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ABSTRACT

The metabolytes from propionic acid metabolism in mouse liver slices have been studied using methods of chromatography for isolation and identification.

Degradation data shows randomization between the α and β carbons of the lactic acid which is formed. When α labeled propionate is the incubation substrate, the activity in the resulting β-hydroxyvaleric acid is principally in the gamma position and the beta and gamma positions are inactive.

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INTRODUCTION

Odd carbon fatty acids are considered to be oxidized in tissue by successive β oxidation in much the same way as even carbon members of the series.[1,2,3,4] The terminal three carbon unit has been investigated to some extent. Atchley[5] demonstrated propionic acid as an end product in the oxidation of valeric acid by a rabbit enzyme system. Malonate inhibition studies with odd carbon fatty acids[6] indicated a small amount of conversion of the three carbon residue to a two carbon unit of the type CH_3-CO. Siegel and Lorber[7] found valeric acid giving much the same metabolic patterns in glycogen in the whole rat as would appear with a mixture of propionic acid and acetic acid.

The intermediates in β oxidation have been investigated[8,9] with enzyme systems. The results indicate comparable rates of oxidation for α-β unsaturated acids and the corresponding saturated acids and formation of identical products. β keto and β hydroxy acids were oxidized to some extent, but indications are that phosphorylated analogues are the actual intermediates.

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The fate of the end product three carbon segment or propionic acid itself is still somewhat in doubt. Lorber et.al. ruled out the possibility of β oxidation to malonate[10] in isotopic experiments. Distribution of activity in this experiment showed an α-β randomization by the time the oxidized propionic acid substrate appeared in rat liver glycogen in the intact rat. A comparable experiment with lactate[11] did not give complete α-β randomization and on these results it is suggested that the oxidation of propionate either does not follow a direct path: propionate to acrylate to lactate to pyruvate or else has a fast side equilibration with symmetrical Krebs cycle intermediates.

In working with isolated rabbit enzymes, Huennekens[12] has supported the theory for a direct pathway, advancing the sequence propionate to acrylate to L-lactate to D-lactate to pyruvate etc. with an α-hydroxy racemase necessary for complete oxidation. The direct path of oxidation has been suggested in the case of mold[13] and muscle tissue[14] previously.

In the present experiments with liver slices it was desired, using methods of chromatography, to show the fairly complete pattern of propionate metabolytes, the substrates being α, β, and carboxyl labeled propionic acid. Sodium bicarbonate-C^{14} and labeled acetate were studied for comparative purposes. It was also hoped, if possible, that some light might be thrown on the mechanism of utilization of the propionic acid by tissue slices.

EXPERIMENTAL

Incubation of Liver Slices. - The incubations were performed with liver slices made by the Deutsch method[15] from year old male non-fasted Bagg albino mice which had been anesthetized and bled before removal of the liver. One gram (wet weight) of liver slices was incubated with approximately

0.01 millimole of radioactive substrate (containing one to five microcuries) in Krebs-Ringer phosphate buffer in one arm Warburg flasks at 37° C. for 2-4 hours. In the malonate inhibition experiment, sodium malonate was present at a 0.005 M concentration in the buffer. The gas phase was air. At the end of the incubation hydrochloric acid was introduced from a hypodermic needle through a rubber syringe cap on the side arm of the vessel. This was mixed with the contents of the flask and the evolved carbon dioxide was collected on sodium hydroxide soaked paper in the center well.

Fractionation of Incubation Mixtures. - The center well papers and well were washed with water, carrier sodium bicarbonate added, and barium carbonate precipitated by the usual method and counted on aluminum disks in a Geiger-Muller counter.

The contents of the main compartment of the flask were homogenized and distilled to drymess *in vacuo* to remove the volatile material. Non-utilized propionic acid in the distillate was determined in one set of experiments by adding a known amount of carrier propionic acid to the sample after incubation was stopped, collecting the distillate from evaporation of the sample, and preparation of the p-bromophenacyl ester of the propionic acid. This was recrystallized to constant specific activity and total activity calculated from the specific activity. The non-volatile residue was taken up in water, centrifuged to remove insoluble protein material and extracted continuously for 18 hours with ether. Aliquots of the ether and water fractions were direct plated and counted in a Nucleometer for total activity.

