The Metabolism of dl-Pipelicolic Acid-2-C\textsuperscript{14} *  

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It has been established that pipelicolic acid is a product of lysine dissimilation in the rat (1), in plants (2), and in Neurospora (3). Its formation in the rat was shown to involve loss of the α-amino group of lysine and not the ε-amino group. This led to the proposal that ε-amino-α-ketocaproic acid is the precursor of pipelicolic acid (1). A similar proposal has been made for the pathway of pipelicolic acid formation in Neurospora (3).

A small conversion of lysine-6-C\textsuperscript{14} to radioactive α-aminoaspartic acid was demonstrated in guinea pig liver homogenate by Borst et al. (4). These authors also indicated that α-aminoaspartic acid is further metabolized to glutaric acid by way of ε-keto-lysine-6-C\textsuperscript{14} as well as dl-ε-aminoaspartic acid-ε-C\textsuperscript{14} give rise to glutarate (5). The above data, and the finding that ε-N\textsuperscript{15}lysine yielded a large amount of N\textsuperscript{15}-labeled pipelicolic acid, as well as a small enrichment of N\textsuperscript{14} in isolated α-aminoaspartic acid, led to the proposal that pipelicolic acid occupies a central role on the catabolic pathway of lysine (3).  

Meister (7) prepared ε-amino-α-keto-caproic acid by deamination of ε-carboxybonyllysine with l-amino acid oxidase from snake venom. After removal of the carboxybonyl group, the product was found to be in equilibrium with its cyclic form, Δ\textsuperscript{3}-piperidine-2-carboxylic acid. This observation was also made by Boulander and Osteux (8) who obtained the material by the action of turkey liver “amino acid dehydrogenase” on L-lysine. Enzyme preparations from many tissues of the rat, including brain, liver, kidney, and muscle, readily reduce Δ\textsuperscript{3}-piperidine-2-carboxylic acid to pipelicolic acid (9). TPNH or DPNH are required for the reaction. This reduction also takes place in microorganisms (9, 3). Thus, there seems to be ample justification for accepting the metabolic pathway: lysine → ε-amino-α-ketocaproic acid → Δ\textsuperscript{3}-piperidine-2-carboxylic acid → pipelicolic acid. On the other hand, no direct evidence appears to have been put forth in support of the later steps of the proposed degradative pathway of lysine, that is, from pipelicolic acid onward. In order to clarify this part of the pathway, dl-pipelicolic acid-2-C\textsuperscript{14} was prepared and its metabolism investigated in the rat, in vivo, and in vitro. The results of these experiments, and of experiments carried out with α-aminoaspartic acid-6-C\textsuperscript{14} and glutaric acid-1,5-C\textsuperscript{14} are reported here.

EXPERIMENTAL  

Synthetic Procedures

Radioactive Substrates—dl-Pipelicolic acid-2-C\textsuperscript{14} was prepared according to Meister (7) by the enzymatic deamination of ε-carboxybonyl-l-lysine-2-C\textsuperscript{14} with dried snake venom (Crotalus adamanteus), followed by hydrogenation of the intermediate product. The dl-pipelicolic acid-2-C\textsuperscript{14} was isolated by fractional elution from a column of Dowex 50\textsuperscript{W} with 2 n HCl. A considerable amount of radioactive by-product came off the column in a radioactive peak before pipelicolic acid. The fractions containing the dl-pipelicolic acid-2-C\textsuperscript{14} were combined, dried, and the residue was recrystallized from ethanol-acetone after treatment with Norit. The final product was shown to be pure by paper chromatography, radioautography, and ion exchange chromatography (Dowex 50). The yield was 96 μc or 12% based on dl-lysine-2-C\textsuperscript{14}. The specific activity of the product was 0.21 mc per mmole.

dl-ε-Aminoaspartic acid-6-C\textsuperscript{14}, synthesized by the method of Rothstein and Claus (10), was generously supplied by Dr. Leon L. Miller; the specific activity of the pure compound was 10 μc per mg.

Glutaric acid-1,5-C\textsuperscript{14} was prepared by the reaction of 0.8 mmole of NaC\textsuperscript{14}O\textsubscript{2} with 0.3 mmole of trimethylamine bromide in 70% ethanol. After being refluxed for 36 hours, the product was hydrolyzed with concentrated HCl, dried, and recrystallized twice from benzene-petroleum ether. The final yield of chromatographically and radioautographically pure product was 17.7 mg or 34%. The specific activity was 1.1 mc per mmole.

