Identification of 3-Methylthiopropionic Acid as an Intermediate in Mammalian Methionine Metabolism in Vitro*

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The oxidative metabolism of methionine by a transaminative pathway was studied in rat and monkey tissue homogenates. Methionine was shown to undergo transamination followed by subsequent oxidation of its α-keto acid, α-keto-γ-methylbutyrate, to carbon dioxide in rat and monkey liver homogenates. Considerable transamination of methionine was observed in monkey kidney homogenates, but little of the α-keto acid was oxidized to carbon dioxide. The addition of saturating levels of S-adenosyl-L-methionine as a diluting pool to the homogenates had no effect on the recovery of radioactive carbon dioxide from L-[methyl-14C]methionine. However, the addition of 3-methylthiopropionate to the incubations inhibited oxidation of 10% L-[methyl-14C]methionine to carbon dioxide by 85% in the rat and by about 50% in the monkey liver homogenate. The expanded 3-methylthiopropionate pool was isolated by anion exchange chromatography to determine if the pool became labeled during the experiment. The isolated radioactive product co-chromatographed with standard 3-methylthiopropionate on paper chromatography. Larger amounts of this isolated radioactive product from rat and monkey liver homogenates were purified by cellulose column chromatography and analyzed by gas-liquid chromatography. The chromatograms that were obtained were essentially identical with the elution profile of a reference sample of 3-methylthiopropionate subjected to the same isolation procedure. Mass spectra of the peak suspected of being 3-methylthiopropionate in the samples obtained from rat and monkey liver homogenates were identical with that of authentic 3-methylthiopropionate. The majority of radioactivity injected into the column was also shown to be associated with 3-methylthiopropionate when the labeled precursor was L-[methyl-14C]- or [15S]methionine, but not when it was L-[1,15]C]methionine. These results show that the expanded 3-methylthiopropionate pool became labeled during the experiments, indicating that 3-methylthiopropionate is an intermediate in methionine catabolism in rat and monkey liver in vitro. This pathway appears to account for a major portion of methionine oxidation in vitro.

Methionine has consistently been shown to be the most toxic amino acid in experiments devised to assess the relative toxicity of dietary amino acids (2-5). The addition of 2% or more of L-methionine to rat diets resulted in elevated plasma and tissue methionine levels, reduction in growth and food intake, and marked tissue abnormalities including lesions of the small intestine, liver, kidney, and pancreas, and, most notably, darkened and enlarged spleens due to accumulation of erythrocytes and deposition of hemosiderin (6-10). The oxidative metabolism of the methyl group of methionine has been extensively studied since it has been postulated to be involved in the severe toxicity of methionine (11).

Experiments by Case et al. (12) showed that the in vitro or in vivo oxidation of the methyl carbon of methionine could not be accounted for via the intermediate formation and metabolism of choline. Other studies (13) showed that metabolism of the methionine methyl carbon probably by the glycine methyltransferase (E.C. 2.1.1.20) enzyme (14, 15) which gives rise to sarcosine could account for only one-fifth of the oxidation of the methionine methyl group. These results suggested that methionine may be metabolized by a pathway independent of its initial conversion to S-adenosyl-L-methionine, as was subsequently shown (16). They further identified formaldehyde and formate as being two intermediates in the oxidative catabolism of the methionine methyl carbon by this alternate pathway. Transamination of methionine to its keto analog, α-keto-γ-methylbutyrate, and further oxidation to CO2 has been shown to account for a substantial amount of methionine methyl carbon oxidation in a rat liver homogenate system (17). Methionine was recently shown to be a better substrate than leucine for a leucine transaminase purified to apparent homogeneity from rat liver mitochondria (18). Sulfur amino acids have also been shown to be effective amino donors in glutamine transaminase (19, 20) and histidine-pyruvate aminotransferase (21). Recently (2), our laboratory studied the effects of feeding to rats 3-methylthiopropionate, the expected decarboxylation product of the keto acid of methionine. The incorporation of 2.5% 3-methylthiopropionate into a 10% casein diet resulted in growth depressions and markedly darkened spleens that were similar to those observed in rats fed a quantity of L-methionine equimolar to the level of 3-methylthiopropionate fed. These results suggested that an intermediate(s) in the transamination pathway may be responsible for the observed growth and tissue effects noted when animals are fed high levels of methionine.

The experiments presented here extend earlier observations (17) and show that, in addition to metabolism via the transsulfuration pathway, methionine may be oxidized by a transamination pathway as shown in Fig. 1. This oxidative pathway has also been observed in monkey liver and kidney homogenate preparations. Finally, we demonstrate for the first time that 3-methylthiopropionate is an intermediate in this pathway in both the rat and monkey and that this pathway represents a significant route of methionine oxidation in tissue homogenate preparations.
3-Methylthiopropionic Acid in Mammalian Methionine Metabolism

Fig. 1. Metabolic scheme for the catabolism of methionine via the proposed transamination pathway or via the transsulfuration pathway. TCA, tricarboxylic acid.

