and DeRobertis (6). The observed effects *in vivo*

Figure 1. Schematic Description of Compartmentation of Amino Acid Metabolism in Brain. The relative sizes of the pools are not known. Solid lines represent the preferred metabolic pathways. Broken lines represent minor pathways. Cit, citrate; αKG, α-ketoglutarate; OAA, oxaloacetate; Glu, glutamic acid; Gln, glutamine; GABA, γ-aminobutyric acid.


ADMINISTERED METABOLITE
GLUCOSE
GLYCEROL
PYRUVATE
LACTATE
D-β-HYDROXYBUTYRATE
ACETOACETATE
ACETATE
BUTYRATE
LEUCINE
PHENYLALANINE
ETHANOL
AcCoA
AcCoA
HCO3-
OAA
Cit
OAA
Cit
αKG
Gln
GABA
Glu
GABA
αKG
Gln
Glu
Glu
NH3
"Energy" Cycle
GABA
"Synthetic" Cycle

with the isotopically labelled compounds, which were best explained by two tricarboxylic acid cycles, and the observations of heterogeneous mitochondrial populations seem to be re-enforcing concepts. The subcellular distributions of many enzymes involved in metabolism of compartmentalized substances of brain have been published (7, 8), but an unambiguous anatomical explanation for the glutamate-glutamine labelling pools has not yet been elucidated.

In 1971, Cremer (9) made the surprising observation that administration of ^{14}C-acetoacetate to rats gave brain glutamic acid/glutamine ratios of specific radioactivity similar to those obtained with ^{14}C-glucose, opposite from the ratios observed with ^{14}C-acetate. The present work is based on this observation.

The rationale behind this study is that if the glutamate-glutamine compartments elucidated by isotopic labelling studies in brain are due to mitochondrial heterogeneity, and an effective resolution of these mitochondrial populations is achieved by existing techniques, knowledge of where acetate, through the action of acetyl CoA synthase (EC 6.2.1.1), is activated to acetyl CoA for entry into the Krebs cycle, and acetoacetate, through the action of 3-keto acid transferase, and acetoacetyl CoA thiolase (EC 2.8.3.5 and EC 2.3.1.16, respectively) is converted to acetyl CoA, might give insight concerning the location of the large and small compartments in brain. This approach allows a substantial reduction in complexity of data, since only three reactions need be followed through brain fractionation, and the results must yield information about the sites of acetyl CoA generation. Since both acetate and acetoacetate label glutamate, some of this acetyl CoA must enter a tricarboxylic acid cycle, and assuming the site of synthesis of acetyl

CoA and the site of condensation into tricarboxylic acid cycle intermediates to be the same, a definition of the subcellular fractions involved in the isotopically defined compartmental effects may be possible.

Studies of brain compartments, and localization of the tricarboxylic acid cycles, are important not only from an academic standpoint, but also may provide an understanding of the metabolic and pharmacological phenomena of compartmented substances. Ammonia toxicity (and related monosodium glutamate effects), carbon dioxide fixation, fluoroacetate toxicity, amino acid neurotransmission functions, and α-aminobutyrate and ethanol metabolism are all intricately connected to compartmentalization of tricarboxylic acid cycles in brain (5, 10, 11).

CHAPTER II

MATERIALS AND METHODS

Materials

The lithium salt of Coenzyme A and the sodium salt of ATP were purchased from Calbiochem. Enzyme grade Tris and $MgSO_4$ were purchased from Schwarz/Mann. Diketene, from Aldrich Chemical Company, was 95% pure. 3-Hydroxy acyl CoA dehydrogenase and acetylcholine were from Sigma. Tergitol-NPX was from Union Carbide. All other materials were of reagent grade.

Methods

Cerebral hemispheres were obtained from male Holtzmann rats immediately after decapitation; the cerebellums were discarded. Four brains were used in the investigations of the mitochondrial sub-fractions. For experiments with the homogenate and primary fractions, single brains were processed separately. The brains were weighed, placed in ice-cold 0.32 M sucrose and minced, and the sucrose solution was decanted. The minced brains were resuspended in 0.32 M sucrose (10 ml/g brain) and homogenized for two minutes in a Potter-Elvehjem vessel. The pestle of the vessel had been shaved to a total pestle diameter/vessel diameter clearance of 0.47 mm and eight passes of the pestle were made in the two minutes. The speed of the homogenizing pestle was 940 rpm. Subcellular fractions were then prepared by

the method of DeRobertis et al. (12). This procedure consisted of differential centrifugation to separate the nuclei, mitochondria, microsomes and supernatant fractions, and used a discontinuous sucrose density gradient to resolve the mitochondrial populations. All pelleted fractions were resuspended in cold 0.32 M sucrose.

Mg-Acetoacetyl CoA Synthesis. 12 mg of lithium CoA was dissolved in 10 ml of glass-distilled water at 4° and 20 µl of 95% diketene was added with stirring. After 30 minutes 0.5 ml of 500 mM $MgSO_4$ was added with stirring, the pH of the solution was brought to 8.0-8.5 by addition of 1 N KOH, and stirring was continued for another 30 minutes. The pH was then adjusted to 5.0 with dilute HCl and the solution was stored at 4° overnight. The above protocol was necessary since unhydrolyzed diketene absorbs at 313 nm. During storage the excess diketene was hydrolyzed and an orange precipitate was formed. The solution was then filtered through a coarse fritted funnel and the precipitate discarded. The Mg-acetoacetyl CoA was stored at 4° until use. The preparation was a substrate for 3-hydroxy acyl CoA dehydrogenase, which was used for determination of the mM absorptivity at 313 nm, found to be 17.5 at pH 8.7.

Tissue Solubilization. The resuspended tissue fractions, at protein concentration of less than 15 mg/ml, were brought to 0.9% tergitol-NPX by adding a 10% aqueous solution of the detergent at pH 7.4. The samples were shaken and incubated in ice for 30 minutes before assay.

Protein Estimation. Protein was estimated by the biuret reaction (13) on non-solubilized fractions against a blank containing equivalent amounts of 0.32 M sucrose, using bovine serum albumin as a standard.

Acetyl CoA Synthase Assays. In a final volume of 0.48 ml, the indicated quantities of the following reagents were added: potassium acetate, 40 µmoles; disodium ATP, 24 µmoles; $MgSO_4$, 40 µmoles; NH_2OH, 200 µmoles; and Tris-Cl, 170 µmoles (pH 8.7). After addition of 0.1 ml of solubilized tissue fraction having a protein concentration of 5-15 mg/ml, the mixture was incubated for 10 minutes at 37°. The reaction was then started with 20 µliters of a solution 40 mM in lithium CoA (0.8 µmole), incubated for one hour at 37°, and terminated with 0.9 ml of a solution 0.6 N HCl, 0.2 M trichloroacetic acid and 10% (w/v) $FeCl_3$. The precipitated protein was removed by centrifugation in a clinical centrifuge and the absorbancy at 540 nm of the supernatant measured with a Hitachi Perkin-Elmer Coleman 124 spectrophotometer. Blanks containing only protein, buffer and hydroxylamine were run for each assay and the blank absorbancy was subtracted from each assay with substrates. Controls omitting a single substrate showed no color development when ATP was omitted, 10-20% of total assay when CoA was omitted, and variable amounts of color when acetate was omitted. These results are probably due to endogenous CoA, which would recycle due to NH_2OH trapping, and endogenous acetate and acetate arising from hydrolysis of acetylcholine and other acetylated compounds during solubilization.

The assay results were linear with respect to time (up to 90 minutes) and protein (up to 15 mg/ml original fraction). In the presence of 200 µmoles of NH_2OH, no difference in standard acetyl CoA, prepared by the method of Utter (29), color yield as ferric ion-hydroxamate complex was noted either with or without solubilized brain homogenate added simultaneously with the NH_2OH. This implies that the NH_2OH is more efficient in trapping the acetyl CoA than the acetyl CoA

hydrolase is in hydrolyzing the thioester. Therefore the problem of enzymatic hydrolysis, which would be present if acetyl CoA accumulation was measured, is circumvented. Although no change was observed when the concentration of each substrate was doubled, halving their concentrations in separate assays led to a decrease in color, especially with ATP. Using standard acetyl CoA prepared as above, the ferric ion-aceto-hydroxamate complex has a mM absorptivity of 0.93. This assay was developed incorporating principles mentioned by Jones and Lippman (14), Webster (15), and Tucek (16).

Supernatant Assays. Due to the unavoidable dilution during preparation of the various fractions the activity of the enzymes was difficult to quantitate in the supernatant portion from a regular fractionation. Therefore in separate experiments the 54,000g supernatants of 10% brain homogenates were prepared directly and assayed.

Assays on Acetoacetate-Activating Enzymes. Both enzymes for acetoacetate activation were estimated by measuring the absorbance of the magnesium complex of the enol tautomer of acetoacetyl CoA. For acetoacetyl CoA thiolase assays, 10 µliters of solubilized tissue fraction were added to a mixture containing 416 µmoles of Tris-Cl and 20 µliters (0.8 µmole) of 40 mM CoA, final volume 0.95 ml. The pH of the reaction was 8.7. Then 50 µliters of Mg acetoacetyl CoA was added and the change in absorbancy at 313 nm was recorded. The acetoacetyl CoA added gave an initial absorbance of about 0.8. Assuming a millimolar absorptivity of 17.5 for acetoacetyl CoA at pH 8.7 (see above), the concentration of acetoacetyl CoA was about 50 µM. In the transferase reaction, 50 µliters of 0.52 M sodium succinate (26 µmoles) was substituted for CoA. Both assays, monitored continuously on a Hitachi

Perkin-Elmer Coleman 124 spectrophotometer equipped with a Coleman 165 recorder, were linear with respect to time for at least two minutes and with respect to protein to 0.3 mg, after which turbidity due to tissue extract caused difficulty in quantitation (this represented about 30 µliters of tissue extract). These assays were developed using principles reported by others (17, 18, 19). Attempts to show acetoacetyl CoA synthase using the method for acetyl CoA synthase by substitution of acetoacetate for acetate, failed. Tildon and Sevdalian (19) were also unable to find this activity in brain. A recent report (20) of this activity in brain cytosol, using a coupled acetoacetyl CoA thiolase-citrate synthasemalic dehydrogenase assay, shows that the proposed activity is less than 1% (0.4 µmoles/hr/g brain) of the succinyl CoA transferase-acetoacetyl CoA thiolase activation system and therefore probably represents a very minor pathway of acetoacetate activation. For discussion purposes, transferase-thiolase will be considered to be the acetoacetate-activating system of brain.

Marker Enzyme Assays. Succinate dehydrogenase was assayed by the succinate-dependent reduction of ferricyanide method of Slater and Banner (21). Acetylcholinesterase was measured by the method of Augustinsson (22).

CHAPTER III

RESULTS

Solubilization of Activities

Solubilization curves, as a function of tergitol-NPX concentration, are shown in Figure 2. These studies were done on brain homogenate, at about 15 mg biuret protein per ml. Since 0.9% provided good activity for all enzymes, this was the concentration used.

Marker Enzyme Studies

In order to show that the homogenization, differential centrifugation and sucrose density gradient used herein provided subfractions of rat brain similar to those studied by DeRobertis et al. (6), thereby allowing classification according to their fractionation scheme, marker enzymes were assayed in the same manner; the results are presented in Table I. This table indicates that the present system represents the same tissue fractions as in the original study. These results are further supported by very similar protein yields reported in (8). Since the assays were originally performed (6) on nonsolubilized fractions, the assays for marker enzymes reported here were run without Tergitol solubilization.

Subcellular Distribution of Acetyl CoA Synthase

The acetyl CoA synthase data are presented in Table II, expressed

Figure 2. Tergitol-NPX solubilization studies. Enzyme activities expressed as percent of maximum activity observed. Curve A, Activity of acetoacetyl CoA thiolase. Curve B, Activity of succinyl CoA transferase. Curve C, Activity of acetyl CoA synthase.