The water fraction was reduced in volume and passed through a Dow A-1 anion exchange resin to remove salt and eluted with 2 N hydrochloric acid; recovery was 70-80%. The eluate, which contained 75-90% of the water soluble activity, was further analyzed. Two dimensional chromatograms on Whatman No. 1 filter paper were made of the salt-free eluate, using phenol saturated with water at 24°, and 40 wt. % n-butanol-25 wt. % propionic acid-35 wt. % water as the solvent systems. Two dimensional chromatograms were also made of the ether extract with a solvent consisting of 60 vol. % n-propanol-40 vol. % 12 M ammonium hydroxide replacing the phenol. Less streaking of acids occurred in this system and mono- and dibasic acids separated very well in this basic solvent, thus it is particularly effective in differentiating lactic and succinic acids which have similar R_f values in both phenol and butanol-propionic acid solvents. Radioautographs were made of the chromatograms. Relative activities in radioactive compounds on a chromatogram were determined by counting with a thin-window Geiger-Muller counting tube.

Identification of Compounds. - Positions of various compounds on the paper in the solvent systems used were determined as follows: amino acids by spraying with a ninhydrin solution in 95% ethanol and subsequent heating at 100°, sugars by spraying with an aniline-phthalic acid reagent;[16] acids by spraying with bromcresol green (0.2% bromcresol green in ethanol and 0.001 N sulfuric acid was used after ammoniacal propanol was the solvent; 0.2% bromcresol green in the basic form in ethanol was used after butanol-propionic acid was the solvent); urea as a white spot upon spraying with 10% mercuric nitrate and then with 2 N sodium hydroxide. Identification of a radioactive spot in the case of an amino acid or a sugar, for which

the color tests are quite sensitive was considered to be satisfactory if the radioactive area coincided exactly with the colored area when rechromatographed with a known carrier. The spots identified as glucose and fructose were also heated with 6 N hydrochloric acid for 18 hours and yielded on chromatography a radioactive spot coincident with carrier levulinic acid. Several other radioactive spots obtained from the hydrolysis were unidentified.

The indicator spray test for acids was not considered sensitive enough to establish identity of the major acid spots. The activity coinciding with lactic acid was acetylated and rechromatographed to confirm the presence of the hydroxy function. Recrystallization of activity with carrier zinc lactate to constant specific acitivity was considered further proof of identity.

Separation and identification of C_3-C_6 hydroxy and keto acids using the solvent systems described above is unsatisfactory since these compounds tend to have nearly similar R_f values (0.75-0.90). Also, when the amount of radioactivity is small (less than 5000 dis./min.), the time required for a radioautograph is unduly long. The use of a silica column with chloroform-butanol as the solvent system[17] was found to be particularly useful. Carrier compounds including α-hydroxybutyric acid, β-hydroxy-valeric acid, and α-ketobutyric acid were chromatographed with the major unknown compound from the ether extract using chloroform-butanol of increasing butanol content as the developing solvent. Fractions (1.0-2.0 ml.) were obtained of the eluate, titrated with 0.01 N sodium hydroxide, and the C^{14} activity determined by direct plating techniques. Coincidence of the radioactivity curve with the

titration curve is evidence that two acids are identical. The curves obtained coincided when β-hydroxyvaleric acid was used as the carrier acid, Figure 2. Constant specific activity was obtained when the p-toluidide (m.p. 128° C.) made with β-hydroxyvaleric acid together with the radioactive acid was recrystallized. Further evidence of the identity of the unknown acid was obtained by acetylating the radioactive compound together with β-hydroxyvaleric acid and with α-hydroxybutyric acid and then rechromatography on a silicic acid column; the radioactivity coincided with β-acetyl valeric acid (Figure 2).

