STUDIES IN VITRO ON THE METABOLISM OF GLUTARIC ACID-3-C^{14}

By

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OF GLUTARIC ACID-3-C^{14}

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CHAPTER I

INTRODUCTION

In nature glutaric acid has been shown to exist in sugar diffusion juice (1), plants (2, 3), sugar beets (4), wood distillation products (5), tobacco smoke (6), coal (7), urine (8), and ale (9). In vitro studies showed that glutaric acid acted as competitative inhibitor of glutamic dehydrogenase (10), fumarase (11), and succinic acid dehydrogenase (12), and inhibited the growth of certain plant tissues when it was present in the culture media (13, 14). Animal experiments presented evidence that glutaric acid was nephropathic (15) and enhanced the morbidity and mortality of animals infected with certain bacteria (16).

The interest in glutaric acid metabolism started in 1913 when Ringer (17) postulated the conversion of lysine to glutaric acid on the basis of the observation that neither lysine nor glutaric acid is glucogenic. This suggestion was confirmed thirty years later by Neuberger and Sanger (18).

In 1948 Borsook et al. (19) isolated radioactive α-ketoglutaric acid and glutaric acid when α-aminoadipic acid-6-C^{14} was incubated with guinea pig liver homogenates. Since then extensive studies on the metabolism of labeled lysine in intact animals (20, 21, 22) and liver homogenates have provided evidence for the following pathway of lysine catabolism:

lysine $\rightarrow$ α-keto-ε-amino caproate $\rightarrow$ Δ'-dehydropipecolate $\rightarrow$ L-pipercolate $\rightarrow$ L-α-aminoadipate $\rightarrow$ α-ketoadipate $\rightarrow$ glutarate

By isolating radioactive glutaric acid from the urine after the administration of DL-tryptophan-7a-C^{14} or 3-hydroxyanthranilic acid-H^{3}, Gholson et al. (23) showed that glutaric acid was a product of tryptophan metabolism.

Glutaric acid is also known to be formed during L-xylose fermentation by Aspergillus niger (24).

The main interest in glutaric acid metabolism started when Rothstein and Miller (25) isolated urinary acetate-1-C^{14}, acetoacetate-1-C^{14}, and glucose-3,4-C^{14} following the administration of glutaric acid-1,5-C^{14} to normal and phlorhizinized rats. On the basis of the labeling patterns, these authors postulated two possible pathways:

1. Glutaric acid $\longrightarrow$ butyrate $\longrightarrow$ acetoacetate $\longrightarrow$ acetate.
2. Glutaric acid $\longrightarrow$ β-ketoglutarate $\longrightarrow$ acetoacetate $\longrightarrow$ acetate.

Subsequently the same authors suggested that α-ketoglutarate and acetate were the products of glutarate catabolism (21). After administering lysine-6-C^{14} to dogs, Miller and Bale (26) were able to isolate liver and plasma protein glutamic acid having 71 per cent of the activity in the γ-carboxyl and 25 per cent in the α-carboxyl group. This again suggested to them that glutarate was converted, in part, directly to α-ketoglutarate.

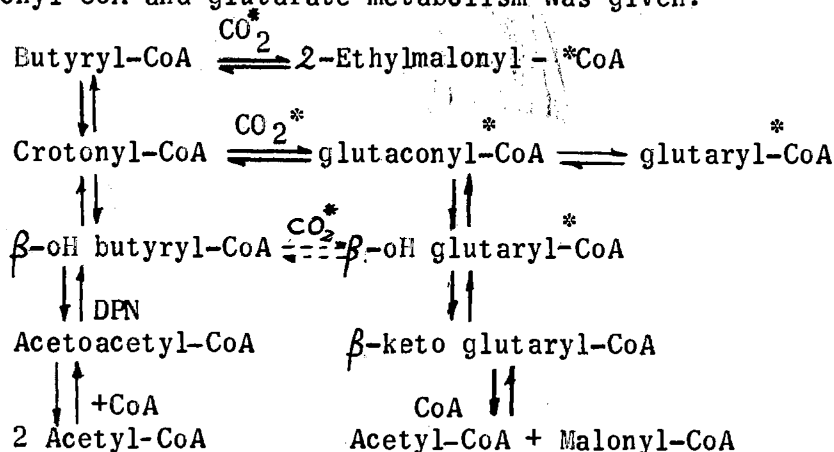
From the studies of Rothstein, Miller, and Bale (21, 22, 26) two main pathways were suggested for the degradation of glutaric acid. One route yields acetate, and the other involves a direct conversion to the carbon chain of α-ketoglutarate. In an effort to assess the relative importance of each pathway, Hobbs and Koeppe (27) studied the metabolism of glutaric acid-3-C^{14} in intact rats. Tissue glutamic acid, aspartic acid and alanine were isolated. Each amino acid studied had most of its

activity located in the carboxyl carbons. This strongly suggested that carbon 3 of glutaric acid is metabolized via the carboxyl position of an intermediate of the Krebs tricarboxylic acid cycle since metabolism via the non-carboxyl positions of these intermediates inevitably labels the non-carboxyl positions of the amino acids isolated (28). These authors concluded that glutarate was metabolized exclusively via acetate. A reasonable pathway would be (27): glutrate ⟶ glutaconate ⟶ β-hydroxyglutarate ⟶ acetonedicarboxylic ⟶ acetoacetate ⟶ acetate.

So far all the pathways mentioned above are based on intact animal experiments. In 1960 Rothstein and Greenberg (29) were able to demonstrate that $C^{14}O_2$ was released from glutarate-1,5-C^{14} by rat liver mitochondria. Subsequently the same authors presented some evidence that β-hydroxyglutarate was an intermediate in glutarate catabolism (29, 30).

Later Stern et al. (31,32) suggested that acyl-CoA derivatives were the actual intermediates in glutarate metabolism based on the findings that animal tissue, including liver, possessed a specific glutarate-activating enzyme which catalyzed the conversion of glutarate to glutaryl-CoA in the presence of CoA and ATP.

While studying enzymic carboxylation of crotonyl-CoA, Tustanoff and Stern (33) claimed that they isolated radioactive glutaconyl-CoA, β-hydroxyglutaryl-CoA and malonyl-CoA. A scheme involving both carboxylation of crotonyl-CoA and glutarate metabolism was given:



Recently the same authors presented some evidence suggesting that glutaconyl-CoA was an intermediate (34). However, they were unable to prove that crotonyl-CoA was derived from the decarboxylation of glutaconyl-CoA. The whole scheme still remains obscure.

Glutarate can also be used by microorganisms as a hydrogen source (35, 36). Of interest is the demonstration that extracts of Psudomonas fluorescens convert glutaryl-CoA to CO_2 and two moles of acetyl-CoA (37).

The purpose of this investigation was to further elucidate the nature of intermediates of glutarate metabolism in vitro. Since mitochondria are known to possess all the enzymes for fatty acid oxidation, one approach was to incubate cell-free rat liver mitochondria with glutarate-3-C^{14} and a suspected metabolite, hoping that the added compound would be equilibrated in some manner with similar biologically formed material. When these experiments using glutarate-3-C^{14} and acetonedicarboxylate, glutaconate, β-hydroxyglutarate, ethylmalonate, or butyrate were conducted, the acids isolated did not contain significent radioactivity. On the other hand acetoacetate was found to be labeled primarily in the carbonyl carbon. These results indicated that glutarate is converted to acetoacetate prior to acetate.

As mentioned before, glutaric acid metabolism may resemble fatty acid oxidation. One difficulty in studying fatty acid oxidation is that no intermediate between the fatty acid and the final products of oxidation accumulate (38). This difficulty is encountered both in whole animal experiments and those using cell-free mitochondria (38). Apparently only at the level of the isolated soluble enzymes does it become possible to interrupt the sequence in such a way as to accumulate intermediates.

In the light of these facts, an effort was made to solubilize

mitochondria, but none of the solubilized systems appeared to be active in glutarate degradation.

However some preliminary evidence has shown that the extract of rat liver acetone powder was able to oxidize glutaryl-CoA but not glutarate as measured by Warburg manometric techniques. Further investigation remains to be done.

CHAPTER II

EXPERIMENTAL

A. Preparation of Compounds.

Glutaric acid-3-C^{14} was synthesized from paraformaldehyde-C^{14} and diethylmalonate according to the method of Hobbs and Koeppe (27). The synthesis is summarized in Figure 1.

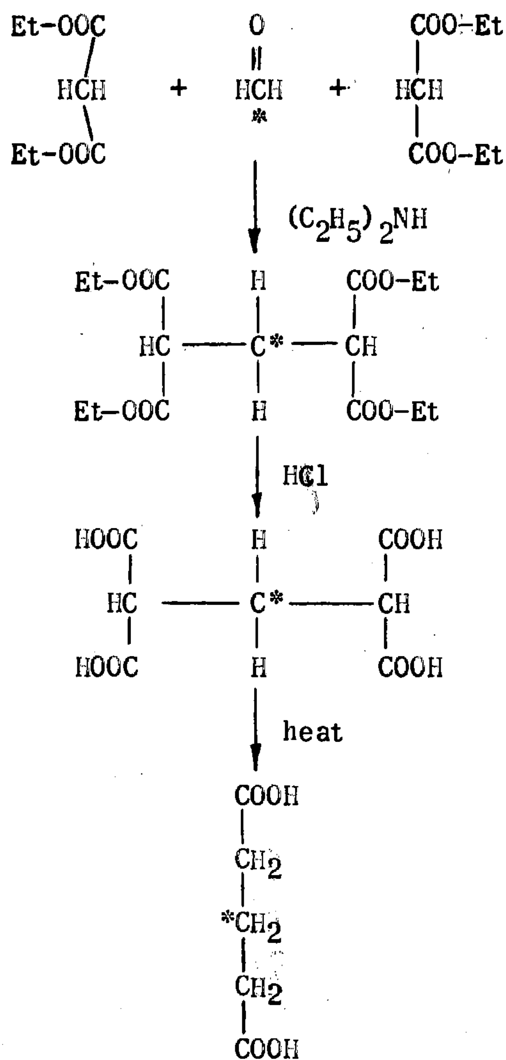


Figure 1. Synthesis of Glutaric Acid-3-C^{14}

Glutaconic acid (trans form) was synthesized by Dr. W. E. Wilson from condensation of disodiomalonic ester and chloroform followed by decomposition of the yellow sodio-derivatives with dilute acid to yield 1,3 dicarbethoxyglutaconic ester which gave glutaconic acid on hydrolysis and decarboxylation (39). The synthesis is summarized in Figure 2. The crystals were purified from ethyl acetate.

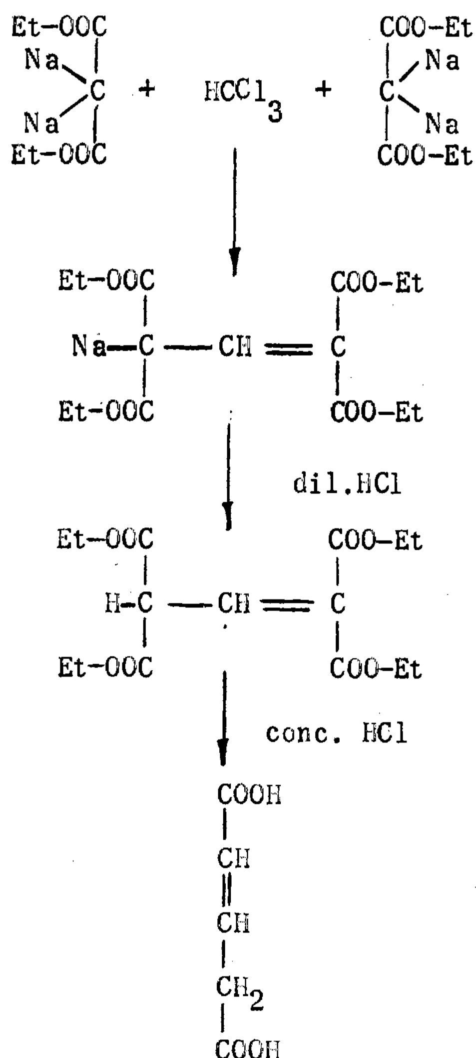


Figure 2. Synthesis of Glutaconic Acid

Acetonedicarboxylic acid was prepared by Isa K. Mushahwar from citric acid according to the method of Adams et al. (40), and recrystallized

from ethyl acetate. Since efficient cooling was required during both the addition of citric acid to fuming sulfuric acid and before final filtration of the acetonedicarboxylic acid, the reaction was run at about $-12^{o}C$ during the five hours of synthesis.