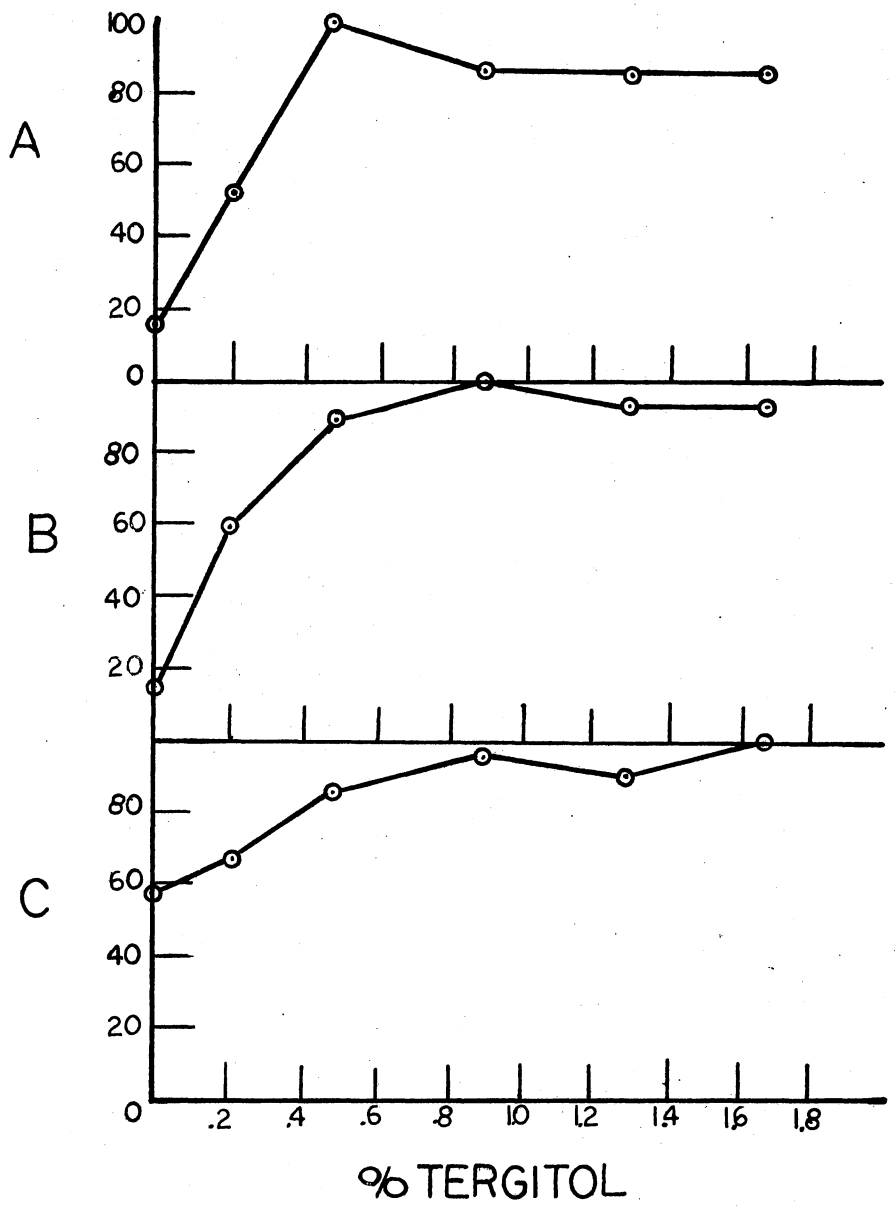

A
B
C
100
80
60
40
20
0
.2
.4
.6
.8
1.0
1.2
1.4
1.6
1.8
% TERGITOL

TABLE I

DISTRIBUTION OF PROTEIN AND MARKER ENZYMES

Fraction	Brain Protein mg/g	Protein %
Homogenate	156 ± 15 - 144[a]	100 - 100
Nuclear	17 ± 3 - 17.4	11 - 12
Mitochondria	65 ± 8 - 61.3	42 - 43
Microsomal	31 ± 6 - 37.5[b]	20 - 26
Supernatant	21 ± 4 - --	14 - --
A-myelin	20 ± 4 - 15.0	31[c] - 28
B-myelin fragments	5 ± 1 - 5.2	8 - 10
C-synaptosomes	13 ± 3 - 6.4	20 - 12
D-nerve endings	15 ± 2 - 12.6	23 - 24
E-glial and cell mitochondria	5 ± 2 - 13.4	8 - 25

Fraction	RSA Acetylcholinesterase[d]
A-myelin	0.3 - 0.15
B-myelin fragments	2.2 - 2.24
C-synaptosomes	2.0 - 2.99
D-nerve endings	1.0 - 0.94
E-glial and cell mitochondria	0.8 - 0.58

Fraction	% SDH[e]	RSA SDH
Homogenate	100 - 100	1.0 - 1.0
Nuclear	5 - 10.3	0.5 - 0.69
Mitochondria	79 - 87.2	2.0 - 1.65
Microsomal	6 - 2.6[b]	0.3 - 0.08
Supernatant	1 - --	0.1 - --
A-myelin	0 - 0	0 - 0
B-myelin fragments	0 - 0	0 - 0
C-synaptosomes	13 - 2	0.5 - 0.3
D-nerve endings	39 - 17	1.6 - 1.3
E-glial and cell mitochondria	16 - 36	2.7 - 2.5

[a] These values, expressed to the right of the data of the present study, are from ref. 12 for comparison.

[b] The microsomes and supernatant were not separated.

[c] All percentages in mitochondrial subfractions are relative to the crude mitochondria, assumed as 100%.

[d] Relative specific activity (RSA) is defined as % enzyme activity/ % protein in fraction.

[e] Succinate dehydrogenase.

TABLE II

SUBCELLULAR DISTRIBUTION OF ACETYL COA SYNTHASE

Fraction	μmoles aceto-hydroxamate-formed/hr-mg protein	μmoles/hr-g wet wt.	%	RSA**	# of repeti-tions
Homogenate	0.13 ± 0.04	17 ± 6	100	1.0	11
Nuclear	0.19 ± 0.19	3 ± 3	16 ± 14	1.5 ± 1.2	5
Mitochondria	0.09 ± 0.04	5 ± 2	39 ± 8	0.9 ± 0.2	11
Microsomes	0.12 ± 0.12	3 ± 3	19 ± 14	0.9 ± 0.7	5
Non-Particulate	0.27 ± 0.05	5 ± 1	32 ± 10	2.4 ± 0.4	6
Average Recovery = 106%					
A	0.05 ± 0.01	0.8 ± 0.3	15 ± 5*	0.5 ± 0.1*	4
B	0.08 ± 0.05	0.5 ± 0.2	4 ± 4	0.5 ± 0.4	5
C	0.05 ± 0.02	0.5 ± 0.2	10 ± 5	0.6 ± 0.3	5
D	0.13 ± 0.02	1.8 ± 0.7	38 ± 8	1.5 ± 0.5	6
E	0.4 ± 0.1	1.5 ± 0.3	30 ± 16	5.2 ± 2.8	6

*All measurements on density gradient fractions are expressed relative to crude mitochondria.

**See Table I.

as means plus or minus the standard error. Excellent recovery of activity was attained. The homogenate shows 0.28 μmoles/min/g tissue, which is in excellent agreement with 0.3 μmoles necessary for acetate labelling of amino acids in compartmental studies by computer simulation done by Van den Berg (10), although the data reported in Table II are the first to show appreciable enzyme in the non-particulate fraction. In the sucrose density gradient, relatively little enzyme is found in fractions A and B (myelin and myelin fragments), and C (synaptosomes). The bulk of the activity sediments with the nerve-ending particles (fraction D) and the glial and perikaryonic mitochondria (fraction E).

Subcellular Distribution of Acetoacetate-Activating System

These data are shown in Tables III and IV. Remarkable correlation of the two activities is apparent, the relative specific activities being almost the same, implying that they cohabit the same compartment. This system showed relatively lighter density than the acetate-activating enzyme in the sucrose density gradient and was almost absent from the non-particulate fraction. The distribution of the acetoacetate enzymes almost coincides with that of succinate dehydrogenase (Table I), implying that they are truly mitochondrial in location.

These assays were run at a higher pH and buffer concentration than is usual for studies in these enzymes because raising these parameters substantially inhibits acetyl CoA hydrolase, a substantial contaminant in brain (see Part II).

The pH change had no effect on either enzyme whereas the higher buffer concentration inhibited succinyl CoA transferase about 50% without affecting the thiolase.

TABLE III

SUBCELLULAR DISTRIBUTION OF ACETOACETYL COA:COASH THIOLASE

Fraction	µmoles/min-g protein	µmoles/min-g wet wt.	%	RSA**	# of repetitions
Homogenate	0.026 ± 0.004	3.7 ± 0.6	100	1.0 ± 0.3	12
Nuclear	0.013 ± 0.008	0.2 ± 0.13	5 ± 4	0.5 ± 0.3	4
Mitochondrial	0.045 ± 0.006	2.3 ± 0.3	64 ± 11	1.7 ± 0.3	10
Microsomal	0.006 ± 0.003	0.2 ± 0.06	4 ± 2	0.2 ± 0.1	4
Non-Particulate	0.013 ± 0.006	0.26 ± 0.2	7 ± 3	0.5 ± 0.2	7
A	0.013 ± 0.006	0.2 ± 0.06	8 ± 3*	0.5 ± 0.8	8
B	0.02 ± 0.02	0.13 ± 0.06	4 ± 3	0.5 ± 0.5	8
C	0.04 ± 0.013	0.4 ± 0.2	19 ± 8	0.9 ± 0.2	8
D	0.06 ± 0.02	0.8 ± 0.26	35 ± 10	1.6 ± 0.5	8
E	0.08 ± 0.04	0.3 ± 0.2	16 ± 8	2.1 ± 0.9	8

* See Table II.

**See Table I.

TABLE IV

SUBCELLULAR DISTRIBUTION OF SUCCINYL COA:ACETOACETATE TRANSFERASE

Fraction	µmoles/min-mg protein	µmoles/min-g wet wt.	%	RSA**	# of repetition
Homogenate	0.045 ± 0.013	6.4 ± 2.1	100	1.0	12
Nuclear	0.026 ± 0.013	0.3 ± 0.1	4 ± 3	0.5 ± 0.3	4
Mitochondria	0.05 ± 0.02	2.7 ± 0.6	66 ± 14	1.6 ± 0.3	11
Microsomal	0.01 ± 0.003	0.17 ± 0.05	3 ± 1	0.18 ± 0.06	4
Non-Particulate	0.03 ± 0.01	0.5 ± 0.14	8 ± 3	0.56 ± 0.17	7
Average Recovery = 81%					
A	0.01 ± 0.01	0.13 ± 0.13	5 ± 3*	0.15 ± 0.12	8
B	0.02 ± 0.01	0.06 ± 0.06	3 ± 2	0.3 ± 0.2	8
C	0.045 ± 0.01	0.52 ± 0.20	19 ± 8	0.9 ± 0.2	8
D	0.10 ± 0.04	1.2 ± 0.06	42 ± 13	1.7 ± 0.4	8
E	0.12 ± 0.06	0.45 ± 0.2	18 ± 11	2.4 ± 1.0	8
Average Recovery = 87% off gradient					

* See Table II.

**See Table I.

CHAPTER IV

DISCUSSION

Since ^{14}C-acetate labels the small compartment of brain, and ^{14}C-acetoacetate the large compartment, a major goal of these efforts was to elucidate any differences in the subcellular location of activation into the tricarboxylic acid cycle of the two compounds. To do this, an assay for acetyl CoA synthase was developed. This assay gives an activity in brain of 17 μmoles/hr/g brain compared to the value of 6.6 μmoles/hr/g brain reported by Niedle *et al.* (7). In their assays ^{14}C-acetate, with all other substrates, was incubated with 0.5% Triton X-100-treated tissue fractions and after 1 hr the reaction was stopped with acid. The acidified mixture was evaporated, thus volatilizing the unreacted ^{14}C-acetic acid; the nonvolatile radioactivity was assumed to be ^{14}C-acetyl CoA. However, if the presence of acetyl CoA hydrolase is considered (See Part II), the assumption that acetyl CoA would accumulate quantitatively is incorrect, especially with substantial acetate concentrations. CoA inhibition of the hydrolase, which is certain to occur, would be decreased somewhat by acetylation of CoA by the synthase. However, even if 90% inhibited the hydrolase activity would still be greater than that of the synthase. The acetyl CoA synthase assay developed in this study circumvents the hydrolase problem.

Another study on brain acetyl CoA synthase was performed by Tucek

(16). These results are not directly comparable since the experiments were done on ovine and guinea pig brains. In their assay system the acetyl CoA formed was trapped by choline acetyltransferase as acetylcholine, which was then measured by its effect on frog rectus abdominus muscle. The method reported here removes the tedious bioassay and the need for an acetylcholinesterase inhibitor.

Since a third study on the acetyl CoA synthase of brain, performed by Schuberth (24), was done on acetone powders of brain, direct comparison of results is not possible. The levels reported (24) for rat brain were only one tenth those we find, probably due to acetone precipitation, etc.

Studies of the acetoacetate utilizing system have been far more extensive than those on acetate activation, probably because the ketone bodies have been proposed as energy substrates for brain (23). Several values for total brain levels have been proposed; for thiolase (μmoles/min/g brain) 3.6 ± 0.3 (26), 1.8 (27), and 2.1 (18) as opposed to 3.7 ± 0.6 found in the present study, and for the succinyl CoA transferase (also μmoles/min/g brain) 2.1 (18) and 4.8 (27), whereas herein reported as 6.4 ± 2.1. In no system other than ours was Tergitol-NPX used. This along with the higher ionic strength in the buffer system (which inhibits the hydrolase) used probably explains the somewhat higher results.

The results indicate two major differences. First, 32% of acetate activation occurs in the non-particulate fraction, whereas acetoacetate activation appears to be essentially mitochondrial. Secondly, the relative magnitude of the rates of activation indicate that acetoacetate can be metabolized at a 10-fold greater rate. This agrees with the hypothesis of Berl and Clarke (5) of an "energy" tricarboxylic cycle

having access to the "large" glutamate compartment, since ketone bodies have been shown by Owen et al. (23) to be utilized as significant energy sources in brain. They would logically enter the "energy" cycle.

However, the finding that a large part of acetate activation occurs in the non-particulate fraction does not necessarily help to clarify its role in glutamic acid compartmentation, since to label glutamate there must be access to a tricarboxylic acid cycle. Further evidence that cytoplasmic metabolism plays some role in the "small" compartment is provided by the recent finding of D'Adamo (27) that N-acetyl aspartate is hydrolyzed in the cytoplasm since the aspartyl portion of this molecule is known to label the "small" compartment (1). The finding of Utter (29), that in rat liver cytosol, an ATP-stimulated acetyl CoA hydrolase exists may explain why this activity has not been previously reported.