Degradation of Alanine, Lactic Acid and β-Hydroxyvaleric Acid. - To determine the distribution of radioactivity in the metabolytes alanine, lactic acid, and β-hydroxyvaleric acid they were degraded.

Alanine was degraded by the ninhydrin method of Van Slyke and McFadden.[18] The acetaldehyde and carbon dioxide obtained were distilled *in vacuo* into a flask containing p-nitrophenylhydrazine (cooled in liquid nitrogen), the reaction vessel was replaced by a flask containing sodium hydroxide, and the carbon dioxide distilled back into it by warming. By this method one is able to determine on one sample the amount of C^{14} in the carboxyl and in the α+β carbons of alanine.

Lactic acid after several recrystallizations with carrier zinc lactate was oxidized with 2 M chromic trioxide in 0.5 M sulfuric acid at 100°[19] and the carbon dioxide determined as barium carbonate. Our experiments indicated that as much as 10% of the α-carbon of synthetic lactic acid may be oxidized to carbon dioxide by this procedure. The acetic acid obtained on degradation of lactic acid was steam distilled and degraded by the Schmidt reaction[20]

to yield carbon dioxide and methyl amine; the methyl amine obtained was oxidized by basic potassium permanganate at 100° to yield carbon dioxide.

β-Hydroxyvaleric acid was oxidized in the same manner as lactic acid to yield propionic acid as the main radioactive product with a small amount of carbon dioxide and acetic acid. The acetic acid and propionic acid were separated and identified by chromatography on a silica column in the same manner as was described for the hydroxy and keto acids. The propionic acid was degraded by the Schmidt reaction to carbon dioxide and ethyl amine. It was also degraded by conversion to lactic acid[21] which was degraded as described above.

RESULTS AND DISCUSSION

The amount of propionate metabolized by the liver slices varied with time of incubation, freshness of the slices, etc., but ran from about 30 to 50%. In a two-hour experiment with carboxyl labeled propionate, 66% of the activity was recovered as propionic acid, 17% as carbon dioxide, 10% in non-volatile water soluble compounds, and less than 0.5% in the insoluble protein residue. A 3 hour incubation gave corresponding values of 59% propionic acid, 23% carbon dioxide, 12% water soluble, and less than 0.5% insoluble protein.

The distribution of activity in various fractions and metabolytes is shown in Tables I and II and in the chromatograms illustrated in Figure 1. As would be expected with propionate as substrate, the major portion of the activity was found in glucose and the glycogenic compounds, alanine and lactic acid. Fructose, glutamic acid, aspartic acid, malic acid and urea also contained significant amounts of activity. The other major

component was identified as β-hydroxyvaleric acid. When acetate was the substrate, about half of the non-volatile water soluble activity was β-hydroxybutyric acid.

Degradations of alanine and lactic acid are shown in Table III. In experiments by Lorber, et.al.[10] and Siegel,[7] on whole animals, a randomization was found between the 1, 2, 5 and 6 carbon atoms of glycogen when propionic acid or propionic producing material (valeric acid) was fed. Our results show a similar randomization in the α-β positions of lactic acid formed from propionic acid in vitro. Such randomization could occur in two ways. Either there is a direct addition of carbon dioxide to propionic acid forming succinic acid (the reverse of a reaction observed in some bacteria[22]) which is then oxidized through fumaric and malic acids leading to a symmetrical distribution in the lactic, or a direct oxidation to pyruvate is accompanied by an extremely rapid equilibration with symmetrical intermediates in the tricarboxylic acid cycle.

The degradation of the β-hydroxyvaleric acid is shown in Table IV. When α-labeled propionate is the substrate, the γ position of the hydroxy valeric acid contains activity while the β and Δ positions are inactive. This would indicate that a direct propylation has occurred between propionate and a C_2 fragment before the α,β carbons of the propionate have become randomized as in the formation of lactic acid. The formation of β-hydroxy valeric acid is analagous to the formation of acetoacetic acid and β-hydroxy butyric acid from acetate and might be considered the reverse of β-oxidation of a fatty acid.

