Enzymatic Reactions in the Degradation of 5-Aminovalerate by 
Clostridium aminovalericum*

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The anaerobic degradation of 5-aminovalerate to valerate, acetate, propionate, and ammonia by Clostridium aminovalericum was shown to involve the following intermediates: glutaric semialdehyde, 5-hydroxyvalerate, 5-hydroxyvaleryl-CoA, 4-pentenoyl-CoA, 2,4-pentadienoyl-CoA, trans-2-pentenoyl-CoA, L-3-hydroxyvaleryl-CoA, 3-ketovaleryl-CoA, acetyl- and propionyl-CoA and the corresponding acylphosphates, valeryl-CoA, and possibly 3-pentenoyl-CoA. With exception of the enzyme presumably reducing 2,4-pentadienoyl-CoA to 3-pentenoyl-CoA, enzymes catalyzing the formation and utilization of the above intermediates were demonstrated in extracts. Trans-2-pentenoyl-CoA was shown to be the immediate precursor of valeryl-CoA. The reduction of 2-pentenoyl-CoA was found to be coupled to the oxidation of 4-pentenoyl-CoA to 2,4-pentadienoyl-CoA. Several enzymes catalyzing the above reactions were partially purified and some of their properties determined. A high pressure liquid chromatography method of identifying and estimating most of the above mentioned CoA thiolesters was developed.

5-Aminovalerate is a common product of the anaerobic degradation of protein hydrolysates by several Clostridium species (1, 2). This amino acid is formed mainly by the reduction of proline and ornithine, which are used as oxidants by anaerobic bacteria. Nothing was known about the further bacterial degradation of 5-aminovalerate in the absence of oxygen until Hardman and Stadtman (3, 4) isolated a bacterium, Clostridium aminovalericum, that specializes in the utilization of this compound as an energy source. They showed that the degradation can be described as an oxidation of 1 mol of 5-aminovalerate to acetate, propionate, and ammonia coupled with a reduction of a 2nd mol to valerate and ammonia. They further established by the use of 5-amin[2-14C]valerate that the cleavage of the C5 chain in the oxidative reaction occurs between carbon atoms 2 and 3, forming unlabeled propionate and [2-14C]acetate.

We have investigated the pathway of 5-aminovalerate degradation in a new strain of C. aminovalericum and have identified most of the intermediates and participating enzymatic reactions.

MATERIALS AND METHODS

14C Distribution in Products of 5-Amino[1,5-14C]valerate Fermentation—To establish that strain T2-7 degrades 5-aminovalerate in the same way as does Hardman and Stadtman’s strain of C. aminovalericum (4), 5-amin[1,5-14C]valerate was fermented by a growing culture of T2-7, and the products and 14C distribution were determined. The specific radioactivity of valerate, 4120 cpm/μmol, was approximately the same as that of the substrate, 3810 cpm/μmol, whereas the specific activities of acetate, 1810 cpm/μmol, and propionate, 1736 cpm/μmol, were about half as large. Stepwise degradation of the labeled valerate (data not shown) established that 14C was distributed almost equally between carbon atoms 1 and 5 as in the substrate. Carbon atom 1 of propionate contained little 14C, whereas the acetate derived from carbon atoms 2 and 3 of propionate contained 98.5% of the 14C of this compound. These results show that the carboxyl group of propionate is derived from carbon 3 rather than carbon 5 of 5-aminovalerate. We conclude that strain T2-7 of C. aminovalericum, like the Hardman and Stadtman strain, oxidizes 1 mol of 5-aminovalerate to acetate and propionate by means of a β-oxidation while reducing a 2nd mol to n-valerate.

Conversion of 5-Aminovalerate to Glutaric Semialdehyde—An active and relatively stable enzyme catalyzing a transamination between 5-aminovalerate and α-ketoglutarate was found in cell-free extracts (Table III). Early experiments were done with [U-14C]α-ketoglutarate and unlabeled 5-aminovalerate or another amino acid, and the amount of [14C]glutamate formed was measured. 5-Aminovalerate was found to react much more rapidly than other amino acids. For example, the relative rates with 25 mM 5-aminovalerate, 6-aminohexanoate, L-alanine, 4-aminobutyrate or DL-2-aminobutyrate were 100, 31, 20, 5, and 4, respectively. Neither DL-3-aminobutyrate nor L-aspartate reacted at a significant rate. These results indicated the presence of a transaminase specialized for reaction with 5-aminovalerate.

The transamination product of 5-aminovalerate was detected and quantitated by using 5-amin[1,5-14C]valerate as a substrate. When a sample of the reaction solution was subjected to paper isophasis at pH 4.4, the radioactive product migrated as an anion and was separated from other reactants. The paper containing the product was cut out and...
its $^{14}$C content estimated. With this assay, conditions were developed for the preparation of the product on a 100 μmol scale (see "Materials and Methods").

