STUDIES ON THE METABOLISM OF GLUTARIC ACID-3-C^{14} AND α,γ-DIAMINOBUTYRIC ACID-2-C^{14} IN INTACT RATS

by

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PART I

CHAPTER I

INTRODUCTION

In nature glutaric acid is known to occur in sugar beets (1), sugar diffusion juice (2), wood distillation products (3), urine (4), tobacco smoke (5), plants (6, 7), coal (8) and ale (9).

The interest in glutaric acid metabolism started in 1907 when Baer and Blum (10) presented evidence to the effect that the subcutaneous injection of glutaric acid greatly reduces the amount of sugar and nitrogen in the urine, and also greatly diminishes or causes the disappearance of urinary acetone and acetoacetic acid in phlorhizin glycosuria. This conclusion however was contradicted by Ringer (11) who showed that glutaric acid has no inhibitory effect on the action of phlorhizin, that it does not reduce the sugar or nitrogen elimination in phlorhizin glycosuria, and that it does not diminish the acetone bodies in the urine. A year later Ringer (12) postulated the conversion of lysine to glutaric acid on the basis of the observation that neither lysine nor glutaric acid is glucogenic, a suggestion that was confirmed thirty years later by Neuberger and Sanger (13).

On incubating guinea pig liver homogenates with α-aminoadipic acid-6-C^{14}, Borsook *et al.* (14) in 1948 isolated radioactive α-ketoglutaric acid and glutaric acid. This was followed by an extensive

research on lysine metabolism. Studies on the metabolism of labeled lysine in homogenates of guinea pig liver, in dogs (15) and in rats (16, 17) since then, have provided evidence for the present pathway of lysine breakdown to glutaric acid.

In 1959, Gholson et al. (18) showed glutaric acid to be a product of tryptophan metabolism by isolating radioactive urinary glutaric acid after metabolite overloading experiments using DL-tryptophan-7a-C^{14} or 3-hydroxyanthranilic acid-H^3 with glutaric acid.

Besides its formation from lysine and tryptophan, glutaric acid is formed during the fermentation of L-xylose by Aspergillus niger (19).

The initial work on glutaric acid metabolism in humans was done by Weitzel (20) who showed that glutaric acid did not appear in the urine after its administration, and thus concluded that it is metabolized. This investigation was followed by the studies of Rothstein and Miller (21) who concluded on the basis of the labeling patterns in urinary acetate, acetoacetate and glucose after the administration of glutaric acid-1,5-C^{14} to normal and phlorhizinized rats, that decarboxylation to butyrate or oxidation to β-ketoglutarate followed by decarboxylation to acetoacetate were the most likely routes of catabolism of glutaric acid in the rat. Subsequently, on the basis of metabolite overloading experiments, the same authors suggested that α-ketoglutarate and acetate were the products of glutarate catabolism (16). Miller and Bale (22), following the administration of lysine-6-C^{14} to dogs, obtained from liver and plasma proteins glutamic acid having 71 per cent of the activity in the γ-carboxyl and 25 per cent in the α-carboxyl. This again suggested to them that the carbon chain of glutarate was converted, in part, to that of α-ketoglutarate.

Thus, from the above-mentioned studies of Rothstein, Miller, and Bale (16, 17, 21, 22), two pathways were suggested for the metabolism of glutaric acid. One of these involved direct conversion of the carbon chain of glutarate to that of α-ketoglutarate; the other involved the formation of acetate. In an effort to assess the relative importance of each pathway, Hobbs and Koeppe (23) studied the metabolism of glutaric acid-3-C^{14} in intact rats and found that the labeling patterns found in isolated glutamic acid, aspartic acid, and alanine were not compatible with a conversion to the carbon chain of α-ketoglutarate and indicated that glutarate was metabolized primarily via acetate. A reasonable hypothesis for the conversion to acetate would be (21, 23): glutarate ⟶ glutaconate ⟶ β-hydroxyglutarate ⟶ acetonedicarboxylate. In this manner glutaric acid-3-C^{14} would yield acetonedicarboxylic acid labeled in the carbonyl position. Subsequent decarboxylation to acetoacetate followed by cleavage to acetate would account for the formation of carboxyl labeled acetate. Also it has been suggested by others (24, 25) that acetoacetate might be formed from glutarate via ethylmalonate.

The objective of this investigation was to further elucidate the metabolism of glutaric acid in rats by acquiring some information about the nature of the intermediates in the oxidation of glutaric acid-3-C^{14} to acetate. The approach was to study the metabolism of glutaric acid-3-C^{14} in the rat by using the "metabolite overloading" technique (16, 17). This method consists of administering a labeled substance (glutaric acid-3-C^{14}) by intraperitoneal injection, together with a large dose of suspected metabolite. "The assumption that the injected compound is equilibrated in some manner with similar biologically formed

material is borne out by the finding that large doses of some non-radioactive metabolites, after being partly excreted in the urine, were found to be radioactive. It is therefore reasonable to assume that some "sampling" of the biologically formed material does take place. It is possible that some compounds do not equilibrate well, and hence negative results are not necessarily significant"(16).

When metabolite overloading experiments using glutaric acid-3-C^{14} and acetonedicarboxylate, β-hydroxyglutarate or ethylmalonate were conducted, urinary acetonedicarboxylate, β-hydroxyglutarate and ethylmalonate did not contain significant radioactivity. On the other hand, overloading with acetoacetate resulted in the formation of urinary acetoacetate labeled chiefly in the carbonyl carbon. These results indicate that glutarate is converted to acetate via acetoacetate.

CHAPTER II

EXPERIMENTAL

A. Synthesis of Compounds Used

Glutaric acid-3-C^{14} was synthesized by Dr. S. P. Bagchi from paraformaldehyde-C^{14} and diethylmalonate according to the method of Hobbs and Koeppe (23). The synthesis is summarized in Figure 1. The two preparations used had specific activities of 330 and 368 μc per millimole.

Acetonedicarboxylic acid was prepared from citric acid according to the method of Adams et al. (26), 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° C during the five hours of synthesis. Better yields were obtained when this low a temperature was maintained.

β-Hydroxyglutaric acid was prepared by Dr. R. E. Koeppe in 70 per cent yield by the reduction of acetonedicarboxylic acid (sodium-salt) in aqueous solution with two moles of sodium borohydride (27). A more detailed description of this synthesis of β-hydroxyglutaric acid has been published (28).

Sodium acetoacetate was prepared by hydrolysis of acetoacetic ester at room temperature with 0.5 N sodium hydroxide according to the method of Ceresol (29). After hydrolysis the solution was lyophylized to remove ethanol.

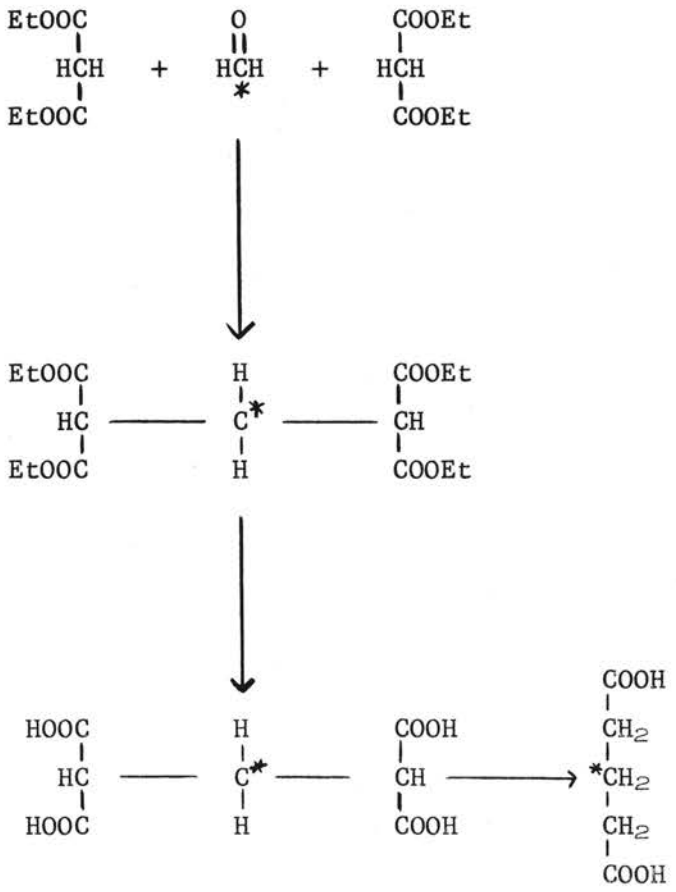


Figure 1. Synthesis of Glutaric Acid-3-C^{14}

Ethylmalonic acid was prepared by Dr. R. E. Koeppe according to the method of Weiner (30).