The malonate inhibition experiment showed a lowering of the amount of propionate metabolized to carbon dioxide and also of the water soluble compounds formed.

SUMMARY

The metabolytes from propionic acid metabolism in mouse liver slices have been studied using methods of chromatography for isolation and identification.

Degradation data shows randomization between the α and β carbons of the lactic acid which is formed. When α labeled propionate is the incubation substrate, the activity in the resulting β-hydroxyvaleric acid is principally in the gamma position and the beta and gamma positions are inactive.

ACKNOWLEDGMENT

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Table I

General Distribution of Activity

Substrate	Time of incubation hrs.	% as CO_2	Non-volatile % non-ether Extractable	Water Soluble % Ether Extractable
1-C^{14} propionate 2.6 x 10^7 dis./flask	2	18.0	5.9	.6
	2	31.0	7.7	2.3
	4	43.0	10.7	.8
2-C^{14} propionate 3.3 x 10^7 dis./flask	2	4.1	7.4	.6
	2	4.6	8.1	1.9
	4	8.3	13.3	2.7
	4	6.8	10.1	.9
Unlabeled Na propionate 1.0 mg.	4	101.0	3.2	<.1
0.2 mg. $NaHC^{14}O_3$ 4.47 x 10^7 dis./min.	4	98.0	2.7	<.1
1-C^{14} Acetate 2.84 x 10^7 dis.	1	2.4	0.9	1.1
	2	4.5	1.1	2.0
2-C^{14} Acetate 6.28 x 10^7 dis.	1	1.5	1.9	1.0
	2	2.0	1.2	2.1

Conditions: 1 mg. Na propionate/flask in phosphate buffer at pH 7.1
All values as % of added activity.

Table II

Distribution of Activity
in Chromatogramed Compounds

Compound	*Propionate	Inactive Propionate(2) $NaHC^*O_3$	*Acetate
Alanine	25	15	20
Lactic	10	10	2
Glucose	42	20	2
Fructose	4	1	1
β-Hydroxyvaleric	8	—	—
β-Hydroxybutyric	—	—	50
Urea	1(1)	50	3(1)
Glutamic Acid	3	1	15
Aspartic Acid	2	1	4
Malic	1	—	1
Succinic	1	—	—
Unidentified spots	2-3	3-4	4-5

Relative amounts of activity in various compounds determined by counting on chromatogram with Scott tube; values show only approximate distribution as variation was large between experiments.

1. Would appear mostly in effluent from ion exchange separation which is not chromatographed.

2. Values obtained from chromatogram of water soluble material which extracts into alcohol when evaporated to dryness.

Table III

Degradation of Alanine and Lactic Acid

Compound Degraded	Substrate	% of Activity in Compound			
		Carboxyl	α + β	α	β
Alanine	Propionate 1-C^{14}	99	2	-	-
	Propionate 2-C^{14}	15	85	-	-
	Inactive Propionate + $NaHC^{14}O_3$	85	5	-	-
Lactic Acid Synthetic -2-C^{14}		10	-	-	-
Lactic Acid	Propionate 1-C^{14}	93	5	-	-
	Propionate 2-C^{14}	18(1)	-	44	35
	Propionate 3-C^{14}	18.5(1)	-	39	39
	Propionate 2-C^{14}+ .005 M Malonate	18(1)	-	49	31

2-4 hour incubations
All values calculated on specific activities.

(1) Values indicate upper limit since some of the other two carbons are oxidized.

Table IV

Degradation of β-Hydroxy Valeric Acid

Substrate	Chromic Acid Oxidation*		Degradation of Propionic Acid in Steam Distillate**			
			Schmidt		Propionic → Lactic	
	CO_2	Steam Distillable	COOH	α + β	α	β
Propionate-1-C^{14}	20	66	92	2 ± 5	–	–
Propionate-2-C^{14}	6	77	2 ± 5			
	2	68	2 ± 5		105	2

(*) % of activity in β-hydroxy valeric acid.
(**) % of activity in Propionic acid.