β-Hydroxyglutaric acid was prepared by Dr. R. E. Koeppe in 70 per cent yield by the reduction of sodium salt of acetonedicarboxylic acid in aqueous solution with two moles of sodium borohydride (41, 42).

Sodium acetoacetate was prepared by hydrolysis of acetoacetic ester at room temperature with 0.5 N sodium hydroxide according to the method of Ceresol (43). After hydrolysis the solution was lyophilized to remove ethanol.

Ethylmalonic acid was prepared by Dr. R. E. Koeppe according to the method of Weiner (44).

Crotonic acid (obtained from K & K Laboratories, Inc.) was recrystallized from hot petroleum ether. This crystalline product melted at $72^{o}C$.

Glutaryl anhydride was prepared according to the method of Fieser and Martin (45). A mixture of 0.5 mole of glutaric acid and 1.5 moles of acetyl chloride was gently refluxed on the steam bath until all the acid dissolved; this generally required two hours. The solution was allowed to cool undisturbed and was finally chilled in an ice bath overnight. The crude crystals were collected on a Buchner funnel and washed with two portions of n-hexane. The crystalline product was purified by dissolving in hot benzene followed by the addition of equal volume of normal hexane. The recrystallized material was filtered with suction and air dried. The glutaryl anhydride thus obtained had a melting point of $56^{o}C$. C^{14}-labeled glutaryl anhydride was prepared from glutaric acid-3-C^{14} by the same method except that after refluxing on a steam bath the mixture was

evaporated to dryness under anhydrous condition and the product was purified as described above.

Glutaryl-coenzyme A was prepared (46) by dissolving 2 μmoles of coenzyme A preparation in 0.2 ml. of ice cold water. To this solution 3μ moles of glutaryl anhydride was added, followed by sodium bicarbonate until the pH was 7-7.5. The mixture was kept in an ice-bath and shaken frequently. The reaction appeared to be complete within one hour, as shown by the disappearance of the sulfhydryl group by the nitroprusside test (47). At 0° the glutaryl-coenzyme A was stable at pH 7-7.5 for several hours.

Glutaconyl anhydride could not be easily prepared by conventional methods (45, 48). Instead a mixed anhydride of ethyl hydrogen carbonate and the acid was used (49). To prepare the mixed anhydride, 1 mmole of anhydrous glutaconic acid and 1 mmole anhydrous pyridine (redistilled over barium hydroxide) were dissolved in 5 ml. anhydrous ether. The mixture was cooled to 0° to -5° in an ethanol-ice bath. Then 1 mmole of ethyl chloroformate was added dropwise with efficient stirring. After standing an hour at 0° the insoluble pyridine hydrochloride was removed by centrifugation. To determine the content of the mixed anhydride an aliquot of the ether solution was added to 0.5 ml. of neutral 2 N hydroxylamine solution. The mixture was shaken. After 5 minutes a suitable aliquot was used for quantitative measurement of the hydroxamic acid by the method of Lipmann and Tuttle (50). The yield of the mixed anhydride was about 80 per cent.

Pantetheine was prepared by the reduction of pantethine with sodium borohydride. 0.2 ml. of pantethine solution (200 mg/ml) and 1.2 ml. distilled water were mixed with 12 mg. of sodium borohydride. The mixture

was incubated at room temperature for an hour. Then the pH was brought to 2 with 3 N HCl. A suitable aliquot was taken for quantitative determination of free sulfhydryl group by the nitroprusside test (47). The yield was 95 per cent.

Glutaryl and glutaconyl pantetheine were prepared by mixing 1 ml. of the panthetheine (pH 8.0) and 30 μ moles of acid anhydride. The mixture was allowed to stand for ten minutes. Then the pH was adjusted to 6.0 with 1 N HCl. The aqueous layer was extracted three times with an equal volume of ether. An aliquot of the aqueous layer was taken for hydroxamic acid determination (50). The yield of glutaconyl pantetheinate was about 60 per cent. These low yields were due to the occurrence of side reactions in which the sulfhydryl groups added to the double bonds of the anhydride and thiol esters (51).

B. Preparation of Enzymes.

Mitochondria were isolated from rat liver according to the method of Schneider and Hogeboom (52, 53) with isotonic sucrose containing 0.002 M Tris buffer (pH 7.3). Adult albino rats were fasted overnight to remove glycogen from the liver. The rats were killed by decapitation. Unless otherwise specified all the following manipulations were carried out in the temperature range of 0^{0} to 5^{0}. The livers were cooled in a beaker placed in an ice bath and were sliced with a scissors into a pulp. The liver pulp was then weighed and homogenized in 9 parts of ice cold 0.25 M sucrose in a Potter-Elvehjem homogenizer. The homogenate was first centrifuged at 600 x g to sediment nuclei, unbroken liver cells and erythrocytes. The mitochondria fraction was obtained by centrifuging the homogenate at the speed of 8500 x g. The fraction containing

mitochondria was washed twice with 5 volumes of 0.25 M sucrose and finally was suspended in 2 ml. of isotonic sucrose per gram of original tissue. The liver mitochondria have a half-life time of several hours at best (54). The isolation procedures are summarized in Figure 3.

Sonic extracts of mitochondria were obtained by exposing the mitochondria suspension obtained above to sonic oscillation in a 10 Kc Raytheon Sonic Oscillator at an energy input of 1 ampere for various time intervals. Ice cold water was circulated through the oscillator during the operation. The treated suspension was centrifuged for ten minutes at 1,000 x g. The supernatant was used for enzyme assay.

Another soluble enzyme system was prepared by extracting acetone powders of rat liver mitochondria with dilute phosphate buffer (0.05 M; pH 7.3). The acetone powders were prepared according to the methods of Drysdale and Lardy (55). The twice washed mitochondria were suspended in a small amount of isotonic sucrose and homogenized with a large glass, Potter-Elvehjem homogenizer into 20 volumes of cold acetone ($-10^{o}C$). The protein precipitate was separated by centrifugation at -5° to -10° and rehomogenized into an additional 20 volumes of cold acetone. After centrifugation the residue was suspended in 20 volumes of cold, anhydrous, peroxide-free ether and centrifuged. The precipitate was rapidly spread in thin layers on filter paper and dried at room temperature. The soluble enzymes were prepared by extracting 1 gm. of acetone powder with 10 ml. dilute neutral buffer for one hour at 0°. The insoluble material was removed by centrifugation at 15,000 x g. for 10 minutes.

Acetone powder of rat liver was prepared by mixing diced fresh rat liver with 20 volumes of cold acetone ($-15^{o}C$) in a Servall Omni-Mixer at full speed for 2 minutes. The mixture was poured into a medium size

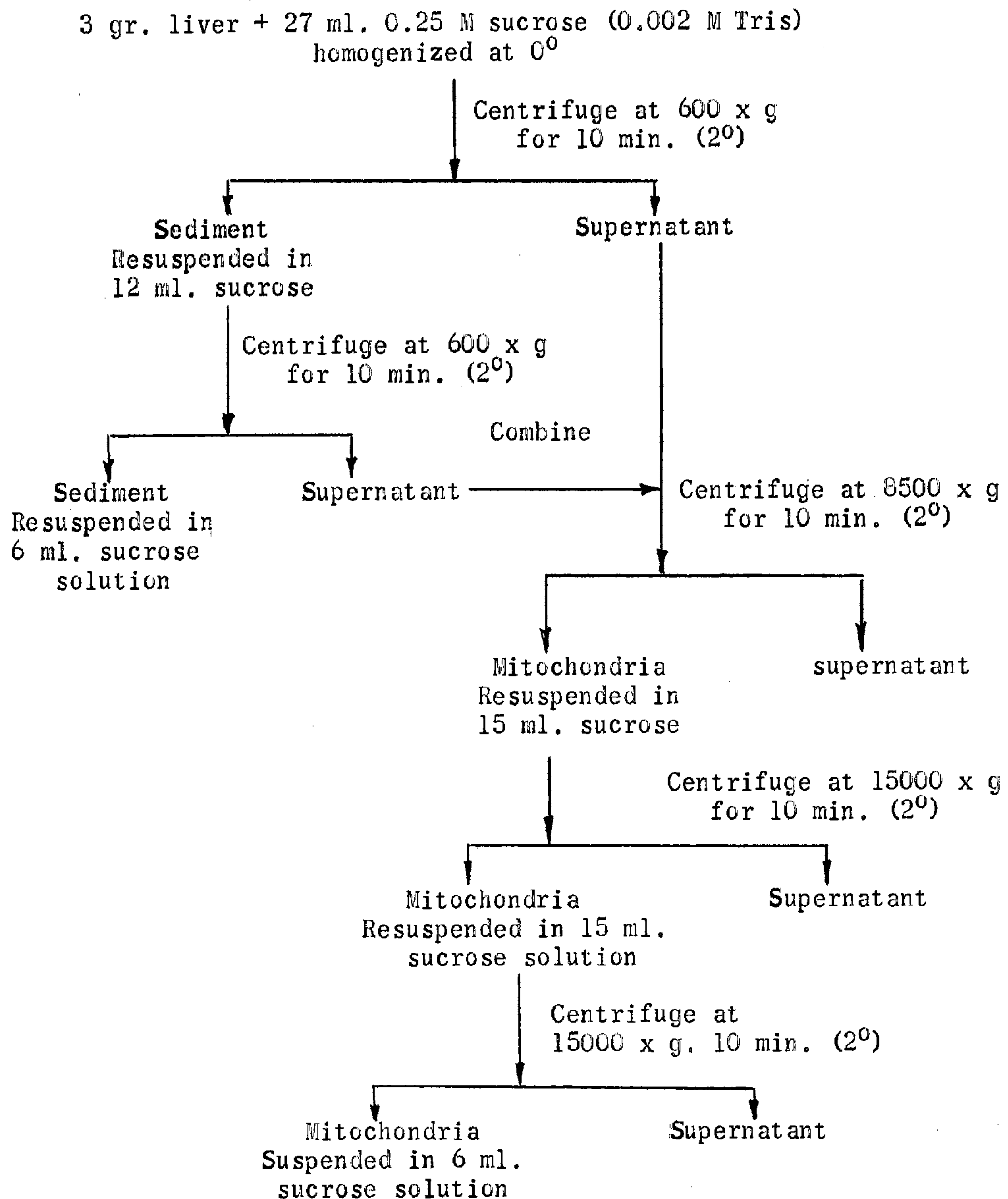


Figure 3. Isolation of Mitochondria.

Buchner funnel equipped with a filter paper. The liquid was removed by suction and the residue was washed several times with cold acetone. The filter cake was smeared on a watch glass and stirred several times with a flat spatula. A fine fluffy acetone powder was obtained. A 10 gm. liver will yield about 3 gm. of acetone powder. One gm. of acetone powder was extracted with 10 ml. 0.05 M neutral phosphate buffer for one hour at 0^{0}. The insoluble material was removed by centrifugation.