Other Substrates—dl-ε-Aminoaspartic acid was synthesized essentially by the method of Waalkes et al. (11), based upon the β-bromination of ethyl hydrogen adipate in thionyl chloride. The procedure was simplified by treating the bromination product with aqueous sodium bicarbonate and isolating the resulting crude 2-bromo-5-carbethoxyvaleric acid by ether extraction of the crude product. The Dr.-dl-pipelicolic acid-2-C\textsuperscript{14} was isolated by fractional elution from a column of Dowex 50\textsuperscript{W} with 2 n HCl. A considerable amount of radioactive by-product came off the column in a radioactive peak before pipelicolic acid. The fractions containing the dl-pipelicolic acid-2-C\textsuperscript{14} were combined, dried, and the residue was recrystallized from ethanol-acetone after treatment with Norit. The final product was shown to be pure by paper chromatography, radioautography, and ion exchange chromatography (Dowex 50). The yield was 96 μc or 12% based on dl-lysine-2-C\textsuperscript{14}. The specific activity of the product was 0.21 mc per mmole.

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dl-Pipelicolic acid was resolved from commercial dl-material by means of the (-)-tartaric acid salt (3). l-ε-Aminoaspartic acid was prepared by the action of acylase upon the m-chloracetyl derivative (12).

Experimental Methods

C\textsuperscript{14}O\textsubscript{2} Expiration—A female Sprague-Dawley rat (190 g), fasted for 24 hours, was treated by intraperitoneal injection with a...
neutralized solution containing 2.5 μc (1.25 mg) of DL-pipecolic acid-2-C14·HCl and 4 mg of inert \( \Delta^1 \)-pipecolic acid·HCl. A second animal was treated similarly with 0.66 μc of \( \Delta^1 \)-pipecolic acid-2-C14 (see below) and 8 mg of carrier DL-pipecolic acid. The animals were placed in an all-glass metabolism cage, and the expired CO₂ was collected in 20% KOH for a total of 11 and 8 hours, respectively. Aliquots of this solution were treated with BaCl₂ and the resulting BaCO₃ was filtered, dried, and weighed. The material was plated on aluminum planchets for counting. Counts were corrected to disintegrations per minute at infinite thinness. 

Urine—The urine was filtered and diluted to 50 ml with water. Samples of suitable volume were plated, counted, and the results extrapolated to infinite thinness.

Isolation of Glutaric Acid Derived in Vivo from DL-Pipecolic Acid-2-C14—The procedure was the same as that previously used to isolate glutaric acid derived from L-lysine-6-C14 (6). A neutralized solution (NaOH) containing 350 mg of glutaric acid and 8.1 μc (4.5 mg) of DL-pipecolic acid-2-C14·HCl was injected intraperitoneally into a female Sprague-Dawley rat which had previously been fasted for 24 hours. From the ether extract of the acidified urine were obtained 53 mg of once recrystallized (benzene) glutaric acid. The product was then recrystallized alternately from hot benzene and ethyl acetate-petroleum ether; four consecutive recrystallizations did not change the specific activity.

Isolation of "D"-Pipecolic Acid-2-C14 from Urine—The urine was isolated from the glutamate experiment above was neutralized, concentrated under reduced pressure to 10 ml and deaslated electrolytically. It was then fractionally eluted from a Dowex 50 column (14 × 500 mm) with 1 N HCl. The pipecolic acid was located by counting a small aliquot of each fraction. The appropriate fractions were concentrated, treated with Norit, filtered, and dried. Paper chromatography of the product (butanol-acetic acid-water; 4:1:1) showed only one ninhydrin-positive spot \( R_f \) 0.42 which matched exactly the radioactive area. The total amount of recovered material was 0.66 μc.