B. Animal Experiments

In each of the experiments performed, young male albino rats were used. The labeled glutaric acid and the compound used to overload were dissolved in 1 to 2 ml. of water. These solutions were administered by intraperitoneal injection after adjusting the pH to 7 to 8. Both the sodium acetoacetate solutions and the acetonedicarboxylic acid solutions were cooled in an ice bath before neutralization to pH 7 to 8.

After injection, the animals were placed in an all-glass metabolism cage which was swept with air. Purina dog checkers and water were supplied ad libitum throughout the experiments which varied in length from 9 to 53 hours. Table I summarizes the data from the individual experiments.

The expired carbon dioxide was trapped in two gas washing bottles containing respectively 250 ml. and 100 ml. of 2 N sodium hydroxide. The contents of both bottles were mixed and made up to 500 ml. with carbon dioxide-free water and 2 ml. aliquots were used for C^{14} assay according to the method of Anthony and Long (31).

The urine was collected in a separate flask attached to the urinofecal separator at the bottom of the metabolism chamber. During the collection period, the urines from Rats 127, 131, 139 and 140 were frozen to prevent the decomposition of the urinary acetonedicarboxylic and acetoacetic acids.

Rats 127 and 131 received glutarate-3-C^{14} and 2.5 to 2.6 millimoles of sodium acetonedicarboxylate by intraperitoneal injection. In the case of Rat 127 the two were administered simultaneously, while Rat 131

TABLE I

SUMMARY OF EXPERIMENTS PERFORMED

Rat No.	Weight in gm.	Catabolite acid administered	Amount catabolite injected (mmoles)	Glutaric acid-3-C^{14} given (μc)	Exhaled $C^{14}O_2$ per cent of injected dose	Duration of experiment (hours)
127	280	Acetonedicarboxylate	2.5[a]	23.6	7.9	43
131	370	Acetonedicarboxylate	2.6[a]	11.0	36	9[c]
133	320	β-Hydroxyglutarate	2.5[b]	12.8[b]	56	53
141	253	β-Hydroxyglutarate	2.0[b]	11.8[b]	47	25
139	170	Acetoacetate	2.0	4.7	58	13
140	123	Acetoacetate	2.0	12.8	42	13
143	326	Ethylmalonate	2.5	9.44	50.7	24

[a]The glutaric acid-3-C^{14} was given 15 minutes before the acetonedicarboxylic acid.

[b]Given in two doses, 3.5 hours apart.

[c]This animal died of suffocation. The exact time of death is not known.

received the glutarate-3-C^{14} 15 minutes before the acetonedicarboxylate. The urine was frozen during the collection period. Acetonedicarboxylic acid was isolated from the urine of both rats, all steps being conducted in the cold room. The urine collected at the end of each experiment was filtered and diluted to a volume of 30 ml. An aliquot (0.1 ml.) of this was used for C^{14} analysis. The diluted urine was passed through a 2.5 x 17 cm. column of Amberlite IR-120 (H^+ form). The effluent and washings were combined and lyophylized to dryness. The residue was chromatographed on a Celite column as described below. The acetonedicarboxylic acid peak came shortly after that of glutaric acid as is shown in Figure 2.

Rats 133 and 141 received by intraperitoneal injection, glutarate--3-C^{14} and 2 to 2.5 millimoles of sodium β-hydroxyglutarate in two equal doses, 3.5 hours apart. The urine collected at the end of each experiment was filtered and diluted to a volume of 25 ml. An aliquot (0.1 ml.) of this was used for C^{14} analysis. The urine was passed over a 2.5 x 17 cm. Amberlite IR-120 (H^+ form) column and the effluent and washings concentrated to dryness under vacuum. The residue was fractionated over Celite as described below. β-Hydroxyglutaric acid is eluted slowly from Celite, the peak coming considerably after that of malonic acid as is shown in Figure 2.

Rats 139 and 140 were fasted for 48 hours prior to the intraperitoneal administration of glutarate-3-C^{14} and about 2 millimoles of sodium-acetoacetate. The urines were collected (frozen) and treated as described under degradation procedures.

Rat 143 received by intraperitoneal injection, glutarate-3-C^{14} and 2.5 millimoles of ethylmalonic acid. The urine collected over the period

of 24 hours was filtered and diluted to 25 ml. An aliquot (0.1 ml.) was used for C^{14} analysis. The remaining volume was passed over a 2.5 x 17 cm. Amberlite IR-120 (H^+ form) column and the effluent and washings concentrated to dryness under vacuum. The residue was acidified with 3 drops of 25 per cent sulfuric acid and fractionated over Celite as described below.

C. Chromatographic Procedures

1. Column Partition Chromatography

The technique of Phares et al. (32) was employed for the purification of the organic acids used; namely, acetic, acetoacetic, glutaric, acetonedicarboxylic, β-hydroxyglutaric, ethylmalonic and butyric acids. Chromatographic columns (0.9 x 30 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 (approximately 2-3 ml.) containing one of the above-mentioned organic acids obtained as described under the different isolation procedures, was transferred quantitatively through a long capillary tube to the top of the Celite and tamped down. The flow rate during elution 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. Suitable corrections were made for the blank titrations (0.001 ml. of 0.1 N NaOH per 5 ml. of effluent). Phenol red was used as indicator. Figure 2 shows a composite diagram of the position

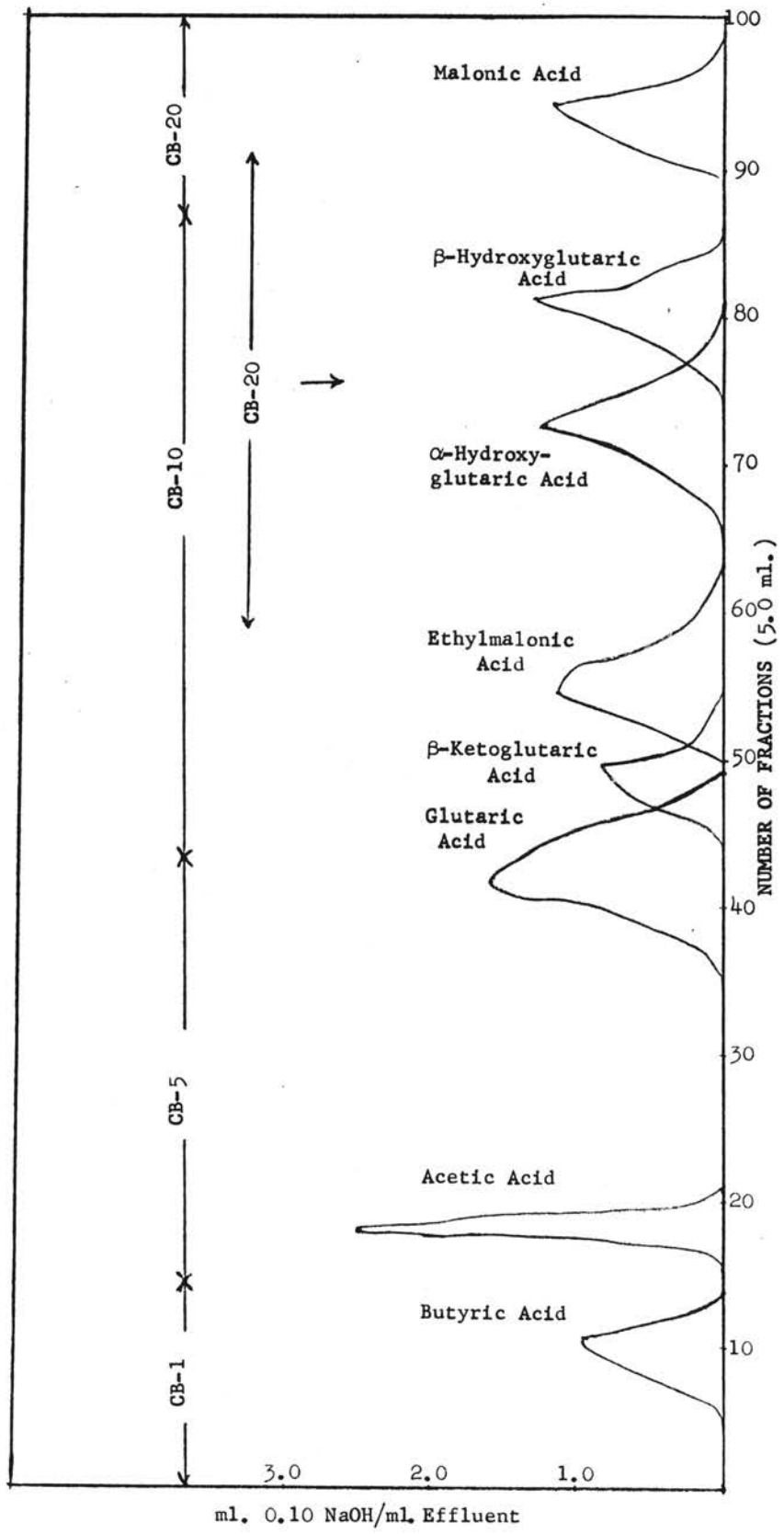


Figure 2. Celite Column Chromatography (Composite Diagram).

of the different acids on a standard chloroform-butanol chromatogram with a column 0.9 by 30 cm. Elution began with 1 per cent butanol in chloroform (CB-1) and continued with increasing amounts of butanol in chloroform as indicated (Figure 2).