C. Experiments in Vitro.

Most in vitro experiments were carried out by conventional Warburg techniques (56). The manometers and flasks were calibrated by using a micrometer type calibrator described by Lazarow (57, 58). Incubations were made in air. The duration of the experiments varied from 2 to 3 hours. The various mixtures (TableIII) were added to the main compartment of the manometer cups. Substrate was added from the side arm after temperature equilibration had been attained (about 10 minutes) and after the taps of the manometers were closed. The final volume of the incubation mixture was 3.0 ml. excluding alkali in the center well (0.2 ml. 20% KOH). $C^{14}O_2$ was trapped in the alkali. Manometric readings were taken during the incubation period. The oxygen uptake values quoted in the text have been corrected for the thermobarometer values. In some instances incubation was done in the Schmidt apparatus (Figure 6). The incubation mixtures were added to the pear-shaped flask which was kept in a constant temperature water bath. CO_2-free air was swept through the apparatus. Care was taken not to reduce the pressure within the apparatus. Carbon dioxide evolved was trapped in 5 ml. of 1 N sodium hydroxide.

After incubation the $C^{14}O_2$ absorbed in alkali was assayed for

radioactivity. The incubation mixtures were treated with an equal volume of 95 per cent ethanol, allowed to stand at least 2 hours and centrifuged. In experiments 5, 6, and 7 (Table III) these solutions were made alkaline with 0.1 N NaOH and allowed to stand for 0.5 hour to insure hydrolysis of any CoA esters (59). The supernatant solutions were then treated with 0.3 to 1.2 mmoles of carrier acid. For keto and hydroxy acids, all the solutions were concentrated to dryness either by an air jet at room temperature (for β-hydroxyglutarate) or by lyophilization (for the keto acids). In the case of crotonic acid and glutaconic acid, the mixture was acidified and extracted with ether in an ether extractor overnight. The ether was evaporated under an air jet. The residues were used for isolation and degradation as described below.

Development of a spectrophotometric measurement of dye reduction by the enzyme in the presence of substrate presented very great difficulties. The first method used evolved measuring reduction of the dye in the presence of enzyme and substrate in a recording spectrophotometer. However, the rates of reduction obtained were negligible. The large amount of oxidant which was added in these assays may inhibit the enzymes (55). To circumvent the possible inhibition by large amounts of electron acceptor, a method reported by Drysdale and Lardy (55) was used. The dye was titrated into the reaction mixture only as rapidly as it was reduced by the enzyme. In this method, 6 μ moles of KPO_4 buffer, 3 μ moles of $MgCl_2$, 3 μ moles of ATP, 2.5 mg. of bovine plasma albumin, 1 μ mole of substrate, and enzyme were combined in a 5 ml. beaker. The mixture was incubated at 37° for three minutes. The solution was then stirred with a small magnetic stirring rod and 2,6-dichlorophenol indophenol solution

containing 5 μ moles of dye per ml. was added from a micro pipet at a rate sufficient to maintain a very pale blue tinge in the solution. The quantity of dye solution added in 15 minutes was recorded.

D. Chromatographic Procedures.

1. Column Partition Chromatography.

The organic acids used, namely β-hydroxyglutaric acid, acetone-dicarboxylic acid, glutaconic acid, ethylmalonic acid, butyric acid, glutaric acid, crotonic acid, and acetic acid, were isolated and purified by the techniques of Phares et al. (60). Chromatographic columns (30 x 1 cm.) were packed with Celite by the addition of a slurry containing 5 gm. of Celite (which had previously been thoroughly mixed with 3 ml. of 0.5 N sulfuric acid) suspended in a 10 per cent solution of acetone in n-hexane. A 3 to 5 cm. layer of anhydrous sodium sulfate was packed on top of the Celite column. Prior to use the column was washed with 50 ml. of chloroform saturated with 0.5 N sulfuric acid.

Each residue as described above was dissolved in 1 ml. of water and was transferred quantitatively to the top of the Celite column. The column was then eluted with 1, 5, 10, and 20 per cent butanol in chloroform. The flow rate was about 1 ml. per minute. Fractions were collected with the aid of an automatic fraction collector and were titrated with 0.1 N sodium hydroxide. Phenol red was used as indicator. Figure 4 shows a composite diagram of the position of the different acids.

As shown in Figure 4 glutaconic acid could not be separated from glutaric acid in the chloroform-butanol solvent system. Resolution of these two acids was obtained by using an ether-benzene system. In a trial run a mixture of twenty mg. of each acid was dissolved in one

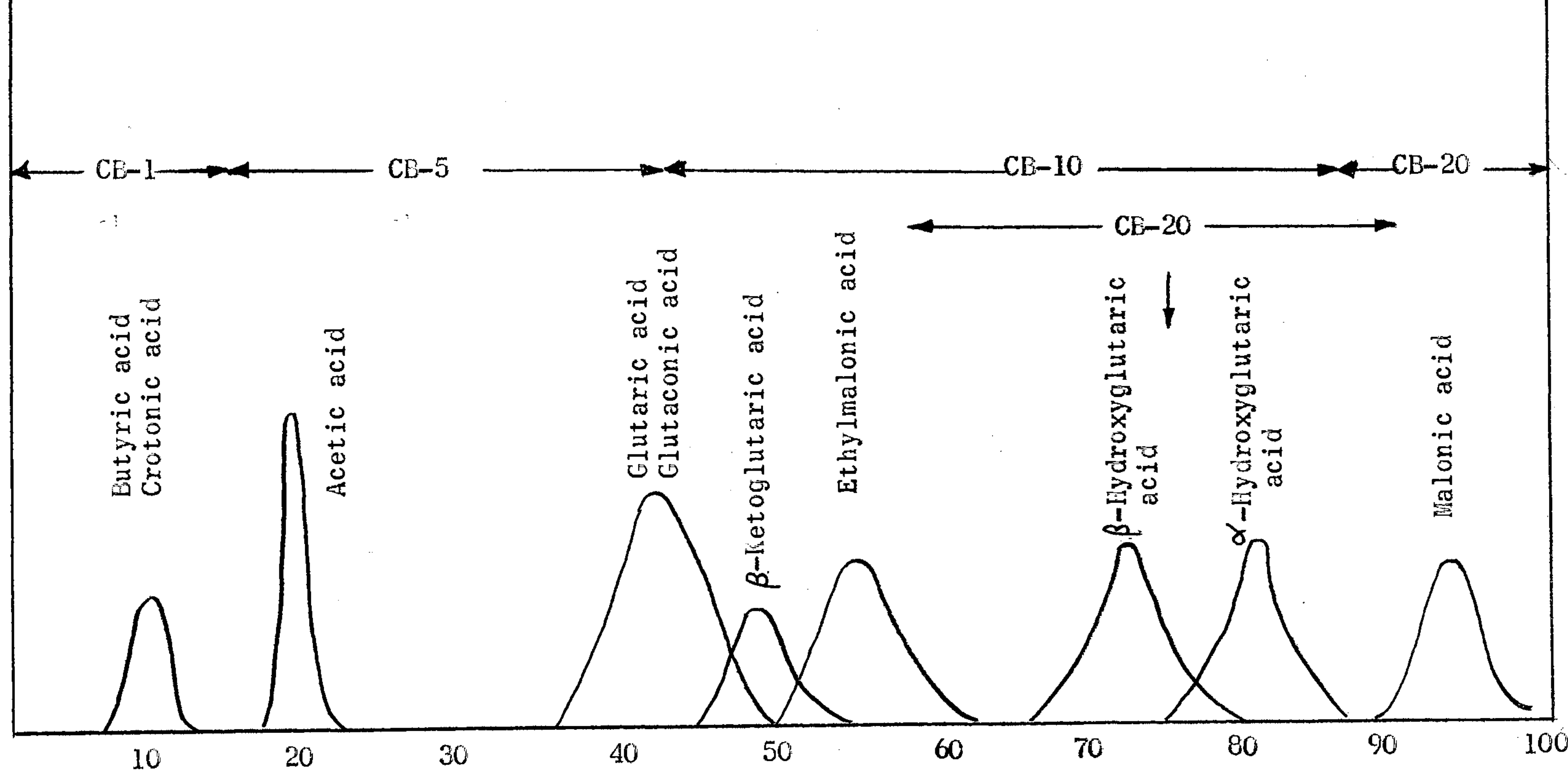


Figure 4. Celite Column Chromatography

ml. of water along with about 0.3 μc glutarate-3-C^{14}. The solution was transferred on to a Celite column (100 x 1 cm.), on top of which was packed about 4 gm. of anhydrous sodium sulfate and the column was eluted with 15 per cent ether in benzene. Fractions (9.5 ml.) were collected and every fourth fraction was titrated with 0.1 N sodium hydroxide after adding 1 ml. water. Glutaconic acid started coming off around fraction 60 and glutaric acid in fraction 77. One ml. of water was added to each fraction and the fractions were allowed to equilibrate for 12-14 hours. The glutaconic acid peak was located by diluting 0.5 ml. of the aquous layer of each fraction in a cuvette with 3 ml. of water. Optical density was then measured at 220 mμ(absorption maximum of glutaconic acid). Glutaric acid was identified by plating 0.2 ml. of the aquous layer on planchets, drying them under an infrared lamp. and determining radioactivity by a Geiger-Muller type counter. Figure 5 shows the position of the acids on an ether-benzene chromatogram.

2. Paper Partition Chromatography

The ascending paper chromatography technique was used to identify the different organic acids used. The acids were spotted on Whatman No. 1 paper and developed in different solvent systems (Table I). In the case of acidic systems the chromatogram was steamed to vaporize adsorbed acid and dried under an infrared lamp.

The R_f's of the acids in different systems are shown in Table II. Unsaturated acids, crotonic acid and glutaconic acid were brominated with bromine in carbon tetrachloride. The differences in R_f's between the brominated and the untreated acids provided a separation of glutaric and glutaconic acid. Unsaturated acids can also be identified by spraying with acidic permanganate solution.