Materials and Methods

Isotopes and Chemicals—14C Glucose was purchased from New England Nuclear Corp. and was used without further purification. 15NH4Cl (Aldrich Chemical Co.) was purchased from EM Science. [3-14C]Methionine and [1,1-13C]methionine were purchased from Amersham/Seearle Corp. The methionine radioisotopes were purified the day before an experiment by cation exchange chromatography of an aliquot on an analytical cation exchange column (4.6 × 12 cm) packed with Dowex 50-X8-400 (sodium form) and then lyophilized as has been described previously. Radiochemical purity was shown to be greater than 98% by chromatography of an aliquot on an analytical cation exchange column (0.6 × 125 cm) by established procedures (22). [methyl-14C]- and [1,13C2]-Keto-γ-methionylbutyrate were prepared 2 days before use by incubating the corresponding labeled methionine with L-aminolevulinic acid oxidase and isolating the desired product by anion exchange chromatography as described in a separate communication (17).

3-Methylthiopropionate was kindly provided as the free acid by Dr. Rudolf Fahrnrich of Digital Chemical Co. The methionine propionate was obtained by saponification of its methyl ester purified the day before an experiment by cation exchange chromatography as described in a separate communication (17).

Animals and Diets—Male rats of the Holtzman strain weighing 150 to 300 g were used in all experiments. They were individually housed in stainless steel cages with wire mesh bottoms in a room with a controlled 12-h light-dark cycle. All animals were offered water and a commercial lab chow ad libitum.

Rhesus monkeys (Macaca mulatta) were housed in individual cages at the Wisconsin Regional Primate Research Center, Madison, Wis. They were fed Purina monkey chow and had free access to water. These animals were participants in a number of different experiments. Our laboratory was notified when fresh tissue samples could be obtained.

Tissue Preparation—Rats were killed by decapitation and the livers excised, placed on ice, and a 20% homogenate made in 0.25 M sucrose with a Potter-Elvehjem tube fitted with a Teflon pestle. Monkey livers were obtained either after sodium pentobarbital anesthetization or exsanguination and immediately placed on ice. Homogenates were then prepared as described above for rats. In one experiment, monkey kidneys were obtained and a homogenate prepared exactly as described for monkey and rat liver preparations.

Incubation Conditions—Two-milliliter samples (0.4 g of tissue) of the homogenates were placed in 50-ml Erlenmeyer flasks and incubated under flowing oxygen for 1 h at 37°C in a shaking water bath. Except where noted, the incubation mixture contained the following in a final volume of 5 ml: 20 mM potassium phosphate buffer (pH 7.5), 1 mM NAD, 1 mM ATP, 5 mM MgCl2, and 10 mM 13C-labeled methionine or α-keto-γ-methylbutyrate. When methionine was the substrate, 10 mM sodium pyruvate or 10 mM α-keto-γ-methylbutyrate was included as the cosubstrate for transamination since prior studies have shown that these two keto acids to be the most active substrates for the highest activity for transamination of methionine in rat liver preparations (17). Water and 0.5 M sucrose were added to give a final volume of 5 ml and 1,400 osmotic equivalents per flask. Reaction mixtures were boiled for 1½ min prior to the addition of the labeled substrate served as blanks. Incubations were killed after 1 h by the addition of 1 ml of 2 N perchloric acid. Control studies showed the reaction to be linear for at least 1½ h of incubation period. The CO2 evolved during the incubations was trapped in 3 ml of an ethanol-ammoniummethylcellulose solution (1:2 v/v). The amount of radioactivity in aliquots of the trapping solution was determined by liquid scintillation spectrometry using the organic fluid described by Jeffay and McAtee (23). Sufficient amount of carrier was added to give a counting error of less than 1%. Counting efficiency of each sample was determined by using an automatic external standard. The perchloric acid-killed samples were centrifuged at 15,000 × g for 15 min to sediment the proteins. The supernatants were neutralized with 6 N potassium hydroxide, centrifuged, lyophilized, and stored at −80°C until they could be analyzed. In some experiments, the keto acid formed from methionine during the incubation was recovered as the phenylhydrazine derivative from an aliquot of the supernatants. The precipitated phenylhydrazine was dissolved in ethanol and an aliquot counted for radioactivity as described (17).