Insights into a two-cycle model of compartmentation provided by the present work are that the synaptosomes (and thereby presumably the synapse in intact tissue) do not represent a major site of acetate activation but have a proportionally larger ability to utilize aceto-acetate. Therefore some evidence is provided for the thesis that the synapse is part of the "large" compartment, and that acetate activation in this structure is only a small portion of total tissue capacity. Perhaps this portion is involved in acetylcholine synthesis.

Further interpretations of the data presented here may be allowable if the idea that the small compartment elements may well also contain the enzyme systems that label the large compartment is considered. This idea arises from consideration of the techniques used in isotopic studies in compartmentation. Normally the radioactive compound is

administered *in vivo*, and after a short period the animal is sacrificed. The brain is then extracted with acid, and the amino acids isolated by ion-exchange chromatography of the acid extract. Therefore, a compound which labelled the small compartment, with its relatively larger pool of glutamine, and much smaller pool of glutamic, still follows in that compartment a direct product-precursor relationship with respect to glutamate and glutamine, but even though the glutamate is labelled equivalently or even perhaps greater than the glutamine, when the brain is homogenized and the two pools are combined, the dilution effect in total glutamate is far greater than in glutamine, thus resulting in a lower specific activity for that compound. Alternatively, when a compound which labels the large compartment is administered, both cycles would then follow product-precursor relationships, and naturally the glutamate would then have a higher specific radioactivity. If mitochondrial populations that activate and utilize acetate also use acetoacetate when available, another population which does not possess acetate activation capacity must exist and actively use acetoacetate. However, if this be the case, a resolution of the two compartments based on the capacity to utilize compounds known to enter the "large" glutamate compartment might be impossible. The only criterion then would be the ability, or lack of same, of a population to activate and utilize acetate. To extend this logic further, the "large" compartment could possess some ability to utilize acetate, as long as the relative ability of the "small" compartment remained high enough. The existence of two separate mitochondrial populations, one exclusively utilizing one set of compounds and the other using a different set, clearly is not required by the isotopic experiments. Conceptually, (See Figure 1),

the view expressed here implies that the "small" cycle could be drawn inside the "large" and compounds feeding into the "large" may also enter the "small".

In the light of this reasoning, the only safe criterion is the ratio of acetate and acetoacetate utilization. Analysis of the data for the mitochondrial populations separated here shows that in fraction C, the synaptosomal fraction, 10% of the mitochondrial acetate activation occurs, whereas 20% of the acetoacetate enzymes are present. Therefore, for purposes of speculation, this fraction may be presumed to represent a component of the "large" compartment. On the other hand, fraction E, considered to arise from the nerve cell body (perikaryonic) and glial mitochondria, has 30% of the mitochondrial acetyl CoA synthase, and only 20% of acetoacetate activation capability, suggesting that fraction E is in the "small" compartment. Although errors implicit in measurements upon tissue extracts preclude definite conclusions, compartmentation of acetate (arising from acetylcholine hydrolysis) activation at a site removed from the synapse might be an effective control of resynthesis of acetylcholine, a reasonable hypothesis supported by the results of the present investigation. Also, a recent study (28) shows that choline acetyltransferase has a similar distribution between crude mitochondria and supernatant (44 and 38% of total, respectively) as the acetyl CoA synthase distribution reported here. Although a great deal of the choline acetyl transferase was found to be occluded in the crude mitochondria, the total activity in whole brain, calculated from their data, for choline acetylation is about 7 μmoles/hr/g brain, the same order of magnitude as the acetyl CoA synthase activity of 17 μmoles/hr/g brain reported here. Unfortunately, the

acetyl CoA synthase assay developed here lacks the sensitivity requisite for differentiation of occluded and particulate forms, since dilution of tissue activity is inevitable during hypoosmotic shock to release occluded activities. Therefore, at present it is not possible to state that acetate activation and acetylcholine synthesis capabilities have similar subcellular distribution in brain. The possibility remains intriguing, however.

Recently Van Den Berg (10) postulated a model of brain compartmentation involving five (or more) separate tricarboxylic acid cycles, with at least two cycles in the old "large" compartment and at least three cycles in the old "small" compartment. This extremely complex model is tempting in the light of the acetate activating system's distribution, since the cytoplasm, the synaptic and nerve-ending particles, and the glial and perikaryonic mitochondria present three separable locations of acetate utilization. Unfortunately, with or without the computer, much more data are needed before we can transverse from speculation to reality.

CHAPTER V

SUMMARY

The subcellular distributions of acetyl CoA synthase, acetoacetyl CoA thiolase, and succinyl CoA transferase in rat brain have been determined. An assay for acetyl CoA synthase was developed which was unaffected by acetyl CoA hydrolase. The activities of these enzymes are 0.28, 3.7 and 6.4 μmoles per minute per gram brain weight respectively. Detergent (Tergitol) is required for release of the activities, all of which are present in varying amounts in all mitochondrial populations isolated. The two acetoacetate enzymes have parallel distributions, whereas the acetate activating enzyme is (percentagewise) less in the synaptosomal mitochondrial subfraction.

The results support the idea that acetoacetate is used in the "energy" cycle for brain and is partially found in synaptosomes, and that part of the "small" glutamate compartment of brain is glial and/or perikaryonic. The data were interpreted in both a two and five tricarboxylic acid cycle hypothesis for a partial explanation of brain compartmentation. Also presented herein was the observation that in contrast to the acetoacetate system, about one third of acetate activation occurs in the cytoplasm.

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PART TWO

STUDIES ON RAT BRAIN ACYL COENZYME A HYDROLASE (SHORT CHAIN)

CHAPTER I

INTRODUCTION

The generation and effective utilization of short-chain fatty acyl CoA compounds in biological systems is a necessity for life. The importance of these substances in synthetic and energy-yielding reactions makes a rationale for the existence and metabolic role of specific acyl CoA hydrolases difficult. Nonetheless, the history of studies upon properties and control of these systems gives little information other than proof of presence.

The presence of short chain-acyl CoA hydrolases was first deduced by Gergely, Hale and Ramakrishnan in 1952 (1). They found that the presence of succinyl CoA hydrolase and acetyl Coenzyme A hydrolase was necessary for activity of 2-ketoglutarate and pyruvate dehydrogenase assay systems, respectively, in pig heart preparations. They also presented evidence that two separatable enzymes catalyze hydrolysis of succinyl and acetyl CoA.

In 1959 Srere, Seubert and Lynen reported that porcine brain possessed several acyl CoA hydrolases, and isolated a fraction which hydrolyzed acetyl CoA (2). Succinyl CoA was not hydrolyzed by this fraction. The presence of palmityl CoA hydrolase in both porcine and neonatal rat brain was also demonstrated and the acetyl CoA hydrolase and palmityl CoA hydrolase activities were separable by heating at 65° for three minutes, the acetyl CoA hydrolase activity being destroyed by

this treatment.

Acetyl CoA hydrolase was reported to contaminate purified choline-acetyl transferase of rabbit brain by Severin and Artenie in 1967 (3).

Rat brain acyl CoA hydrolases were studied in 1967 by Anderson and Erwin (4). The short chain hydrolases were reported to be cytosolic and destroyed by a pH 5.1 acetic acid precipitation of the longer-chain hydrolases.

Knowles, Jarrett, Filsell and Ballard, in 1974, studied acetyl CoA hydrolases of various tissues and reported the enzyme to be mainly mitochondrial and in highest concentration in rat liver, but ubiquitous in tissues studied (5). The liver enzyme was significantly increased by starvation and had a K_m of 0.7 mM for acetyl CoA.

In 1973 Mahan observed acetyl CoA hydrolase as a contaminant in rat brain pyruvate carboxylase preparations (6).

The purpose of the present study was to characterize the activity in brain which hydrolyses acetyl CoA and to learn how to prevent or minimize interference by this enzyme in other enzyme assays using short chain thioesters. There being no standard system for assaying this activity makes comparison of reported results difficult; moreover, all determinations of activity reported so far suffer from a lack of sensitivity or from interference by substances present in tissue preparations. Resolution of these problems was also sought.

CHAPTER II

MATERIALS AND METHODS

Materials

DTNB, ADP, Ficoll, 2-mercaptoethanol, Tergitol-NPX, DL-carnitine chloride, choline chloride, acetylcholine chloride, N-acetylaspartic acid, 3',5'-cyclic AMP, phosphoenolpyruvate, fructose 1,6-diphosphate, reduced lipoic acid, lipoamide and epinephrine were from Sigma Chemical Co. CoA and ATP were purchased from Calbiochem. Sucrose, mannitol, $MgSO_4$, $(NH_4)_2SO_4$, and Na_2EDTA were obtained from Schwartz/Mann. Alcohol dehydrogenase was from Worthington. Octanoyl CoA and palmityl CoA were from P-L Biochemicals, Inc. dl-Alpha-lipoic acid (disulfide form) was obtained from DuPont, biotin from Nutritional Biochemical Corporation, and 1-^{14}C acetate from New England Nuclear.

Methods

Preparation of Reagents. Acetyl CoA was prepared by the action of acetic anhydride on free CoA in 0.1 M potassium bicarbonate using a 15% excess of the anhydride as suggested by Utter (7). Other thioesters were prepared in a similar manner except acetoacetyl CoA, which was prepared as in Part I, and benzoyl CoA, which was prepared by reaction of benzoyl chloride with CoA.

Enzyme Assays. During purification the assay medium used was 20 µliters of enzyme (up to 2 units/ml) in 0.96 ml of 200 mM Tris, 250

mM sodium acetate, 5 mM $MgSO_4$, 1 mM Na_2EDTA, 100 μM DTNB, 10% glycerol, pH 8.1. An initial absorbancy, due to reaction of 2-mercaptoethanol with DTNB, of about 0.6 was allowed to fully develop before the hydrolase reaction was started by addition of 20 μliters of 12 mM (240 μmoles) acetyl CoA. The reaction was run at 25° in a Hitachi Perkin-Elmer recording spectrophotometer fitted with a 124-801 scale expander.

For analysis of product effects two additional assay buffers were developed. The first, designated R, was 200 mM Tris-Cl and 100 μM DTNB. The second, designated A, was 40 mM Tris-Cl, 250 mM sodium acetate, and 100 μM DTNB. Both assays were run at 25° and pH 8.1. In all assays with DTNB the mM absorptivity of the DTNB chromogen at 412 nm was assumed to be 11.4.

In an assay where reduced sulfydryl groups had to be maintained, the absorption of the thioester group at 232 nm was utilized. In the CoA inhibition studies the reaction was run in 40 mM Tris-Cl, 250 mM sodium acetate, 1 mM dithiothreitol, and 10 mM $MgCl_2$ at pH 8.1. The assay at 232 nm is approximately 20% as sensitive as that using DTNB. Consequently larger amounts of enzyme were required to obtain data which still possessed larger inherent errors due to low absorbance and interference by protein absorbance at 232 nm. The latter made it impossible to use enough enzyme to give changes in absorbancy equivalent to the DTNB assay.

Tissue Distribution Studies. Male Holtzmann rats were used. The rat was decapitated and various tissues excised, placed in ice-cold AGX-1 (See Table I) and homogenized with a tight-fitting Potter-Elvejehm homogenizing vessel. Heart, lung and leg muscle, however, were homogenized using a shaved teflon pestle, the vessel inner diameter

to pestle diameter clearance being 0.47 mm. Ten volumes of buffer per gram of tissue were used. One ml of the homogenate was then treated with 0.1 ml 10% Tergitol-NPX, pH 7.4, stirred gently, and placed in ice for 30 minutes. Since no optimal concentration of Tergitol was determined for any tissue other than brain, the activities reported must be considered as minimal. Protein was estimated by the biuret reaction (8).

Protein Estimation During Purification. The method of Lowry et al. was used, modified for 5 to 25 µg of protein, monitored at 750 nm (9). Blanks with appropriate buffer were run for each sample.

Data Analysis. Least-squares fits of kinetic data were done on a conversational programming system through an IBM 2741 communications terminal interfaced to an IBM 360/65 computer. The program and method used have been described by Kelly (17).

CHAPTER III

EXPERIMENTAL AND RESULTS

Purification of Rat Brain Short Chain Acyl CoA Hydrolase

Much of the initial phase of this work is based on the findings of Mahan (6), who reported that lyophilization released acetyl CoA hydrolase from mitochondria and that the activity was co-purified with pyruvate carboxylase. The preparation described here is, therefore, similar to the purification of pyruvate carboxylase through the Celite stage, but with appropriate modifications to optimize yield of acetyl CoA hydrolase.

Rat brain mitochondria were isolated according to the procedure of Clark and Nicklas (10). All centrifugations were carried out either in a Servall RC 2-B refrigerated centrifuge or a calibrated Servall SS-1 tabletop centrifuge in the cold room. All mitochondrial isolations were performed at 4° using 50-ml polyethylene centrifuge tubes in an SS-34 head.

Male Holtzmann rats were decapitated using a guillotine and the cerebral hemispheres excised and placed in cold isolation medium consisting of 0.25 M sucrose, 10 mM Tris, pH 7.4, 5 mM 2-mercaptoethanol and 0.5 mM Na_2EDTA. The brains were then minced with surgical scissors and the original isolation medium carefully decanted. A normal preparation consisted of 32 rats, killing usually required 60 to 90

minutes. The minced pieces were divided into two roughly equal portions by pouring half of them into a Thomas size C homogenizer. Fifty ml of isolation medium was added to the homogenizer and the minced brains were homogenized by two passes at 640 rpm using a pestle with standard 0.2 mm clearance. The homogenate obtained was poured into a glass 250-ml graduated cylinder and the process repeated for the rest of the brains. Then the mixture of the two homogenates was brought to 240 ml by addition of cold isolation medium.

