Metabolism of 2-Methylstearic Acid in the Rat

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The intestinal absorption and the metabolism of 2-methyl[1-¹⁴C]-octadecanoic acid were found to be very similar to those of the ordinary, long, straight chain fatty acids. For the first time, however, a small amount of metabolites from 2-methylstearic acid could be isolated from the urine and identified as 2-methyladipic (1.1 %) and 2-methylsuccinic acid (0.6 %).

In extensive studies on dogs Weitzel¹ investigated the effect on metabolism of monoalkylsubstitution in the α -position of long chain fatty acids. He found that 2-ethylstearic acid was excreted in the urine to 1.2 % as 2-ethyladipic acid. Analogously he showed that 2-propylstearic acid and 2-butylstearic acid gave rise to 2-propyladipic acid (5.2 %) and 2-butyladipic acid (6.5 %), respectively. With the methods used Weitzel found that, in contrast to these higher fatty acids with comparatively large α -substituents, α -methylsubstituted acids (fed in the ester form) did not produce any short dicarboxylic acids in the urine^{2,3}.

Continuing the investigation on the metabolism of long, branched chain fatty acids started with 2,2-dimethylstearic acid⁴ and 2,2-dimethylnonadecanoic acid⁵, we have now studied 2-methyl[1-¹⁴C]stearic acid. It was given *per os* to intact rats or rats with thoracic duct fistulas. The isotopic distribution in the neutral fat, phospholipids and unesterified fatty acids of the lymph has been investigated. In addition the isotope excretion has been followed in the expired carbon dioxide, the feces and the urine. The metabolites were isolated by partition chromatography and identified by repeated recrystallizations with inactive, synthetic material.

When the 2-methyl[1-¹⁴C]stearic acid was fed to unanesthetized rats with lymph fistulas⁶, about 60 % of the absorbed activity was recovered in the lymph lipids. This is about the same figure as those found in similar experiments with other, long chain, branched or unbranched fatty acids. The main transport form of the 2-methyl[1-¹⁴C]stearic acid was as glycerides (89—96 % of the total lymph fat activity). Only small amounts of cholesterol esters were present, and just a few per cent of the acid (Table 2) was found to be in unesterified form (3.7 %) or incorporated into phospholipids (4.0 %).

The specific activities of the unesterified fatty acids were in three experiments with 2-methyl[1-¹⁴C]stearic acid 75, 36 and 14 % of the specific activities of the neutral fat fatty acids (Table 2). These figures were always found to be less than 100 % for ordinary straight chain fatty acids, whereas the corresponding values for the 2,2-dimethyl[1-¹⁴C]nonadecanoic acid were 200—300 %. Thus the distribution of the lymph activity between neutral fat and unesterified fatty acids was the same as for ordinary straight chain fatty acids⁷ or 15,15-dimethylpalmitic acid^{*}, and it differs from fatty acids with two methyl groups in the α -position (*i. e.*, 2,2-dimethylstearic acid⁴, 2,2-dimethylnonadecanoic acid⁵ and 2,2,17,17-tetramethylstearic acid⁸).

The feces were collected daily from each rat and their isotope contents determined after saponification and extraction of the acids with ether. In thirteen experiments between 3 and 31 % (mean 13 %) of the fed activity could be recovered in the feces during four days after the administration of 2-methyl[1-¹⁴C]stearic acid.

Almost all the active material in the feces was identified as the 2-methylstearic acid. Consequently, throughout this investigation, the absorbed activity has been calculated as the difference between the fed activity and the activity recovered in the feces. — However, a small amount of the fecal activity (in one experiment less than 0.2 %) chromatographically appeared as 2-methylsuccinic and 2-methyladipic acid, identical with the urinary metabolites of 2-methyl stearic acid (see below). Earlier, 2,2-dimethylnonadecanoic acid has been shown to produce dicarboxylic acids in the feces as well as in the urine⁵.