Chromatographic Procedures—Ion exchange chromatography of methionine and its degradation products was performed using a glass column (34 cm) packed with 34 cm of Dowex 1-X8-400 (acetate form) and eluted with a constant volume (600 ml) water/hydrochloric acid (1 N) gradient. The column flow rate was maintained at 0.5 ml per min. The eluate was monitored for radioactivity with a Packard flow cell and collected in a fraction collector. Fractions corresponding to radioactive peaks were combined, neutralized, and lyophilized.

Paper chromatography was run either in the ascending or descending direction on Whatman No. 1 paper and developed with butanol-acetic acid-water (12:3:5 v/v/v). Compounds were detected on the dried papers by exposure to iodine vapors. The papers were then cut into 1-cm strips and added to scintillation vials. Two milliliters of water and 15 ml of aqueous scintillation fluid (24) were added to the vials and counted as described above.

Column chromatography was used for the purification of larger amounts of isolated compounds. A glass column (2.8 × 58 cm) was packed with Whatman CF11 cellulose powder. The column was developed with butanol-acetic acid-water (12:3:5 v/v/v) with a flow rate of 1 ml per min. Fractions (12 ml) were collected and aliquots counted for radioactivity.

Gas liquid chromatography was performed on a Packard model 419 gas chromatograph equipped with dual flame ionization detectors and fitted with 5 foot, 2 mm inner diameter glass columns packed with 10% SP-1200 (5% H2O) on 80 to 100 mesh Chromosorb W AW (Supelco, Inc., Bellefonte, PA). The column temperature was maintained at 170°C and the detector and injection port temperatures were 175°C and 170°C, respectively. Nitrogen was used as a carrier gas at a flow rate of about 35 ml per min.

A stream splitter was fitted to the column just prior to the detector so that 90% of the effluent would be diverted and collected with a Packard model 852 gas fraction collector. The inlet tube that lead to the fraction collector was maintained at 180°C. The effluent was trapped in glass wool-filled cartridges inserted into a turntable which was manually advanced. The well in the turntable was filled with dry ice and ethanol to insure adequate condensation of the compounds in the column effluent onto the glass wool. The glass wool was expelled from the cartridge into a scintillation vial, water and aqueous scintillation fluid added, and the radioactivity determined as described above. The glass cartridge was also counted, but it was found to contain negligible amounts of radioactivity.

A Varian 2170 gas chromatograph was used for gas chromatography-mass spectrometry studies. The column length and packing and column, injection port, and detector temperature were the same as described above. Helium was used as a carrier gas at a flow of about 30 ml per min. The gas chromatograph was connected to a Dupont 21-491 B mass spectrometer. The system was of all glass construction and used a jet separator. The spectrometer scanned at 2 s per decade with a new scan being initiated every 10 s. Data were collected and processed on an AEI DS-50 data system (AEI Scientific Apparatus, Elmsford, NY). Peaks were detected by plots of selected ion currents or of total ion current versus scan number.

1 Supported by National Institutes of Health Grant RR00167.
RESULTS

The results of a number of studies of methionine and α-keto-γ-methylthiobutyrate catabolism in rat and monkey tissue homogenates are shown in Table I. It is apparent that, in addition to rat liver, methionine undergoes transamination and subsequent oxidation in the monkey liver homogenate as well. The amount of radioactivity recovered in the phenylhydrazine derivative is an underestimation of transamination since once the keto acid is formed, it may be further oxidized as shown in Table I. A more correct estimation of transamination would be the sum of the radioactivity recovered as the phenylhydrazide and $^{14}CO_2$ when L-[1-14C]methionine is used as the radioactive substrate. From the phenylhydrazide data, it appears that the most effective amino group acceptor for transamination in the monkey tissue is the keto acid of methionine. This was also found to be the case with rat tissue preparations (17). When L-[35S]- or [1-14C]methionine were used as substrates, they yielded recoveries of labeled α-keto-γ-methylthiobutyrate as the phenylhydrazine derivative that were similar to those obtained with L-[methyl-14C]methionine.

Another interesting observation from these initial studies was the presence of a considerable capacity for transamination (but apparently not subsequent oxidation) of the methionine methyl carbon in monkey kidney preparations, as evidenced by a considerable recovery of radioactivity as the phenylhydrazone, but very little as $^{14}CO_2$. These results are again in agreement with recent work (17) with the rat showing marked transamination of methionine, but little capability of rat kidney preparations to oxidize α-keto-γ-[methyl-14C]methiothiobutyrate.

As was observed with the rat liver homogenate (16), the oxidation of the methionine methyl carbon in the monkey liver homogenate is not inhibited by the addition of saturating levels of S-adenosyl-L-methionine. While saturating levels of S-adenosyl-L-methionine in the rat did not inhibit methionine oxidation, the amount of radioactivity recovered as sarcosine was reduced by 75%, indicating that metabolism of the methionine methyl carbon via sarcosine was not an important route in the homogenate system. This remains to be tested in the monkey.