The homogenate was centrifuged at 2000g for 3 minutes; supernatant solutions were decanted and pooled. The pellets were washed twice by suspending in approximately 100 ml cold isolation medium, rehomogenizing and centrifuging as before. The supernatants were combined with the first one and centrifuged for 8 minutes at 12,500g.

This mitochondrial pellet was suspended in 8 ml of a 3% Ficoll solution containing 120 mM mannitol, 30 mM sucrose, 0.25 mM Na_2EDTA, and 2.5 mM 2-mercaptoethanol and the suspension gently layered on 20 ml of 6% Ficoll with 240 mM mannitol, 60 mM sucrose, 0.5 mM Na_2EDTA and 5 mM 2-mercaptoethanol. Six like tubes prepared using equal amounts of mitochondria were then centrifuged at 11,500g for 30 minutes. About half of the supernatant was decanted and the remaining solution was swirled gently to remove a white layer which usually formed above the tan mitochondria. This solution was poured off and discarded. The pellets were suspended in 20 ml of 10^{-4}M Na_2EDTA, lyophilized and the residue stored *in vacuo* at -29°. The enzyme is not stable; in one instance storage at this stage for 3 weeks resulted in a 95% loss of activity upon extraction. Normally this procedure was used for overnight storage only.

The lyophilized mitochondria were extracted with 25-30 ml of AXG-1, a buffer system consisting of 200 mM Tris-acetate supplemented with sodium acetate so that total acetate was 250 mM, 5 mM $MgSO_4$, 1 mM Na_2EDTA, 5 mM 2-mercaptoethanol, 10% glycerol (v/v), pH 7.4 (See Table I). The mitochondria were homogenized as before and centrifuged at 54,000g for 1 hour in a refrigerated Beckman Spinco model L preparative ultracentrifuge using a Type 30 head. The supernatant was then brought to 38% ammonium sulfate (221 g/liter) by the addition of solid ammonium sulfate at 0-4° over a one-hour period. The enzyme was in the pellet obtained by a 10,000g centrifugation for 20 minutes at 4°.

In some preparations the addition of ammonium sulfate up to 38% saturation did not precipitate the acetyl CoA hydrolase activity. The supernatant above the 38% pellet was routinely assayed using DTNB; at times as much as 70% of the acetyl CoA hydrolase activity was not precipitated. This problem was solved by addition of ammonium sulfate to 50% saturation, which always pelleted all the activity. The pellet could then be extracted with 200 mM Tris-Cl, 5 mM $MgSO_4$, 5 mM 2-mercaptoethanol and 38% ammonium sulfate without solubilizing the enzyme, which is also insoluble in the AXG-2 system (see below).

The precipitated enzyme was then resuspended in AXG-2, a system similar to AXG-1 but also containing 38% ammonium sulfate. The suspension volume was minimized, 2 ml being optimum for the normal 32-rat-brain procedure. This enzyme solution was placed on a 1 x 7 cm column of Celite prepared after the procedure of King (8), as modified by Mahan (6). The Celite had been previously washed extensively with AXG-2. The top centimeter of the column was slurried and a linear gradient of 25 ml AXG-2 and 25 ml AXG-1 was applied. Column flow was

TABLE I

BUFFER SYSTEMS

Name	Composition	Use
AXG-pH 7.4	200 mM Tris 250 mM Acetate 10% glycerol 5 mM 2-mercaptoethanol 5 mM $MgSO_4$ 1 mM Na_2EDTA	Purification and storage
AXG-1	As above, but pH 8.1	Assay buffer
AXG-2	AXG-1 with 38% ammonium sulfate	Celite column
HA-I	25 mM KPO_4 150 mM ammonium sulfate 5 mM 2-mercaptoethanol pH 7.4	Hydroxyapatite
HA-II	HA-I with 200 mM ammonium sulfate	Hydroxyapatite
R	200 mM Tris-Cl pH 8.1	Activation studies
A	40 mM Tris-Cl 250 mM Acetate pH 8.1	Activation studies

regulated at 0.25 ml/min by a Beckman solution metering pump. Fractions of 1.5 ml size were collected, the entire procedure being carried out in a cold room at 4°.

Tubes containing activity by DTNB assay were pooled and concentrated by the colloidion-bag technique (18). The enzyme solution, concentrated to about 5 ml, was then dialyzed 10 hours against HA-I. Stability was marginal in this buffer and the yields after dialysis were highly variable.

The preparation was then placed on a 6 ml Bio-Gel HTP hydroxyapatite column poured in a 10 ml syringe barrel over pyrex wool at room temperature. The column was washed with 5 volumes of HA-I before application of enzyme. In this and all steps of this procedure the buffer was kept on chipped ice and the column held at room temperature. Efforts to run this column in the cold room or to use buffer at room temperature led to poor yield of enzyme. Also attempts to use a gradient system with this column gave poor yields.

The column was washed with two 5-ml aliquots of HA-I, two 5-ml aliquots of an equal mixture of HA-I and HA-II, and 5-ml portions of HA-II until all enzyme activity was eluted. Characteristically the enzyme emerged in the second 5-ml HA-II wash and continued until approximately the sixth HA-II wash. Fractions containing activity were pooled, brought to 10% glycerol immediately and concentrated by the colloidion-bag technique to a final volume of 2 ml. The buffer used during concentration was AXG-I.

In preparations in which more than 5 units of enzyme was obtained, or if two successive batches of enzyme off hydroxyapatite could be combined to more than 5 units, the enzyme after concentration was

applied to a 2.3 x 71.5 cm column of Sephadex G-200 equilibrated with AXG-I in a 4° cold room. The operating pressure of the column was adjusted so that a flow rate of about 5 ml/hr was achieved; 5 ml fractions were collected. This rate was obtained when about 10 cm of water pressure was applied to the column head. The void volume of this column was found using Blue Dextran to be 99 ml.

Acetyl CoA hydrolase, which eluted from this column at a V_e/V_o of 1.1 to 1.3, was located by DTNB assay. Spectrophotometric readings at 280 nm or 220 nm were impractical because of a high background, probably due to reduced sulfhydryl reagent. The fractions containing activity were pooled and concentrated as before.

Comments on Purification. The preparation of acetyl CoA hydrolase as described here gives variable yields of enzyme. In some preparations, especially before development of the AXG buffers, complete loss of activity was sometimes observed at almost every phase of the preparation. The AXG systems allow some degree of confidence in manipulation and, with the enzyme from G-200, give absolute stability for at least four months. Indeed, in one case where through carelessness a colloidion bag was allowed to go dry, the first extraction of the enzyme showed only 20% or original activity, but after a week in AXG-I in the refrigerator all activity was recovered.

Other methods of purification tried without success were heating at 58° for three minutes, polyethylene glycol precipitation, DEAE-cellulose and CoA affinity columns. All showed either inordinate loss of activity or little gain in purity. The CoA affinity columns tried were CoA linked to Sepharose 4B through the adenine amino group with a hexane spacer and CoA linked in a thioester bond with a hexanoic acid

spacer. The former bound the enzyme but activity was not eluted by pH change, ionic strength gradient or substrate. The thioester column proved to be a substrate for the enzyme as evidenced by a 260/280 ratio in the eluate of 5. Precipitation by organic solvents such as ethanol or acetone or acidification with acetic acid to pH 5.1 also destroyed activity.

Considering the kinetic and physical properties adduced herein, the possibility remains that ATP affinity columns or hydrophobic column chromatography would be of significant value in purification of this enzyme. However, these were not attempted.

The major deficiencies of the preparation are the ion-exchange step and the lyophilization. Both are variable and the data presented in Table II are the best obtained in many separate preparations. However, during the course of these studies several observations were made which may optimize yield. Purity in all steps was remarkably constant.

First, in application of enzyme to hydroxyapatite best results were obtained if the smallest possible volume of enzyme was used, that is, the activity was as concentrated as manipulation allowed. Also, the longer the column was prewashed with HA-I the better the results. The hydroxyapatite columns were never reused.

Second, the time between lyophilization and extraction is critical and 10^{-4}M Na_2EDTA may not be the best agent for lysis of mitochondria for liberation of acetyl CoA hydrolase. No studies were done on this. However, in view of the loss of 87% of homogenate activity during isolation, mitochondrial extraction and subcellular distribution studies are in order before attempting extensive studies needing large amounts

TABLE II

PURIFICATION OF RAT BRAIN ACETYL COA HYDROLASE

Fraction	Units/mg protein	Total units	% Yield	Fold purification
Homogenate (0.9% Tergitol)	0.04	250	100	1.0
Mitochondria (lyo. & extracted)	1.8	32	13	45
Reverse solubilization off Celite	5.0	14	6	125
Hydroxyapatite	30	6	3	750
Sephadex G-200	55	4.2	1.7	1375

of enzyme.

Miscellaneous Properties. Dialysis of the enzyme against cold 0.25 M sucrose in 10 mM Tris-Cl caused total loss of activity, implying that ionic strength rather than osmolarity is the principal basis for the aforementioned stability.

A single experiment indicated that at high buffer concentrations (400 mM) divalent calcium tended to inhibit the activity. However, this was not repeated.

Glycerol appears to have no effect on the catalysis of acetyl CoA hydrolysis, but has a stabilizing activity which was not extensively studied.

The enzyme is present in brains of 6-day-old rats, but since this activity was released by lyophilization, a method which predated the more accurate Tergitol-NPX solubilization, the absolute activity is unknown.

The necessity of sulfhydryl reagent during purification appears to be absolute, but during storage of enzyme from G-200 complete oxidation of sulfhydryl groups could occur without loss of enzyme activity.

During the enzymatic hydrolysis of acetyl CoA, no substrate for β-OH-acyl CoA dehydrogenase was evolved.

Physical Properties of Acetyl CoA Hydrolase

Purity. The activity as isolated from hydroxyapatite showed at least 4 bands on polyacrylamide disc-gel electrophoresis (12), with one band in predominance. However, some material did not enter the gel, remaining at the interface. This preparation showed slight lactate

dehydrogenase activity, not present in the enzyme passed through Sephadex G-200. Because of the small amount of enzyme obtained from G-200, polyacrylamide gel electrophoresis was not attempted.

pH and Ionic Strength Studies. These studies were conducted with a 38% ammonium sulfate precipitate of an extract of lyophilized mitochondria. The pH studies used phosphate buffers. In the light of later studies, the validity of these observations must be questioned since phosphate is an inhibitor of the enzyme. However, an optimum pH of 8.1 was observed with Tris. Since the enzyme shows good activity at this pH, the question remaining is whether or not the Tris optimum is broader than the apparent sharp peak of activity found at about pH 8.

Ionic strength studies (Figure I) showed maximum enzyme activity in the range of 100 to 200 mM buffer. For further data see metal ion studies.

Metal Ion Studies. Metal ions appear to enhance stability rather than catalysis. In Figure 2 are presented results from a study of the effect of divalent calcium upon enzyme stability. The enzyme was incubated in a 1.0 ml cuvette for the times indicated, then DTNB was added, a baseline recorded and acetyl CoA added. The results show that in a 50 mM buffer system the stability of the enzyme is greatly increased by the presence of Ca^{++}. Examination of the zero time assays show that the metal ion also abolishes inhibition by low ionic strength, an effect which was repeatable at all stages of purification. However, kinetic studies with Ca^{++} and Mg^{++} (which stabilizes like Ca^{++}) at 200 mM buffer concentrations, showed no effect at any acetyl CoA concentration, except for an insignificant inhibition.

The effect of alkali metal ions was also studied. KI, LiCl, and

Figure 1. Ionic strength effects on the activity of acetyl CoA hydrolase. The substrate was 240 μM acetyl CoA. The open circles are Tris-Cl varied as indicated, the closed circles 20 mM Tris-Cl, KCl to indicate millimolarity. Enzyme used was off Celite.

