PROPYLIC ACID METABOLISM IN MOUSE LIVER SLICES

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September 1951

Berkeley, California
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(*)

ABSTRACT

The metabolites 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 $\alpha$ and $\beta$ carbons of the lactic acid which is formed. When $\alpha$ labeled propionate is the incubation substrate, the activity in the resulting $\beta$-hydroxyvaleric acid is principally in the gamma position and the beta and gamma positions are inactive.

(*) The work described in this paper was sponsored by the U. S. Atomic Energy Commission.

For publication in the Journal of Biological Chemistry.
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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. The terminal three carbon unit has been investigated to some extent. Atchley 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 indicated a small amount of conversion of the three carbon residue to a two carbon unit of the type CH₃-CO₂⁻. Siegel and Lorber 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 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 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 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 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 and muscle tissue previously.

In the present experiments with liver slices it was desired, using methods of chromatography, to show the fairly complete pattern of propionate metabolites, the substrates being α, β, and carboxyl labeled propionic acid. Sodium bicarbonate-C 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 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 micro-
curies) 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-Müller counter.

The contents of the main compartment of the flask were homogenized
and distilled to dryness 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 evapora-
tion 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
Nucleospectrometer 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 rechroma-
tographed with a known carrier. The spots identified as glucose and fruc-
tose 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 coin-
ciding 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 activity was considered further
proof of identity.

Separation and identification of C₃-C₆ 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 was found to be particularly useful. Carrier
compounds including α-hydroxybutyric acid, β-hydroxy-valeric acid, and α-keto-
butyric 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 $^{14}$C 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 acetylation of 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 metabolites alanine, lactic acid, and β-hydroxyvaleric acid they were degraded.

Alanine was degraded by the ninhydrin method of Van Slyke and McFadden.\textsuperscript{18} The acetaldehyde and carbon dioxide obtained were distilled \textit{in vacuo} into a flask containing p-nitrophenylhydrzone (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\textsubscript{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° C.\textsuperscript{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.\textsuperscript{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 acid21 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 metabolites 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 hydroxyvaleric acid contains activity while the β and δ positions are inactive. This would indicate that a direct propylation has occurred between propionate and a C2 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 analogous 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 metabolites 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

The authors wish to express their appreciation to Dr. E. L. Bennett for his interest in this work.
Table I

General Distribution of Activity

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Time of incubation</th>
<th>% as CO₂</th>
<th>Non-volatile Extractable</th>
<th>Water Soluble Extractable</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>hrs.</td>
<td></td>
<td>% non-ether</td>
<td>% Ether</td>
</tr>
<tr>
<td>1-(^{14})C propionate</td>
<td>2</td>
<td>18.0</td>
<td>5.9</td>
<td>.6</td>
</tr>
<tr>
<td>2.6 x 10^7 dis./flask</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>31.0</td>
<td>7.7</td>
<td>2.3</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>43.0</td>
<td>10.7</td>
<td>.8</td>
</tr>
<tr>
<td>2-(^{14})C propionate</td>
<td>2</td>
<td>4.1</td>
<td>7.4</td>
<td>.6</td>
</tr>
<tr>
<td>3.3 x 10^7 dis./flask</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>4.6</td>
<td>8.1</td>
<td>1.9</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>8.3</td>
<td>13.3</td>
<td>2.7</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>6.8</td>
<td>10.1</td>
<td>.9</td>
</tr>
<tr>
<td>Unlabeled Na propionate</td>
<td>4</td>
<td>101.0</td>
<td>3.2</td>
<td>4.1</td>
</tr>
<tr>
<td>1.0 mg.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.2 mg. NaH(^{14})CO₃</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4.4 x 10^7 dis./min</td>
<td>4</td>
<td>98.0</td>
<td>2.7</td>
<td>4.1</td>
</tr>
<tr>
<td>1-(^{14})C Acetate</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.8 x 10^7 dis.</td>
<td>1</td>
<td>2.4</td>
<td>0.9</td>
<td>1.1</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>4.5</td>
<td>1.1</td>
<td>2.0</td>
</tr>
<tr>
<td>2-(^{14})C Acetate</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6.28 x 10^7 dis.</td>
<td>1</td>
<td>1.5</td>
<td>1.9</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>2.0</td>
<td>1.2</td>
<td>2.1</td>
</tr>
</tbody>
</table>