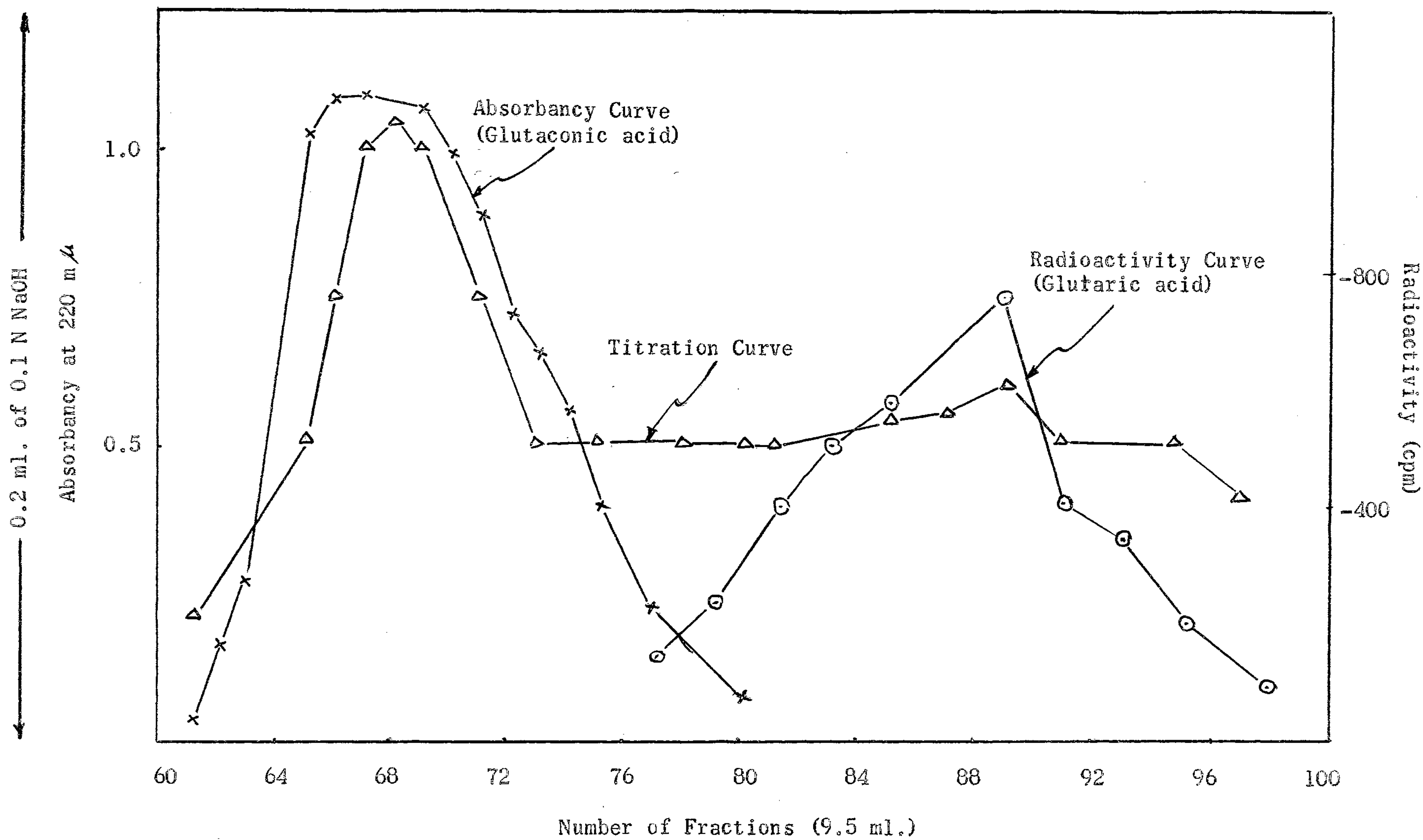


Figure 5. Separation of Glutaric Acid and Glutaconic Acid by Celite Column Chromatography

TABLE I

COMPOSITION OF DIFFERENT SOLVENT SYSTEMS FOR PAPER CHROMATOGRAPHY

Solvent Systems	Composition	Spraying Agents
1	0.015% bromophenol blue and 0.05% sodium acetate in ethyl acetate : Acetic acid : water (6:2:2 v/v)	--
2	n-Propanol : conc. NH_3 : water (6:2:2v/v)	0.2% ninhydrin and 0.05% ascorbic acid in ethanol (61)
3	1 N NH_4OH in 78% ethanol	"
4	n-Butanol : Acetic acid : water (9:1:2.5v/v)	0.04% bromocresol green
5	n-Butanol : Formic acid : water (9:1:25v/v)	"
6	Acetic acid : ether : benzene (1:5:5v/v)	"
7	Benzene : water : Acetic acid (2:1:1v/v) and use the benzene layer	"

TABLE II

R_f VALUES OF ORGANIC ACIDS IN DIFFERENT SOLVENT SYSTEMS

Acids	Solvent Systems[+]						
	1	2	3	4	5	6	7
Acetonedicarbxsylic acid		0.30	0.18	0.62	0.63		
Acetoacetic acid		0.30	0.18				
Butyric acid		0.58	0.48				
β-Hydroxybutyric acid	0.87			0.75			
Crotonic acid				0.73			
Br-crotonic acid*	0.95			0.77	0.83		
Ethylmalonic acid				0.81			
Glutaconic acid	0.91	0.37	0.32	0.75	0.82	0.68	0.62
Glutaric acid	0.91	0.37	0.32	0.75	0.81	0.72	0.74
Br-glutaconic acid*	0.83			0.63	0.77		
Hydroxyglutaric acid				0.52			
β-Hydroxyglutaric acid	0.72	0.38	0.32	0.52	0.54		

+ For composition see Table I.

* Brominated unsaturated acids.

E. Degradation Procedures.

1. Degradation of Acetoacetic Acid.

Acetoacetic acid was decomposed by heat to carbon dioxide and acetone in the Schmidt apparatus (Figure 6). The lyophilized sodium acetoacetate obtained as described above was dissolved in 2 ml. of water and was transferred to a 5 ml. pear-shaped flask. While keeping the flask in an ice-bath, the contents were acidified to pH 3 with 25 per cent sulfuric acid. The flask was quickly attached to the Schmidt apparatus and the cold solution was swept with carbon dioxide-free air for about five minutes to remove preformed carbon dioxide. Then the flask was heated in a boiling water bath. The decarboxylation of acetoacetic acid appeared to be completed within 10 minutes. The heating and sweeping was continued for an additional 0.5 hour. Evolved carbon dioxide (carbon 1 of acetoacetic acid) was trapped in the second trap containing 5 ml. of 1 N carbon dioxide-free sodium hydroxide and liberated acetone (carbons 2, 3, and 4 of acetoacetic acid) was trapped in the first trap containing 5 ml. of ice-cold acidified water. The carbon dioxide in the alkali was assayed for C^{14}.

To the aqueous acetone solution was added dropwise 2, 4-dinitrophenylhydrazine reagent (62). The dinitrophenylhydrazone precipitate was removed by filtration and was recrystallized from 67 per cent hot ethanol to constant melting point (126°) and specific activity. The purified crystals were assayed for C^{14}.

Acetone was then recovered from the purified dinitrophenylhydrazone by steam distillation from 2 N HCl. To the aqueous acetone solution was added 1 ml. 10 N sodium hydroxide and then iodoform reagent (potassium iodide 20 gm., iodine 10 gm. in 80 ml. water) dropwise until a faint

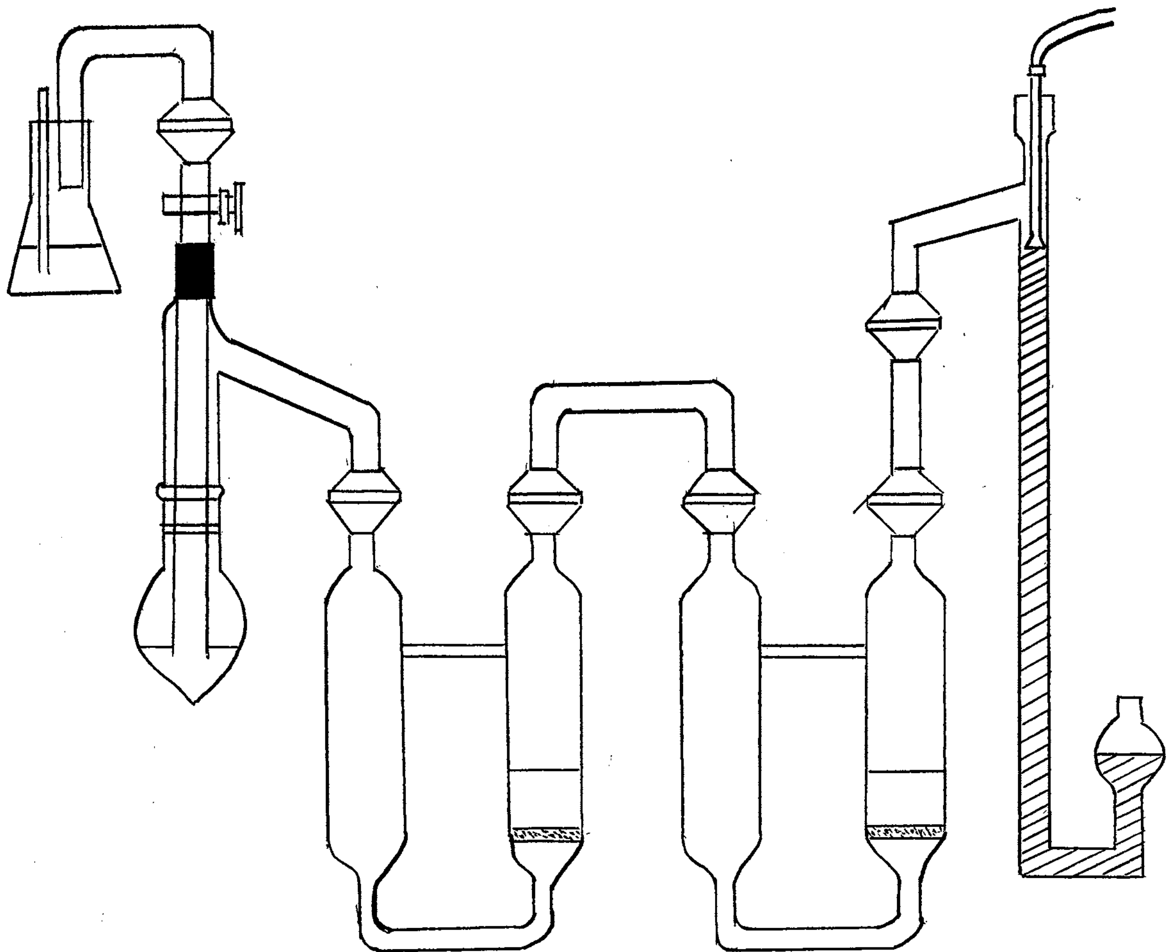


Figure 6. The Schmidt Apparatus.

color of iodine persisted for 2 to 3 minutes. The iodoform precipitate (carbons 2 and 4 of acetoacetic acid) was collected by filtration and recrystallized from hot 95 per cent ethanol. The filtrate obtained was dried over a hot plate. The dried material was acidified and steam distilled. The volatile acetic acid (carbons 2, 3, and 4 of acetoacetic acid) and excess iodine were collected in 200 ml. of distillate. Acetic acid was extracted with ether. The ether extract was then made alkaline and dried by an air jet at room temperature. The dried sodium acetate was dissolved in a small amount of water, transferred quantitatively to a pear-shaped flask and dried overnight at 110^{0}.

Sodium acetate was decarboxylated by the Schmidt reaction (63). The dried material in the flask was chilled in an ice bath and 0.6 ml. of 100 per cent sulfuric acid was added to each millimole of sodium acetate. The glass-stoppered flask was heated briefly over a small flame to dissolve the sodium acetate and then chilled in an ice bath. For each millimole of acetate, 100 mg. of sodium azide was added in one portion and the flask was attached to the Schmidt apparatus. The air inlet to the reaction flask was kept closed while the temperature of the water bath was raised to 70^{0} over a period of one hour. The air inlet was then opened and carbon dioxide-free air was allowed to sweep slowly through the system for one hour while heating at 70^{0} or above. Carbon dioxide was trapped in alkali. The methyl amines produced were trapped in 1 N HCl and the resulting hydrochloride was oxidized to CO_2 by the method of Van Slyke et al. (64, 65).

Figure 7 outlines the entire degradation procedure.