The transamination product derived from 5-aminoovalerate was expected to be glutaric semialdehyde. Since an authentic sample of this compound was not available initially, the product was characterized by various chemical and physical properties. The compound was shown to be an acid that migrated about half as fast as α-ketoglutarate toward the anode in paper isoelectric at pH 4.4, indicating the presence of a single carboxyl group. Determination of the aldehyde content of the best preparation by the method of Dixon and Kornberg (26) showed the presence of approximately one aldehyde group (0.96 and 0.89 mol) per acid equivalent. The compound evidently contained the entire carbon chain of 5-aminoovalerate, since no other radioactive product was formed from 5-amino$^{[1,5-^{14}C]}$valerate. Oxidation of the product with hypiodite gave glutaric acid, which was identified by HPLC.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Specific activity</th>
<th>Temperature</th>
<th>pH</th>
</tr>
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<tbody>
<tr>
<td>5-Aminoovalerate-α-ketoglutarate transaminase</td>
<td>4,800-11,000</td>
<td>37</td>
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<td>5-Aminoovalerate-pyruvate transaminase</td>
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<td>5-Hydroxyovalerate dehydrogenase (DPN)</td>
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<td>25</td>
<td>25</td>
<td>7.2</td>
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<tr>
<td>DPNH oxidase</td>
<td>3-14</td>
<td>25</td>
<td>7.2</td>
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<tr>
<td>TPNH oxidase</td>
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<td>37</td>
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<td>3-Ketovaleryl-CoA thiolase</td>
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<td>Phosphotransacetylase</td>
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<td>Acetokinase</td>
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<td>37</td>
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<tr>
<td>3-Pentenoyl-CoA-2-pentenoyl-CoA isomerase</td>
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<td>7.5</td>
</tr>
<tr>
<td>Acet-CoA thiol esterase</td>
<td>53</td>
<td>37</td>
<td>7.6</td>
</tr>
</tbody>
</table>

*Reverse reaction.
2 Spectrophotometric assay.
3 Based on HPLC data.
4 This is probably another name for valeryl-CoA dehydrogenase.

with 0.5 mM pyridoxal phosphate in the presence of 5 mM 2-mercaptoethanol. The optimal pH for transamination is 8.2 with half-maximal rates at pH 6.5 and 9.8. $K_m$ values for 5-aminoovalerate and 2-ketoglutarate are 4.0 and 4.7 mM, respectively, when the cosubstrate concentration is 20 mM.

The transaminase was partially purified to a specific activity of 15.5 units/mg (see "Materials and Methods"). The purified enzyme was used mainly for preparing glutaric semialdehyde. It was also shown to convert 4-aminobutyrate and 6-aminohexanoate, by transamination with 2-ketoglutarate, to products, presumably succinic semialdehyde and adipic semialdehyde, respectively, that are reduced by NADH in the presence of purified 5-hydroxyvalerate dehydrogenase.

**Glutamic Dehydrogenase**—Extracts contained a very active NAD-dependent glutamic dehydrogenase (Table III) that presumably is responsible for the reoxidation of glutamate formed in the conversion of 5-aminoovalerate to glutaric semialdehyde and for the consequent formation of ammonia and NADH.

**Reduction of Glutaric Semialdehyde by 5-Hydroxyvalerate Dehydrogenase**—Extracts contained a 5-hydroxyvalerate dehydrogenase catalyzing the reduction of glutaric semialdehyde by NADH and the oxidation of 5-hydroxyvalerate by DPN (Table III). Partial purification of this activity (see "Materials and Methods") gave a moderately stable preparation with a specific activity of 1.3 units/mg, which was suitable for estimating glutaric semialdehyde and for reducing small amounts of the aldehyde to 5-hydroxyvalerate. This enzyme preparation also reduced adipic semialdehyde and succinic semialdehyde at 12-15% of the rate with glutaric semialdehyde. Propionaldehyde or 2-ketoglutarate was not reduced by the en-

*The abbreviations used are: HPLC, high pressure liquid chromatography; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid).*
zyme at a significant rate. NADPH did not replace NADH in the reduction of glutaric semialdehyde.

Some kinetic properties of the enzyme were determined in extracts. When assayed by the oxidation of NADH in the presence of glutaric semialdehyde, the rate of reaction was linear with enzyme concentration up to about 0.2 A\textsubscript{340} per min. The optimal pH was close to 6.0. The rate declined with increasing pH and was about half-maximal at pH 8.2. In the oxidation of 5-hydroxyvalerate by NAD, the pH optimum was about 10.2 in ethanolamine buffer and the half-maximal rate was obtained at pH 9.1. Apparent K\textsubscript{m} values for glutaric semialdehyde at pH 6.8 and for 5-hydroxyvalerate at pH 10.2 were found to be 0.80 and 3.8 mM, respectively. At concentrations above 15 mM, 5-hydroxyvalerate inhibited moderately.

Conversion of 5-Hydroxyvalerate to 5-Hydroxyvaleryl-CoA by a CoA Transferase—Extracts contain a relatively stable enzyme that catalyzes a reversible transfer of the CoA moiety from acetyl-CoA to 5-hydroxyvalerate to form acetate and 5-hydroxyvaleryl-CoA (Table III). Three enzymes for the assay were developed, an ionophoretic radiochemical assay measuring the formation of 5-hydroxyvaleryl-CoA and two spectrophotometric assays, one measuring the utilization of acetyl-CoA, the other measuring the formation of acetyl-CoA in the reverse reaction. An enzyme catalyzing the above reaction was purified about 24-fold to a final specific activity of 95 units/mg (see "Materials and Methods").

The only 3\textsuperscript{H}-labeled product formed in significant amounts from 5-hydroxy-[5-\textsuperscript{3}H]valerate and acetyl-CoA, catalyzed by the step 2 CoA transferase, was identified as 5-hydroxyvaleryl-CoA by comparison with the authentic compound in HPLC. In addition, the product of alkaline hydrolysis of the isolated 3\textsuperscript{H}-labeled thiol ester was identified as 5-hydroxyvalerate by HPLC.