2. Paper Partition Chromatography

Aliquots of the eluates from the different chromatographic columns used, as well as of the various diluted animal urines were chromatographed separately on Whatman No. 1 paper in a n-butanol-acetic-acid-water (9-1-2.5) system, using the ascending technique. After development, each chromatogram was steamed to vaporize adsorbed acetic acid and dried under an infrared lamp. The dried chromatograms were sprayed with bromcresol green solution (100 mg. of bromcresol green per 250 ml. of water) and dried. Table II shows the Rf's of the different acids tested by this method.

D. Degradation Procedures

1. Degradation of Acetonedicarboxylic Acid

The acetonedicarboxylic acid peak obtained as described above was lyophilyzed to dryness. The dried material was dissolved in 2 ml. of water, acidified with 2 drops of 25 per cent sulfuric acid, and heated in the Schmidt apparatus (Figure 3). Evolved carbon dioxide was trapped in 5 ml. of carbon dioxide-free 1 N sodium hydroxide, and the liberated acetone was trapped in 5 ml. of ice-cold distilled water. Figure 4 outlines the whole degradation procedure.

TABLE II

R_f VALUES OBTAINED WITH n-BUTANOL-ACETIC ACID-WATER (9-1-2.5)

Ethylmalonic Acid	0.81
Glutaconic Acid	0.75
Glutaric Acid	0.75
Acetonedicarboxylic Acid	0.62
α-Hydroxyglutaric Acid	0.52
β-Hydroxyglutaric Acid	0.52
Citric Acid	0.42
Sulfuric Acid	0.27

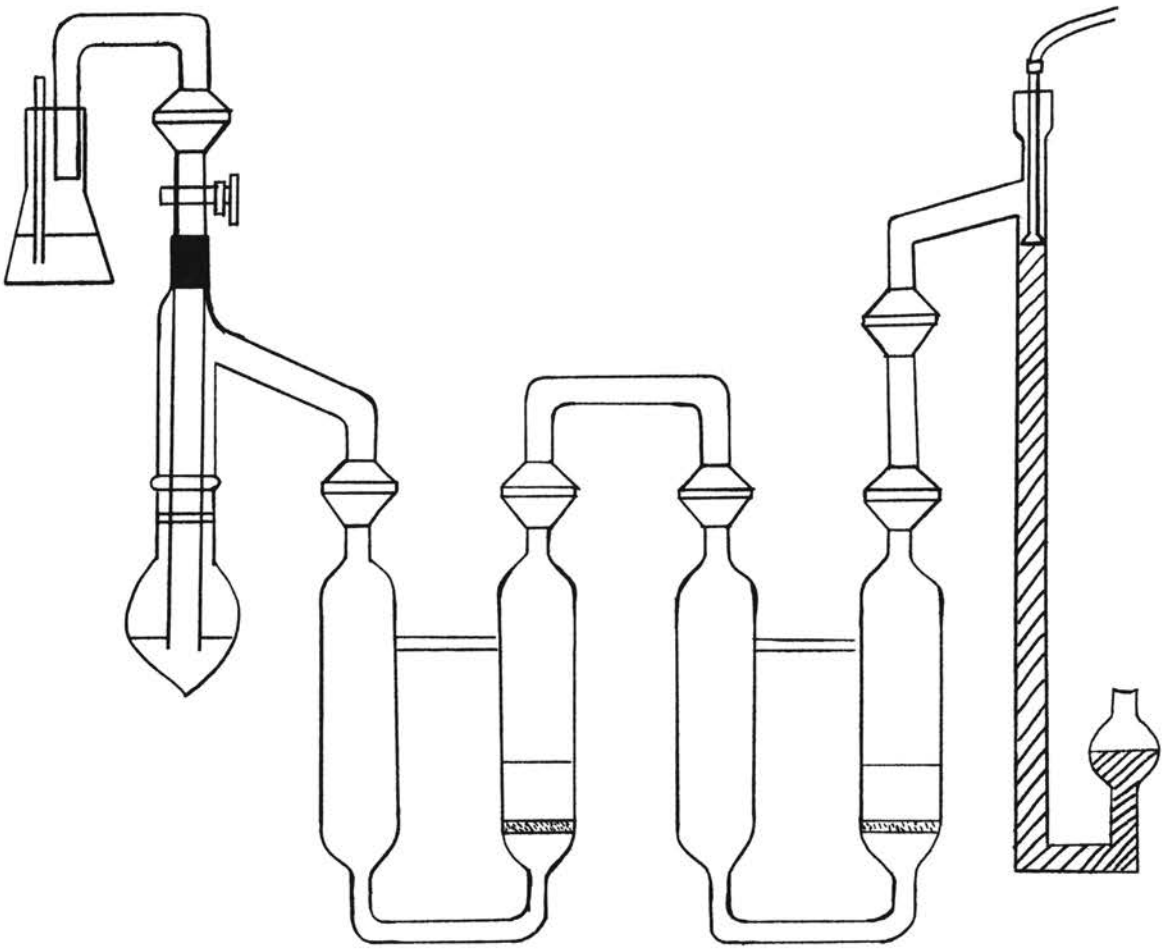


Figure 3. The Schmidt Apparatus

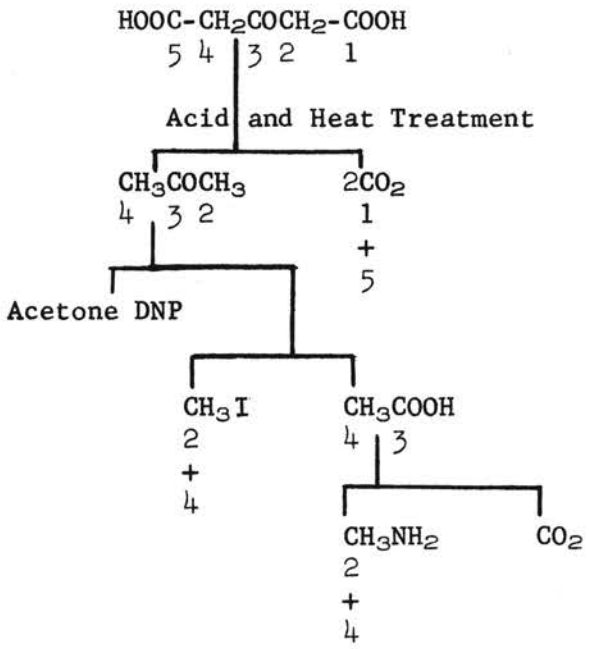


Figure 4. Degradation of Acetonedicarboxylic Acid

To half of the aqueous acetone solution was added dropwise 2,4-dinitrophenylhydrazine reagent (3 gm. of 2,4-dinitrophenylhydrazine in 15 ml. of concentrated sulfuric acid). This solution was then added, with stirring, to 20 ml. of water and 70 ml. of 95 per cent ethanol. The phenylhydrazone precipitate was recrystallized from 3 ml. of 67 per cent hot ethanol. The melting point of the crystals after the second crystallization was 126°.

To the other half of the aqueous acetone solution was added 10 N sodium hydroxide (0.8 ml.) and then iodoform reagent (potassium iodide 20 gm., iodine 10 gm., in 80 ml. water) dropwise until no more iodoform precipitated. The iodoform (carbons 2 and 4 of acetonedicarboxylic acid) was removed by filtration and crystallized from hot 75 per cent ethanol. The melting point of the crystals after the second crystallization was 119°.

The iodoform supernatant obtained above was dried over a hot plate, acidified with 5 ml. of 25 per cent sulfuric acid and steam distilled. The volatile acetic acid and excess iodine were collected in 250 ml. of distillate and acetate was extracted with ether. To the ether extract was added 8 ml. of 10 N sodium hydroxide and ether was removed by a jet of air. The dried material was dissolved in 2 ml. of water, transferred quantitatively to a pear-shaped flask and dried overnight at 110°.