Degradation of Isolated Glutarate—The glutaric acid was decarboxylated by the action of sodium azide in 100% sulfuric acid (Schmidt reaction). The evolved CO₂ was trapped in CO₂ in 0.25 ml removed. To this were added 20 mg of Na₂CO₃ and the carbon-succinate. Incubations were carried out in 25-ml Erlenmeyer flasks were combined and fractionally eluted from Dowex 50. The ammonium fractions were located (ninhydrin), combined and dried. Two-thirds of the material was treated on 4 papers (8 inches wide) by high-voltage electrophoresis (pyridine-acetic acid buffer, pH 6.4; 2000 volts) under toluene (14). After location and elution of the \( \alpha \)-aminoadipic acid, 2050 c.p.m. were obtained. Approximately one-half of this material was treated with Na₂S in 100% H₂SO₄. After removal of the sulfate as BaS₀₄, the product was desalted by elution from Dowex 1 (OH⁻) with 2 N HCl. Aliquots of the g-ml fractions were dried. Two-thirds of the material was treated on 4 papers (8 inches wide) by high-voltage electrophoresis. Ornithine was located on the paper and eluted. On the anode side, ninhydrin color spots indicated the presence of a small amount of unchanged \( \alpha \)-aminoadipic acid and a trace of glutamic acid. The ornithine was well clear of any ninhydrin-positive contaminants except \( \alpha \), \( \gamma \)-diaminobutyric acid, derived from glutamic acid. The ornithine was chromatographed (descending) on a large sheet of Whatman No. 1 paper (butanol-pyridine-water; 1:1:1) for 24 hours. After drying the paper, the development was repeated. The ornithine band was then located about 10 cm from the origin by spraying of selected areas of the paper with ninhydrin. Its position was clear of contaminating \( \alpha \), \( \gamma \)-diaminobutyric acid by at least 2.5 cm. After elution, the ornithine solution was filtered, treated with Norit, dried on a polyethylene planchlet, and counted.

The other one-half of the \( \alpha \)-aminoadipic acid from the electrophoresis procedure was chromatographed as above for 2 periods of 24 hours in butanol-acetic acid-water; 4:1:1. The aminoadipate was located, eluted, dried, and counted. Contaminating glutamic acid was more than 5 cm above the product.

Results and Discussion

Fig. 1 shows the CO₂ expiration results. The oxidation of pipecolic acid-2-C14 appears to occur at a moderate rate. Rothstein and Miller (6), who used Wistar rats, determined that lysine-6-C14 yielded 42% of the dose as CO₂ in 8 hours under similar conditions. According to the proposed metabolic pathway, both amino acids should yield glutaric acid (carboxyl-
had a constant specific activity of 30 c.p.m. per mg. This did not change significantly through 3 recrystallizations. From the isolated glutarate showed that 82% of the activity resided in the carboxyl groups. Unfortunately, the NaN₃ used in the reaction had not been recrystallized (15) and had a high BaCO₃ blank. Treatment of the chloroform extract of the material labeled in such a manner as to support the proposed metabolic pathway for lysine catabolism. The glutarate isolated from the urine of the rat given pipecolic acid-2-C₁⁴ along with a trapping agent (99 c.p.m. of a-aminoadipic acid) was indeed radioactive. The 53 mg obtained had a constant specific activity (through 4 recrystallizations) of 10.2 c.p.m. per mg. If the glutarate was formed according to the equations given in Fig. 2, it should be labeled with Cl₁₄ entirely in the carboxyl groups. Decarboxylation of the isolated glutarate resulted in the same apparent carboxyl activity (82%). Thus, there appears to be little doubt that pipecolic acid is converted, at least in part, to glutaric acid in the intact rat: A—Δ, dl-pipecolic acid-2-C₁⁴ (percentage of dose based on L-isomer); O—O, n-pipecolic acid-2-C₁⁴.