Specificity of the step 2 CoA transferase with respect to acetyl-CoA compounds was determined by using 5-hydroxy-[5-\textsuperscript{3}H]valerate and acetyl-CoA, catalyzed by the step 2 CoA transferase, was identified as 5-hydroxyvaleryl-CoA by comparison with the authentic compound in HPLC. Subsequently, this product began to decrease as 2,4-pentadienoyl-CoA and 2-pentenoyl-CoA were identified. Eventually, the two products were identified as 2,4-pentadienoic acid and its CoA thiol ester by HPLC comparison with the authentic compounds.

As illustrated above, experiments in which unbuffered extracts are allowed to act on CoA thiol esters are complicated by the formation of a variety of products, both free acids and thiol esters, in relatively low yields. Furthermore, extracts contain many UV-absorbing compounds that interfere with the identification and estimation of unlabeled CoA thiol esters by HPLC. To obtain readily interpretable data with this analytical technique it was necessary to use at least partially purified enzymes as well as highly purified CoA thiol esters as substrates. Since we could not initially devise assays and purification methods for individual enzymes involved in 5-hydroxyvaleryl-CoA degradation, we used a partially purified (step 2) valeryl-CoA dehydrogenase preparation (see "Materials and Methods") that contained several enzymatic activities acting on various CoA thiol esters and lacked the objectionable features of extracts.

Interconversion of 5-Hydroxyvaleryl-CoA and 4-Pentenoyl-CoA and Oxidation of the Latter to 2,4-Pentadienoyl-CoA—When 0.1 mM 4-pentenoyl-CoA was incubated with 96 µg/ml step 2 valeryl-CoA dehydrogenase in air, most of the substrate was rapidly converted to 5-hydroxyvaleryl-CoA (Fig. 2). The specific activity of the enzyme, calculated from data for a 2-min incubation, was 347 milliunits/mg. After 6 min 71.5% of the 4-pentenoyl-CoA had been converted to 5-hydroxyvaleryl-CoA. Subsequently, this product began to decrease as 2,4-pentadienoic acid and an as yet unidentified CoA derivative,
possibly 3-keto-4-pentenoyl-CoA, accumulated. In a similar
substrate at pH 7.5.

pentenoyl-CoA and X-C5-CoA, an unidentified compound,
specific activity of the isomerase in cell-free extracts varied
from 135 to 184 units/mg when 0.1 mM 3-pentenoyl-CoA was
established when either 3-[1-14C]pentenoyl-CoA or X-[1-14C]
C5-CoA replaced 2-[1-14C]pentenoyl-CoA as substrate and
also when extensively purified step 3 3-pentenoyl-CoA:2-
pentenoyl-CoA isomerase was used in place of extract. The
specific activity of the isomerase in cell-free extracts varied
from 135 to 184 units/mg when 0.1 mM 3-pentenoyl-CoA was
the substrate at pH 7.5.

Properties of the Isomerase—The isomerase has a high
catalytic activity. Step 3 isomerase, which still contains sub-
stantial protein impurities, has a specific activity of 21,400
units/mg. The pure enzyme probably has a specific activity
at least 3 times larger. The enzyme is relatively stable at
−15 °C unless highly diluted. It is active from at least 6.7 to
9.0; the activity declines as the pH increases over this range.
Tris buffer has been used somewhat arbitrarily at pH 7.5 for
the assay. Potassium phosphate buffers (50 mM) are unsatisfactory,
since they inhibit 80–90% at pH 6.8–7.4. In addition to acting on unsaturated C5-CoA thioesters, the enzyme readily converts vinylacetyl-CoA to crotonyl-CoA. It does not hydrate the double bond of 2-pentenoyl-CoA.

Demonstration That X-[1-14C]C5-CoA Is Not 4-Pentenoyl-CoA—Since X-[1-14C]C5-CoA, prepared by the oxidation of [1-14C]valeryl-CoA (see "Materials and Methods"), has the same retention time as 4-pentenoyl-CoA in gradient elution on a reverse phase HPLC column and is reduced catalytically by H2Pd to n-valeryl-CoA, it was originally thought to be 4-pentenoyl-CoA. This conclusion was proved to be incorrect by showing that X-[1-14C]C5-CoA and authentic 4-pentenoyl-
CoA were converted to different products when incubated with step 2 valeryl-CoA dehydrogenase. 100 μM authentic 4-
pentenoyl-CoA, 3.2 μM of enzymatically prepared X-[1-14C]
C5-CoA, 3566 cpm/nmol, 100 mM Tris, pH 7.8, and 4.8 μg of
step 2 valeryl-CoA dehydrogenase were incubated 5 min at
37 °C, and the 14C and A260nm distribution patterns of the
products were determined following HPLC. The 14C and ab-
sorbance patterns for products derived from the two sub-
strates were entirely different. The main UV-absorbing prod-
ucts derived from authentic 4-pentenoyl-CoA were 5-hydroxy-
valeryl-CoA and 2,4-pentadienoyl-CoA; these products con-
tained no 14C. The main 14C-labeled products derived from X-
[1-14C]C5-CoA were valeryl-CoA (62%), 2-pentenoyl-CoA (20%); 3-pentenoyl-CoA (3.3%), and 3-hydroxyvaleryl-CoA (3.3%); about 2.5% of the X-C5-CoA remained. None of the labeled products showed an appreciable UV absorbance be-
cause of their low concentrations. We conclude that X-C5-
CoA and 4-pentenoyl-CoA are different compounds.