The major and heretofore unobserved finding from these initial studies was that, while S-adenosyl-L-methionine did not inhibit recovery of $^{14}CO_2$ from methyl-labeled methionine, the addition of 10 to 20 mM 3-methylthiobutyropionate to the homogenate inhibited $^{14}CO_2$ recovery substantially in the rat liver and monkey liver and kidney. The conversion of α-keto-γ-[methyl-14C]methiothiobutyrate to $^{14}CO_2$ in the monkey liver homogenate was virtually completely inhibited (98%) by 3-methylthiobutyropionate, indicating that the inhibitory effect may be on the transamination pathway of methionine metabolism. The dilution of methionine methyl carbon oxidation by varying levels of 3-methylthiobutyropionate in rat and monkey liver homogenates is shown in Fig. 2. In the rat, maximal inhibition was at 2.5 mM, whereas a 3-methylthiobutyropionate concentration of >5 mM was required for maximum inhibition in the monkey liver homogenate. The degree of inhibition by the addition of 10 to 20 mM 3-methylthiobutyropionate in both

![TABLE I](image)

### Table I

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Amino acceptor</th>
<th>$^{14}$CO$_2$</th>
<th>$^{14C}$-Phenylhydrazide</th>
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<tbody>
<tr>
<td><strong>Rat liver</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 mM [Me-14C]Met</td>
<td>10 mM pyruvate</td>
<td>1.63 ± 0.03</td>
<td>13.4 ± 0.46</td>
</tr>
<tr>
<td>10 mM [Me-14C]KyMB</td>
<td>10 mM pyruvate</td>
<td>4.47 ± 0.25</td>
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</tr>
<tr>
<td>10 mM [Me-14C]Met</td>
<td>10 mM pyruvate</td>
<td>0.25 ± 0.02</td>
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</tr>
<tr>
<td><strong>Monkey liver</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 mM [Me-14C]Met</td>
<td>10 mM pyruvate</td>
<td>0.25 ± 0.02</td>
<td>3.21 ± 0.00</td>
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<tr>
<td>10 mM [Me-14C]Met</td>
<td>10 mM aKyMB</td>
<td>0.32 ± 0.09</td>
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<td>10 mM [Me-14C]Met</td>
<td>10 mM aKyMB</td>
<td>0.42 ± 0.04</td>
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<td>10 mM [Me-14C]Met</td>
<td>10 mM aKyMB</td>
<td>0.51 ± 0.05</td>
<td>16.87 ± 1.54</td>
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<td>10 mM [Me-14C]Met</td>
<td>10 mM aKyMB</td>
<td>0.43 ± 0.04</td>
<td>16.67 ± 1.50</td>
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<td>10 mM aKyMB</td>
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<td>16.57 ± 0.08</td>
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<td>10 mM [Me-14C]Met</td>
<td>10 mM aKyMB</td>
<td>0.28 ± 0.01</td>
<td>16.19 ± 0.10</td>
</tr>
<tr>
<td>10 mM [Me-14C]Met</td>
<td>10 mM aKyMB</td>
<td>11.81 ± 0.06</td>
<td>16.87 ± 1.54</td>
</tr>
<tr>
<td>10 mM [Me-14C]KyMB</td>
<td>10 mM aKyMB</td>
<td>1.22 ± 0.02</td>
<td></td>
</tr>
<tr>
<td><strong>Monkey kidney</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 mM [Me-14C]Met</td>
<td>10 mM aKyMB</td>
<td>0.09 ± 0.00</td>
<td>23.17 ± 0.22</td>
</tr>
<tr>
<td>10 mM [Me-14C]Met</td>
<td>10 mM aKyMB</td>
<td>0.04 ± 0.00</td>
<td>21.32 ± 0.19</td>
</tr>
</tbody>
</table>

- The keto acid added to the reaction mixture as the amino group acceptor.
- $^{14}$CO$_2$ = S-adenosyl-L-methionine.
- $^{14C}$-Phenylhydrazide = 3-methylthiobutyropionate.
- Number of observations.

Results are reported as the mean ± S.E.
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Indeed, 3-methylthiopropionate. This radioactive peak was collected, neutralized, lyophilized, and extracted with 95% ethanol, diluted with water (50%), and applied to a cellulose column to isolate larger amounts of the purified labeled compound. A representative chromatogram from a monkey liver sample is shown in Fig. 4. Again, the chromatograms of rat liver samples were qualitatively identical and for brevity are not shown. A sharp band of radioactivity was obtained when samples were developed in the ascending direction with the same solvent system.