Schmidt Reaction on Sodium Acetate (33)

The dried material in the flask was chilled in an ice bath and 0.6 ml. of 100 per cent sulfuric acid was added for 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 (Figure 3). The air inlet to the reaction flask was kept closed while the temperature of the water bath was raised to 70° 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° or above. The sodium hydroxide solution in the carbon dioxide trap was then transferred to a combustion tube for the determination of carbon dioxide and radioactivity.

2. Degradation of β-Hydroxyglutaric Acid

The β-hydroxyglutaric acid peak obtained as described above was dried on a rotary evaporator, acidified and run over another Celite column for further purification. One-fourth of this purified peak was transferred to a combustion tube, dried in an oven at 90°, and the

residue assayed for C^{14}. To the rest (0.8 millimoles) of the β-hydroxyglutaric acid peak was added 65 ml. of distilled water, 10 ml. of 50 per cent sulfuric acid and 35 ml. of 10 per cent mercuric sulfate (34). The mixture was heated to boiling, treated with 8 ml. of 5 per cent potassium dichromate solution, and heated under reflux for 90 minutes. This procedure converted the middle three carbons of β-hydroxyglutaric acid to the mercury complex of acetone (34). At the end of the refluxing period the solution was cooled to room temperature and the yellowish-orange precipitate was removed by filtration and dried at 110° for one hour. The dried mercury-acetone complex was placed in 8 ml. of 2 N hydrochloric acid in a pear-shaped flask. This was attached to a Schmidt apparatus (Figure 3) and heated gently to boiling. The liberated acetone was trapped in 8 ml. of ice-cold distilled water. The phenylhydrazone derivative and iodoform were prepared and assayed as described above.

3. Degradation of Acetoacetate

Acetoacetic acid was decomposed into carbon dioxide and acetone (Figure 5) by the method of Van Slyke (34). This method was used for urinary acetoacetate in the metabolic overload experiment.

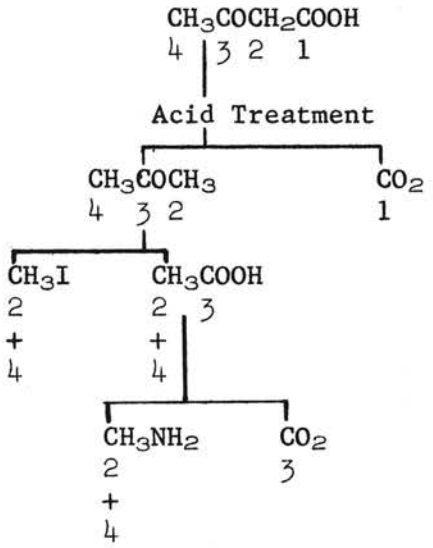


Figure 5. Degradation of Acetoacetic Acid

The urine, collected over a 13-hour period in a flask cooled in a dry ice-acetone mixture, was filtered and diluted to a volume of 25 ml. An aliquot (0.1 ml.) was removed for C^{14} analysis. The remaining urine was transferred to a 250 ml. volumetric flask containing 50 ml. of 20 per cent copper sulfate solution. Immediately a 10 per cent calcium hydroxide suspension (approximately 50 ml.) was added until the solution was alkaline (pH 8 to 9). After standing at room temperature for one hour and at 5° for two hours, the mixture was filtered. The filtrate was cooled to 3° in an ice bath, adjusted to pH 2 and aerated for 20 minutes with carbon dioxide-free air to remove any dissolved carbon dioxide. After the addition of 10 ml. of 10 per cent mercuric sulfate the solution was heated under a reflux for one hour while the aeration was continued. The liberated carbon dioxide (from carbon 1 of acetoacetate) was trapped in 1 N sodium hydroxide (5 ml.). The acetone-mercury complex was removed by filtration, washed with 100 ml. of water and dried at 110° for one hour. This was converted to acetone as described above.

4. Degradation of Ethylmalonic Acid

The ethylmalonic acid peak (Figure 2) obtained as described above was pooled. An aliquot (0.1 millimole) was transferred to a combustion tube for C^{14} analysis. The rest of the 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 (Figure 3) and heated in a mineral oil bath to 180°. The liberated carbon dioxide was trapped in 1 N sodium hydroxide (5 ml.) and the butyric acid was trapped in ice-cold acidified water (5.0 ml.). The butyric acid was

purified by Celite chromatography. The liberated carbon dioxide and purified butyric acid were assayed for radioactivity.

E. Radioactivity Determinations

Carbon analyses and C^{14} assays involved manometric measurement of CO_2 (35) and determination of radioactivity with a vibrating reed electrometer (36).

CHAPTER III

RESULTS AND DISCUSSION

A. Oxidation of Glutaric Acid-3-C^{14}

The experimental data are presented in Tables I, III and IV.

The extent of the oxidation of glutaric acid-3-C^{14}, as measured by $C^{14}O_2$ production, is shown in Table IV. It is apparent from Table IV that acetonedicarboxylic acid and β-hydroxyglutaric acid inhibit glutaric acid oxidation. In Rat 128, Table III, a dose of acetonedicarboxylic acid, slightly greater than that given Rats 127 and 131, diminished the $C^{14}O_2$ excretion virtually to zero, while the untreated Rat 129 converted 71 per cent of a dose of glutaric acid-3-C^{14} to $C^{14}O_2$ in two hours. As a result of this observation, Rat 131 was given glutaric acid-3-C^{14} fifteen minutes before acetonedicarboxylic acid to assure the presence of labeled metabolites; and subsequently acetonedicarboxylic acid toxicity studies were run as shown in Table III. These studies show that acetonedicarboxylic acid, in doses of one milligram per gram body weight, does not greatly depress the oxidation by rats of acetate-1-C^{14}, alanine-1-C^{14}, or glutamic acid--5-C^{14}.

The release of $C^{14}O_2$ from glutaric acid-3-C^{14} by intact rats has already been reported by Hobbs and Koeppe (23). Oxidation of glutaric acid--1,5-C^{14} by liver mitochondria has been reported by Rothstein and Greenberg (37), and by Bagchi et al. (28).

TABLE III

ACETONEDICARBOXYLIC ACID TOXICITY EXPERIMENTS

Rat No.	Rat weight	Milligram acetone-dicarboxylic administered	Labeled acid administered	μc acid administered	Per cent $C^{14}O_2$ exhaled	Duration of experiment (hrs)
128	390	453	Glutaric acid-3-C^{14}	13.5	0	42
129	385	None	Glutaric acid-3-C^{14}	2.36	81.3	23
136 (i)	420	347	DL-Alanine-1-C^{14}	15.2	73.5	18
(ii)		None	DL-Alanine-1-C^{14}	14.35	124	25 1/2
142 (i)	245	None	Sodium acetate-2-C^{14}	13.26	57	24
(ii)		245	Sodium acetate-2-C^{14}	13.26	67.8	24
(iii)		None	Glutamic acid-HCL-5-C^{14}	7.0	49	24
(iv)		247	Glutamic acid-HCl-5-C^{14}	7.0	42	24
(v)		254	Glutaric acid-3-C^{14}	9.44	44	21 1/2

Table IV shows that the administration of acetonedicarboxylic acid or β-hydroxyglutaric acid with glutaric acid-3-C^{14} facilitated a more rapid urinary excretion of C^{14}. Whereas Rat 129 (Table III), given labeled glutaric acid alone, excreted less than 10 per cent of the dose in the urine in 24 hours, the treated rats excreted from 18.6 to 32.2 per cent of the dose as shown in Table IV. In the case of Rat 127, most of the urinary C^{14} was located in the glutaric acid peak.

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

As shown in Table IV, all efforts to demonstrate the formation in vivo of acetonedicarboxylic acid, β-hydroxyglutaric acid, or ethylmalonic acid from glutaric acid-3-C^{14} failed. Although the β-hydroxyglutaric acid peaks (Figure 2) obtained from the urine had small amounts of activity, the acetone prepared from one of these peaks had negligible activity. Thus, these results supported by similar studies in vitro (28) do not agree with the postulate (21, 23) that these acids are intermediates in glutaric acid catabolism. However, the failure of glutaric acid-3-C^{14} to label acetonedicarboxylic acid, β-hydroxyglutaric acid or ethylmalonic acid does not disprove the previous postulates (21, 23), since exogenous acid may not equilibrate with that formed endogenously (38, 39, 40).