Properties of X-C5-CoA—Although X-[1-14C]C5-CoA was eluted with or closely adjacent to 4-pentenoyl-CoA and 2,4-
pentadienoyl-CoA in gradient elution from a reverse phase LC-18 column, it could be distinguished from these compo-
ounds by the retention time of the acid obtained by alkaline
hydrolysis of the thioester. On an Aminex HPX-87H column
the unknown acid was retained 3.5 min longer than 4-pente-
noic acid; on a reverse phase LC-18 column eluted isocratically
it was retained about 0.4 min longer than 4-pentenoic acid
and 0.6 min shorter than 2,4-pentadienoic acid (Table I, Miniprint). The similarity in retention times of X-C5-CoA and its hydrolysis product with the known unsaturated acids and thiol esters indicates that X-C5-CoA contains a mono-
unsaturated C5 acyl group. This was firmly established by
showing that the 4C-labeled acid derived from X-[1-14C]C5-
CoA was reduced by H2Pd to n-valeric acid. The unsaturated acid has not been positively identified for want of suitable reference compounds, but it probably is cis-3-pentenoic acid (see "Discussion").

Formation of 3-Hydroxyvaleryl-CoA from 2-Pentenoyl-
CoA—When step 2 valeryl-CoA dehydrogenase was incubated with any of the three compounds involved in the equilibria mentioned above, an additional compound, eluted just after 5-hydroxyvaleryl-CoA in HPLC, accumulated slowly. With a dehydrogenase concentration of 96 μg/ml, the yield of this product after a 5-min incubation was relatively low, about half that of 3-pentenoyl-CoA. However, with a higher de-
hydrogenase concentration (480 μg/ml) and a 60-min incubation
time, the yield reached 68% (Fig. 3). The product was identi-
fied as 3-hydroxyvaleryl-CoA by its retention time in HPLC.

![Graph](image-url)
Enzymatic Reactions in 5-Aminovalerate Degradation

Fig. 3. Conversion of 2-pentenoyl-CoA to 3-hydroxyvaleryl-CoA, 3-pentenoyl-CoA, and X-C6-CoA. The reaction solution contained 0.11 mM 2-[1-14C]pentenoyl-CoA, 5000 cpm/nmol, 48 µg of step 2 valeryl-CoA dehydrogenase, and 0.1 M Tris chloride, pH 7.8, in a total volume of 100 µl. After the indicated incubation times at 37 °C, a 30-µl aliquot was taken for estimation of UV (324 nm)-absorbing and radioactive compounds by HPLC gradient elution.

Fig. 4. Conversion of 2-pentenoyl-CoA to valeryl-CoA and other products from 2-pentenoyl-CoA and 4-pentenoyl-CoA. The reaction solution contained 0.11 mM 2-[1-14C]pentenoyl-CoA, 7280 cpm/nmol, 0.14 mM 4-pentenoyl-CoA, 0.1 M Tris chloride, pH 7.8, and 24 µg of step 2 valeryl-CoA dehydrogenase in 140 µl. After incubation for the indicated times at 37 °C, a 44-µl aliquot was used for estimation of radioactive compounds by HPLC gradient elution.

In a separate experiment it was shown that the isolated product was oxidized quantitatively by NAD in the presence of β-hydroxyacyl-CoA dehydrogenase thus establishing the 1(+)-configuration of the acyl group. In other experiments the specific activity of the 2-pentenoyl-CoA hydratase was found to range from 9 to 17 milliunits/mg in cell-free extracts.

Formation of Valeryl-CoA and Other Products from 2-Pentenoyl-CoA and 4-Pentenoyl-CoA—When step 2 valeryl-CoA dehydrogenase was incubated with 2-pentenoyl-CoA or 4-pentenoyl-CoA alone, no valeryl-CoA was formed. But when both compounds were provided as substrates valeryl-CoA was a major product of the reaction. Fig. 4 gives data on the time course of substrate utilization and 14C-labeled product formation from 2-[1-14C]pentenoyl-CoA when incubated with excess unlabeled 4-pentenoyl-CoA and a moderate level of dehydrogenase. A relatively rapid conversion of 48% of the 2-pentenoyl-CoA to valeryl-CoA occurred during the first 2 min of incubation, accompanied by a much smaller formation of 3-pentenoyl-CoA, X-C6-CoA and 3-hydroxyvaleryl-CoA. At 7 min, valeryl-CoA reached a maximum concentration and thereafter declined as a result of air oxidation. 3-Pentenoyl-CoA and X-C6-CoA declined slightly after an initial rise in synchrony with 2-pentenoyl-CoA. As seen earlier, 3-hydroxyvaleryl-CoA continued to increase slowly but continuously.

The HPLC A244 patterns showed the changes in 2-pentenoyl-CoA, valeryl-CoA, and 3-hydroxyvaleryl-CoA peaks expected from the 14C pattern of Fig. 4. In addition, they showed a large progressive decrease in 4-pentenoyl-CoA and corresponding increases in 2,4-pentadienoyl-CoA and 5-hydroxyvaleryl-CoA. A rough estimate of the molar quantities of 2,4-pentadienoyl-CoA formed, based on A244 data, gave values of 67 and 75% of those for valeryl-CoA formation after 2 and 7 min incubation, respectively.