Fig. 3 Separation of metabolites from L-[methyl-14C]methionine by anion exchange column chromatography. The labeled substrate was incubated in a rat liver homogenate system containing 2.5 mM 3-methylthiopropionate for 1 h and the neutralized and concentrated supernatant applied to a Dowex 1-X8-400 column (34 x 0.6 cm) and eluted as described under “Materials and Methods.” Column flow rate was 0.5 ml/min, and fraction volumes were 2 ml. For clarity, although each fraction was counted, only those fractions containing significant amounts of radioactivity are marked as data points in the figure.

Lyophilized samples of the radioactive peak from the anion exchange chromatography (Fractions 25 to 30, Fig. 3) were also extracted with 95% ethanol, diluted with water (50%), and applied to a cellulose column to isolate larger amounts of the purified labeled compound. A representative chromatogram from a monkey liver sample is shown in Fig. 4. Again, the chromatograms of rat liver samples were qualitatively identical and for brevity are not shown. A sharp band of radioactivity eluted after 20 fractions with a small but distinct peak following. The large peak that appeared after 50 fractions was not always present indicating that it perhaps may have been a degradation product formed during chromatography or that it may have been due to sporadic contamination from the sharp peak (Fractions 21 to 23, Fig. 3) that eluted just in front of this 3-methylthiopropionate-dependent peak during anion exchange chromatography.

The main portion of the peak at Fractions 20 to 25 (Fig. 4) minus the leading and trailing edges was combined and evaporated at 50°C under reduced pressure. A standard solution of 3-methylthiopropionate was added to some developments with the rat and monkey liver homogenate system 3-methylthiopropionate and an intermediate, then radioactive 3-methylthiopropionate should be produced during the incubation. Experiments with rat and monkey liver homogenates were performed to test this hypothesis using L-[methyl-14C]methionine. Since 2.5 mM and 7.5 mM 3-methylthiopropionate were found to give maximal inhibition of 14CO2 recovery from methyl-labeled methionine in the rat and monkey liver homogenate, respectively, these levels were used in these experiments as a trapping pool. After a 1-h incubation, the reactions were killed by adding 1 ml of 2 N perchloric acid to the 5-ml incubation mixture and the supernatants recovered, neutralized with 6 N potassium hydroxide, re-centrifuged, and lyophilized. A representative chromatogram from a rat liver homogenate sample separated on a Dowex 1 column is shown in Fig. 3. Samples chromatographed from monkey liver homogenates showed qualitatively identical elution profiles. The radioactivity that eluted at the front is almost entirely due to unreacted methionine. The sharp peak that eluted at Fraction 21 has not been identified but is due to a nonenzymatic reaction of the substrate since it was also found after chromatography of a boiled tissue blank. The broad peak that eluted between Fractions 36 and 52 co-chromatographed with 3- keto-γ-methylbutyrate, α-keto-γ-methylbutyrate, a possible contaminant, had an RF of 0.70, which was clearly separated from the radioactive spot detected at an RF of 0.82. Therefore, the majority of the recovered radioactivity was not likely to have been due to contamination by the α-keto acid of methionine. Similar results were obtained when samples were developed in the ascending direction with the same solvent system.

The lyophilized samples from both the rat and monkey experiments (Fractions 25 to 30, Fig. 3) were dissolved in acid and extracted with diethylether. The ether extract was concentrated under nitrogen and aliquots spotted on paper. After development, a major radioactive peak was detected in all samples at an RF of approximately 0.82. A spot with an RF of 0.83 was observed with a standard sample of 3-methylthiopropionate. A minor radioactive spot was sometimes detected at an RF of about 0.60. When the unknown was treated with hydrogen peroxide prior to paper chromatography, the radioactivity at RF 0.82 was decreased, whereas the radioactivity in the spot at RF 0.60 was elevated. When care was taken to prevent the samples from oxidation, however, the amount of radioactivity recovered at RF 0.60 was minimal. When 3-methylthiopropionate was treated with hydrogen peroxide before chromatography, an iodine-positive spot was detected with an RF of about 0.60, indicating that the unknown co-chromatographed with standard 3-methylthiopropionate and with its oxidation product, the sulfoxide or sulfone derivative. α-Keto-γ-methylbutyrate, a possible contaminant, had an RF of 0.70, which was clearly separated from the radioactive spot detected at an RF of 0.82. Therefore, the majority of the recovered radioactivity was not likely to have been due to contamination by the α-keto acid of methionine. Similar results were obtained when samples were developed in the ascending direction with the same solvent system.