In attempting to demonstrate the conversion of pipelic acid to α-aminoadipic acid, experiments were carried out in which dl-pipecolic acid-2-C₁⁴ was incubated with rat liver mitochondria in the presence of excess L-α-aminoadipic acid as a trap. The aminoacidopate, after undergoing the procedure described above, had a constant specific activity of 30 c.p.m. per mg. This did not change significantly through 3 recrystallizations. From the equations above, it would be expected that the material would contain C₁⁴ only in carbon 2. Decarboxylation by the ninhydrin reaction indicated a considerable amount of C₁⁴ in the carboxyl carbon (approximately 40%). This must be attributed to a radioactive impurity, probably glutamic acid. In short, recrystallization to constant specific activity, is not, in this case, a good criterion of radioimpurity. The problem, therefore, as to whether α-aminoadipic acid is truly a product of pipecolic acid breakdown, must be reconsidered in view of the fact that it was first implicated in this connection by this criterion (4). Under these circumstances, the somewhat heroic measures described under "experimental procedures" were undertaken in order to establish with certainty whether α-aminoadipic acid is truly a product of pipecolic acid breakdown. Treatment of 1000 c.p.m. of α-aminoadipic acid (isolated by paper electrophoresis) by the Schmidt reaction, led to the recovery of 146 c.p.m. of pure ornithine after purification of the latter by paper electrophoresis and paper chromatography. Ninhydrin degradation of this material now indicated only negligible amounts of C₁⁴ in the carboxyl group. In addition, the α-aminoadipic acid isolated after paper chromatographic treatment contained 250 c.p.m. These results establish that α-aminoadipic acid is a product of pipecolic acid (and hence, lysine) breakdown. The amount of activity can be calculated roughly. The C₁⁴ isolated in ornithine and α-aminoadipic acid amounts to 0.18 and 0.30% of the starting activity respectively (based on 2.5 × 10⁶ c.p.m. of L-pipecolic acid-2-C₁⁴), and does not take into account the considerable losses involved in spraying part of the chromatograms with ninhydrin for purposes of location before elution, and the losses during the Schmidt reaction. Though the actual activity is probably somewhat larger, this represents an order of magnitude. The small recovery may be due to rapid oxidation of the biologically formed material, coupled with poor equilibration with the externally added aminoacidic acid.

An alternative reason may be the existence of other metabolic pathways which do not involve α-aminoadipic acid. In this regard, it is of interest to note that an as yet unidentified amino acid appears to be a major product of the oxidation of dl-pipecolic acid-2-C₁⁴ by rat liver mitochondria. Ion exchange chromatography of deproteinized incubation mixtures leads to the results shown in Fig. 3. The large peak appears to be that of unutilized pipecolic acid, probably to a large extent the n-isomer. Peak A, which often contains as much as 10% of the starting

An experiment performed under similar conditions with synthetic L-pipecolic acid-2-C₁⁴ in the absence of the externally added aminoadipic acid. Treatment of the chloroform extract of the material labeled by the Schmidt reaction indicated a considerable amount of C₁⁴ in the carboxyl carbon (approximately 40%). This must be attributed to a
activity (based on L-pipecolic acid-2-C$^{14}$), yields a ninhydrin-
positive spot on paper chromatograms.

The material runs just ahead of pipecolic acid in butanol-
acetic acid-water, 4:1:1, in a position which does not coincide
with that of any of the amino acids found in protein. The ma-
terial was first thought to be $\alpha$-aminovaleric acid, but addition
of carrier to peak A followed by chromatography on Dowex 50
did not lead to coincidence of ninhydrin and radioactivity peaks.

The details of these and other experiments, and discussion of
the significance of this material in relation to lysine metabolism
will be the subject of a future communication.

Although ion exchange chromatography, as used here, readily
demonstrates the presence of the unknown amino acid, this find-
ing should not be accepted as a sensitive indication of all the
products formed. Glutamic acid and $\alpha$-aminoadipic acid, for
example, are both labeled, but do not have enough activity to
show up as specific radioactive peaks.

The conversion of pipecolic acid to $\alpha$-aminoadipic acid could
be expected to involve the steps suggested above, and indeed
leaves support to the idea of an intramolecular transamination as
proposed earlier (1). No direct evidence has yet been uncovered
for the formation of $\Delta^{1}$-piperidine-6-carboxylic acid. However,
the analogous but reverse reaction, reduction of $\Delta^{2}$-pyrroline-5-
carboxylic acid, has been demonstrated in numerous tissues of
the rat (9). The six membered ring compound was not tested.