The effects of 2,4-pentadienoyl-CoA and of different concentrations of 4-pentenoyl-CoA on the conversion of 2-[1-14C]pentenoyl-CoA to valeryl-CoA and other products are shown in Table IV. 2,4-Pentadienoyl-CoA had little effect on 2-pentenoyl-CoA utilization except possibly to inhibit the formation of 3-hydroxyvaleryl-CoA. Addition of 50 and 100 µM 4-pentenoyl-CoA caused the formation of 35.3 and 45.9 µM valeryl-CoA, respectively. Evidently, the coupling of 4-pentenoyl-CoA oxidation to 2-pentenoyl-CoA reduction is relatively efficient despite the presence of O2, a competing electron acceptor. In a separate experiment, 5-hydroxyvaleryl-CoA was shown to reduce 2-pentenoyl-CoA as a source of reducing power for the conversion of 2-pentenoyl-CoA to valeryl-CoA, although the efficiency appeared to be lower, 46 as compared to 70% conversion at 50 µM. In this experiment, 2,4-pentadienoyl-CoA and valeryl-CoA were formed in approximately equivalent amounts. Attempts to use NADH or NADPH as reductant for 2-pentenoyl-CoA, either in the presence or absence of purified electron transporting factor from Megasphaera elsdii, gave negative results.

The approximate equilibrium constant for the conversion of 2-pentenoyl-CoA and 4-pentenoyl-CoA to valeryl-CoA and 2,4-pentadienoyl-CoA was calculated from the 7-min incubation data of Fig. 4 on the assumption that equal amounts of the products were formed. The value of 0.7 so obtained is probably somewhat low because equilibrium had not been reached.

The substrate specificity of valeryl-CoA dehydrogenase appears to be rather broad. The relative rates of dye reduction (9) by step 3 enzyme with 0.1 mM valeryl-, 6-heptenoyl-, 4-pentenoyl-, butyryl-, and propionyl-CoA are 100, 76, 75, 63, and 8.9, respectively. 4-Acetamidobutyryl-CoA is oxidized very slowly, if at all.

Evidence That 2-Pentenoyl-CoA Is Reduced to Valeryl-CoA—Identification of the immediate precursor of valeryl-CoA was impossible with step 2 valeryl-CoA dehydrogenase because of contamination by the isomerase that interconverts 2-pentenoyl-CoA, 3-pentenoyl-CoA, and X-C6-CoA. Purification step 3 removed most of the isomerase, and what remained could be mostly inhibited by the use of a phosphate buffer. Table V shows the results of an experiment in which a low level of step 3 dehydrogenase was incubated for 3 min in phosphate-Tris buffer with 4-pentenoyl-CoA and either trans-2- or trans-3-[1-14C]pentenoyl-CoA, and the distribution of 14C among the substrates and products was determined following HPLC. The data show that valeryl-CoA was formed over 3 times more rapidly from 2-pentenoyl-CoA than from


**TABLE IV**

**Effects of 2,4-pentadienoyl-CoA and 4-pentenoyl-CoA on the formation of valeryl-CoA and other products from 2-[/1-14C]pentenoyl-CoA**

The reaction solutions contained 0.1 mM 2-[/1-14C]pentenoyl-CoA, 7400 cpm/nmol, the indicated concentrations of 4-pentenoyl-CoA or 2,4-pentadienoyl-CoA, 0.1 M Tris chloride, pH 7.8, and 4.8 µg of valeryl-CoA dehydrogenase in a total volume of 50 µl. After 5 min incubation at 37 °C the 14C-labeled thiol esters were separated by HPLC and estimated by their 14C contents.

<table>
<thead>
<tr>
<th>CoA thiol ester</th>
<th>2,4-Pentenoyl-CoA (74 µM)</th>
<th>4-Pentenoyl-CoA (50 µM)</th>
<th>4-Pentenoyl-CoA (100 µM)</th>
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<td>Trans-2-pentenoyl</td>
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<td>72.2</td>
<td>46.0</td>
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<td>Trans-3-pentenoyl</td>
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<tr>
<td>Valeryl</td>
<td>0.0</td>
<td>0.0</td>
<td>35.5</td>
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</table>

3-pentenoyl-CoA under conditions permitting a small interconversion of these substrates. We conclude that 2-pentenoyl-CoA is the preferred and possibly the only immediate precursor of valeryl-CoA.

**Possible Reduction of 2,4-Pentadienoyl-CoA**

In several attempts to detect conversion of 2,4-pentadienoyl-CoA to compounds that can be reduced to valeryl-CoA, the former compound was incubated with excess NADH, NADPH, or 4-pentenoyl-CoA in the presence of either step 2 valeryl-CoA dehydrogenase or cell-free extract, and the reaction solution was examined by HPLC. Neither 2-pentenoyl-CoA, 3-pentenoyl-CoA, nor valeryl-CoA was formed in significant amounts.

**Conversion of 3-Hydroxyvaleryl-CoA to Acetate and Propionate**

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**TABLE V**

**Identification of 2-pentenoyl-CoA as the immediate precursor of valeryl-CoA**

The reaction solution contained 0.15 mM 4-pentenoyl-CoA, 0.11 mM either 2- or 3-[/1-14C]pentenoyl-CoA, 30,000 cpm, 80 µM Tris chloride, pH 8.1, 60 mM potassium phosphate, pH 7.9, and 0.72 µg of a very active phosphotransacetylase. An acetokinase catalyzes formation of acetate and propionate. A moderately active thiolase in extracts cleaved 3-ketovaleryl-CoA, the product of reduction of 3-ketovaleryl-CoA, catalyzed by an extract of C. aminovalericum, was oxidized in extracts oxidized L-3-hydroxyvaleryl-CoA to 3-ketovaleryl-CoA and propionate, which presumably reoxidizes glutamate and forms glutaric semialdehyde and glutamate. The same reaction has been reported previously to occur in a lysine-oxidizing Pseudomonas (27). The responsible enzyme was purified about 20-fold from the Pseudomonas and shown to be highly specific for utilization of 5-aminovalerate and α-ketoglutarate. A highly active glutamic dehydrogenase is present in C. aminovalericum that presumably reoxidizes glutamate and forms glutaric semialdehyde and glutamate.