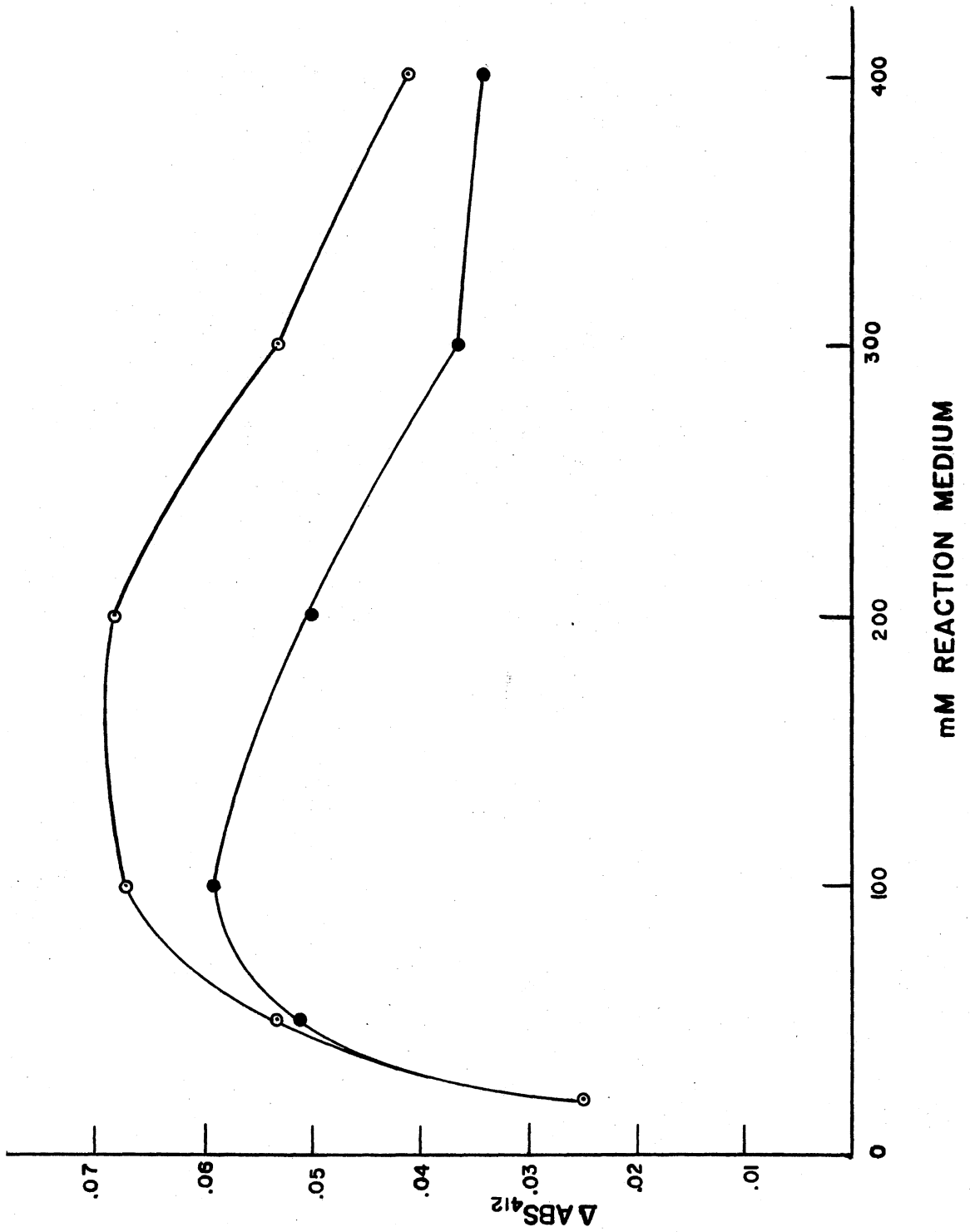
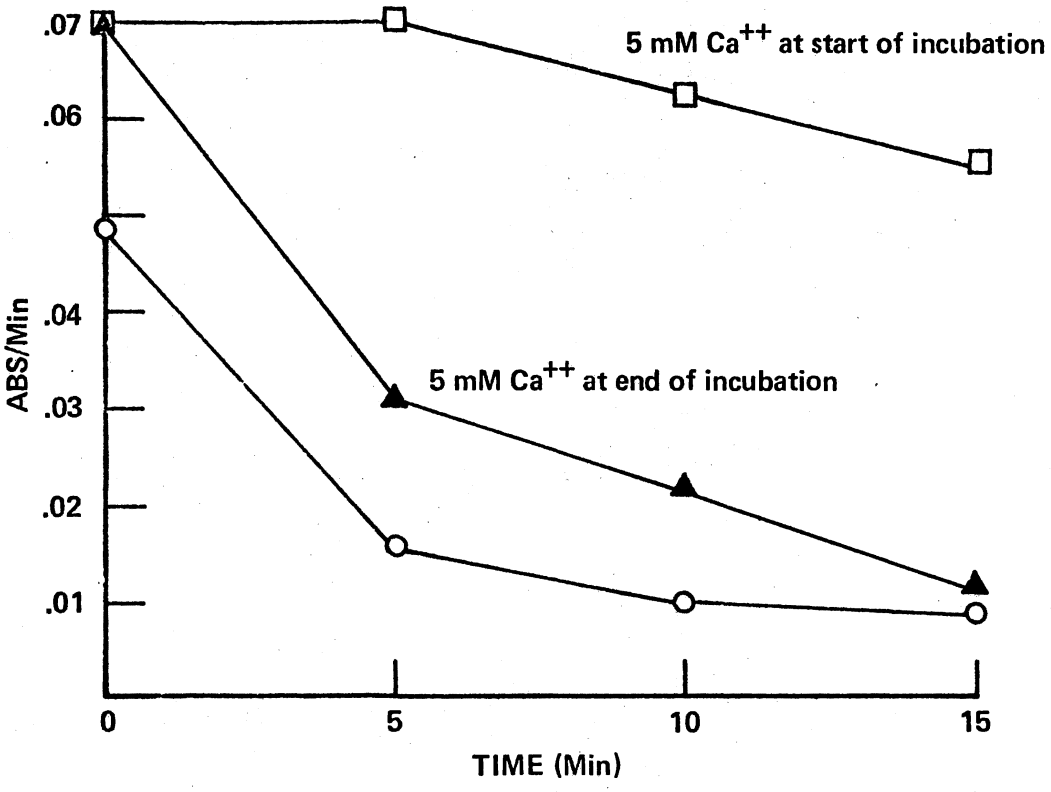

.07
.06
.05
.04
.03
.02
.01
ΔABS412
0
100
200
300
400
mM REACTION MEDIUM

Figure 2. Divalent calcium effects on the stability of acetyl CoA hydrolase at low ionic strength. The enzyme (off Celite) was added to 50 mM Tris-Cl at time 0, incubated for the indicated time, and then DTNB and substrate were added, and the absorbancy at 412 nm recorded for 1 minute. Bottom line (open circles) had no metal.

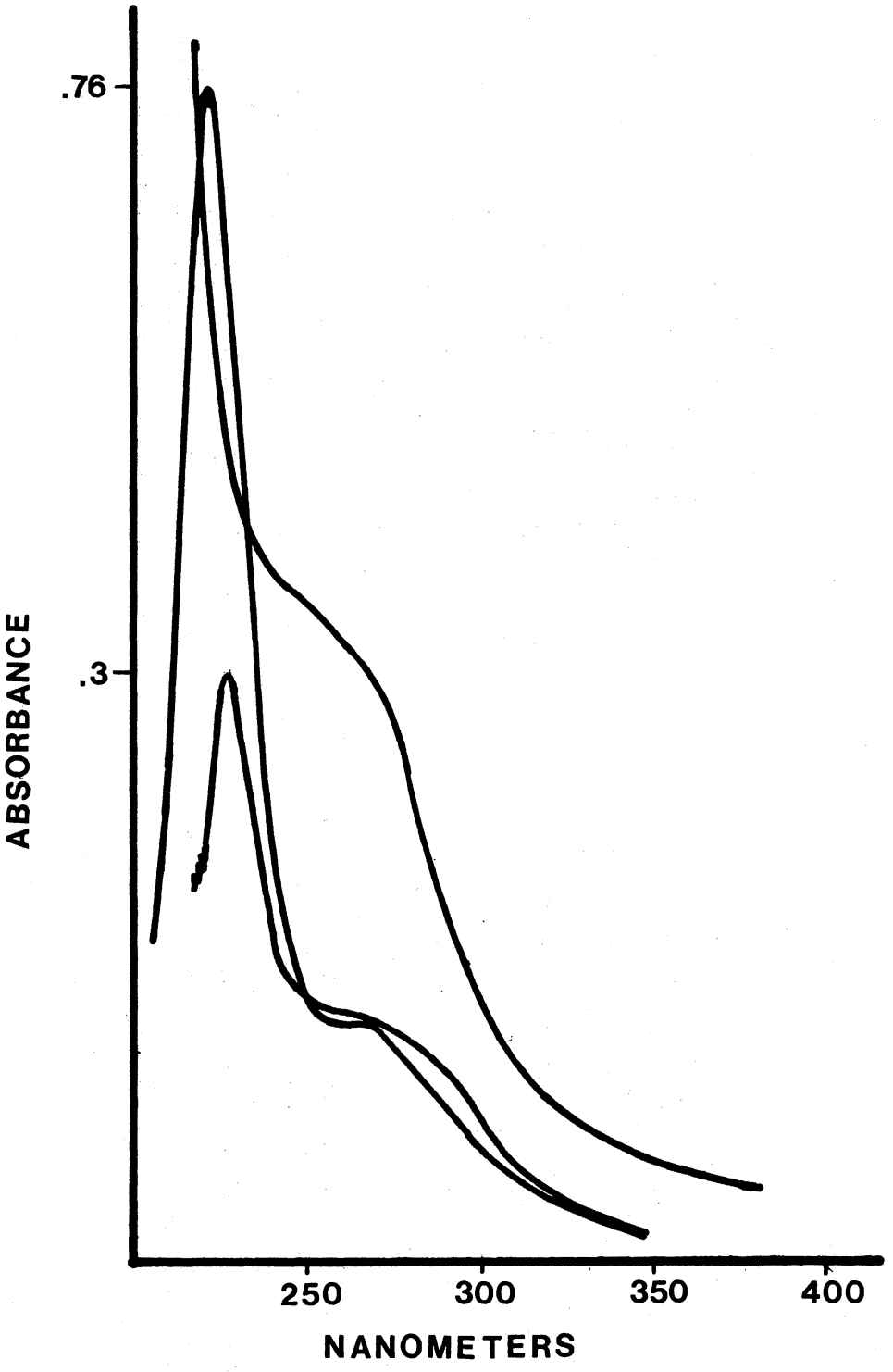

.07
.06
.04
.03
.02
.01
ABS/Min
5 mM Ca++ at start of incubation
5 mM Ca++ at end of incubation
0
5
10
15
TIME (Min)

NaCl all gave about 90% activity at 100 mM salt in 100 mM Tris-Cl, pH 8.1, compared to 200 mM Tris-Cl. One hundred mM KCl in 100 mM Tris-Cl showed equivalent activity to the 200 mM system while $KAsO_4$ and KBr (100 mM in 100 mM Tris-Cl) gave 83% and 76% respectively. Mn^{++} is about 50% as effective in stabilization as Ca^{++} or Mg^{++} in low ionic strength buffer.

Molecular Weight. The molecular weight of acetyl CoA hydrolase has been reported by Mahan (6) to be 160,000 using sedimentation velocity in a sucrose gradient. During purification acetyl CoA hydrolase was eluted from Sephadex G-200 at V_e/V_o of peak activity of 1.1. This is equivalent to the elution volume of yeast alcohol dehydrogenase in the same column. The molecular weight of the yeast alcohol dehydrogenase is 160,000, verifying Mahan's result. V_e/V_o of cytochrome C was 2.2.

Spectral Properties. The enzyme from Sephadex G-200 was examined for ultraviolet absorption as shown in Figure 3. The concentration of protein was about 25 μg per ml. Peaks at 265 and 230 nm were seen with amplitudes which suggested the presence of material other than protein. Two other studies were run, therefore. First, 0.25 ml of the enzyme was mixed with 0.75 ml of 7% perchloric acid. No precipitate was observed but the peaks shifted to 250 and 228 nm. The latter peak was 40% higher than the original peak at 230 nm, which, in view of dilution suggests a great gain in absorptivity at this wavelength. Finally, the enzyme was treated with 0.1% $NaBH_4$ with resultant peak at 260 nm and a very high absorption at the lower wavelength. In all these studies a reference cell of AXG-1 which had been used for dialysis of the enzyme sample and which had been treated like the enzyme was used to correct

Figure 3. Ultraviolet spectrum of acetyl CoA hydrolase off Sephadex G-200. Curve A, untreated enzyme. Curve B, perchloric acid 3:enzyme 1. Curve C, 0.1% $NaBH_4$ with enzyme.


.76
.3
ABSORBANCE
250
300
350
400
NANOMETERS

for sulfhydryl and stabilizer reactions and absorptions.

Inhibition Studies

Inhibitors of acetyl CoA hydrolase discovered in this study are listed in Table III, along with apparent K_i when determined.

ATP Inhibition. In addition to the study of ATP presented in Table III and Figure 4, other studies showed that the metal ion complexes of ATP such as $CaATP^{-2}$ and $MgATP^{-2}$ have no effect, thus accounting for the observation that metal ion relieves ATP inhibition.

ADP Inhibition. Figure 5 shows the effect of ADP on acetyl CoA hydrolase. Also studied with this compound were orthophosphate effects on the enzyme. ADP and orthophosphate inhibitions were additive over a range of acetyl CoA concentrations. Other experiments showed similar metal ion effects as with ATP, but this was not studied extensively.

Coenzyme A Inhibition. These results are shown in Figure 6. Other studies showed that omission of added metal ion had no effect.

Acetate Effects on Acetyl CoA Hydrolase

Discovery. In the course of studying product inhibition of acetyl CoA hydrolase, acetate at 1 to 5 mM was found to have no effect upon the rate of the enzyme reaction. However, upon increasing the concentration of acetate the rate of acetyl CoA hydrolysis increased 3 fold. This effect has since been confirmed in many preparations of acetyl CoA hydrolase, at all stages of preparation, and in various tissues of the rat (see tissue distribution studies). It is observed through increasing the acetate concentration until inhibition due to ionic

TABLE III

INHIBITORS OF ACETYL COA HYDROLASE

Compound	K_i app	Buffer	Comments	Type
Malonic acid	3.2 mM	R	Only studied at low acetyl CoA	Nonclassical
CoA	Variable	-	See Figure 7	Nonclassical
ATP	Variable	-	See Figure 5	Nonclassical
ADP	Variable	-	See Figure 6	Nonclassical
NAD^+ NADH	nd	R	10 mM causes 20% inhibition	nd
Orthophosphate	40 mM	R	-	NC
Citrate	nd	R	10 mM causes about 50% inhibition	nd
dl-alpha lipoic acid	about 5 μM	A	See discussion	NC
Na arsenite	nd	R	50 mM causes 75% inhibition	

Figure 4. ATP inhibition of acetyl CoA hydrolase. Assays performed in buffer A with 1 mM EDTA. The enzyme used was from G-200, and ionic strength was kept constant by addition of NaCl.