C. Labeling in Acetoacetic Acid

As shown in Table IV, glutaric acid-3-C^{14} was converted in vivo to acetoacetic acid containing C^{14} only in the carboxyl and carbonyl carbons. The ratio of carbonyl to carboxyl radioactivity was greater than 3 in both experiments. These results were supported by studies in vitro (28). Similar results have been reported by Crandall et al. (41) who found that octanoic acid-7-C^{14} gave rise in vitro to acetoacetate in which the carbonyl

TABLE IV

DATA OBTAINED FROM RATS GIVEN GLUTARIC ACID-3-C^{14} AND SUSPECTED CATABOLITES

Rat No.	Rat weight	Catabolite acid administered	Amount administered	Glutaric acid-3-C^{14} given	Injection exhaled as CO_2	Duration of experiment	Urine excretion data								
							Total radioactivity	Total catabolite isolated	β-Hydroxyglutarate peak	Acetone from catabolite	Acetate from acetone	Carboxyl of acetate from acetone	Iodoform from acetone	Carboxyl from acetoacetate	Butyrate from ethylmalonate[f]
	g		mmole	μc	%	hrs	μc	mmole	mμc/mmole						
127	280	Acetonedicarboxylic	2.5	23.6	7.9	43	4.4	0.5		0	2.2[a]				
131	370	Acetonedicarboxylic	2.6[b]	11[b]	36	9[c]	2.2	0.9		0					
133	320	β-Hydroxyglutaric	2.5[d]	12.8[d]	56	53	2.8	1.2	9						
141	253	β-Hydroxyglutaric	2[d]	11.8[d]	47	25	3.8	1.2	24.6	0.3[a]					
139	170	Acetoacetic	2	4.7	58	13	0.9	0.36(0.28)[e]		697		483	0.5	135	
140	123	Acetoacetic	2	12.8	42	13	2.3	0.49(0.05)[e]		1740		1355	0	111	
143	326	Ethylmalonic	2.5	9.4	50.8	24	0.7	0.82							0.5

[a] Too low for accurate measurement. These samples may have had no radioactivity.

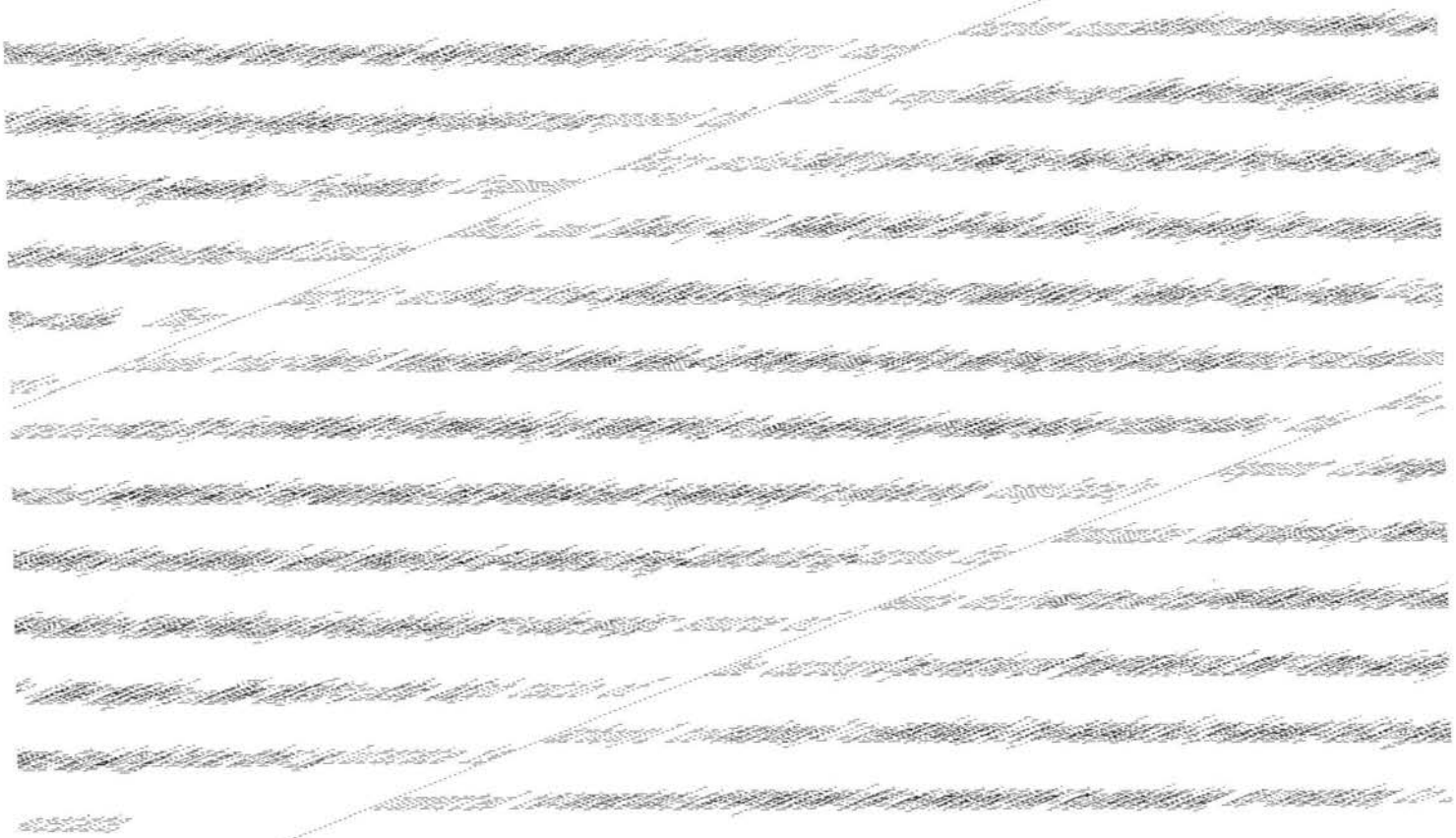
[b] The glutaric acid-3-C^{14} was given 15 minutes before the acetonedicarboxylic acid.

[c] This animal died of suffocation. The exact time of death is not known.

[d] Given in 2 doses, 3.5 hours apart.

[e] Figures are based on CO_2 release; those in parentheses are based on recovery of the mercury-acetone complex.

[f] Purified once on a Celite column.



CHAPTER IV

SUMMARY

When male rats were given glutaric acid-3-C^{14} and either acetonedicarboxylic acid, β-hydroxyglutaric acid, ethylmalonic acid, or acetoacetic acid, urine analysis gave the following results:

1. The three middle carbons of acetonedicarboxylic acid and β-hydroxyglutaric acids contained no detectable C^{14}.
2. The butyric acid obtained from the decarboxylation of ethylmalonic acid contained no significant radioactivity.
3. Acetoacetic acid was labeled in the carbonyl and carboxyl carbons, the carbonyl to carboxyl ratio being at least 3:1.

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

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PART II

THE METABOLISM OF α,γ-DIAMINOBUTYRIC ACID-2-C^{14} IN INTACT RATS

PART II

CHAPTER I

INTRODUCTION

In 1947 two groups of investigators reported almost simultaneously the isolation of a new antibiotic polypeptide from Bacillus polymyxa (1, 2) and called it polymyxin. A month later, a third group (3) reported the isolation of the antibiotic aerosporin from Bacillus aerosporus. Further investigation (4) has shown that the two species, Bacillus polymyxa and Bacillus aerosporus, are identical. Other strains have been found to elaborate related antibiotics, and these are known collectively as polymyxins. At present, a total of five polymyxins are known (4) and are designated polymyxin A, B, C, D and E. Circulin (5), isolated from Bacillus circulans, and polypeptin (6, 7), isolated from Bacillus kremieniewski, also appear to belong to the polymyxin category.

The polymyxins differ somewhat in pharmacological properties but are similar in having a narrow antibacterial spectrum that is limited to certain gram negative bacteria, including Hemophilis influenza, Brucella abortus, Salmonella enteritidis, Escherichia coli, and Proteus vulgaris (2).

The early clinical use of polymyxin D (ca 1950) was subsequently abandoned due to its production of some undesirable side-effects, especially kidney damage. Polymixin D (8), the form then employed clinically, was

found to contain D-serine and was somewhat more toxic than polymyxins B and E which do not contain this amino acid. Polymyxin B is used mainly for topical application. It acts synergistically with a number of other antibiotics like bacitracin, an antibiotic which is effective against gram positive pathogens.