That the inhibition of methionine oxidation by 3-methylthiopropionate may be due simply to a general inhibition of cellular metabolism was ruled out because no inhibition of the oxidation of 10 mM [U-14C]glucose to 14CO2 by 3 methylthiopropionate was observed at any concentration tested (Fig. 2). Thus, the effect of 3-methylthiopropionate on methionine oxidation is most likely specific and not due to a depressed capability for general oxidation.

If the dilution of methionine catabolism in either the rat or monkey liver homogente system by 3-methylthiopropionate is because 3-methylthiopropionate is an intermediate, then radioactive 3-methylthiopropionate should be produced during the incubation. Experiments with rat and monkey liver homogenates were performed to test this hypothesis using L-[methyl-14C]methionine. Since 2.5 mM and 7.5 mM 3-methylthiopropionate were found to give maximal inhibition of 14CO2 recovery from methyl-labeled methionine in the rat and monkey liver homogenate, respectively, these levels were used in these experiments as a trapping pool. After a 1-h incubation, the reactions were killed by adding 1 ml of 2 N perchloric acid to the 5-ml incubation mixture and the supernatants recovered, neutralized with 6 N potassium hydroxide, re-centrifuged, and lyophilized. A representative chromatogram from a rat liver homogenate sample separated on a Dowex 1 column is shown in Fig. 3. Samples chromatographed from monkey liver homogenates showed qualitatively identical elution profiles. The radioactivity that eluted at the front is almost entirely due to unreacted methionine. The sharp peak that eluted at Fraction 21 has not been identified but is due to a nonenzymatic reaction of the substrate since it was also found after chromatography of a boiled tissue blank. The broad peak that eluted between Fractions 36 and 52 co-chromatographed with α-keto-γ-methylbutyrate. The smaller peak that eluted between Fractions 25 and 30 was not observed when 3-methylthiopropionate was omitted from the incubation mixture. Chromatography of standard 3-methylthiopropionate on this same system yielded a single peak, as detected by the nitroprusside reaction, which eluted at approximately the same volume as the 3-methylthiopropionate-dependent peak seen between Fractions 25 and 30 in Fig. 3. Therefore, this radioactive peak was collected, neutralized, lyophilized, and used in further studies to determine whether or not it was indeed 3-methylthiopropionate.

The lyophilized samples from both the rat and monkey experiments (Fractions 25 to 30, Fig. 3) were dissolved in acid and extracted with diethylether. The ether extract was concentrated under nitrogen and aliquots spotted on paper. After development, a major radioactive peak was detected in all samples at an RF of approximately 0.82. A spot with an RF of 0.83 was observed with a standard sample of 3-methylthiopropionate. A minor radioactive spot was sometimes detected at an RF of about 0.60. When the unknown was treated with hydrogen peroxide prior to paper chromatography, the radioactivity at RF 0.82 was decreased, whereas the radioactivity in the spot at RF 0.60 was elevated. When care was taken to prevent the samples from oxidation, however, the amount of
Samples from rat and monkey experiments and the standard 3-methylthiopropionate (for comparison purposes) were dissolved in ether and subjected to gas-liquid chromatography as detailed above. Elution profiles of three samples are shown in Fig. 5. The similarity of the chromatograms from these three samples is striking. A peak was observed with a retention time of about 19% min in all three samples and accounted for about three-fourths of the total peak area. Standard 3-methylthiopropionate treated in the same manner as the experimental samples led to minor products, presumably degradation products from sample breakdown on the column or perhaps from other products formed during the preparative procedure. However, no significant peaks other than that seen with the standard reference were observed in either the rat or monkey liver homogenate samples.

In order to verify that the radioactivity injected into the chromatograph was associated with one or more of the peaks shown in Fig. 5, a portion of the eluate (90%) was diverted with a stream splitter and counted for radioactivity. Also, in addition to using L-[methyl-14C]methionine, L-[15S]methionine and L-[1-14C]methionine were used as substrates in the rat and monkey liver homogenate incubations. Thus the sulfur- and methyl-labeled substrates should yield radioactive 3-methylthiopropionate and the carboxyl-labeled substrate should yield 14CO2 and nonradioactive $m$-methylthiopropionate if the proposed pathway of methionine catabolism is correct (Fig. 1).