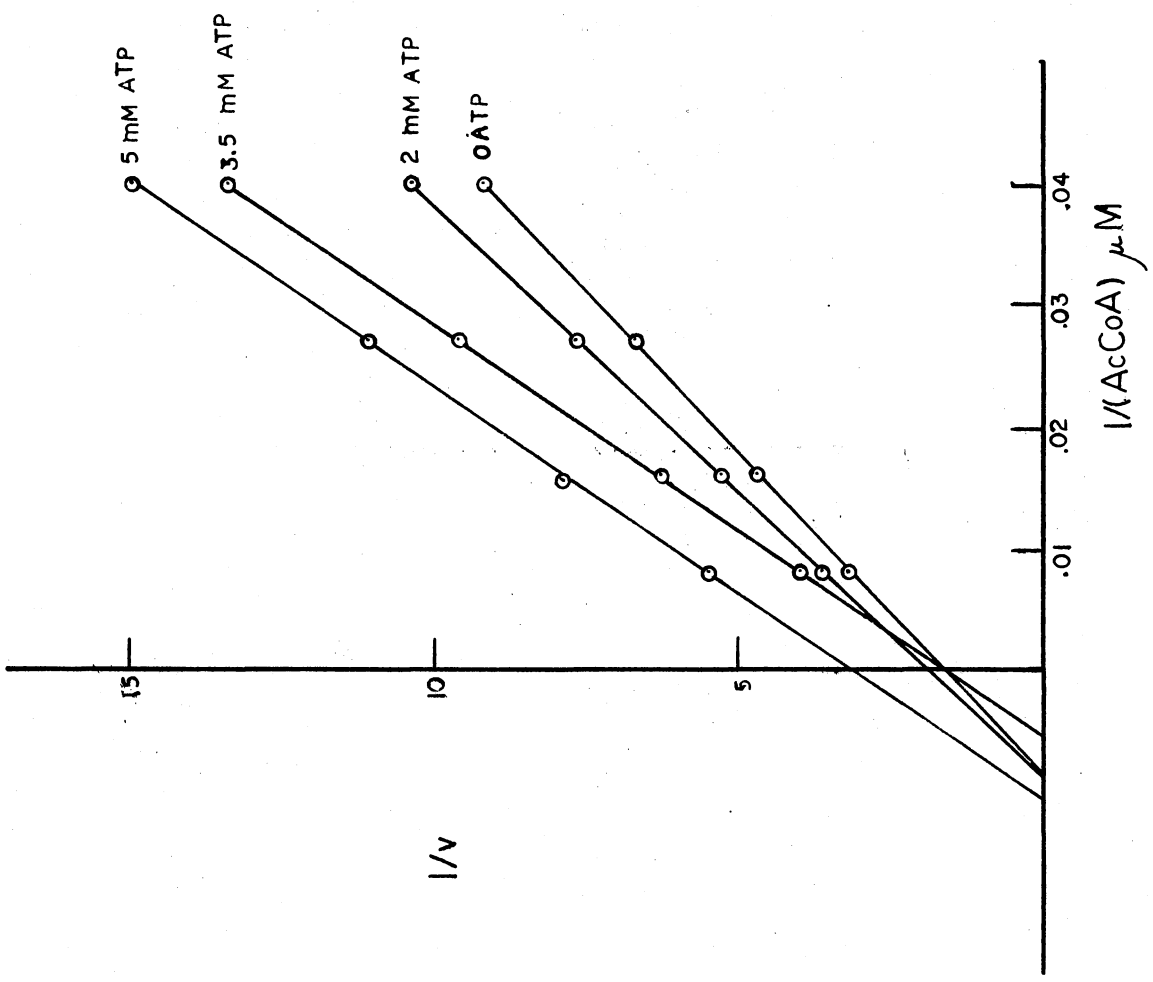

5 mM ATP
3.5 mM ATP
2 mM ATP
0 ATP
15
10
5
1/v
.01
.02
.03
.04
1/(AcCoA) μM

Figure 5. ADP inhibition of acetyl CoA hydrolase. Assays performed in buffer A with 1 mM EDTA. The enzyme was from G-200, and ionic strength was kept constant by addition of NaCl.

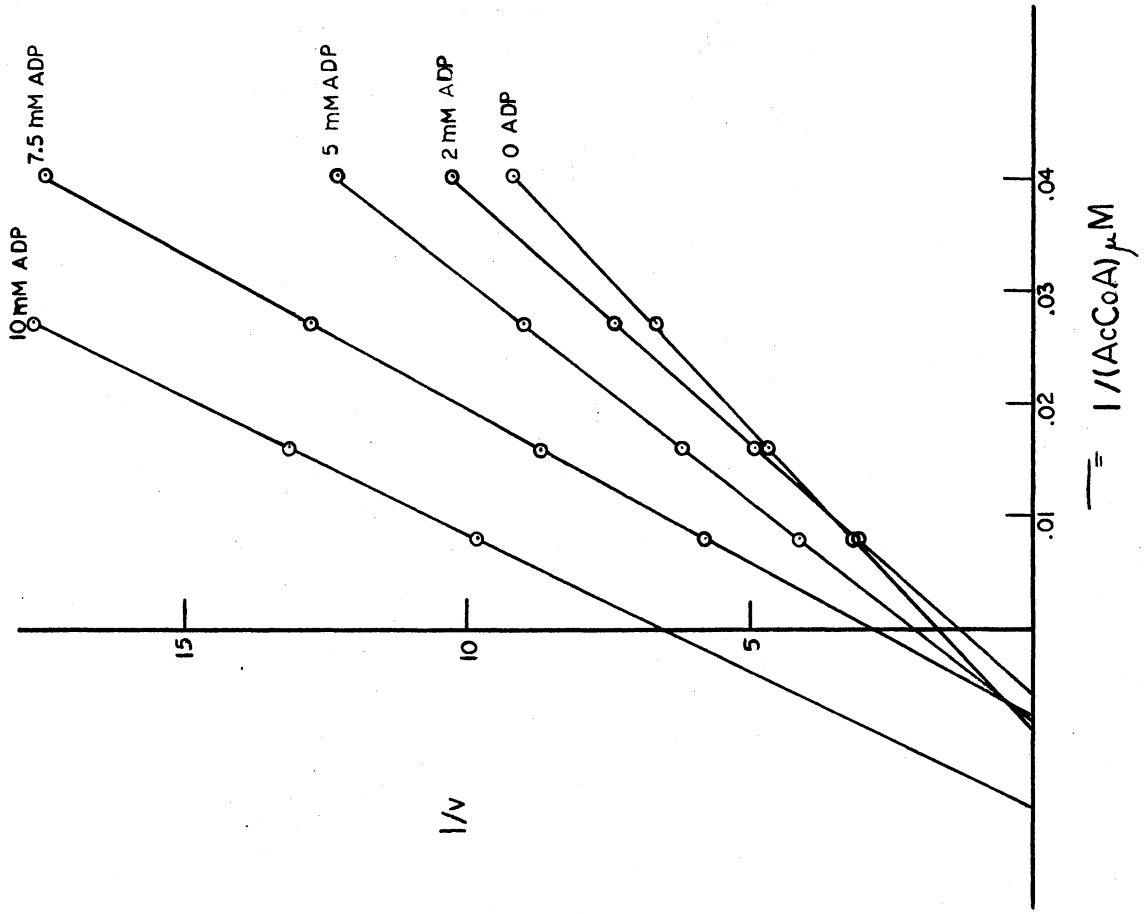
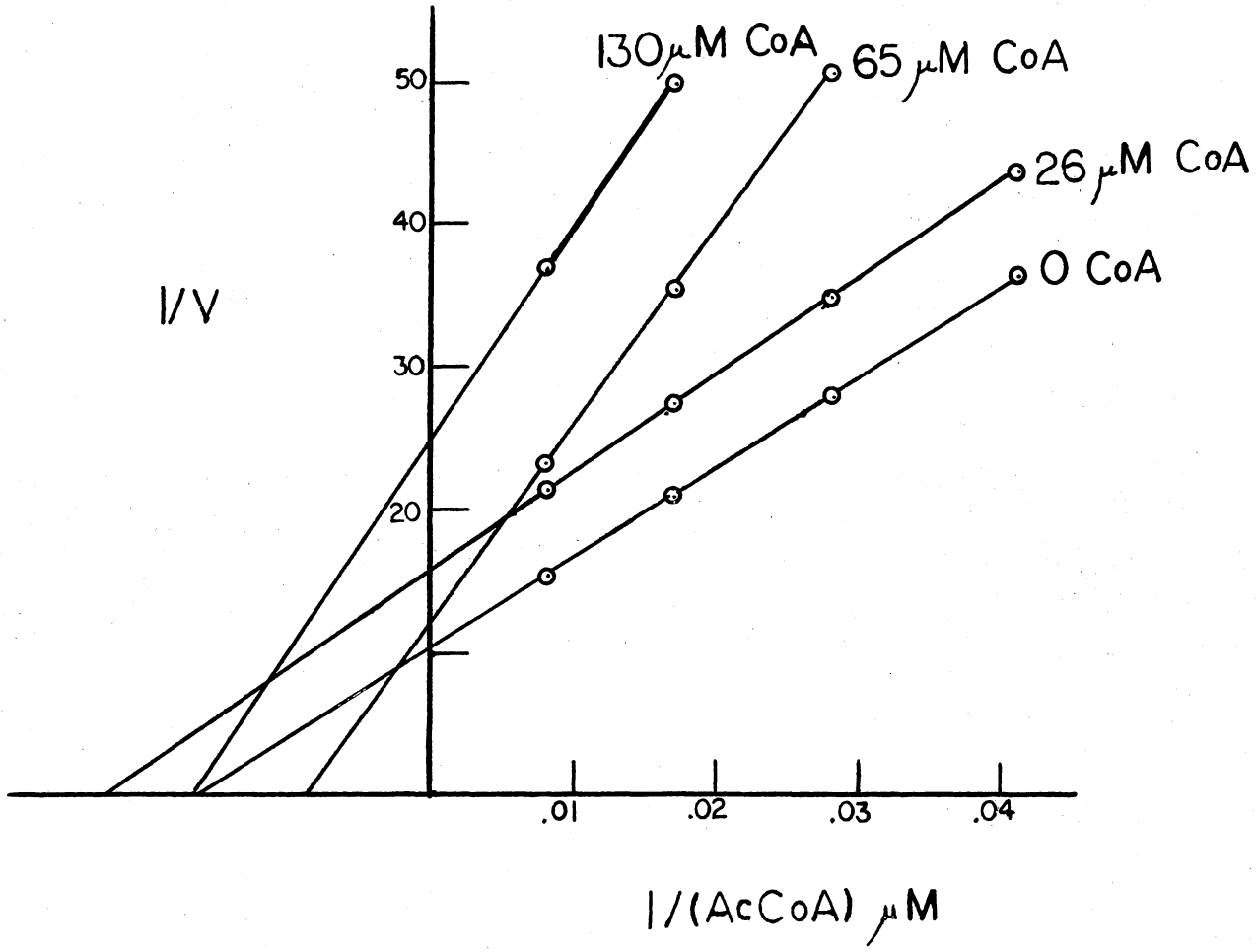

10 mM ADP
7.5 mM ADP
5 mM ADP
2 mM ADP
0 ADP
15
10
5
1/v
.01
.02
.03
.04
1/(AcCoA) μM

Figure 6. Coenzyme A inhibition of acetyl CoA hydrolase.
See methods section for assay details.


130μM CoA
65 μM CoA
26 μM CoA
0 CoA
1/V
50
40
30
20
.01
.02
.03
.04
1/(AcCoA) μM

strength becomes a factor.

Kinetics of Acetate Activation. Kinetic studies on the effects of acetate on acetyl CoA hydrolase are presented in Figure 7.

Specificity of Acetate Activation. Due to this unusual product effect, studies were carried out to determine the nature of the activation by acetate and closely related compounds. These data are presented in Table IV. Further data may be found in alternate substrate studies. All solutions were brought to pH 8.1 by addition of NaOH.

Acetate Inhibition of Succinyl CoA Hydrolysis. During alternate substrate studies an inhibition of succinyl CoA hydrolysis by the G-200 preparation was noted. An R/A ratio of 1.8 was obtained. This effect is discussed in the section on alternate substrate studies.

Tissue Distribution Experiments

Rationale. Tissue distribution of acetyl CoA hydrolase has been studied before (5), but using phosphate buffer at 100 mM in the absence of acetate activation of the enzyme. Using the AXG systems for homogenization and acetate to enhance activity, much higher acetyl CoA hydrolase activities have been found in the various organs. The nonionic detergent, Tergitol-NPX, is an excellent solubilizer for these experiments, the yield of enzyme being a function of Tergitol as shown in Figure 8.

In these studies total activity, A/R for acetyl CoA, and R/A for succinyl CoA were determined. Because these results presented in Table V are from only three rats, individual data rather than averages are presented.

Figure 7. Acetate activation of acetyl CoA hydrolase. Assays performed in 100 mM Tris-Cl, with acetate varied as indicated, and NaCl added so that total salt was 100 mM. Enzyme used was from Sephadex G-200.