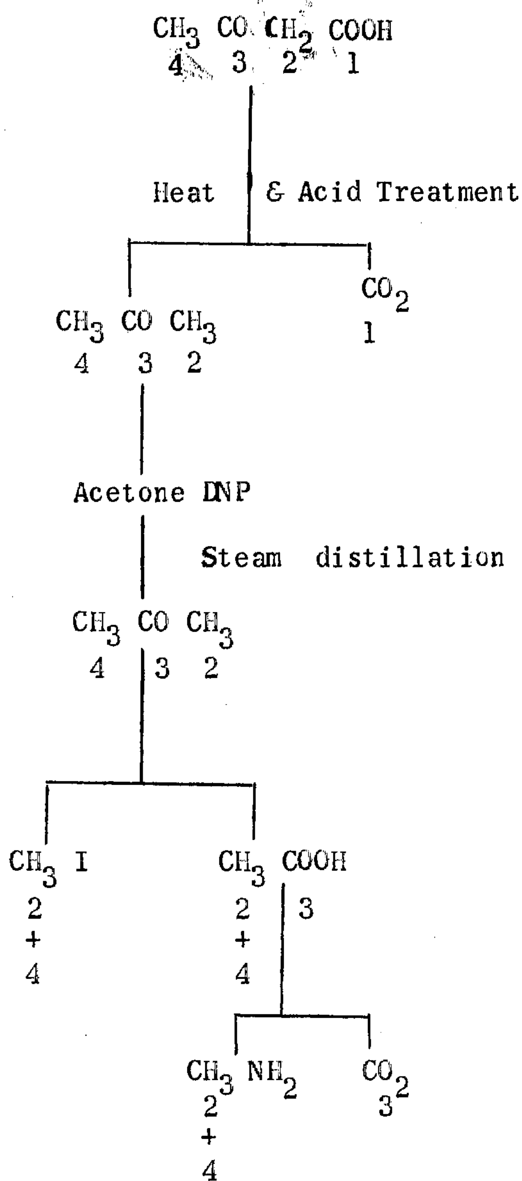


Figure 7. Degradation of Acetoacetic Acid.

2. Degradation of β-Hydroxyglutaric Acid.

The β-hydroxyglutaric acid peak obtained from the Celite column was dried by an air jet at room temperature. Part of the peak was assayed for C^{14}.

To the rest of the β-hydroxyglutaric acid peak was added 125 ml. of distilled water, 10 ml. of 50 per cent sulfuric acid and 35 ml. of 10 per cent mercuric sulfate (66). The mixture was heated to boiling and 5 ml. of 5 per cent potassium dichromate solution was added through the condenser. The refluxing was continued for another 90 minutes. The precipitate was removed by filtration and dried.

The dried mercury-acetone complex was treated with 2 N HCl and steam distilled to recover acetone, which was degraded by the same methods described above.

3. Degradation of Acetonedicarboxylic Acid.

The methods used for degrading acetonedicarboxylic acid were the same as for acetoacetic acid. The acid was decomposed by heat to 2 moles of carbon dioxide (carbons 1 and 5 of acetonedicarboxylic acid) and one mole of acetone (carbons 2, 3, and 4). The acetone was degraded by the methods described above. The whole procedure is shown in Figure 8.

4. Degradation of Ethylmalonic Acid.

The ethylmalonic acid peak was transferred to a pear-shaped flask and dried at 110°. The flask was placed in an ice-bath and its contents acidified to pH 1 with 25 per cent sulfuric acid. Then the flask was attached to the Schmidt apparatus and heated in a mineral oil bath to 180°. The liberated carbon dioxide was trapped in 1 N sodium hydroxide and the butyric acid was trapped in ice-cold acidified water. The butyric acid was purified by Celite chromatography. The liberated carbon dioxide

and purified butyric acid were assayed for radioactivity.

F. Radioactivity Determinations.

Carbon analysis and C^{14} assays involved manometric measurement of carbon dioxide and determination of radioactivity with a vibrating reed electrometer (67).

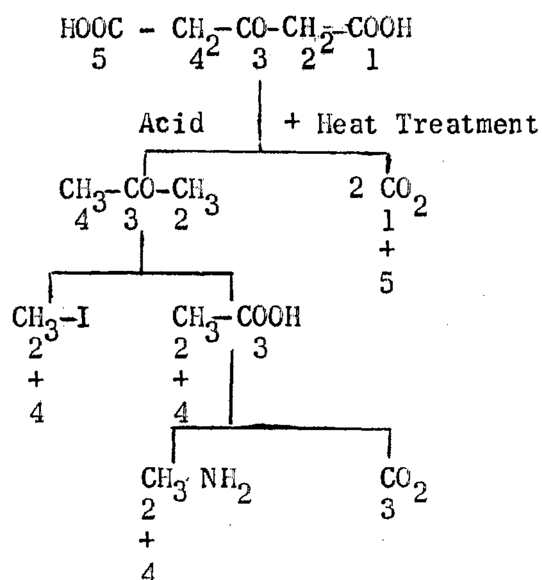


Figure 8. Degradation of Acetonedicarboxylic Acid

In some instances, acetonedicarboxylic acid was reduced to β-hydroxyglutaric acid (42) which was purified on Celite and degraded as described above.

CHAPTER III

RESULTS AND DISCUSSION

A. Oxidation of Glutarate by Mitochondria.

The O_2 uptake by mitochondria in the presence of glutarate and various cofactors was investigated in preliminary experiments. A slight increase over the control values was observed (Figure 9). An increase of O_2 uptake was caused by "sparking" with dicarboxylic acids, namely succinate, fumarate, oxaloacetate, and malate (Figures 9, 10, 11, 12). Pyruvate did not have any "sparking" effect (Figure 13). The sparking phenomenon can be attributed to the generation of ATP during oxidation of the dicarboxylic acids and formation of the condensing partner (oxaloacetate) for acetyl-CoA. Unless acetyl-CoA can be condensed with oxaloacetate to form citrate, the available CoA which is present in catalytic amounts, is tied up. Thus the sparkers may have the dual role of generating ATP and of facilitating a turnover of CoA through the condensation reaction.

The effect of various cofactors on the O_2 uptake are shown in Figures 14, 15, 16, 17. The omission of adenosinetriphosphate (ATP) and $MgCl_2$ from the incubation mixture depressed O_2 uptake practically to zero (Figure 14). This indicates that a reaction similar to that of fatty acid oxidation, formation of thiol esters of CoA, may be involved before the acid is oxidized. The addition of cytochrome c slightly increased the uptake (Figure 15). Coenzyme A failed to increase the uptake (Figure 16). It is interesting to note that over 50 per cent of the total liver

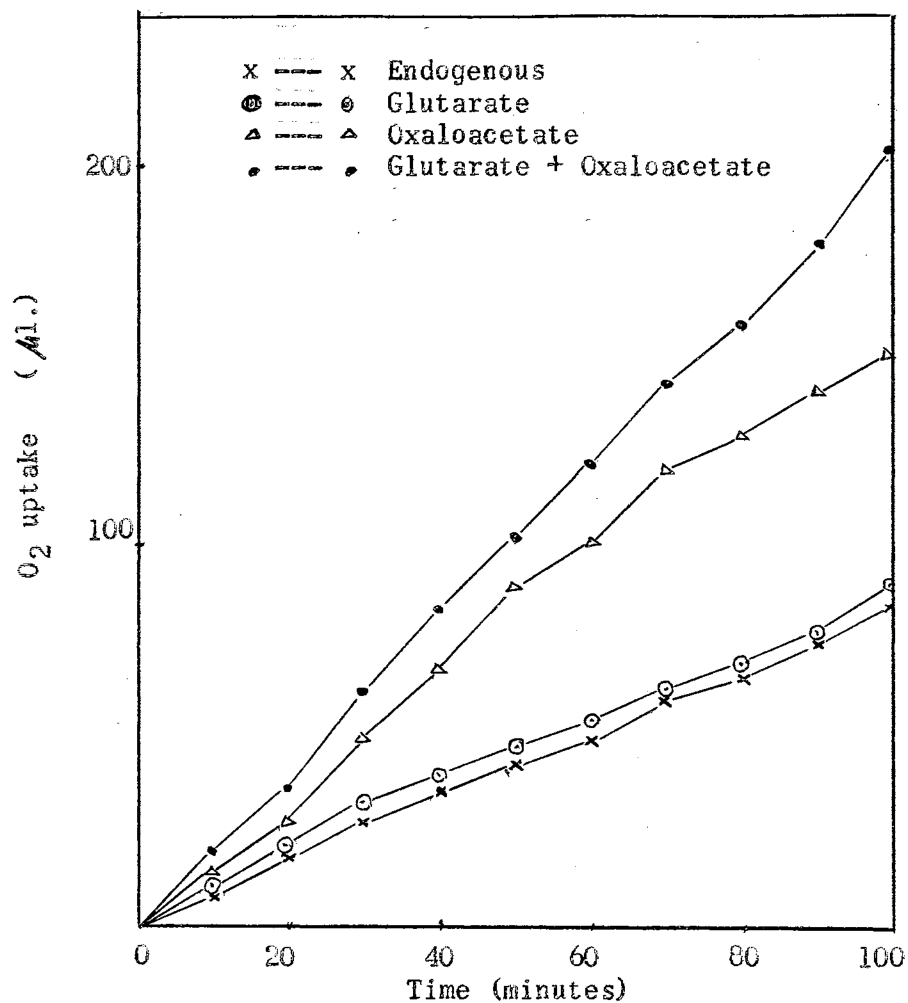


Figure 9. Effect of Oxaloacetic Acid

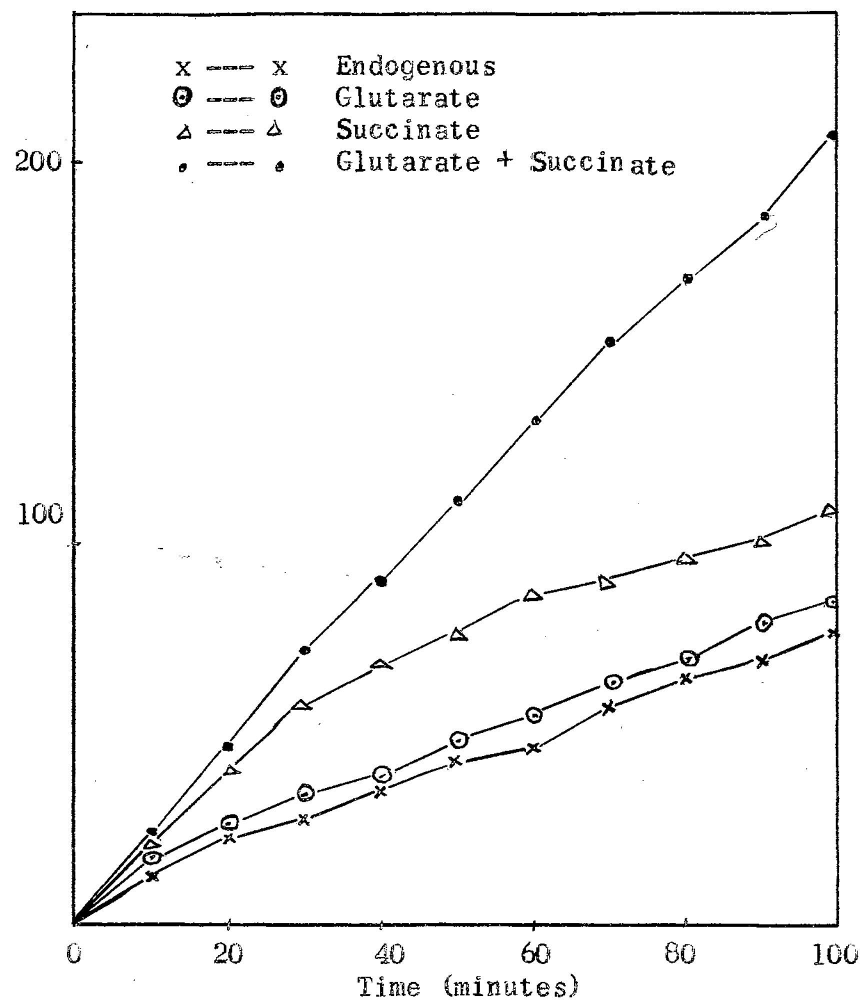


Figure 10. Effect of Succinic Acid

Incubations were conducted at 30° at pH 7.3 in standard Warburg flasks. Each mixture had a final volume of 3 ml., and contained, in μmoles the following: phosphate, 30; $MgCl_2$, 12; ATP, 12; cytochrome c, 0.03; glutarate, 30; dicarboxylic acids, 15. Each mixture also contained the mitochondria from 0.5 g. of rat liver.