These experiments with rat and monkey liver homogenate preparations were performed and the samples processed exactly as above. The distribution of radioactivity recovered upon gas-liquid chromatography of some of the samples is shown in Table II. In both species, the majority of the radioactivity recovered was associated with the peak that eluted at 19% min when either L-[methyl-14C] or [15S]methionine was the substrate. However, in incubations conducted with L-[1-14C]methionine as the substrate, the total radioactivity recovered was considerably less and was distributed more or less over the entire elution profile, with apparently the major-

![Fig. 4. Cellulose column elution profile of partially purified monkey liver supernatant from anion exchange column. The peak from Fractions 25 to 30 (Fig. 3) was neutralized, concentrated, applied to a cellulose powder column (58 X 2.8 cm), and eluted with butanol-acetic acid-H2O (12:3:5). Column flow rate was 1 ml/min, and fraction volumes were 12 ml. Aliquots of each fraction were counted for radioactivity as described under "Materials and Methods." Only those fractions containing significant amounts of radioactivity are shown as data points on the elution profile.

![Fig. 5. Gas-liquid chromatography elution of radioactive compounds isolated by anion exchange and subsequent cellulose column chromatography of supernatants from liver homogenate incubations. A, compound isolated from a rat liver incubation; B, compound isolated from a monkey liver incubation; C, a reference solution of 3 methylthiopropionate subjected to similar concentration procedures.

### Table II

**Distribution of radioactivity recovered during gas-liquid chromatography of isolated metabolites from rat and monkey liver homogenate incubations**

<table>
<thead>
<tr>
<th>Species</th>
<th>Labeled substrate</th>
<th>Column retention time (min)</th>
<th>Percentage of recovery*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0-4</td>
<td>5-8</td>
</tr>
<tr>
<td>Rat</td>
<td>[Me-14C]Met (2)b</td>
<td>5.2 ± 0.1</td>
<td>5.0 ± 1.6</td>
</tr>
<tr>
<td></td>
<td>[15S]Met (2)</td>
<td>2.3 ± 0.4</td>
<td>1.7 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>[L-14C]Met (3)</td>
<td>21.9 ± 0.5</td>
<td>20.6 ± 0.4</td>
</tr>
<tr>
<td>Monkey</td>
<td>[Me-14C]Met (3)</td>
<td>10.9 ± 1.4</td>
<td>5.6 ± 0.8</td>
</tr>
<tr>
<td></td>
<td>[15S]Met (3)</td>
<td>7.3 ± 0.8</td>
<td>2.7 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>[L-14C]Met (2)</td>
<td>10.7 ± 0.9</td>
<td>21.6 ± 5.5</td>
</tr>
</tbody>
</table>

* (Total counts per min recovered/cpm injected) X 100.
* Number of observations.
* Results are reported as the mean ± S.E.
ity of the radioactivity retained on the column. In addition, 2.18 μmol/g/h of 14CO2 were recovered during these incubations with L-[1-14C]methionine, indicating that decarboxylation of the keto acid of methionine had occurred even in the presence of a trapping pool of 3-methylthiopropionate.

Attempts were made to separate the radioactivity corresponding to the peak detected at 19½ min when L-[methyl-14C]- or [35S]methionine were the labeled precursors. The radioactivity remained associated with the peak corresponding to standard 3-methylthiopropionate (19½ min) when the retention times were altered by changing carrier gas flow rate or by increasing or decreasing column temperature. Thus, it appeared that the majority of the radioactivity recovered with these compounds during isolation was associated with the compound that eluted from the column at about 19½ min (Fig. 5).

Further comparison of the unknown compounds with standard 3-methylthiopropionate was carried out by gas chromatographic separation on a column packed and prepared so that the effluent from the column could be analyzed by mass spectrometry. A mass spectrum of the column eluate was taken every 10 s. Analysis of the spectra showed that the peak that eluted from the column at about 19½ min gave a spectrum that was compatible with 3-methylthiopropionate (Fig. 6). Spectral analysis also showed that the radioactive peak isolated after cellulose column chromatography of both the rat and monkey liver tissue samples was also 3-methylthiopropionate (Fig. 5). Spectra of all samples taken at the beginning, middle, and end of the elution of the radioactive peak at 19½ min (Fig. 5) were essentially identical. The material which eluted at the front appeared to be mostly solvent ether, some residual butanol, and unidentified breakdown products of 3-methylthiopropionate. Again no differences in the mass spectra of these compounds eluted in the first few minutes (0 to 5 min) could be detected between the rat and monkey experimental samples and the 3-methylthiopropionate reference standard.

The possibility that the radioactive 3-methylthiopropionate recovered was not due to an enzymatically catalyzed reaction but due to spontaneous decarboxylation of the α-keto-γ-methylionobutyrate during sample preparation was checked by carrying 10 mM α-keto-γ-[14C]methylionobutyrate through the sample preparation procedure. Gas chromatographic analysis of the samples indicated that approximately 0.6% of the radioactivity in the keto acid sample was recovered as 3-methylthiopropionate. This amounts to approximately 15% (8,000 dpm versus 70,000 dpm) of the activity recovered in 3-methylthiopropionate when L-[methyl-14C]- or [35S]methionine is incubated with a liver homogenate. Thus, while nonenzymatic decarboxylation may occur, it is clearly not sufficient to account for the substantial amounts of radioactivity recovered in the 3-methylthiopropionate pool.