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**DISCUSSION**

The above results provide evidence for most of the enzymatic reactions involved in the anaerobic degradation of 5-aminovalerate to acetate, propionate, valerate, and ammonia by C. aminovalericum (Fig. 5). A transaminase transfers the amino group from 5-aminovalerate to α-ketoglutarate to form glutaric semialdehyde and glutamate. The same reaction has been reported previously to occur in a lysine-oxidizing Pseudomonas (27). The responsible enzyme was purified about 20-fold from the Pseudomonas and shown to be highly specific for utilization of 5-aminovalerate and α-ketoglutarate. A highly active glutamic dehydrogenase is present in C. aminovalericum that presumably reoxidizes glutamate and forms glutaric semialdehyde and glutamate.
ammonia and NADH. The latter is used by a dehydrogenase to reduce glutaric semialdehyde to 5-hydroxylvalerate which they react with acetyl-, propionyl-, or valeryl-CoA under the influence of a specialized CoA transferase to form 5-hydroxyvaleryl-CoA and the three fatty acids that are final products of the fermentation. This sequence of reactions is completely analogous to the conversion of 4-aminobutyrate to 4-hydroxybutyryl-CoA catalyzed by enzymes from C. aminobutyricum (28, 29). The latter organism was postulated to convert 4-hydroxybutyryl-CoA to vinylacetyl-CoA, but direct evidence for the reaction could not be obtained with cell-free extracts because of the presence of an active isomerase converting vinylacetyl-CoA irreversibly to crotonyl-CoA. Neither the conversion of 4-hydroxybutyryl-CoA to vinylacetyl-CoA nor the reverse reaction could be demonstrated. The evidence for 4-pentenoyl-CoA hydratase in C. aminovalericum is more direct. 4-Pentenoyl-CoA is readily converted to 5-hydroxyvaleryl-CoA both by extracts and by partially purified hydratase preparations. The product of the reaction was identified by HPLC as well as by enzymatic assay with 5-hydroxyvaleryl-CoA:acetate-CoA transferase. Identification of the product of the reverse reaction is less complete because of the unfavorable equilibrium and the difficulty of separating small amounts of 4-pentenoyl CoA and 2,4-pentadienoyl-CoA by HPLC. Nevertheless, the conversion of 5-hydroxyvaleryl-CoA to 4-pentenoyl-CoA is indicated by these facts that both compounds serve as reductants for the formation of valeryl-CoA and both are oxidized to 2,4-pentadienoyl-CoA.

4-Pentenoyl-CoA is oxidized slowly by oxygen and rapidly in a coupled reaction with 2-pentenoyl-CoA catalyzed by step 2 valeryl-CoA dehydrogenase. The products of the coupled reaction are 2,4-pentadienoyl-CoA and valeryl-CoA. Since the dehydrogenase preparation contains several enzymatic activities it is not certain that the dehydrogenase per se catalyzes the above reaction. However, this seems probable because both the oxidation of 4-pentenoyl-CoA and the reduction of 2-pentenoyl-CoA involve oxidation-reduction reactions at the same carbon atoms. In this connection we have shown that 2-pentenoyl-CoA rather than 3- or 4-pentenoyl-CoA is the immediate precursor of valeryl-CoA.

Extracts of C. aminovalericum have been shown to convert highly tritium-labeled 5-hydroxyvaleryl-CoA to 2-pentenoyl-CoA and valeryl-CoA in low yields. Presumably, this involves 4-pentenoyl-CoA and 2,4-pentadienoyl-CoA as intermediates. A reduction of 2,4-pentadienoyl-CoA to 3- or 2-pentenoyl-CoA by NADPH is known to be catalyzed by enzymes derived from liver mitochondria (30, 31). However, we have been unable so far to demonstrate such a reaction in extracts of C. aminovalericum using either NADH, NADPH, or 4-pentenoyl-CoA as reductant. Nevertheless, we still postulate that, under suitable conditions, 2,4-pentadienoyl-CoA is reduced to a pentenoyl-CoA since this provides a reasonable path to subsequent known reactions. If 3-pentenoyl-CoA is the product of the postulated 2,4-pentadienoyl-CoA reduction, a function is provided for the very active isomerase that converts 3- to 2-pentenoyl-CoA.

The unidentified product, X-C5-CoA, formed from either trans-2- or trans-3-pentenoyl-CoA by the isomerase is probably cis-3-pentenoyl-CoA. This conclusion is based upon the fact that similar isomerases derived from liver mitochondria (32–34) and the slime mold Dictyostelium discoideum (35) interconvert cis- and trans-3-enoyl-CoA and trans-2-enoyl-CoA compounds containing longer chain acyl groups. The Dictyostelium enzyme has been shown to form trans- and cis-3-hexadecenoyl-CoA in a molar ratio of approximately 3, which is close to the value of 3.4 for the ratio of trans-3-pentenoyl-CoA to X-C5-CoA obtained in the equilibrium catalyzed by the C. aminovalericum isomerase.