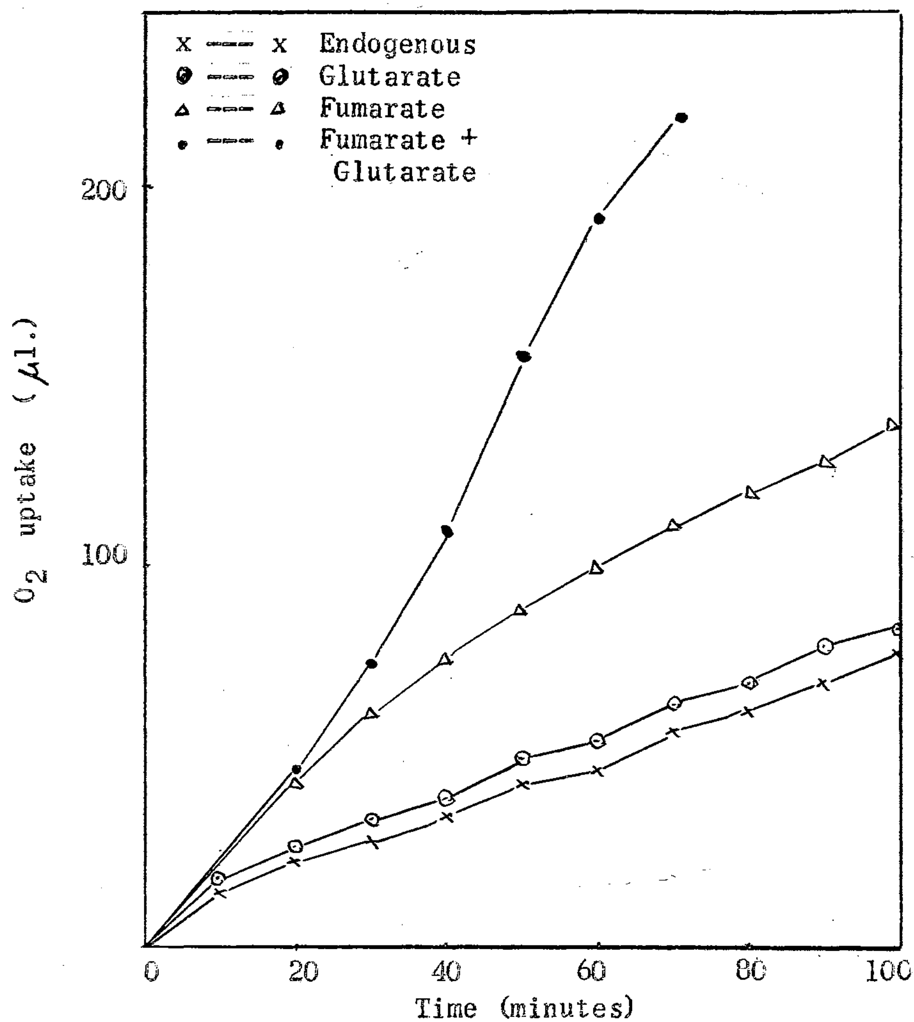


Figure 11. Effect of Fumaric Acid

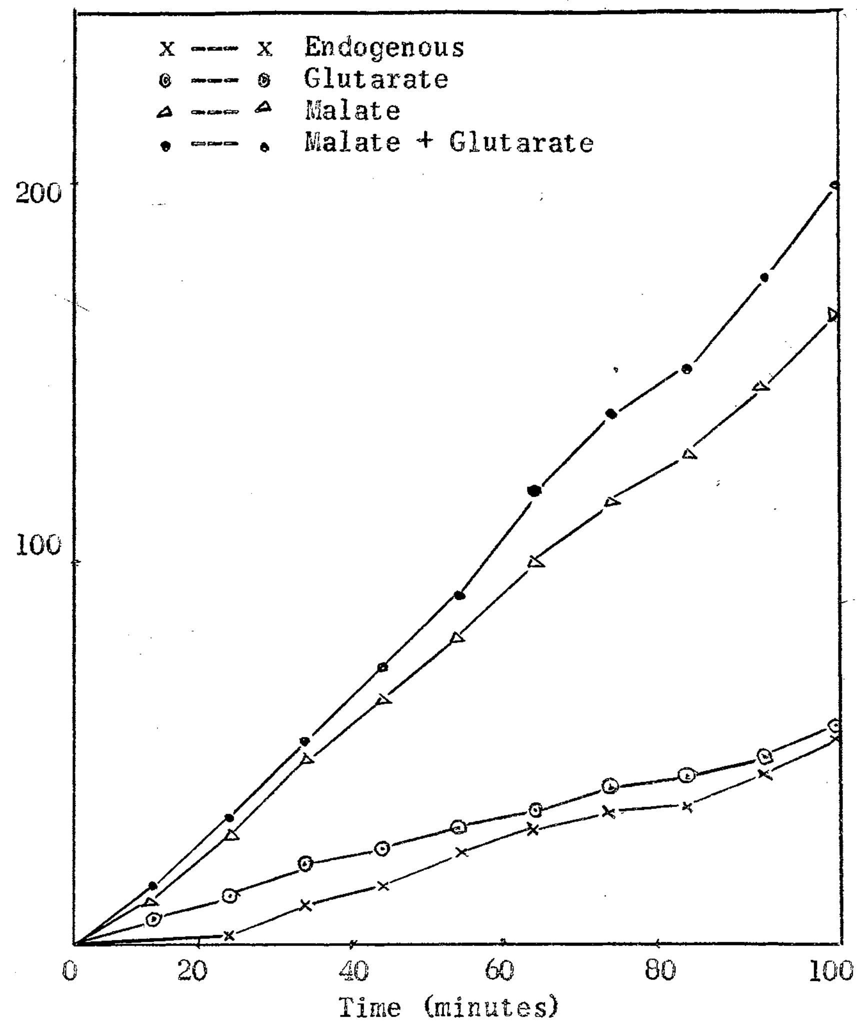


Figure 12. Effect of Malic Acid

The experimental conditions and incubation mixtures were identical with those given under Figures 9 and 10.

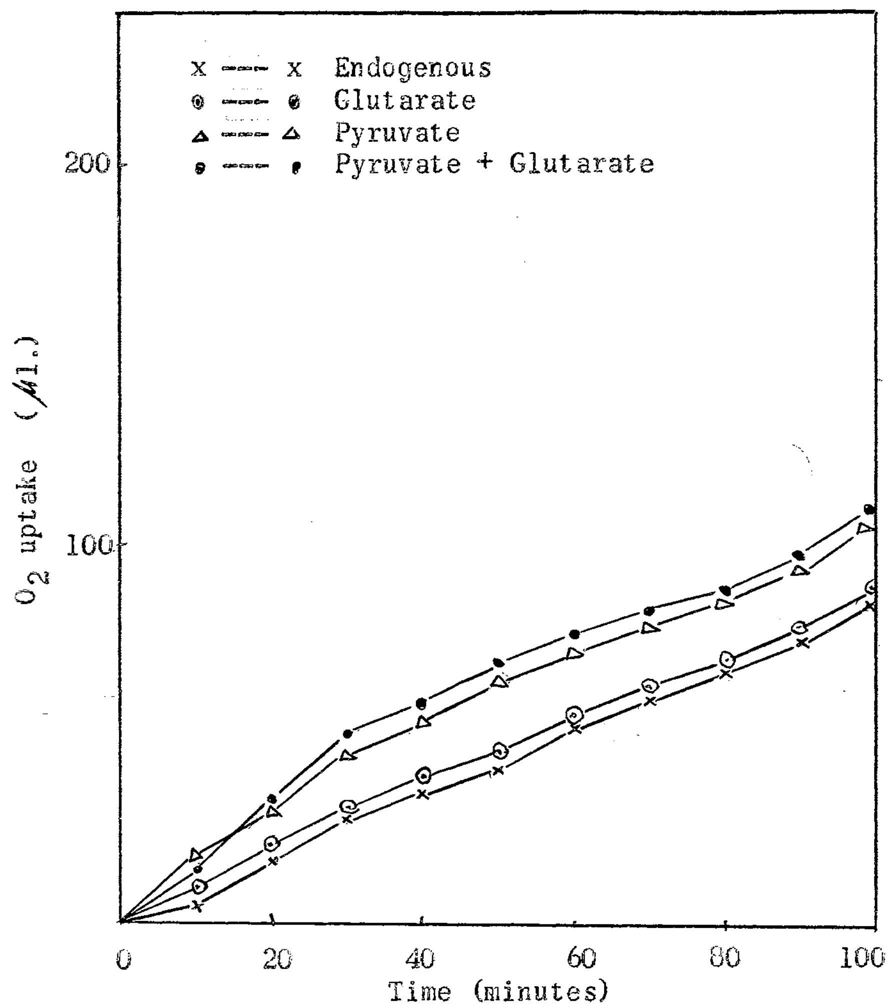


Figure 13. Effect of Pyruvic Acid

The experimental conditions and the incubation mixtures were identical with those given under Figures 9 and 10.

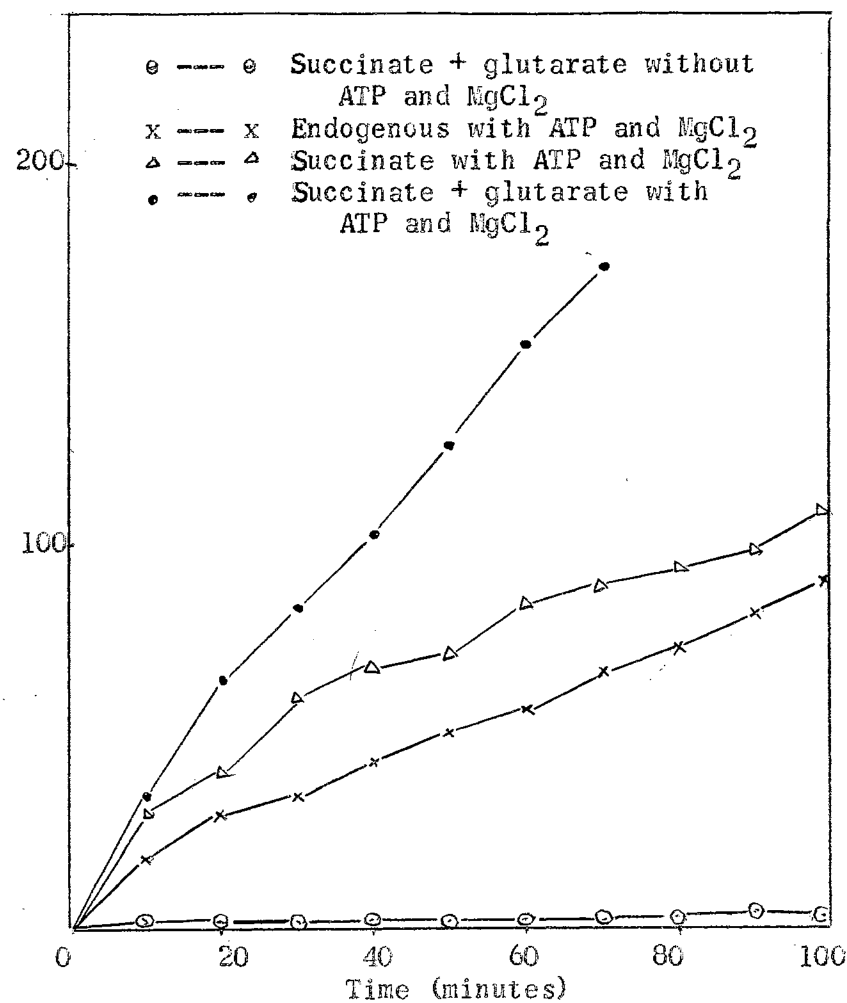


Figure 14. Effect of ATP and $MgCl_2$

The experimental conditions were identical with those given under Figures 9 and 10. Each mixture contained, in µmoles, the following: phosphate, 30; glutarate, 30; succinate, 15.