After feeding carboxyl-labelled 2-methylstearic acid to a rat, about 25 % of the absorbed activity was found in the expired carbon dioxide in two days. The same value was found in a parallel experiment with [1-¹⁴C]stearic acid. From these data one methyl group in the α -position does not seem to hinder the oxidation of the higher fatty acids. In contrast, after administration of 2,2-dimethyl[1-¹⁴C]stearic acid⁴ and 2,2,17,17-tetramethyl[1-¹⁴C]stearic acid⁸, less than 3 % of the absorbed activity was excreted as carbon dioxide. The β -oxidation from the carboxyl end of the molecule is not possible, because the two hydrogen atoms on the α -carbon atom are substituted with methyl radicals. The different metabolism of 2-methylstearic acid and 2,2-dimethylstearic acid is also strongly reflected in the isotope recoveries in the urine.

The urine from rats fed 2-methyl[1-¹⁴C]stearic acid has been found to contain 1.6—3.5 % of the absorbed activity (Table 6). (After feeding [1-¹⁴C]-palmitic acid to rats less than 0.5 % of the activity can be recovered from the urine⁹). By partition chromatography and repeated recrystallizations with synthetic unlabelled compound, two of the active substances have been identified as 2-methyladipic acid (0.8—1.7 % of the absorbed activity) and 2-methylsuccinic acid (0.4—0.9 %). They were excreted partly in conjugated form. The methyl side chain of 2-methylstearic acid thus has some, although a minor effect in impeding the β -oxidation from the carboxyl end.

* To be published.

Fig. 1. Partition chromatography of part (39 mg) of an acid mixture obtained after Kolbe electrolysis of methyl hydrogen succinate and sodium 3-cyanobutyrate. Phase system C. Supporting medium: 4.5 g of hydrophobic kieselguhr.

SYNTHESES

2-Methyl[1- 14 C]octadecanoic acid. Two different methods were used for the preparation of the labelled 2-methyloctadecanoic acids. The comparatively weakly radioactive product (0.13 μ C/mg) was prepared by a Kolbe electrolysis of palmitic acid and sodium 3-[14 C]cyanobutyrate according to Westö $\ddot{o}$ ¹⁰, the strongly active one (6.5 μ C/mg) was made from inactive 2-methyloctadecanoic acid (prepared by the above method) by exchange of the carboxyl group for bromine by degradation of the silver salt (Rottenberg¹¹) and reaction of the 2-bromooctadecane formed with K 14 CN.

Silver 2-methyloctadecanoate (0.50 g) and silver acetate (0.20 g) were thoroughly mixed, pulverized and dried at 0.1 mm Hg over P $_2$ O $_5$. Dry ethyl bromide (4 ml) was added and into the boiling mixture 0.13 ml of dry bromine was dropped. The boiling was continued for half an hour. Part of the ethyl bromide was distilled off, the mixture was filtered, and the filtrate was shaken with potassium iodide solution, water, sodium thio-sulphate solution, and water again. It was dried over anhydrous sodium sulphate and filtered. After removal of the solvent, the residue was dissolved in light petroleum and chromatographed on 2.5 g of alumina. The front band (0.35 g) contained the 2-bromooctadecane.

Crude 2-bromooctadecane (0.11 g) was heated with K 14 CN (0.017 mg, 0.50 mC), ethanol (2.5 ml) and water (0.7 ml) in a sealed glass tube at 95° for 24 h. The nitrile formed was hydrolyzed by heating with an excess of 10 % potassium hydroxide solution (ethanol-water, 4:1) in an autoclave at 130° for 4 h. Part of the ethanol was removed by distillation, and the rest of the solution was acidified with dilute hydrochloric acid and extracted with ether. The ether solution was washed with water, dried with sodium sulphate and evaporated to dryness. The product obtained was chromatographed according to system A (Table 1).