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Experiment 1A is typical of the results obtained, the values of
the early experiments being mostly in the range of 0.4 to 0.7% CO$_{2}$.
ATP is necessary for the reaction. EDTA, in the pres-

3 Personal communication from Dr. D. R. Rao, Dr. V. W. Rod-
well, and G. Thind of this department.

4 EDTA, ethylenediaminetetraacetate; Versene (Dow Chemical
Company) was used in this work.

### Table I

Oxidation of DL-pipecolic acid-2-C$^{14}$ by rat liver mitochondria*

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Additions†</th>
<th>Dose‡ as CO$_{2}$ %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A</td>
<td>ATP + Mg++</td>
<td>0.7</td>
</tr>
<tr>
<td>1B</td>
<td>EDTA</td>
<td>0</td>
</tr>
<tr>
<td>2B</td>
<td>ATP + EDTA</td>
<td>6.3</td>
</tr>
<tr>
<td>3B</td>
<td>ATP + Mg++ + EDTA</td>
<td>10.5</td>
</tr>
<tr>
<td>1C</td>
<td>ATP + Mg++</td>
<td>2.0</td>
</tr>
<tr>
<td>2C</td>
<td>ATP + Mg++ + EDTA</td>
<td>4.5</td>
</tr>
<tr>
<td>3C</td>
<td>ATP + Mg++ + EDTA + CoA</td>
<td>3.4</td>
</tr>
</tbody>
</table>

* Substrate, DL-pipecolic acid-2-C$^{14}$, 3.6 μmoles. Flasks con-
tained, besides other additions, 2 ml of mitochondria and 50
μmoles of potassium phosphate buffer (pH 7.4, 0.1 M).

† ATP, 3 μmoles; Mg++; 6 μmoles; EDTA, 3 μmoles; CoA, 3
μmoles. In all cases, final volume was 3 ml. Incubations were
carried out for 2 hours at 37°C.

‡ Some observations of interest made during the various ex-
periments are as follows: Anaerobic conditions in two experi-
ments yielded CO$_{2}$ at nearly the same rate as similar aerobic
experiments (0.5 to 0.7%). In the B series of experiments, addi-
tion of TPN, DPN, and CoA (1 μMole; 1 μMole, and 3 μMoles,
respectively), both with and without pyruvate and fumarate, re-
duced CO$_{2}$ output by 50%. CO$_{2}$ in particular, always lowered
CO$_{2}$ output. Addition of 3 μMoles each of pyruvate and fumarate
to “spark” the tricarboxylic acid cycle in no case had a noticeable
effect on pipecolic acid oxidation, and did not result in any ob-
ervable change in the ion exchange chromatographs which might
indicate an enhanced formation of glutamate, aspartate, or ala-
nine. Semicarbazide (0.01 M) completely inhibited CO$_{2}$ produc-
tion, although it had no noticeable effect on the formation of
compound A (Fig. 3).

### Table II

Oxidation of DL-α-aminoadipic acid-6-C$^{14}$ and glutaric
acid-1,5-C$^{14}$ by rat liver mitochondria

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Substrate*</th>
<th>Additions†</th>
<th>Dose as CO$_{2}$ %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A</td>
<td>Aminoadipate</td>
<td>ATP</td>
<td>1.3</td>
</tr>
<tr>
<td>2A</td>
<td>Aminoadipate</td>
<td>ATP</td>
<td>1.1</td>
</tr>
<tr>
<td>3A</td>
<td>Aminoadipate</td>
<td>ATP + EDTA</td>
<td>21.5</td>
</tr>
<tr>
<td>4A</td>
<td>Aminoadipate</td>
<td>ATP + EDTA + Mg++</td>
<td>25.1</td>
</tr>
<tr>
<td>1B</td>
<td>Aminoadipate</td>
<td>EDTA</td>
<td>2.8</td>
</tr>
<tr>
<td>2B</td>
<td>Aminoadipate</td>
<td>ATP + EDTA</td>
<td>10.2</td>
</tr>
<tr>
<td>3B</td>
<td>Aminoadipate</td>
<td>ATP + Mg++</td>
<td>19.3</td>
</tr>
<tr>
<td>4B</td>
<td>Aminoadipate</td>
<td>ATP + EDTA + Mg++</td>
<td>21.7</td>
</tr>
<tr>
<td>1C</td>
<td>Glutarate</td>
<td>ATP</td>
<td>1.3</td>
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<tr>
<td>2C</td>
<td>Glutarate</td>
<td>ATP</td>
<td>2.9</td>
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<td>3C</td>
<td>Glutarate</td>
<td>ATP + EDTA</td>
<td>19.2</td>
</tr>
<tr>
<td>4C</td>
<td>Glutarate</td>
<td>ATP + EDTA + Mg++</td>
<td>17.7</td>
</tr>
</tbody>
</table>

* DL-α-Aminoadipic acid 6 C$^{14}$, 1.5 μmoles; glutaric acid 1,5
C$^{14}$, 1.4 μmoles.