Mass spectral analysis of the peak that eluted just prior to 3-methylthiopropionate in some samples (Fig. 5) suggested that it may be the butyl ester of 3-methylthiopropionate, most likely formed during or after cellulose column chromatography. To test this, the butyl ester of 3-methylthiopropionate was prepared as follows. To 5 g of 3-methylthiopropionate, 7 g of butanol was added plus 1 ml of concentrated sulfuric acid and the mixture refluxed for 25 min. After cooling, the reaction mixture was diluted with water, mixed, and the organic layer was separated, collected, anhydrous sodium carbonate added, and the mixture filtered. When a 0.1-μl sample of the filtrate was injected into the gas chromatograph as above, a large peak eluted at 16 min, the same retention time as the compound suspected as being the butyl ester of 3-methylthiopropionate. A smaller peak appeared at 19½ min due to unreacted 3-methylthiopropionate. From this we have concluded that the peak eluting in front of 3-methylthiopropionate at 16 min most likely is the butyl ester of 3-methylthiopropionate. Since it was also observed when the reference solution was chromatographed, it was probably an artifact formed during cellulose column chromatography or subsequent preparation of samples.

**DISCUSSION**

These experiments demonstrate that in both the rat and monkey methionine is catabolized via transamination and subsequent decarboxylation of the keto acid to 3-methylthiopropionate. While this was accomplished by the use of a trapping pool, attempts to isolate 3-methylthiopropionate without a diluting pool have to date been unsuccessful. Our preliminary experiments indicate that the methyl carbon of 3-methylthiopropionate is oxidized to carbon dioxide quite rapidly in vitro (about 3 times the rate of methionine) and probably in vivo as well. As the isotope dilution studies in Fig. 2 indicate, this pathway appears to account for about 85% of the total oxidation of the methionine methyl carbon in the rat liver homogenate system and about 50% of the total in a monkey liver homogenate. These results are consistent with the observations of Case and Benevenga (16) that 75% of methionine oxidation could be inhibited by S-methyl-L-cysteine in a rat liver homogenate, probably by competing with methionine at the transamination step.

To our knowledge, this is the first demonstration of the formation of 3-methylthiopropionate in a mammalian system. The occurrence of the methyl ester of 3-methylthiopropionate has been demonstrated in pineapple juice (25). Another laboratory (26) identified 3-methylthiopropionate in the medium of an ethionine-resistant strain of Neurospora but later showed that this was an artifact produced from α-keto-γ-methylionobutyrate during isolation. In studies with planarian, Arima et al. (27) identified trans-3-methylthiaoacrylic acid and 3-methylthiopropionate when a strain of Streptomyces was incubated in a medium containing L-methionine. They suggested that 3-methylthiopropionate was formed from the deamination or transamination of methionine followed by decarboxylation, and that 3-methylthiopropionic acid was then formed from 3-methylthiopropionate. In studies with *Esche-
richia coli (28), the formation of [35S]3-ethylthiopropionate was demonstrated from either [35S]sulfate or [35S]methionine. Another analog, 3-methylthiopropionamide, has been identified as the product of a pyridoxal phosphate-dependent decarboxylation of methionine by horseradish peroxidase (29, 30).

In some studies in the rat where S-methyl-L-cysteine, a structural analog of methionine, was used, rats injected subcutaneously with this compound excreted methylthioacetic acid in the urine (31). The author suggested deamination and decarboxylation of the substrate as a means whereby this compound might have been formed. The glycine conjugate of methylthioacetic acid was also isolated from the urine. Similarly, when methylthioacetic acid was injected into rats, the glycine conjugate and unchanged methylthioacetic acid were recovered from the urine. The metabolism of this compound warrants further investigation since rats fed high levels of S-methyl-L-cysteine experience growth depressions and tissue alterations similar to that observed with high levels of methionine (10). Studies similar to the ones reported here have been done in the intact animal and liver homogenate system with the straight chain amino acids, α-amino butyrate, norleucine, and norvaline (32-34). After isolating the intermediates it was found that in all cases these amino acids were catabolized first by transamination or deamination to their respective α-keto acids and then by decarboxylation to the free fatty acid.

From the data in Table I and Fig. 2, the transamination pathway of methionine catabolism must be a significant pathway of methionine metabolism. From the data in Table I and Fig. 2, the transamination pathway of methionine metabolism may contribute significantly to methionine catabolism, especially when the capabilities of transsulfuration have been exceeded or impaired.

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