Table V

Malonate Inhibition

Comp.	Malonate	Time	% CO_2	% Fixed in H_2O (After ether ext.)	~% in Ether
C–$\overset{*}{C}$–C	None	3 hr.	5.1%	9.2%	3.0%
	"	3 hr.	8.9%	21.3%	—
	.005 M	3 hr.	1.9%	2.7%	1.5%
$\overset{*}{C}$–C–C	None	3 hr.	8.4%	4.8%	4.1%
	.005 M	3 hr.	3.1%	2.0%	5.0%

Figure 1

A Propionate-2-C^{14} non-volatile, non-ether extractable portion of water soluble activity eluted from Dow A-1 column.

B Propionate-2-C^{14}, ether extractable portion of water soluble activity.

C Inactive propionate + $NaHC^{14}O_3$, non-volatile, non-ether extractable portion of water soluble activity taken up in alcohol to remove salt.

D Propionate-2-C^{14} + malonic acid .005 M, ether extractable portion of water soluble material.

E Acetate-2-C^{14}, non-volatile, non-ether extractable water soluble activity in eluate from Dow A-1 column.

F Acetate-2-C^{14}, ether extractable portion of water soluble activity.

Figure 2

A Activity + α-hydroxy butyric acid.

B Activity + α-ketobutyric acid + β-hydroxyvaleric acid.

C Acetylated activity + α-acetylbutyric acid.

D Acetylated activity + β-acetylvaleric acid.

Activity 0

Acidity 0

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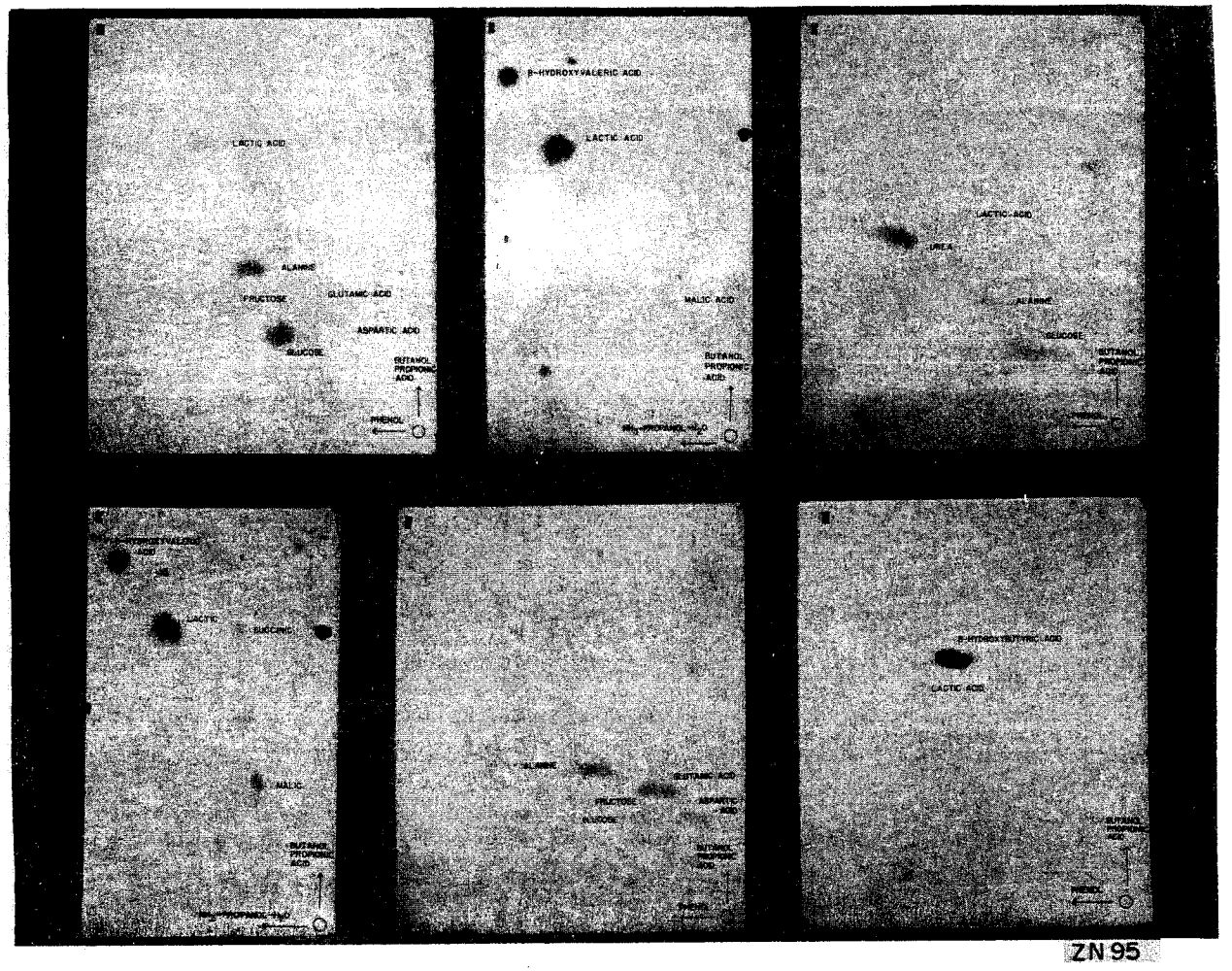
LACTIC ACID
ALANINE
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GLUTAMIC ACID
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GLUCOSE
BUTANOL PROPIONIC ACID
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LACTIC ACID
MALIC ACID
BUTANOL PROPIONIC ACID
LACTIC ACID
UREA
ALANINE
GLUCOSE
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SUCCINIC
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GLUTAMIC ACID
FRUCTOSE
ASPARTIC ACID
GLUCOSE
β-HYDROXYBUTYRIC ACID
LACTIC ACID
BUTANOL PROPIONIC ACID
PHENOL
ZN 95