If the identification of X-C5-CoA is correct, the isomerase, in combination with the HPLC method of analysis, provides the best data on the equilibria among cis-3-, trans-3-, and trans-2-enoyl-CoA compounds. Earlier equilibrium determinations (33, 35, 36) involved the use of either less precise analytical methods, less highly purified enzyme preparations, or only two of the three substrates.

Neither NADH nor NADPH were found to replace 4-pentenoyl-CoA as reductant for 2-pentenoyl-CoA. This was unexpected since C. aminovalericum forms NADH in the oxidation of both glutamate and 3-hydroxyvaleryl-CoA and since other anaerobic bacteria, M. elsdenii (13) and C. aminobutyricum (29), have been shown to use NADH to reduce crotonyl-CoA to butyryl-CoA. The M. elsdenii system was reported to require both butyryl-CoA dehydrogenase and an electron-transporting flavoprotein for this reaction. We confirmed this result, but were unable to utilize NADH or NADPH as a reductant by supplementing C. aminovalericum valeryl-CoA dehydrogenase with active M. elsdenii electron-transporting flavoprotein. This result and the apparently tight coupling between 4-pentenoyl-CoA oxidation and 2-pentenoyl-CoA reduction in C. aminovalericum indicate that the two fatty acid synthesizing systems are significantly different. Since NADH is apparently not used for the reduction of 2-pentenoyl-CoA and the reduction of 2,4-pentadienoyl-CoA could not be demonstrated in extracts of C. aminovalericum, the reaction responsible for the reoxidation of half of the NADH generated in the degradation of 5-aminovalerate remains obscure.

Extracts of C. aminovalericum contain a low level of a relatively stable 2-enoyl hydratase that converts 2-pentenoyl-CoA to 3-hydroxyvaleryl-CoA. We were initially unable to detect this activity in extracts by a coupled reaction with 3-hydroxyacyl-CoA dehydrogenase (37). It was later demonstrated with step 2 valeryl-CoA dehydrogenase by using high enzyme levels, relatively long incubation times, and HPLC to identify 3-hydroxyvaleryl-CoA as a product formed from 2-pentenoyl-CoA. The product was shown to be the L-isomer by oxidation with L-3-hydroxyacyl-CoA dehydrogenase. The observed level of the hydratase is apparently too low to account for the overall rate of 5-aminovalerate degradation, but it is possible that the experimental conditions of the assay were not optimal.

The further conversion of L-3-hydroxyvaleryl-CoA is catalyzed by a highly active NAD-specific dehydrogenase. The resulting 3-keto compound is cleaved by a moderately active 3-ketothiolase to acetyl- and propionyl-CoA. These thiol esters can be converted to the corresponding acylphosphates and free fatty acids by successive actions of phosphotransacetylase and acetokinase, both of which are present in relatively high levels. More than half of the acetyl- and propionyl-CoA can be utilized to generate ATP by these reactions since the remainder and valeryl-CoA are needed to generate 5-hydroxyvaleryl-CoA. The actual amounts of valeryl-CoA directly used in this CoA transferase reaction are not known. At least part of the valeryl-CoA probably reacts with acetate or propionate to form the corresponding CoA thiol esters, which then participate in the formation of 5-hydroxyvaleryl-CoA.

A summary of some of our studies on the degradation of 5-aminovalerate by C. aminovalericum was reported previously (37). Any results or conclusions in that report that disagree with data and conclusions in the present paper should be disregarded.
Enzymatic Reactions in 5-Aminoualerate Degradation

Acknowledgments—We wish to thank Dr. Todd King and Dr. Richard Cathcart for advice on the use of HPLC for the separation of CoA thiol esters, Prof. Horst Schulz for the gift of a sample of 2,4-pentadienoic acid, Prof. D. E. Koehl, Jr. for the use of his HPLC system, and Lora Hedrick for technical assistance.

REFERENCES


SUPPLEMENTAL MATERIAL

Enzymatic reactions in the degradation of 5-aminovalerate

MATERIALS AND METHODS

Isolation and culture of C. ammoniagenes, strain 205. The organism was isolated from sewage sludge after treatment of an aerated sewage containing 35 ml of 5-aminovalerate. The strain was identified as C. ammoniagenes by its ability to grow on a medium containing 5-aminovalerate and sodium thiosulfate, which was the only source of nitrogen. The organism was grown as a batch culture in a modified Nagakura medium (10): 5 mg glucose, 0.5 mg NH₄Cl, 0.5 mg yeast extract, 20 ml peptone broth, 20 ml peptone broth, and 0.5 mg thiosulfate. The medium was adjusted to a pH of 7.4, sterilized, and aerated at 37°C. The cultures were harvested by centrifugation at 3,000 rpm. The supernatant was decanted, the pellet was washed once with 0.9% NaCl, and the residue was resuspended in 0.9% NaCl.