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 Chromatographed Compounds

<table>
<thead>
<tr>
<th>Compound</th>
<th>*Propionate</th>
<th>*Propionate NaHCO₃</th>
<th>*Acetate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>25</td>
<td>15</td>
<td>20</td>
</tr>
<tr>
<td>Lactic</td>
<td>10</td>
<td>10</td>
<td>2</td>
</tr>
<tr>
<td>Glucose</td>
<td>42</td>
<td>20</td>
<td>2</td>
</tr>
<tr>
<td>Fructose</td>
<td>4</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>β-Hydroxyvaleric</td>
<td>8</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>β-Hydroxybutyric</td>
<td>−</td>
<td>−</td>
<td>50</td>
</tr>
<tr>
<td>Urea</td>
<td>1(1)</td>
<td>50</td>
<td>3(1)</td>
</tr>
<tr>
<td>Glutamic Acid</td>
<td>3</td>
<td>1</td>
<td>15</td>
</tr>
<tr>
<td>Aspartic Acid</td>
<td>2</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>Malic</td>
<td>1</td>
<td>−</td>
<td>1</td>
</tr>
<tr>
<td>Succinic</td>
<td>1</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Unidentified spots</td>
<td>2–3</td>
<td>3–4</td>
<td>4–5</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Compound Degraded</th>
<th>Substrate</th>
<th>% of Activity in Compound</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Carboxyl</td>
</tr>
<tr>
<td>Alanine</td>
<td>Propionate 1-^14</td>
<td>99</td>
</tr>
<tr>
<td></td>
<td>Propionate 2-^14</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>Inactive Propionate + NaHCO_3</td>
<td>85</td>
</tr>
<tr>
<td>Lactic Acid</td>
<td>Propionate 1-^14</td>
<td>93</td>
</tr>
<tr>
<td>Synthetic -2-^14</td>
<td>Propionate 2-^14</td>
<td>18(1)</td>
</tr>
<tr>
<td></td>
<td>Propionate 3-^14</td>
<td>18.5(1)</td>
</tr>
<tr>
<td></td>
<td>Propionate 2-^14</td>
<td>18(1)</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Chromic Acid Oxidation CO₂</th>
<th>Steam Distillable</th>
<th>Degradation of Propionic Acid in Steam Distillate</th>
<th>Schmidt</th>
<th>Propionic → Lactic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>COOH</td>
<td>α + β</td>
<td>α</td>
</tr>
<tr>
<td>Propionate-1-C^{14}</td>
<td>20</td>
<td>66</td>
<td>92</td>
<td>2 ± 5</td>
<td>-</td>
</tr>
<tr>
<td>Propionate-2-C^{14}</td>
<td>6</td>
<td>77</td>
<td>2 ± 5</td>
<td></td>
<td>105</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>68</td>
<td>2 ± 5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(*) % of activity in β-hydroxy valeric acid.
(**) % of activity in Propionic acid.
<table>
<thead>
<tr>
<th>Comp.</th>
<th>Malonate</th>
<th>Time</th>
<th>% CO₂</th>
<th>% Fixed in H₂O (After ether ext.)</th>
<th>~ % in Ether</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-C-C</td>
<td>None</td>
<td>3 hr.</td>
<td>5.1%</td>
<td>9.2%</td>
<td>3.0%</td>
</tr>
<tr>
<td>&quot;</td>
<td>3 hr.</td>
<td>8.9%</td>
<td></td>
<td>21.3%</td>
<td>-</td>
</tr>
<tr>
<td>.005 M</td>
<td>3 hr.</td>
<td>1.9%</td>
<td></td>
<td>2.7%</td>
<td>1.5%</td>
</tr>
<tr>
<td>C-C-C</td>
<td>None</td>
<td>3 hr.</td>
<td>8.4%</td>
<td>4.8%</td>
<td>4.1%</td>
</tr>
<tr>
<td>.005 M</td>
<td>3 hr.</td>
<td>3.1%</td>
<td></td>
<td>2.0%</td>
<td>5.0%</td>
</tr>
</tbody>
</table>
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 + NaHCO$^{14}$, 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
REFERENCES

Fig. 1
Fig. 2