The polymyxins are also related chemically. They are strongly basic, cyclic polypeptides. They all yield α,γ-diaminobutyric acid, threonine, and isopelargonic acid, a branched chain saturated, optically active fatty acid (6-methyl-octan-1-oic). Polypeptin is reported to contain an isooctanoic acid (7) rather than isopelargonic acid. Most of the diaminobutyric acid residues in polymyxin have free γ-amino groups (9, 10). The amino acid composition of the various members of the polymyxin complex have been determined (11) and are summarized in Table I.

Studies on the structure of polymyxin B, conducted independently by Hausman and Craig (12) and by Biserte and Dautrevaux (10), were in substantial agreement in suggesting a cyclic octapeptide in so far as no free α-amino groups were detectable. Hausman and Craig (13) have established that commercial polymyxin B is itself a mixture of two components, polymyxins B_1 and B_2. Polymyxin B_1 comprises about 65 per cent of the antibiotic and polymyxin B_2 the remainder. When the same workers (13) subjected polymyxin B to partial acid hydrolysis they obtained a complicated mixture of strongly basic peptides. These were converted to their dinitrophenyl derivatives and were separated by counter current distribution, or were fractionated on Dowex 50 ion exchange resin (10). The identification of the dinitrophenyl derivatives showed that all of the free amino groups were the γ-amino groups of the α,γ-diaminobutyric acid residues. No dinitrophenyl derivatives of α-amino acids were found.

TABLE I

AMINO ACID COMPONENTS OF THE POLYMYXINS

	Polymyxin Type						
Amino Acid Components	A	B	C	D	E	Polypeptin	Circulin
Leucine	D	1L		1D	D	2L	1D
Phenylalanine		1D	L			1D	
Threonine	L	2L	L	3L	L	1L	1L
Serine				1D			
α,γ-Diaminobutyric Acid	L & DL	5L	L	5L	L	3L	5L
Valine						1D	
Isoleucine						1L	

The presence of one free carboxyl group was indicated by titration. These data suggested that polymyxin B_1 is also a cyclic polypeptide. The two possible structures suggested by these studies (10, 13) are shown in Figure 1 and Figure 2.

In addition to the polymyxins, colistin also contains α,γ-diaminobutyric acid. Colistin is an antibiotic peptide which contains five molecules of α,γ-diaminobutyric acid and one molecule of each of the following: L-threonine, L-leucine, D-leucine, and isopelargonic acid (14). Recently, Namiki (15) described a new method for the production and purification of colistin. Also, he showed that the antibiotic has a remarkable inhibitory effect on Shigella dysenteria, Salmonella typhosa, Escherichia coli, and Pseudomonas aeruginosa.

From this brief survey, it is apparent (Table I) that α,γ-diaminobutyric acid is an important component of several useful antibiotics. To our knowledge, prior to this study no investigations were conducted regarding the mammalian catabolism of α,γ-diaminobutyric acid. The aim of this investigation was to study the gross metabolism of α,γ-diaminobutyric acid by rats. The approach used was to synthesize DL-, L-, and D-α,γ-diaminobutyric acids-2-C^{14} by the Schmidt reaction (16) from DL-, L-, and D-glutamic acids-2-C^{14}, and to study their metabolism in rats. After administration to rats, the exhaled $C^{14}O_2$, the excreted C^{14} in urine and the labeling patterns in carcass aspartate, glutamate and alanine (17) were determined.

The results of this study showed (a) that the L-isomer of α,γ--diaminobutyric acid is oxidized more rapidly that the D-isomer, (b) that the urine had a substantial amount of radioactivity, (c) that most of the radioactive components of the urine were probably free or conjugated

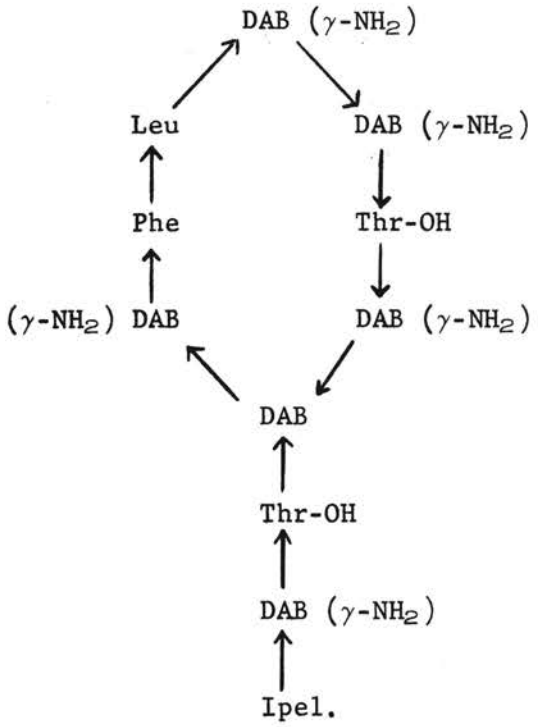

Figure 1.

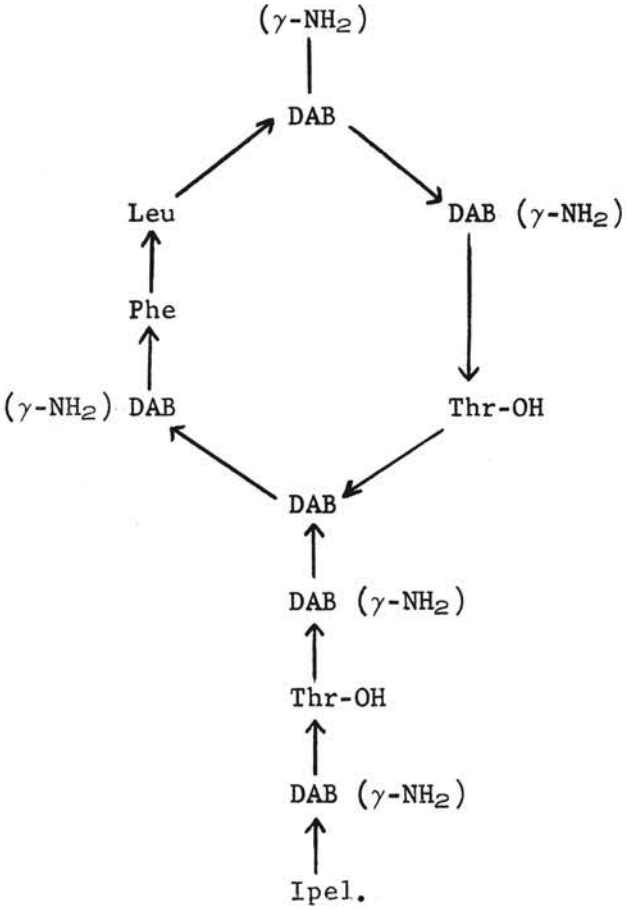

Figure 2.

Polymyxin B (Possible Structures)

α,γ-diaminobutyric acids and (d) that the labeling patterns observed in isolated carcass glutamate, aspartate and alanine do not indicate an obvious route for the oxidation of these compounds.

CHAPTER II

EXPERIMENTAL

A. Synthesis of α,γ-Diaminobutyric Acid-2-C^{14}

DL-, L- and D-α,γ-diaminobutyric acids-2-C^{14} have been synthesized by the Schmidt reaction (16) as modified by Koeppe and Hill (17), from DL-, L- and D-glutamic acids-2-C^{14}. The D- and L-glutamic acids-2-C^{14} were prepared by Dr. W. E. Wilson (18).

In a pear-shaped flask were placed 0.14 millimole of glutamic acid-2-C^{14} and 0.16 millimole of unlabeled glutamic acid. The flask was stoppered and cooled in an ice bath, and then 0.40 ml. of 100 per cent sulfuric acid was added with shaking and warming, until the glutamic acid dissolved. The resulting sulfuric acid solution was chilled in an ice bath. Then 75 mg. of sodium azide were added with shaking to disperse the azide crystals. The flask was quickly attached to the apparatus shown in Figure 3, one trap of which contained an acid permanganate solution while the second trap contained 5 ml. of 1 N carbon dioxide-free sodium hydroxide. The reaction usually proceeded vigorously upon warming the reaction flask to 40° C. When the rapid gas evolution subsided, the water bath temperature was raised to 50° C over a period of one hour and then kept at this temperature for one hour. The bath's temperature was finally raised to boiling over a period of half an hour, during which time the system was swept with carbon dioxide-free air. Then the reaction flask was removed and one drop of fuming sulfuric acid and 35 mg. of sodium

azide were added. The system was closed again and sweeping with carbon dioxide-free air continued for half an hour while the reaction flask was heated at 90° C. Evolved carbon dioxide, which was trapped in alkali, was assayed for radioactivity and found to contain less than 0.01 to 0.03 per cent of the total radioactivity of the glutamic acid used for synthesis.