2-Methyladipic acid. Methyl hydrogen succinate (10.5 g) and sodium 3-cyanobutyrate (1.0 g) were electrolyzed in methanol (400 ml). The electrolysis was carried out as described earlier⁸. The slightly alkaline solution (pH 8) was evaporated to 50 ml. The products of the electrolysis were hydrolyzed in 2 N alcoholic potassium hydroxide for 2 h. The methanol was evaporated and after acidification the hydrolysate was extracted with ether. The acids obtained (4.4 g) were chromatographed three times according to system

C (Table 1). They were eluted in the following order: succinic and 2-methylsuccinic acid (in the front band), adipic, 2-methyladipic acid and 2,5-dimethyladipic acid (*cf.* Fig. 1). The 2-methyladipic acid (0.36 g) was recrystallized repeatedly from water and from benzene-light petroleum; m. p. 61° (equiv. wt. 80.1).

CHROMATOGRAPHIC SYSTEMS

The chromatographic systems used in this investigation are summarized in Table 1. System A was used for the isolation and identification of 2-methylstearic acid. Phase system B was found suitable for the separation of dicarboxylic acids with five to nine carbon atoms. The phases used in system C are more polar than those of system B, which enables also C₄-dicarboxylic acids to be chromatographed. The ordinary partition chromatography (with phase system D) was used as an alternative to the reversed phase system C.

Table 1. Chromatographic systems.

System	According to	Supporting medium	Stationary phase	Moving phase
A	Howard and Martin ¹²	Hydrophobic kieselguhr (Hyflo supercel treated with dimethyldichlorosilane)	Paraffin oil (25 ml)	Acetone: water 7:3, v/v (210 ml: 90 ml)
B	Bergström and Sjövall ¹³ for the separation of free bile acids. Also used by Bergström, Borgström, Tryding and Westöö ⁴ for the separation of dicarboxylic acids	see above	<i>iso</i> -octanol (2-ethylhexanol)- chloroform 1:1, v/v (25 ml: 25 ml)	methanol: water 1:3, v/v (75 ml: 225 ml) (modified from 2:3, v/v)
C	Norman ¹⁴ for the separation of conjugated bile acids	see above	<i>n</i> -butanol (100 ml)	distilled water (300 ml)
D	Isherwood ¹⁵	silica gel prepared according to Nijkamp ¹⁶	0.5 N sulfuric acid (50 ml)	butanol : chloroform 1:9, v/v (30 ml: 270 ml)

The volumes of solvents given in Table 1 for each system were mixed in a separatory funnel and equilibrated at room temperature (23° C) before use. Four ml of the stationary phase were used per 4.5 g of supporting medium. Generally 9 g columns were made with solvent systems B and C, while all the chromatograms with phase system A and D were run on 4.5 g columns. The internal diameter of the chromatographic tubes used was 12 mm. The techniques of fraction collection, titration and determination of ¹⁴C-activity have been described earlier ⁴.

METABOLIC EXPERIMENTS

A 3.6 % stock solution (I) of 2-methyl[1-¹⁴C]stearic acid (specific activity 110 000 cpm/mg) in olive oil was prepared and found to have a specific activity of 3 900 cpm/mg.

Another olive oil solution (II) contained 0.045 % of the strongly active 2-methyl-[1-¹⁴C]stearic acid (s. a. 5 400 000 cpm/mg). The specific activity of this solution was 2 400 cpm/mg. No differences were found in the absorption or metabolism of the two stock solutions.

Radioactivity determinations were performed with a gas flow counter (background effect 30 cpm) generally after direct plating of the samples on aluminium planchets.

The animals used in these investigations were white, adult, male rats weighing about 250 grams.

Administration of 2-methylstearic acid to rats with lymph fistula

Unanesthetized rats provided with a thoracic duct fistula* were fed 1 ml of the olive oil solution containing 2-methylstearic acid (stock solution I). The general procedure for the collection, extraction and separation of the lymph lipids has been reported earlier from this institute^{6,7}. From Table 2 it is seen that the absorption of 2-methyl[1-¹⁴C]-stearic acid varied from 69 to 96 % of the fed amount. However, the percentage of absorbed activity recovered in the lymph was relatively constant, about 60 % in three rats out of four. Almost all the lymph activity (96 % for rat No. 1, Table 2) could be recovered during the first 12 h after the administration, but the values given in the table were determined on 24-h-specimens.

Table 2. Recovery of activity in lymph fatty acids after oral administration of 2-methyl-[1-¹⁴C]stearic acid to rats.