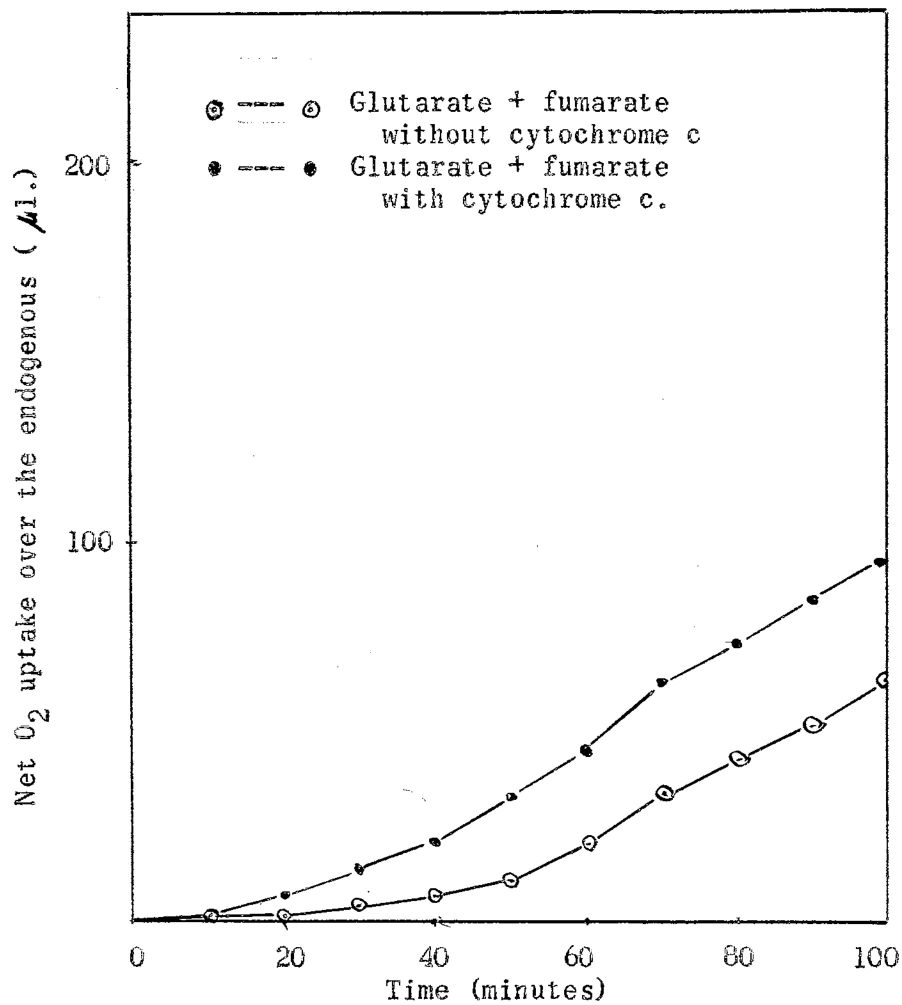


Figure 15. Effect of Cytochrome c

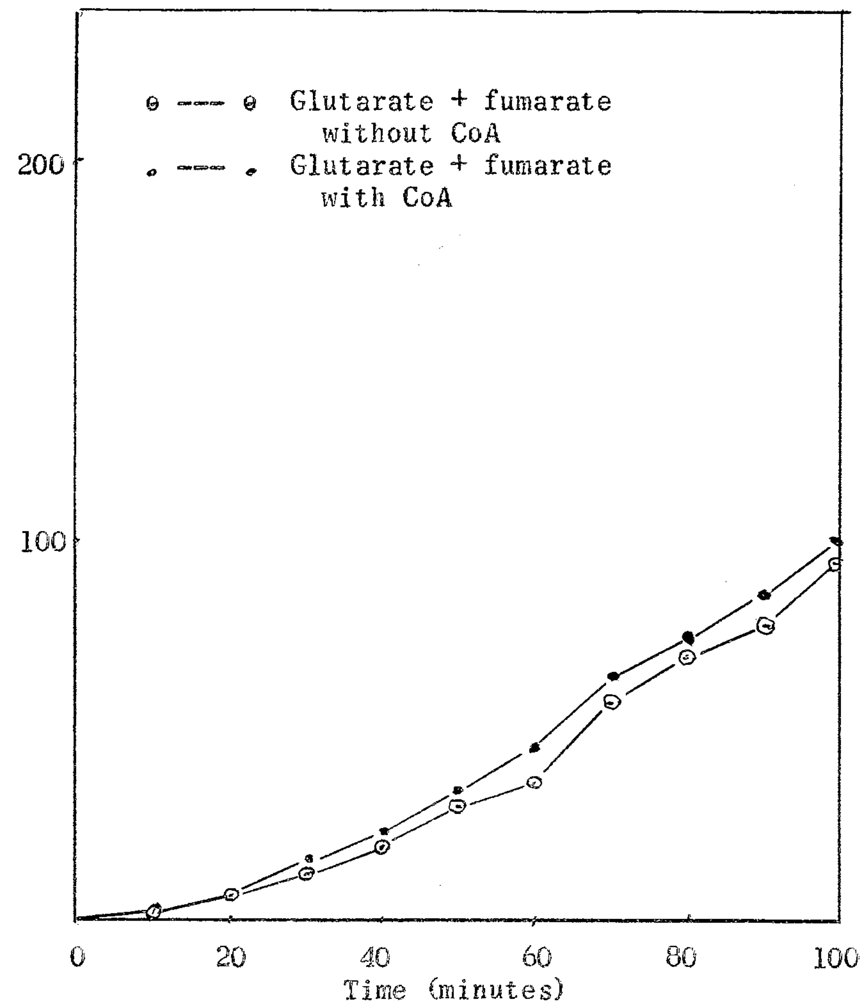


Figure 16. Effect of CoA

The experimental conditions and the incubation mixtures were identical with those given under Figures 9 and 10.

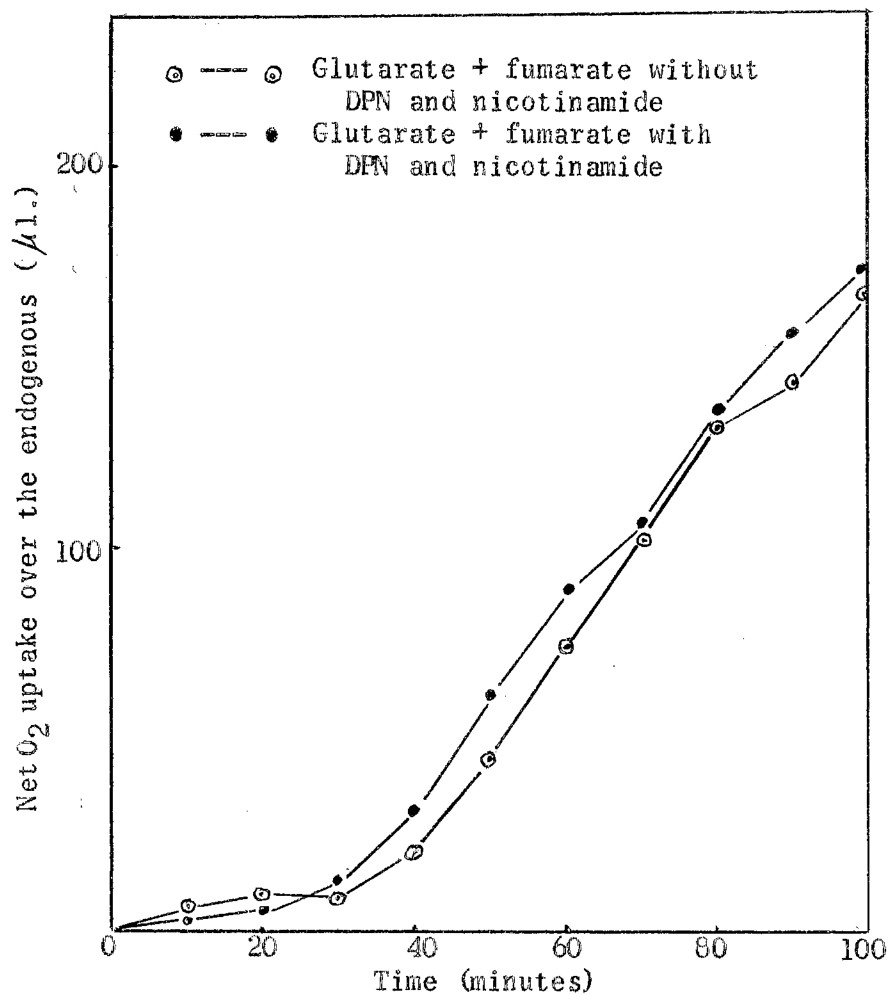


Figure 17. Effect of DPN and Nicotinamide

The experimental conditions and the incubation mixtures were identical with those given under Figures 9 and 10, except that 3 μmoles of DPN and 36 μmoles of nicotinamide were used.

CoA is in the mitochondria and it does not readily exchange with that in the external medium (68). The addition of DPN and nicotinamide also failed to enhance the uptake (Figure 17).

The oxidation of glutarate-3-C^{14} by mitochondria, as measured by $C^{14}O_2$ production, is shown in Experiments 1a and 3a of Table III.

The addition of the nuclear or soluble supernatant fractions to the mitochondrial preparations did not increase $C^{14}O_2$ production significantly (Table IV). The reason for conducting these experiments is that localization of a complete group of metabolic reactions in a particular particle gives no reliable indication that any of the enzymes concerned are mainly situated in that particle; it merely indicates that only in that particle are all the enzymes present together. For instance, in the case of a system depending on four enzymes A, B, C, and D, if A and B are situated mainly in the microsomes while C and D are situated mainly in the supernatant, and small amounts of all four enzymes are in the mitochondria, it is only in the mitochondria that the system will be detected (68). The "sparking" effect of fumarate is shown in Experiments 1a and 1b of Table III.

As shown in Table III a 10-fold excess of acetonedicarboxylic acid with respect to glutaric acid (Experiments 2a and 2b), sharply depressed glutarate oxidation. When the ratio of these acids was decreased (Experiments 4a, 4b and 4c), the inhibition of $C^{14}O_2$ production was decreased.

Glutarate oxidation is moderately inhibited by the presence of β-hydroxyglutaric acid (Table III, Experiments 2a and 2c).

Studies in vivo also indicate that acetonedicarboxylic acid and β-hydroxyglutaric acid inhibited glutarate oxidation (42).

TABLE III

OXIDATION OF GLUTARIC ACID-3-C^{14} BY RAT LIVER MITOCHONDRIA

Incubations were conducted at 30° at pH 7.3 for 3 hours in standard Warburg flasks. Each mixture had a final volume of 3 ml, and contained, (exceptions are described in footnotes) in μ moles, the following: phosphate, 30; $MgCl_2$, 12; ATP, 12; cytochrome c, 0.03; sucrose, 250; Tris buffer, 2. Each mixture also contained the mitochondria from 0.5 g of rat liver and 36 μ moles of glutaric acid-3-C^{14} (2.36 μc).