Fig. 1

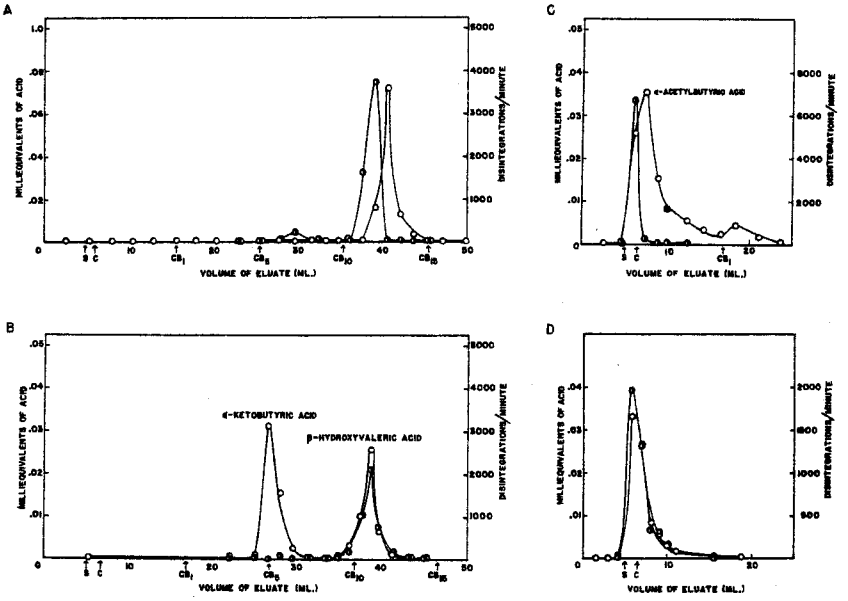
A
B
C
D
MILLIEQUIVALENTS OF ACID
DISINTEGRATIONS/MINUTE
VOLUME OF ELUATE (ML.)
α-KETOBUTYRIC ACID
β-HYDROXYVALERIC ACID
α-ACETYLBUTYRIC ACID

Fig. 2