Rat No.	Per cent of administered activity absorbed	Per cent of absorbed activity recovered in lymph fatty acids	Per cent of lymph fat activity found as		
			unesterified fatty acids	neutral fat	phospholipids
1	85.3	61.2	96.5		3.5
2	69.3	62.5	5.0	88.7	6.3
3	95.7	63.2	2.4	95.6	2.0
4	91.9	45.1	3.7	92.1	4.2

Two of the experiments (Rats No. 3 and 4) complete an investigation by Borgström and Tryding on the free fatty acid content of rat thoracic duct lymph⁷. Table 3 summarizes the results of these two experiments. — Cholesterol esters could be present only in very small amounts, as only traces of the lymph fat activity could be eluted with light petroleum from a silicic acid column¹⁷.

Table 3. Weight of different lymph fat fractions and specific activity of their fatty acids.

Rat No.	Neutral fat		Free fatty acids		s.a. of free fatty acids in % of s.a. of neutral fat fatty acids
	Weight mg	Spec. activity	Weight mg	Spec. activity	
3	690	70	48.4	25	36
4	784	50	22.6	7	14

Isotope recovery in feces after feeding 2-methyl-[1-¹⁴C]stearic acid to rats

The feces from the rats were collected daily during at least four days after the oral administration of the labelled acid. After homogenization they were hydrolyzed with 5 N alcoholic potassium hydroxide. The alcohol was evaporated and the acidified mixture was extracted three times with three volumes of peroxide-free ether. In the feces from twelve animals between 8 and 31 % of the fed activity was recovered (*cf.* Tables 2 and 4).

Table 4. Isotope recovery in the urine from rats fed 2-methyl[1-¹⁴C]stearic acid.

Rat No.	Per cent of administered activity absorbed	Per cent of absorbed activity recovered in the urine		
		total	chromatographically identified as	
			2-methylsuccinic acid	2-methyladipic acid
5	89.5	1.9		
6	73.3	1.6	0.5	0.9
7	94.2	2.7	0.8	1.1
8	91.1	3.1	0.8	1.5
9	84.8	3.5	0.9	1.7
10	93.2	2.3	0.4	1.0
11	80.9	2.2	0.5	0.8
12	97.0	2.3	0.6	1.1

Fig. 2. Recovery of ^{14}C in expired carbon dioxide after feeding 2 methyl- $[1-^{14}\text{C}]$ stearic acid (circles) and $[1-^{14}\text{C}]$ stearic acid (squares) to rats.

Fig. 3. Chromatography of part of an ether extract of urine from Rat No. 11. The titration peaks are caused by ordinary urine metabolites, as no carrier substances were added. Phase system B. Supporting medium: 9 g of hydrophobic kieselguhr; — titration curve, --- c.p.m./fraction. Band I contains α -methylsuccinic acid and conjugated products.

Fig. 4. Rechromatography of band I, Fig. 3. Phase system D. Supporting medium: 4.5 g of silica gel. Band I represents α -methylsuccinic acid. Band III and IV contain conjugated metabolites.

In one additional experiment the isotope contents of the feces were determined during 15 days after the administration of 1 ml of the olive oil solution (I) containing 3.6 % of 2-methyl[1- 14 C]stearic acid. The feces from the first day after the feeding contained 8 % of the fed activity. During the second, third and fourth day 4.3, 3.4 and 1.3 % respectively appeared in the feces. In ten days the total isotope recovery was 21 %. In the next five days only 0.8 % of the fed activity was found in the feces.

The feces excreted by a rat during the first day after the administration of 2-methylstearic acid was saponified and extracted as described above. The ether extract contained 5.7 % of the fed activity. When half of this extract was chromatographed according to system A, 95 % of the activity was found unchanged in the band of 2-methylstearic acid, which appeared at 60 ml on a 4.5 g column. The other half of the ether extract was subjected to partition chromatography according to system C.

A small amount (less than 0.2 %) of the activity was found at the positions of 2-methylsuccinic and 2-methyladipic acid.