Experiment No.	Trapping acid added		$C^{14}O_2$ formed	Degradation data				Butyric acid	Glutaconic acid
				Carboxyl carbons	Acetone, carbons 2, 3, 4	Acetate carboxyl, carbon 3	Iodoform, carbons 2+4		
		moles	% C^{14} added to incubation mixture						
1a	None		1.8						
1b[a]	None		8.2						
2a[b]	None		5.4						
3a	None		1.4	0.06[c]	0.74[c]				
4a[a]	None		3.5						
2b[b]	Acetonedicarboxylic	60	0.6	0.04	0.46				
4b[a]	Acetonedicarboxylic	60	1.0	0	0.15				
4c[a]	Acetonedicarboxylic	10	2.5	0.04	0.42				
4d	Acetonedicarboxylic	10	0.6	0.05	0.46				
5a	Acetonedicarboxylic	10	0.4		0[d]				
5b	Acetonedicarboxylic	10	0.4		0[d]				

Table III (continued)

Experiment No.	Trapping acid added		$C^{14}O_2$ formed	Degradation data: Carboxyl carbons	Acetone, carbons 2, 3, 4	Acetate carboxyl, carbon 3	Iodoform, carbons 2+4	Butyric acid	Glutaconic acid
		moles	% C^{14} added to incubation mixture						
2c[b]	β-Hydroxy glutaric	60	2.8						
6a	β-Hydroxy-glutatic	60	1.0	0.03	0				
6b	β-Hydroxy-glutaric	60	0.9	0.04	0				
5c[e]	Acetoacetic	35	0.3	0.13	0.41	0.35	0		
5d[e]	Acetoacetic	35	0.3	0.12	0.40	0.34	0		
7a	Acetoacetic	17	1.3	0.07		0.42	0		
7b	Acetoacetic	34	0.7	0.10		0.57	0		
7c	Acetoacetic	51	0.5	0.09		0.51	0		
8a[a]	Acetoacetic	17	1.9	0.08	0.53				
8b	Acetoacetic	17	0.6	0.14	0.71				
9	Butyric	90	0.17					0.05	
10	Ethylmalonic	60	0.33	0				0	
11+	Glutaconic	60	1.5						0
12*	Glutaconyl pantetheirate	60	0						0

[a] Fumarate, 1.5 moles per flask was added. [b] Instead of 36μmoles, 6μ moles of gluratic acid were added.
[c] From acetonedicarboxylic acid added as carrier.
[d] Acetone was prepared from B-hydroxyglutatic acid obtained from the reduction of acetonedicarboxylic acid (See "Experimental Procedure").
[e] Instead of 36 moles, 42 moles of glutaric acid were added. + 50 μM glutarate-1,5-C^{14} (2.2 μc) added instead of glutarate-3-C^{14}. * 70 μM glutaryl-pantetheine (567 mμC) added instead of glutarate-3-C^{14}

TABLE IV

EFFECT OF THE NUCLEAR OR SOLUBLE SUPERNATANT FRACTIONS ON THE OXIDATION OF GLUTARIC ACID-3-C^{14} BY RAT LIVER MITOCHONDRIA

Incubations were conducted at 30° at pH 7.3 for 3 hours in standard Warburg flasks. Each mixture contained in μmoles the following: phosphate, 30; $MgCl_2$, 12; ATP, 12; cytochrome c, 0.03; sucrose, 250 to 500; Tris buffer, 2. Each mixture also contained the mitochondria and nuclear fraction (or soluble supernatant) from 0.5 g rat liver and 36 μmoles of glutarate-3-C^{14} (2.36 μc). The total volume was 3 ml.

Fractions of rat liver	$C^{14}O_2$ formed % C^{14} added to incubation mixture
Mitochondria	1.44 %
Mitochondria + Neuclear fraction	1.87 %
Mitochondria + soluble supernatant	1.31 %

B. Labeling in Acetonedicarboxylic Acid, β-Hydroxyglutaric Acid, Ethylmalonic Acid, Butyric Acid, and Glutaconic Acid.

As shown in Table III, all efforts to demonstrate the formation in vitro of acetonedicarboxylic acid or β-hydroxyglutaric acid labeled in carbon 3 from glutarate-3-C^{14} failed also no radioactivity was found in ethylmalonic acid, glutaconic acid, and butyric acid (Table III).

When acetonedicarboxylic acid was added to mitochondrial preparations as trapping agent, labeled acetone was isolated (Table III, Experiments 2b, 3a, 4b, 4c, and 4d). However, this labeled acetone did

not come from acetonedicarboxylic acid, since reduction with $NaBH_4$, isolation of the resulting β-hydroxyglutaric acid, and subsequent preparation of acetone from the latter yielded a product devoid of radioactivity (Table III, Experiments 5a and 5b). It should be noted that in the crucial experiments (Nos. 5 and 6) care was taken to hydrolyze any CoA esters before isolating and degrading the various acids. The β-hydroxyglutarate peaks obtained from mitochondrial incubations had small amounts of activity, but the acetone prepared from these peaks had no activity.

As noted, when glutaryl pantetheinate and glutaconyl pantetheinate (Table III, Experiment 12) were used as substrate and trapping agent, no radioactivity was found in glutaconate and expired CO_2. A reasonable explanation could be that the pantetheine derivatives could not penetrate through the mitochondrial membrane.

C. Labeling in Acetoacetic Acid.

Glutarate-3-C^{14} was readily converted in vitro to acetoacetate containing C^{14} only in the carboxyl and carbonyl carbons (Table III). The ratios of carbonyl to carboxyl radioactivities were greater than 3. Studies in vivo gave rise to the same results (42). Ratios of this magnitude are obtained only when the terminal 2-carbon unit of a fatty acid is appropriately labeled. Crandall et al. (69) found that octanoic acid-7-C^{14} produced acetoacetate in which the carbonyl carbon contained three times as much isotope as did the carboxyl carbon. These investigators proposed that fatty acids may give rise to two types of active acetyl groups. The terminal two carbon unit (CH_3CO-) of a fatty acid chain serves predominantly as a source of the acetyl moiety of acetoacetate and the two-carbon fragments derived from the remainder of the chain ($-CH_2CO-$) are the major source of the carboxyl portion of the keto acid.

Subsequent cleavage and resynthesis of acetoacetate does not completely randomize the carbonyl and carboxyl carbons. Therefore our results indicate that the carbon chain of glutarate remains intact during its conversion to acetoacetate.

D. Oxidation of Glutarate by Soluble Enzyme Systems.

As shown above (section B) none of the acids, acetonedicarboxylic acid, β-hydroxyglutaric acid, glutaconic acid, ethylmalonic acid, and butyric acid, contained significant radioactivity. The results do not support the postulates (25, 26, 27, 30, 33) that these acids are intermediates in glutarate catabolism. However, the data should not be construed as conclusive evidence against such a catabolic route since equilibration between endogenous and carrier acid may not have occurred (71, 72, 73). The difficulty may be that, as in fatty acid oxidation, no intermediate between the acid and the final product of oxidation accumulates when intact animals or cell-free mitochondria are used (38). Probably only at the level of the isolated soluble enzymes does it become possible to interrupt the sequence in such a way as to accumulate intermediates.

In the light of these facts, an effort was made to solubilize the mitochondria by sonic oscillation. The effect of duration of sonication on the rupture of mitochondria is shown in Table V. None of the sonicated preparations appeared to be active in glutarate oxidation. The O_2 uptake was practically zero. When glutarate-3-C^{14} was used no $C^{14}O_2$ and acetoacetate-C^{14} were detected.

Extracts of rat liver acetone powder were able to oxidize glutaryl-CoA, but not glutarate, in the presence of phenazine methosulfate as

measured by O_2 uptake (Table VI). When glutaryl-3-C^{14}-CoA and glutaconate were incubated with the extracts, no radioactivity was detected in CO_2. After the incubation mixture was treated with alkali, the isolated glutaconate contained no detectable C^{14}. Dialysis of the extracts caused loss of activity. All the efforts to show the formation of labeled glutaconate failed. If cis glutaconate were formed during glutarate oxidation it might not equilibrate with the trans isomer which was added to the incubation mixture.

TABLE V

EFFECT OF DURATION OF SONICATION

Mitochondria from 0.5 g rat liver was suspended in 1 ml. water or 0.25 M sucrose; after sonication the insoluble part was removed by centrifuging at 2,000x g. 0.3 ml. of the supernatant was used for colorimetric measurement by Biuret Method (70). Each tube contains: 0.3 supernatant, 1.7 ml. 0.9 % NaCl and 8.0 ml. Biuret reagent. Optical density was measured at 540 m .

Preparations	Fold of increase of O.D. over blank		
	30 sec.	5 min.	15 min.
Mitochondria suspended in H_2O	1.95	2.14	2.7
Mitochondria suspended in 0.25 M sucrose	1.3	1.85	91.0

Development of a colorimetric assay method has been rather unsuccesful. Initially a method was used by measuring reduction of either cytochrome c or 2,6-dinitrophenol indophenol in the presence of enzyme and substrate. However, the rates of reduction obtained were negligible.

The large amount of oxidant may inhibit the enzyme (55). When the titration method (55) was used, a slight increase of dye uptake over the control was observed. However, the method was not adequate as an assay system for enzyme activity.

Further investigation remains to be done.

TABLE VI

OXIDATION OF GLUTARATE BY EXTRACTS OF ACETONE POWDER OF RAT LIVER

Incubations were conducted at 37° at pH 7.3 in standard Warburg flasks. Each mixture had a final volume of 3 ml, and contained, in μ moles, the following: phosphate, 60; $MgCl_2$, 12; ATP, 12; Cytochrome c, 0.03; coenzyme A, 2. 2 mg. of phenazine methosulfate (PMS) were used.

Substrate	O_2 uptake in 100 minutes (μl)
Endogenous	127
Endogenous + PMS	148
Glutarate (60 μmoles)	120
Glutarate (60 μmoles) + PMS	150
Glutaryl-CoA (2 μmoles)	131
Glutaryl-CoA (2 μmoles) + PMS	196

CHAPTER IV

SUMMARY

When rat liver mitochondria were incubated with glutaric acid-3-C^{14} and either glutaconic acid, acetonedicarboxylic acid, β-hydroxyglutaric acid, ethylmalonic acid, butyric acid, or acetoacetic acid, the following results were obtained:

1. Glutarate-3-C^{14} was oxidized by mitochondria as measured by O_2 uptake and the release of $C^{14}O_2$. Oxidation was enhanced by "sparking" with one of dicarboxylic acids, fumaric, succinic, malic, or oxalacetic.

2. Acetoacetic acid was labeled in the carbonyl and carboxyl carbons, the carbonyl to carboxyl ratio being at least 3:1.

3. The three middle carbons of acetonedicarboxylic and β-hydroxyglutaric acids contained no detectable C^{14}.

4. The butyric acid obtained from the decarboxylation of ethylmalonic acid contained no significant radioactivity.

5. The glutaconic acid and butyric acids contained no detectable C^{14}.

These results indicate that glutarate is converted to acetate via acetoacetate. No insight was gained concerning the intermediates in this conversion.

When extracts of rat liver acetone powder were used, the following results were obtained:

1. Glutaryl CoA, but not glutarate, was oxidized, in the presence of phenazine methosulfate, as measured by O_2 uptake.

2. Attempts to develop a colorimetric assay procedure have been rather unsuccessful.

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