The contents of the flask were poured into a 150 ml. beaker and titrated to pH 6.0 (pH meter) with a hot saturated barium hydroxide solution. Precipitated barium sulfate was removed by centrifugation. The supernatant was adjusted to pH 3.0 with 2 N hydrochloric acid. The resulting solution (90 ml.) was passed over a Dowex-I column (OH^- form; 13 x 1.5 cm.). The column was washed with distilled water until the effluent reaction was neutral, then it was eluted with 0.1 N acetic acid solution until the acetate band was approximately 8 cm. deep. Frequent ninhydrin tests (all negative) were run on the eluate during this elution period. Then the Dowex-I column was mounted over an Amberlite IR-45 column (acetate form; 13 x 1.5 cm.). The columns were eluted with approximately 30 ml. 0.1 N acetic acid solution, 3.0 ml. fractions being collected on an automatic fraction collector. On cold runs this method was shown to effect the separation of α,γ-diaminobutyric acid from glutamic acid, the latter being eluted 6 to 10 fractions after the last fraction of α,γ-diaminobutyric acid. Fractions containing ninhydrin-positive material were pooled in a beaker and the pH of this solution was adjusted to about 1 with 2 N hydrochloric acid solution. The beaker was then placed on a hot plate (70 to 80°), and its contents were evaporated to dryness by a jet of air.

The material in the beaker was dissolved in approximately 1.5 ml. of distilled water. The pH was brought up to 3.5 to 3.8 with a few

drops of 25 per cent pyridine. After the solution had been cooled in the refrigerator for an hour, α,γ-diaminobutyric acid monohydrochloride was crystallized by the addition of approximately 10 ml. of absolute ethanol. After standing in the deep freeze over night, the crystals were removed by filtration, washed with ethanol, acetone and anhydrous ether, and dried in a dessicator over phosphorous pentoxide. The yield of DL- and L-α,γ-diaminobutyric acid-2-C^{14} monohydrochloride was approximately 70 per cent. A 50 per cent yield of the labeled D-isomer was obtained.

Paper chromatography of the compound in an 80 per cent phenol-water mixture containing 1 per cent ammonium hydroxide (19) gave a single ninhydrin spot with an R_f value of 0.74, identical to that of authentic α,γ-diaminobutyric acid. All of the radioactivity on the chromatogram was shown by the paper strip scanning technique to be located in the area occupied by the ninhydrin spot.

The melting points of the synthesized DL-, and L-diaminobutyric acids-2-C^{14} ranged from 245 to 247° C, and that of D-diaminobutyric acid-2-C^{14} was 237° C. The labeled D-isomer appeared to be less pure.

B. Determination of the Purity of L- and D-Diaminobutyric Acids-2-C^{14}

The procedure for determining the purity of each isomer of α,γ-diaminobutyric acid-2-C^{14} was that of Minthorn and Koeppe (20). This procedure employs repeated crystallization to a constant specific activity of a mixture of enantiomorphs in which one (unlabeled) is present in a large excess of the other (labeled). These crystallizations would be expected to lead to a purification of the more abundant isomer; hence, the presence of isotope in the purified abundant isomer would represent contamination of the labeled antipode.

When the synthesized L-α,γ-diaminobutyric acid-2-C^{14} was diluted about 500-fold with unlabeled D-α,γ-diaminobutyric acid it was found that, after seven crystallizations, the picrate derivative of D-α,γ-diaminobutyric acid had a specific activity of 1.56 microcuries per millimole. This corresponds to a maximum contamination of 1.6/96.6, or 1.7 per cent of the synthesized L-α,γ-diaminobutyric acid-2-C^{14} by D-α,γ-diaminobutyric acid-2-C^{14} (Table II).

When the synthesized D-α,γ-diaminobutyric acid-2-C^{14} was diluted about 300-fold with unlabeled L-α,γ-diaminobutyric acid, the recrystallized picrate derivative of L-α,γ-diaminobutyric acid had a specific activity of 0.9 microcuries per millimole. This indicated that the greatest possible contamination of the synthesized D-α,γ-diaminobutyric acid-2-C^{14} by L-α,γ-diaminobutyric acid-2-C^{14} was 0.9/69.0, or 1.4 per cent.

C. Animal Experiments and Isolation of Amino Acids

Intraperitoneal injections of the isomers of α,γ-diaminobutyric acid-2-C^{14} and collection of exhaled $C^{14}O_2$ and excreted urine were carried out as previously described under Part I, Chapter II. Table III summarizes the data from the individual experiments.

After the rat was killed, "carcass" and liver protein powders were prepared (21). "Carcass" refers to the entire animal, except liver, including the washed gastrointestinal tract.

The carcass protein from Rats 151 and 159 was hydrolyzed by boiling under reflux for 24 hours with 10 volumes of 6 N hydrochloric acid. After repeated vacuum distillation, the hydrolysates were treated with charcoal (Darco G-60) (22).

The various amino acids were then fractionated and isolated by displacement chromatographic techniques as described by Partridge and Brimley (23) as modified by Koeppe et al. (21).

TABLE II

SPECIFIC ACTIVITIES AFTER SUCCESSIVE RECRYSTALLIZATIONS OF L- AND D-α,γ-DIAMINOBUTYRIC ACID-2-C^{14} DILUTED 300 TO 500-FOLD WITH CARRIER D- OR L-α,γ-DIAMINOBUTYRIC ACID

No. of recrystallization	μc/mmole[1]			
	L^{14} + D	L^{14} + L	D^{14} + L	D^{14} + D
α,γ-Diaminobutyric Acid-2-C^{14}				
1		95.7	45.6	69.3
2	49.0	96.6	12.3	71.2
6	12.1			
7	9.2			
Picrate Derivative on 2				
1		93.2	1.3	66.8
2			1.2	68.5
3			0.9	
Picrate Derivative on 7	1.6			

[1]Corrected for dilution with carrier. Therefore the results give the relative specific activities of each precipitate.

TABLE III

A SUMMARY OF THE EXPERIMENTAL DATA

Rat No.	Weight in gm.	Compound administered	Microcuries injected	Per Cent microcuries exhaled	Per Cent microcuries in urine	Duration of experiment (hrs)
151	135	DL-α,γ-Diaminobutyric Acid-2-C^{14}	11.4	15.3	48.2	24
157	106	DL-α,γ-Diaminobutyric Acid-2-C^{14}	5.7	10.2	26.8	24
159 A	157	L-α,γ-Diaminobutyric Acid-2-C^{14}	2.6	27.7	18.9	24
B	157[1]	L-α,γ-Diaminobutyric Acid-2-C^{14}	14.4	18.8[2]	22.6	24
168[3]	150	D-α,γ-Diaminobutyric Acid-2-C^{14}	0.536	6.7	46.3	27

[1]Administered 24 hours after first dose.

[2]Based on second injection administered.

[3]Female rat.

The isolated aspartic acid, glutamic acid and alanine were characterized by paper chromatography and ninhydrin-released CO_2. The chromatography was performed on paper impregnated with 0.1 M phosphate buffer (pH 7) by using 76 per cent aqueous ethanol as the developing solvent. All the samples were recrystallized to constant specific activity with the exception of carcass alanine of Rat 151.

D. Degradation Procedures

The degradation of glutamic acid was carried out by the method of Mosbach et al. (24) as modified by Koeppe and Hill (21).

The degradation of alanine was carried out as described by Koeppe, Minthorn and Hill (25).

Aspartic acid was decarboxylated with ninhydrin (26).

Carbon analyses and C^{14} assays were accomplished by the use of procedures discussed in Part I, Chapter II. These involved manometric measurement of carbon dioxide and determination of radioactivity with a vibrating-reed electrometer.

E. Characterization of Excretory Products in Urine

The urine collected at the end of each experiment was filtered and diluted to a 25 ml. volume. An aliquot (0.1 ml.) of this was used for C^{14} analyses. The diluted urine was passed through a Dowex-I column (OH^- form, 20 x 1.5 cm.). The column was washed with 250 ml. of distilled water. Both the effluent and the washings were combined and dried on a hot plate with the aid of a jet of air. The dried material was made up to a known volume and an aliquot of it was withdrawn and assayed for C^{14}. The Dowex-I column was then eluted with approximately 200 ml. of 1 N acetic acid, and the eluate was lyophylized to dryness. The residue was dissolved in 25 ml. of water and an aliquot was assayed for C^{14}.