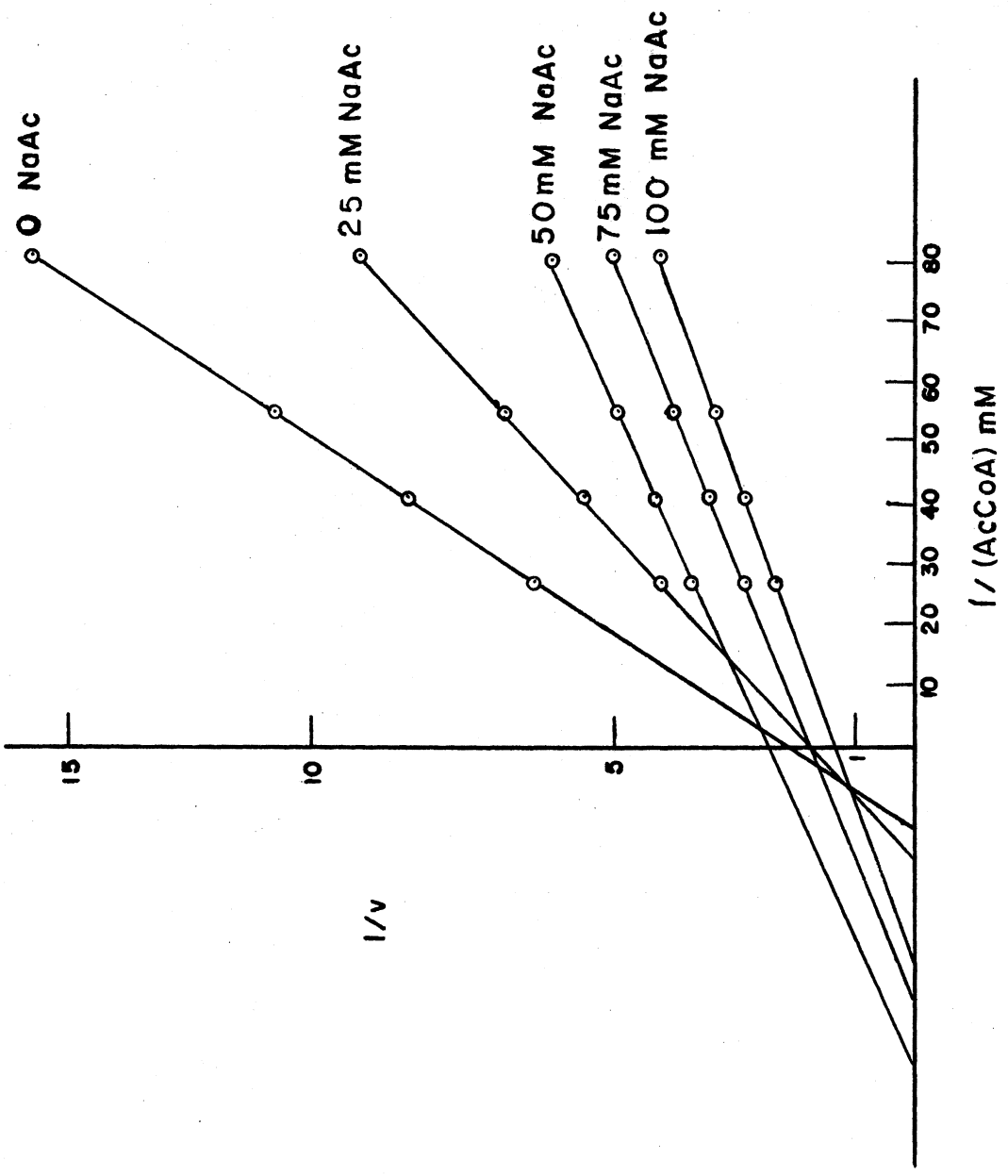

0 NaAc
25 mM NaAc
50mM NaAc
75mM NaAc
100 mM NaAc
1/v
15
10
5
1
10
20
30
40
50
60
70
80
1/(AcCoA) mM

TABLE IV

STUDIES ON PRODUCT ACTIVATION IN ACETYL COA HYDROLASE

Compound	Concentration	Fold Activation	Enzyme Source
Tris-Cl	200 mM	1.0	Off G-200
Tris-Cl Naacetate	100 mM 100 mM	2.7	Off G-200
Tris-Cl Nafluoroacetate	100 mM 100 mM	1.6	Off G-200
Tris-Cl Acetaldehyde	200 mM 100 mM	1.0	Off G-200
Tris-Cl Liacetoacetate	200 mM 100 mM	1.1	Off HA
Tris-Cl 3-OH-Butyrate	200 mM 100 mM	1.4	Off HA
Tris-Cl Napropionate	40 mM 250 mM	1.7	Off HA
Tris-Cl Nabutyrate	40 mM	1.1	Off HA

Figure 8. Solubilization of acetyl CoA hydrolase with Tergitol-NPX. See text for discussion.

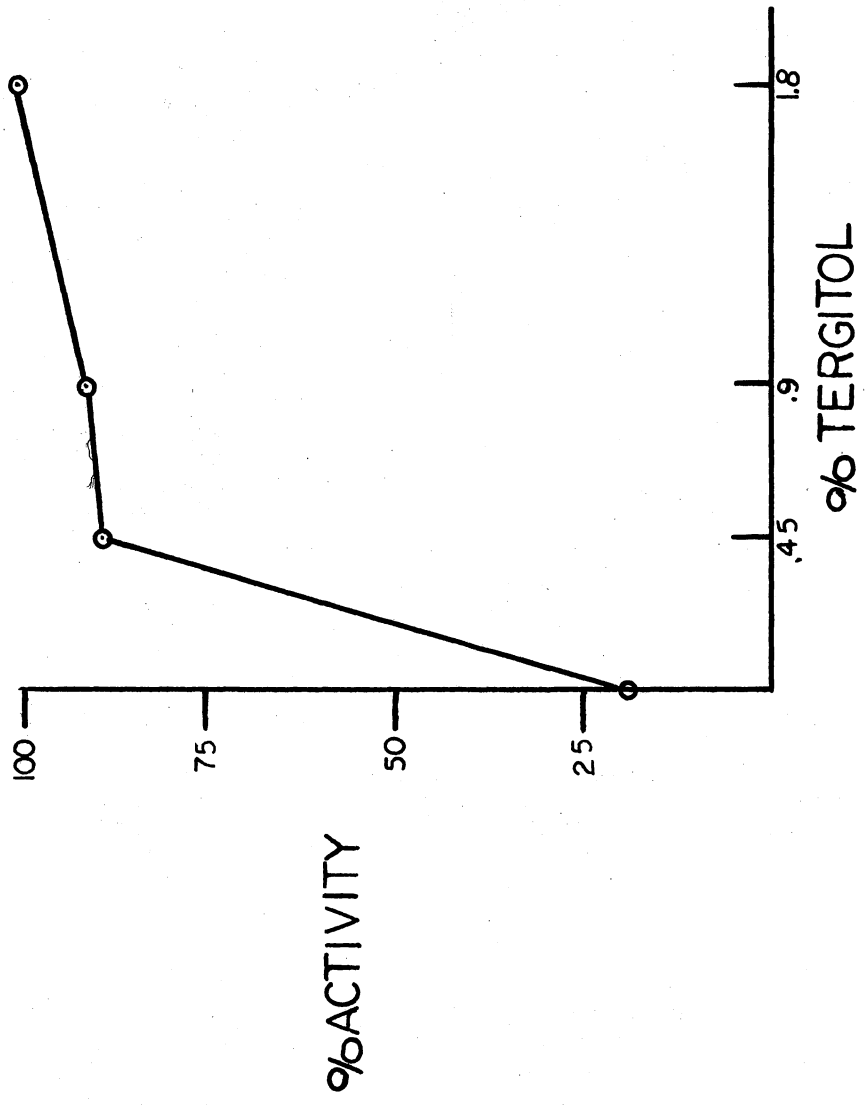

100
75
50
25
%ACTIVITY
.45
.9
1.8
% TERGITOL

TABLE V

CHARACTERISTICS AND DISTRIBUTION OF ACETYL COA HYDROLASE IN VARIOUS RAT TISSUES

Tissue	Activity**	A/R, Acetyl CoA	R/A, Succ CoA
Cerebrum	8.5, 12.2, 10.5(s)†	3.4	1.3
Liver	3.1, 4.2, 6.1(s)†	1.6	1.4
Kidney	11.9, 11.9, 13.5(s)†	2.8	1.2
Heart	5.5	2.0	1.2
Leg Muscle	0	-	-
Lung	2.7	2.9	1.0*
Testes	2.9, 2.9(s)	nd	nd
Cerebellum and Pons	7.3	nd	nd

*Very low activity.

**Activity expressed in micromoles/min/g tissue.

†Rat fasted 24 hours.

Alternate Substrate Studies

Rationale. To define a role for acetyl CoA hydrolase, a knowledge of substrates utilized by the enzyme is needed. Although only acetyl CoA was used to follow the purification of the enzyme, the purified preparation retained the ability to hydrolyze other thioesters. These experiments are summarized in Table VI.

Alternate product activation was shown in cases, although the effect seems to be quite specific for acetate. In all systems stability of the thioesters at pH 8.1 was checked and appropriate corrections made. Substrate stability was a significant problem only with succinyl CoA, where hydrolysis due to pH sometimes equalled hydrolysis by enzyme.

Palmityl CoA, benzoyl CoA, and acetyl thiocholine were not substrates of the enzyme, whereas malonyl and octanoyl CoA were substrates at approximately 100 μM concentrations.

Kinetic parameters varied greatly as a complex function of product concentration, ionic strength, and metal-ion concentration. The values obtained are listed in Table VII along with reaction parameters. Although earlier studies on acetyl CoA hydrolysis by the enzyme from Celite suggested a lower K_m (about 20 μM), the higher value observed with the purer enzyme from G-200 was obtained in 2 separate preparations and is the value of choice.

dl-Alpha Lipoic Acid Studies

The oxidized form of lipoic acid proved to be the most potent inhibitor of acetyl CoA hydrolase yet encountered. In Figure 9 is shown the noncompetitive inhibition due to lipoate. Analysis of the

TABLE VI

ALTERNATE SUBSTRATE AND ALTERNATE PRODUCT EFFECTS ON ACETYL COA HYDROLASE

Fold Activation of Hydrolysis of → by ↓	AcCoA[a]	PropCoA[b]	ButCoA[c]	AcAcCoA[d]	SuccCoA[e]
Naacetate 250 mM	2.8	1.3	1.1	1.3	0.7
Napropionate 250 mM	1.7	1.5	nd	nd	nd
Nabutyrate 250 mM	1.1	nd	1.4	nd	nd

[a] Acetyl CoA, 250 μM.

[b] Propionyl CoA, 250 μM.

[c] Butyryl CoA, 250 μM.

[d] Acetoacetyl CoA, 50 μM.

[e] Succinyl CoA, 200 μM.

TABLE VII

KINETIC CONSTANTS

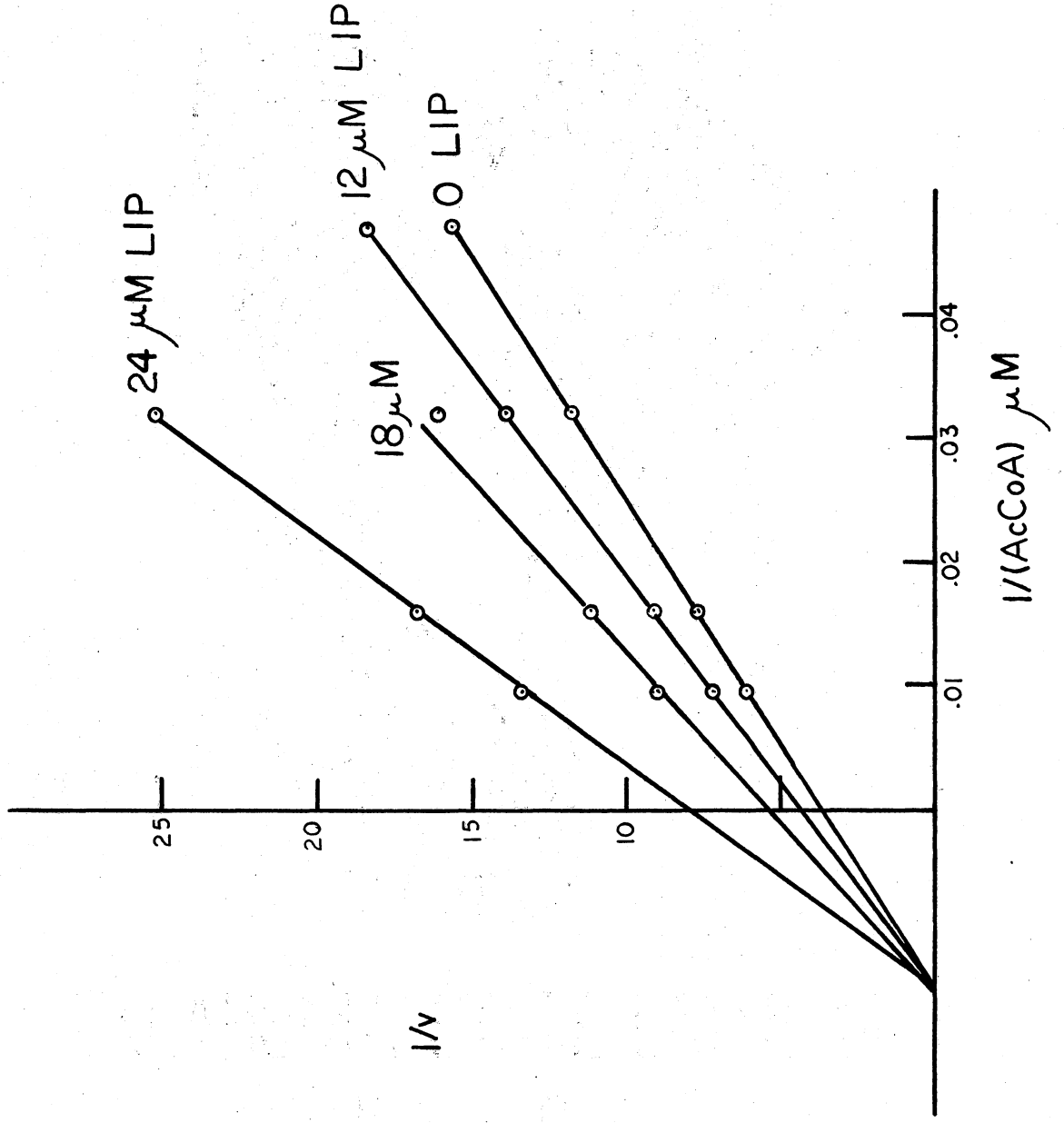
Substrate	Apparent K_m [a]	Apparent V_m [b]	Enzyme	Buffer
Acetyl CoA	15-70	0.05	Off G-200	A
Succinyl CoA	96	0.02	Off Hydroxyapatite	R
Propionyl CoA	50	0.04	Off Hydroxyapatite	AXG pH 7.4
Butyryl CoA	15	0.01	Off Hydroxyapatite	AXG pH 7.4
Acetoacetyl CoA	20	0.02	Off Celite[c]	R

[a] K_m in μM.