† ATP, 3 μmoles; Mg++; 6 μmoles; EDTA, 3 μmoles. In addi-
tion, each flask contained 2 ml of mitochondria, and 50 μmoles of
potassium phosphate buffer (pH 7.4, 0.1 M). Total volumes were
adjusted to 3 ml. Incubations were 2 hours at 37°C.

ence of ATP, boosted CO$_{2}$ production enormously (5B), even
without Mg$^{++}$. Addition of Mg$^{++}$ to a combination of ATP
and EDTA, or EDTA to ATP plus Mg$^{++}$ always produced an
additional increase in oxidation. It may thus be surmised that
at least one of the enzymes in the pathway is sensitive to heavy metal inhibition which may be nullified by the addition of either EDTA or Mg++. The water used in the early experiments was ordinary distilled water. Later experiments (including the C series) were carried out with distilled water passed through deionizing resin. Under these conditions, similar though less striking results were obtained.

The mitochondria appeared to work equally well when prepared in 0.25 M sucrose and then taken up in KCl. The "debris" fraction, precipitated by centrifugation for 10 minutes at 600 X g, was extremely active in oxidizing proline, as indicated by O2 uptake in the Warburg manometric apparatus (16), but no significant oxidation was observed with pipecolic acid. However, the more sensitive method of measuring C4O2 production was not undertaken.

The results of experiments carried out with DL-α-aminoacidic acid-6-C14 and glutaric acid-1,5-C14 are shown in Table II. These results follow the same general pattern observed with the mitochondrial oxidation of pipecolic acid-2-C14. It would seem that the EDTA and Mg++ act in one of two ways: either by helping to retain the structural integrity of the mitochondria, or by reversing a heavy metal inhibition. In this regard, in the oxidation of α-aminoacidic acid-6-C14 as in Table II (ATP present), increasing concentrations of Mg++ (0.6 μmole per ml to 2 μmole per ml) yielded corresponding increases in C4O2 production (9.3 to 19.1%) but greater concentrations had no further effect. EDTA achieved maximal oxidation (17%) at a concentration somewhere between 0.3 and 1 μmole per ml. Increasing the concentration to 33 μmole per ml in the mitochondrial incubation mixture did not further raise CO2 output. More important, it did not inhibit the oxidation, indicating that if Mg++ is necessary, as one would expect in view of the ATP requirement, the metal must be firmly bound inside the mitochondrial membrane. These conclusions are, of course, only tentative. In dealing with an entity as complex as the intact mitochondrion, effects such as those observed here may not be readily explainable in the light of present knowledge.

As can be seen, the oxidation of α-aminoacidic acid-6-C14 and glutaric acid-1,5-C14 proceeds at an extremely rapid rate. The products of the reaction have not been identified with certainty, but from the mitochondrial oxidation of α-aminoacidic acid, radioactive glutarate was isolated, as expected, by a trapping procedure similar to that described earlier. From glutarate-1,5-C14, similar trapping experiments with inert β-hydroxyglutarate led to the preliminary identification of the latter as a radioactive spot on paper chromatograms. This suggests that glutarate may be metabolized via acetoenecarboxylic acid and acetoacetyl-CoA, in keeping with previous proposals (17). Until more critical proof is obtained, however, this must remain a tentative conclusion.

**SUMMARY**

1. Pipecolic acid has been shown to form α-aminoacidic acid and glutaric acid in the rat, establishing its position on the main pathway of lysine metabolism.

2. Rat liver mitochondria readily oxidize pipecolic acid, α-aminoacidic acid, and glutaric acid to CO2 in the presence of ATP if either Mg++, ethylenediaminetetraacetate, or both are added.

**Acknowledgment**—The authors wish to thank Miss Alice Kells for her capable technical assistance.

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The Metabolism of dl-Pipecolic Acid-2-C\textsuperscript{14}
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