The remaining volume was passed over an Amberlite IRC-50 column (acetate form, pH 4.7; 25 x 1.0 cm.). The column was washed with 250 ml. of distilled water. Both the effluent and the washings were combined and dried. The dried material was dissolved in water and assayed for C^{14}. The IRC-50 column was then eluted with approximately 200 ml. of 0.2 N hydrochloric acid. The eluate was dried, made up to volume, and assayed for radioactivity.

Aliquots from the different effluents and eluants obtained from the Dowex-I and the Amberlite IRC-50 columns were chromatogramed separately on Whatman No. 1 paper in an 80 per cent phenol-water mixture containing 1 per cent ammonium hydroxide (19). After development, the chromatogram was dried, sprayed with 0.2 per cent ninhydrin and dried again. Strips from the chromatogram containing the ninhydrin-positive spots were cut out and run on a paper strip scanning instrument to see whether all of the radioactivity on the chromatogram was located in an area identical with the ninhydrin spots.

To two volumes (10 ml. each) of the Dowex-I eluant from the urine of Rat 151 were added separately 2 millimoles of L-α,γ-diaminobutyric acid and 2 millimoles of D-α,γ-diaminobutyric acid. Successive crystallizations, as well as picrate derivatives, were made as described under the determination of the purity of L- and D-α,γ-diaminobutyric acids.

CHAPTER III

RESULTS AND DISCUSSION

A. Gross Distribution of C^{14}

The gross distribution of C^{14} in the tissues and excretory products of these rats following intraperitoneal administration of DL-, L-, and D-α,γ-diaminobutyric acid-2-C^{14} is shown in Table IV. Both isomers are oxidized to CO_2 by rat tissue. The presence of large amounts of C^{14} in urine suggests that the ability to oxidize α,γ-diaminobutyric acid is limited.

B. Excretion of $C^{14}O_2$

A general summary of the experimental data is presented in Table III (page 40). Data relating to the rate of excretion of $C^{14}O_2$ may be found in Tables III and V. From Tables III and V it may be seen that the rate of excretion of $C^{14}O_2$ by rats receiving DL-α,γ-diaminobutyric acid-2-C^{14} is somewhat slower than the rate by rats receiving the L-isomer. If one considers that 50 per cent of the injected dose of DL-α,γ-diaminobutyric acid-2-C^{14} is L-α,γ-diaminobutyric acid-2-C^{14}, then one might conclude that most of the exhaled radioactivity of the rats receiving DL-α,γ-diaminobutyric acid-2-C^{14} is due to the oxidation of the L-isomer. On the basis of this assumption, D-α,γ-diaminobutyric acid would be expected to be metabolized more slowly than its enantiomorph. The presence of only 7 per cent of the administered radioactivity in the exhaled CO_2 of Rat 168 substantiates this expectation. It should also be

TABLE IV

GROSS DISTRIBUTION[1] OF C^{14} IN TISSUES AND EXCRETORY PRODUCTS OF RATS ADMINISTERED α,γ-DIAMINOBUTYRIC ACIDS-2-C^{14}

	Rat 151[2]	Rat 159	Rat 168[3]
Rat weight g.	135	157	150
Test dose mg.	5.2	27.2	1.2
Administered μc	11.4	17.0	0.536
	Per Cent C^{14} Administered Dose		
Exhaled CO_2	15.2	20.6[4]	6.7
Urine	48.2	22.4[4]	46.3
Carcass[5] protein	1.2	1.5	
Liver protein	0.2	0.3	
Liver glycogen	0.2	0.3	
Total	65.0	44.9	53.0

[1]Twenty-four hours after the first administration of the isotope, with the exception of Rat 159 which received two doses of L-α,γ-diaminobutyric acid-2-C^{14} 24 hours apart.

[2]Injected DL-α,γ-diaminobutyric acid-2-C^{14}.

[3]Injected D-α,γ-diaminobutyric acid-2-C^{14}.

[4]Based on total dose injected.

[5]Refers to the entire animal, except liver, including the washed gastrointestinal tract.

TABLE V

EXHALATION OF $C^{14}O_2$ AFTER INJECTION OF α,γ-DIAMINOBUTYRIC ACID-2-C^{14} ISOMERS INTO RATS

Rat No.	α,γ-Diamino-butyric acid-2-C^{14} isomer administered	Amount in μc	The cumulative exhalation of $C^{14}O_2$ as per cent of the injected dose		
			First hour	Second hour	Twenty-four hour
151	DL	11.4	0.4	3.3	15.3
157	DL	5.7	0.4	1.3	10.2
159A	L	2.6	1.5	5.4	33.5
159B[1]	L	14.4	0.7	2.7	21.7
168	D	0.5	0	0.2	7.0

[1]Given 24 hours after the administration of the first dose.

noted that a small dose of L-α,γ-diaminobutyric acid-2-C^{14} is oxidized almost three times as rapidly as a larger dose. This observation is in agreement with the earlier postulate that the ability of rat tissue to oxidize α,γ-diaminobutyric acid is rather limited.

C. Distribution of C^{14} in Carcass Glutamate and Alanine After Administration of DL- and L-α,γ-Diaminobutyric Acid-2-C^{14}

The labeling patterns obtained in carcass glutamic acid and carcass alanine after injection of DL-, and L-α,γ-diaminobutyric acid-2-C^{14} are shown in Tables VI and VII. As may be observed, radioactivity is distributed throughout the entire molecule of both glutamate and alanine. Comparison of these labeling patterns with those given by known metabolic intermediates (17, 21) does not indicate an obvious route for the oxidation of these compounds.

D. Nature of the Urinary Excretion Products

A preliminary study on the nature of the urinary excretion products in the urine of rats receiving the isomers of α,γ-diaminobutyric acid-2-C^{14}, has been performed as described in Part II, Chapter II.

Studies to date have shown that these compounds are either free α,γ-diaminobutyric acids or conjugated α,γ-diaminobutyric acids. Chromatograms obtained from the Amberlite IRC-50 eluants had a radioactive peak having an R_f identical to that of authentic α,γ-diaminobutyric acid, namely 0.73 to 0.75. In another study, it has been found by employing the technique described on page 37 that the eluant from Dowex-I of Rat 151 contains seven times as much D-α,γ-diaminobutyric acid-2-C^{14} as it does L-α,γ-diaminobutyric acid-2-C^{14}. This is compatable with the observation that the L-isomer is oxidized more rapidly than its enantiomorph. However, complete identification of the urinary excretion products will require further investigation.

TABLE VI

LABELING PATTERNS IN CARCASS GLUTAMIC ACID, ASPARTIC ACID, AND ALANINE AFTER ADMINISTRATION OF DL-α,γ-DIAMINOBUTYRIC-2-C^{14}

	Rat 151	
	mμc per mmole	per cent total
Glutamic acid		
Total	3.01	
Carbon 1	1.23	40.9
Aspartic acid		
Total	2.00	
Carbons 1 and 4	0.92	46.0
Alanine		
Total	2.13	
Carbon 1	0.62	29.1
Carbon 2	0.84	39.4
Carbon 3	0.50	23.5
Sum	1.96	92.0

TABLE VII

LABELING PATTERNS IN CARCASS GLUTAMIC ACID, ASPARTIC ACID AND ALANINE AFTER ADMINISTRATION OF L-α,γ-DIAMINOBUTYRIC ACID-2-C^{14}

	Rat 159	
	mμc per mmole	per cent total
Glutamic acid		
Total	3.47	
Carbon 1	1.34	38.6
Carbon 2	0.62	17.9
Carbon 3	0.61	17.6
Carbon 4	0.24	6.8
Carbon 5	0.42	12.1
Sum	3.23	93.0
Aspartic acid		
Total	2.57	
Carbons 1 and 4	1.62	63.0
Alanine		
Total	1.94	
Carbon 1	0.77	39.7
Carbon 2	0.65	35.5
Carbon 3	0.24	14.4
Sum	1.66	89.6

CHAPTER IV

SUMMARY

DL-, L-, and D-α,γ-Diaminobutyric acids-2-C^{14} were synthesized by the Schmidt reaction from DL-, L-, and D-glutamic acids-2-C^{14}.

When these compounds were injected intraperitoneally into rats, radioactivity was found in the exhaled carbon dioxide, in the excreted urine, in the proteins, and in liver glycogen. Further analyses and degradations gave the following results:

1. The L-isomer of α,γ-diaminobutyric acid is oxidized more rapidly than the D-isomer.
2. The excreted urine had a substantial amount of radioactivity.
3. Most of the radioactive components of the urine were probably either free or conjugated α,γ-diaminobutyric acids.
4. The labeling patterns observed in isolated carcass glutamate, aspartate and alanine do not indicate an obvious route for the oxidation of these compounds.

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