5-Aminovalerate-3-[14C]thioester (5-atom 1-14C). A reaction mixture containing 5 ml of 5-atom 14C was added to 5 ml of 5-atom 14C, followed by 5 ml of 5-atom 14C. Incubation was continued for 20 hr at 37°C. After the reactions were completed, the products were precipitated and washed as previously described. The yield of the 5-atom 14C was determined by measuring the optical density at 540 nm. The reaction was stopped by the addition of 0.5 ml of 5 mol/L HCl. The reaction was stopped by the addition of 0.5 ml of 5 mol/L HCl. The reaction was stopped by the addition of 0.5 ml of 5 mol/L HCl. The reaction was stopped by the addition of 0.5 ml of 5 mol/L HCl.
9002 Enzymatic Reactions in 5-Aminoualerate Degradation

Abstract (127.5-2.7.1.1) was amended by following the decrease in orthophosphate plus nitrate concentration (10) when the latter was incubated with 407 and enzyme. Lycopersicum esculentum 3.4 mL of 50 mM phosphate buffer, pH 7.4, 50 mM potassium chloride, 0.5 mM of EDTA, and 0.1% Triton X-100 were incubated for 2 h at 37°C with enzyme.

**Materials and Methods**

All purification steps were done at 4°C.

**Assay of 5-Aminoualerate Oxidoreductase** (EC 1.1.2.1) activity was measured in a solution (100 ml) containing 1 mM ascorbate, 50 mM potassium phosphate buffer, pH 8.0, 0.1% Triton X-100, and enzyme. The initial rate of ascorbate oxidation was determined.

**Enzyme Assay**

**Analytical Methods**

The concentrations of all bisulfide solutions were generally estimated by using EPR to measure the amount of hydrogen peroxide formed upon reaction with potassium ferricyanide (22). 5-Aminoualerate catabolism and its catabolites were estimated by gas chromatography and paper chromatography. The oxygen uptake was measured by the Warburg manometric technique (23). The 5-Aminoualerate content was determined by spectrophotometry using a Perkin-Elmer spectrophotometer at 275 nm.

**Results and Discussion**

The results of this study suggest that the 5-Aminoualerate catabolism may involve a number of different enzymes including 5-Aminoualerate dioxygenase, 5-Aminoualerate reductase, and 5-Aminoualerate oxidoreductase.
Enzymatic Reactions in 5-Aminovalerate Degradation

Table II

Retention times of CoASH and CoA thioesters in high pressure liquid chromatography

Reversed-phase LC-18 column. Eluent: 50 mM potassium phosphate pH 2.5-3.0/Me gradient flow rate: 0.8 ml/min.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Retention time (min.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CoASH</td>
<td>9.5</td>
</tr>
<tr>
<td>n-valeryl-CoA</td>
<td>29.4</td>
</tr>
<tr>
<td>2-hydroxyvaleryl-CoA</td>
<td>29.4</td>
</tr>
<tr>
<td>3-hydroxyvaleryl-CoA</td>
<td>31.4</td>
</tr>
<tr>
<td>4-hydroxyvaleryl-CoA</td>
<td>32.6</td>
</tr>
<tr>
<td>a-ketovaleryl-CoA</td>
<td>34.0</td>
</tr>
<tr>
<td>2-pentenoyl-CoA</td>
<td>35.5</td>
</tr>
<tr>
<td>3-pentenoyl-CoA</td>
<td>38.5</td>
</tr>
<tr>
<td>2,4-pentadienoyl-CoA</td>
<td>39.0</td>
</tr>
<tr>
<td>trans-2-pentenoyl-CoA</td>
<td>41.0</td>
</tr>
<tr>
<td>cis-2-pentenoyl-CoA</td>
<td>41.0</td>
</tr>
<tr>
<td>2-pentenoyl-CoA</td>
<td>41.0</td>
</tr>
<tr>
<td>4-pentenoyl-CoA</td>
<td>44.0</td>
</tr>
</tbody>
</table>

1 See Materials and Methods for details.
2 The absolute retention times vary by as much as 1% on different occasions but the relative values are almost constant.
3 Unlabeled CoA is retained by the column.

The esters labeled with $^{14}C$ or $^{13}C$ were monitored by estimating the radioactivity in fractions collected at 0.5 min intervals. The amount of an unlabeled CoA compound was estimated from its peak height at 270 nm, corresponding to 8.10 $B_{2}$, and full scale on the recorder was 0.2. Peak heights per unit area were determined with different standards. From $^{14}C$-acetyl-CoA, $^{14}C$-valeryl-CoA, $^{14}C$-hydroxyneryl-CoA and $^{14}C$-pyruvyl-CoA the values are 82.0, 90.5, 92.0, 98.0 and 99.6, respectively.

$^{14}C$-labeled precursors and $^{13}C$-labeled substrates were degraded unequivocally by the Schmidt reaction (25) to determine the distribution of $^{13}C$.

Identification of inactivated esters, labeled with $^{14}C$ or $^{13}C$, as CoA compounds was done by selective reversible isotope effects. A neutral solution of an isotope-labeled CoA ester is 50 mM MgCl2 and containing 1% to 5% maltose. After no reaction was detected, the solution was filtered through a 0.45 μm filter. The sample was analyzed by HPLC and mass spectrometry. The yields of esters lost from $^{14}C$- or $^{13}C$-isovaleryl-CoA was 99%.

Retention time was estimated by the method of Lawry et al. (15) using deuterium or methyl ester as a standard.

Isotopic enrichment was estimated by isotope reduction with D-glucose. The reaction mixture contained 50 μM reducing compounds, 10 mM MgCl2, 50 mM HEPES (pH 7.4), and 500 μM sodium glucose dehydrogenase. After 1.5 h, the evolution of CO2 was estimated and expressed as a percentage of theoretical production. The deuterium oxide was deuterated for 30 min to estimate deuterium content. The deuterated esters were identified by mass spectroscopy and cleanup with HPLC.