[b] V_m in mM^{-1} min^{-1}.

[c] This activity is retained in enzyme from Sephadex G-200.

Figure 9. Lipoic Acid inhibition of Acetyl CoA Hydrolase. Assays performed in buffer A using DTNB. Lip = dl alpha lipoic acid, which was used in absolute alcohol solution such that 10 µl gave the concentrations indicated.


24 μM LIP
18μM
12μM LIP
0 LIP
1/v
25
20
15
10
.01
.02
.03
.04
1/(AcCoA) μM

least-squares intercepts givea a K_i of about 5 μM.

Related compounds have been tried on acetyl CoA hydrolase with poor results. The reduced form of dl-lipoic acid was an inhibitor of acetyl CoA hydrolase when assayed by measuring absorption at 232 nm of the thioester bond, since the reduced lipoic acid would react with DTNB. Owing to the low sensitivity of this assay, the data must be interpreted with caution. Further study is needed. The amide of lipoic acid proved to be a very poor inhibitor, inhibition not being apparent until the concentration reached approximately 0.5 mM; this was not studied further. Hydrolysis of propionyl and succinyl CoA was significantly inhibited by dl-alpha lipoic acid. This was shown on single assays with 250 μM substrate and 5 μM lipoate, using enzyme from hydroxyapatite.

Miscellaneous Results

Several compounds tested with acetyl CoA hydrolase showed little or no effect (Table VIII). These studies were done because one of the functions of a hydrolase might be to transfer groups to water or another acceptor. Several possible acceptor molecules (Figure IX) did not affect the rate of acetyl CoA hydrolysis.

TABLE VIII

SUBSTANCES LACKING SIGNIFICANT EFFECT UPON ACCOA HYDROLASE

Compound	Concentration	Buffer	Enzyme
Adenosine	1 mM	R	Off Celite
Palmityl CoA	0.08 mM	R	Off HA
Acetylthiocholine	1 mM	R	Off HA
Ethyl acetate	1%	R	Off HA
RNase	0.01 mg	A	Off G-200
Na_2EDTA	10 mM	R	Off Celite
Guanosine	1 mM	R	Off Celite
Choline chloride	1 mg	R	Off Celite
N-Acetylaspartate	1 mg	R	Off Celite
Aspartate	1 mg	R	Off Celite
KCl*	100 mM**	R/2	Off G-200
KI	100 mM	R/2	Off G-200
LiCl	100 mM	R/2	Off G-200
NaCl	100 mM	R/2	Off G-200
$KAsO_4$	100 mM	R/2	Off G-200
dl-Carnitine-HCl	10 & 100 mM	R/2	Off G-200
l-Epinephrine	0.1 mM	R	Off G-200
GABA	1 mM	R	Off G-200
l-Lysine	1 mM	R	Off G-200
Seramine	1 mM	R	Off G-200
l-Glutamine	1 mM	R	Off G-200
3',5'-Cyclic AMP	0.03 mM	R	Off HA
Acetaldehyde	100 mM	R	Off G-200
Phosphoenolpyruvate	1.2 mM	R	Off HA
Fructose diphosphate	1 mM	R	Off HA
Biotin	0.001 mg	A	Off Celite
Pyridoxal phosphate	0.01 mM	R	Off HA
Glutathione***	0.05 mg	A	Off HA
Sodium fluoride	10 mM	R	Off Celite
Oxalacetic acid	10 mM	R	Off Celite
Thiamine Pyrophosphate	0.1 to 1 mM	A,R	Off HA
Ethanol	10%	A,R	Off HA

*See discussion.

**All materials added over 1 mM were brought to pH 8.1 before assay.

***Substrate was acetoacetyl CoA.

CHAPTER IV

DISCUSSION

Purification

The scheme reported herein is the first described for a brain short chain thioesterase, and, despite its shortcomings, with diligence will supply a stable, reproducible enzyme preparation. Assuming (for purpose of calculation only) that the enzyme obtained from Sephadex G-200 with a specific activity of 55 is pure, there is about 0.2 mg enzyme per gram cerebrum. Since this project resulted from other studies on rat brain, cerebrum was the primary source of enzyme. Further characterization will require many more rats or some new and better source of enzyme.

Physical Properties

The role of metal ions in the reaction is not well defined by these studies. Is there stabilization due to metal-ion protein interaction at low ionic strength or is this only part of the consequences of this interaction? The enhancement of stability and activity at lower ionic strength, abolished by higher ionic strength, and the lack of kinetic effects of metal ions at higher ionic strengths, pose questions beyond the scope of the present study.

Also unexplained are the phenomena observed during the spectral studies of the enzyme from G-200. Before meaningful conclusions can be made from data presented in the scans, much more must be known regarding

purity of the preparation. What are the factors that cause these spectra? These questions remain unanswered.

The purification procedure presented herein gives low yields; however, recent work (7) implies that the mitochondrial activity purified here may not be the only such activity in brain, a deduction reinforced by the findings cited above (4) for a cytosolic activity. A stability system has been developed which allows storage of the purified enzyme for four months without measurable loss, and much of the initial losses of enzyme observed during the preliminary studies can be avoided by using the AXG systems, minimizing the storage of lyophilized mitochondria, and concentrating the protein before application to hydroxyapatite.

Inhibition Studies

Acetyl CoA hydrolase was inhibited by a wide range of compounds which can be divided into two general categories. The first group which includes malonate, citrate, and orthophosphate, has high K_i's and was not studied extensively. The second set was composed of compounds possessing cofactor activity in other cellular reactions. In this group are ATP, ADP, NAD, NADH, CoA, and dl-alpha lipoic acid. Of these, four compounds were studied in some detail. Both ATP and ADP are non-competitive inhibitors, although classical plots were not obtained. CoA was shown to be a product inhibitor with uncompetitive characteristics, but again departure from simple Michaelis-Menton kinetics was observed. dl-Alpha lipoic acid showed classical noncompetitive kinetics. In defense of the data it may be stated that at no time did the fitted lines deviate more than 8% from the original data. Unfortunately,

insufficient data about the system exists to postulate a physiological role for any of the observed effects.

Substrate and Product Studies

The activity of acetyl CoA hydrolase is stimulated by high acetate concentrations. This effect is at least twofold that of other product-substrate interactions studied, although hydrolysis of other short-chain thioesters is significantly activated by the organic acid product. The activation of acetyl CoA hydrolysis by acetate shows irregular kinetics, which are not readily explained. However, this effect permits a sensitive assay for acetyl CoA hydrolase in crude tissue preparations. Moreover, acetate activation was shown to occur in varying amounts in several tissues. A previous study (5) showing much lower values for acetyl CoA hydrolase in all tissues is suspect in light of the present data since several techniques used in that study raise doubt as to its validity. First, the tissues were lyophilized to release activity, as opposed to the use of detergent here; lyophilization causes some loss of activity. Second, the use of 0.1 M phosphate at pH 7.0 is questionable, since phosphate is an inhibitor (K_i~40 mM) of purified acetyl CoA hydrolase and preliminary pH studies show an activity optimum of 8.1 in enzyme derived from brain. Clearly their observation (5) that brain can hydrolyze at most 0.6 μmoles/min/g brain is much too low. In the present study the lowest activity observed was 8 μmoles/min/g brain.

The high concentrations necessary for acetate activation seem to preclude a physiological role for this effect. However, Whittaker (16) has calculated the concentration of acetylcholine in synaptosomes to be 200 mM, suggesting that after neurotransmission and acetylcholine

hydrolysis, at least momentarily, high concentrations of acetate may occur. Studies on the subcellular distribution of the enzyme are necessary to determine if the activity is associated with synaptosomes.

Pragmatic Observations

Several useful observations have been made in the course of this study. First of all, in order to limit the deleterious effects of acetyl CoA hydrolase on studies with pyruvate carboxylase it is advisable to allow the lyophilized mitochondria to remain in vacuo and frozen as long as possible. Also, storage in lower ionic strength buffers at low protein concentrations when feasible, will lower hydrolase activity.

The inhibitor of choice is lipoic acid, subject to considerations of solubility and the effect of trace amounts of ethanol on the system under study. CoA can also be used, e.g. in the acetyl CoA synthase assay system 0.8 mM CoA was used, a concentration which totally inhibits the hydrolase.

Another benefit is the quantitative reaction with short-chain thioesters, a proven fact since the hydrolase reaction and the citrate synthase reaction yield similar color with DTNB. Thus the hydrolase can be used to quantitatively determine short-chain thioesters more rapidly and with greater sensitivity than by the classical hydroxamate test, even in the presence of labile esters such as acetylcholine.

The lipoic acid inhibition of acetyl CoA hydrolase may explain the conflicting data of Seaman (13), who reported a lipoic acid activation of acetyl CoA synthase, and Gal (14) who did not observe this effect in an acetone powder of liver. Inhibition of the hydrolase by lipoate

could give an apparent activation of acetyl CoA synthase and acetone fractionation (see purification comments) destroys hydrolase activity. Thus the apparent conflict may be reconciled by the present observations.

Unanswered Questions and Speculations

The inhibition observed with lipoic acid suggests two possible physiological roles for the enzymes. First, this cofactor may be catalytically involved in the hydrolase reaction, albeit in reduced form as opposed to the inhibitory disulfide. An experiment remaining to be done is to see if the observed arsenite inhibition is reversed by 2,3-dimercaptopropanol, which would imply vicinal sulfhydryl action in catalysis. The other possibility is that the oxidized lipoate inhibits by binding at another site than acetyl CoA, which in light of the finding of Ebner et al. (15) that UDP-galactose hydrolase is inhibited by tetrahydrofolic acid, suggesting that control of hydrolase activity may be by coenzymes produced in excess of prosthetic necessity. However, much more data are necessary to elevate this idea from the realm of speculation.

Other questions are: (a) Does the preparation here reported represent a single or several activities? and (b) Is acetyl CoA hydrolase an artifact of tissue manipulation? If not, what is its role in the living cell? To answer the first question, the experiment remaining to be done is polyacrylamide electrophoresis of the preparation, followed by activity staining, possibly with DTNB, and variation of substrate. This would require a substantial investment of time and animals. However, until it is done one can only speculate that several

similar hydrolases may possibly explain the nonclassical kinetics observed.

Concerning the second question, the best argument for a physiological role for the hydrolase is that the activity liberated by lyophilization and detergent solubilization shows similar acetate activation effects. Also, Utter (7) has shown a cytosolic acetyl CoA hydrolase which is cold-labile and activated by ATP. These observations imply that short-chain thioester hydrolysis is a vital factor, although its precise role has yet to be defined.

CHAPTER V

SUMMARY

A short-chain acyl CoA hydrolase has been purified 1375-fold from rat brain and some of its kinetic and physical properties have been studied. The enzyme has a molecular weight of 160,000 and shows a pH optimum of 8.1 and an ionic strength optimum between 100 and 300 mM. The enzyme after passage through G-200 shows absorbance peaks at 266 and 230 nm. Divalent metal ions stabilize and activate it at low ionic strength, but lose this effect at higher ionic strengths.

The activity with respect to acetyl CoA is product-activated by acetate, an effect which is relatively specific and which follows non-classical kinetic patterns. This effect was also found in enzyme from other tissues, with rat kidney showing activation similar to brain.

The enzyme is inhibited by CoA, adenine nucleotides, orthophosphate, malonate, and citrate. The best inhibitor found is dl-alpha lipoic acid, with a K_i of 5 μM.

Other substrates for the system are propionyl CoA, butyryl CoA, succinyl CoA, acetoacetyl CoA, malonyl CoA, and octanoyl CoA. Michaelis constants for these substrates are below 100 μM (15-96 μM range), and the maximum velocities vary over a six-fold range, with acetyl CoA having the highest and butyryl CoA the lowest. A small amount of product activation was shown for the other substrates (10-70%), but of lesser extent than for the acetyl CoA system (300%).

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