Isotope recovery in expired carbon dioxide after oral administration of carboxyl-labelled 2-methylstearic and stearic acids

A male rat weighing 250 g (Rat No. 5, Table 4) was given through a stomach tube 1 ml of the olive oil solution (I), corresponding to 0.3 ml per dm² of body surface (Lee¹⁸).

The isotope content of the expired carbon dioxide was determined with the technique described earlier⁴. The accumulation curve is shown in Fig. 2.

Another 250 g rat from the same litter was fed 1 ml of an olive oil solution of [1- 14 C]-stearic acid with a specific activity of 130 000 cpm/mg*.

* Kindly placed at our disposal by Professor S. Bergström.

Fig. 5*. Partition chromatography of metabolites in hydrolyzed urine together with unlabelled 2-methylsuccinic acid (5.4 mg). Phase system D. Supporting medium: 4.5 g of silica gel. — titration curve, --- c.p.m./fraction.

Fig. 6*. Rechromatography of the material from the chromatogram shown in Fig. 5 and a parallel chromatogram together with synthetic 2-methyladipic acid (2.4 mg). Phase system C. Supporting medium: 9 g of hydrophobic kieselguhr; — titration curve, --- c.p.m./fraction.

In four days 31.8 % of the fed activity was recovered in the feces. The CO_2 of the expired air was collected during six, twenty-four and forty-eight hours after the administration of the acid. From Fig. 2 it is seen that the $^{14}\text{CO}_2$ -excretion in per cent of the absorbed activity was about the same for the two acids.

Isotope recovery in urine and identification of isotopic urinary compounds after the administration of 2-methyl[1- ^{14}C]stearic acid

In order to obtain the urine completely free from fecal radioactive impurities a plastic funnel was sewn on to the cutis around the penis of the rat. The urine was collected into a flask with toluene. The activities recovered in the urine collected for 3 days after the administration of 2-methyl[1- ^{14}C]stearic acid are given in Table 4. More than 75 % of the total urinary activity was excreted during the first day. The urine was acidified with hydrochloric acid and extracted three times with two volumes of peroxide-free ether. After that the urine was either extracted with butanol or continuously with ether for 24 h.

* See footnote p. 436.

Fig. 7*. Chromatography of an ether extract of hydrolyzed urine from Rat No. 10 together with unlabelled 2-methyladipic acid (4.0 mg). Phase system C. Supporting medium: 9 g of hydrophobic kieselguhr; — titration curve, --- c.p.m./fraction.

Fig. 8*. Rechromatography of material from the active band II in a chromatogram with phase system C together with synthetic 2-methyladipic acid (2.9 mg). Phase system B. Supporting medium: 9 g of hydrophobic kieselguhr; — titration curve, --- c.p.m./fraction.

The urine from Rat No. 11 was divided into two equal parts. One of these was extracted three times with two volumes of ether. The extract (17.6 mg) contained 0.8 % of the absorbed activity. Part of the ether extract was chromatographed according to system C as shown in Fig. 3. Two distinct active bands were found containing about the same amount of activity.

The other half of the urine was hydrolyzed with 2 N potassium hydroxide before acidification and analogous ether extraction.

The ether extract of the hydrolyzed urine contained 30 % more activity than the corresponding extract of unhydrolyzed urine. This finding indicates the existence of conjugated active products. The chromatogram showed two main active peaks as for the unhydrolyzed urine. However, the relation between the active bands Nos. I and II (Fig. 3) was changed. In the chromatogram of the hydrolyzed urine the second active

* The isotope and titration curves do not coincide exactly in the chromatograms of this investigation (see Figs. 5—8). The ^{14}C -labelled dicarboxylic acids seem to be somewhat less polar than the corresponding unlabelled compounds. Thus the ^{14}C -labelled acids are inclined to come out a little more slowly from the 9 g columns with the reversed phase systems B and C than does the unlabelled material (Figs. 6—8). In the ordinary partition chromatographic system (D) the 2-methyl[1- ^{14}C]succinic acid tends to move more rapidly than the added unlabelled carrier (Fig. 5). These findings are in good agreement with the recent reports by Piez and Engle^{19,20} on the ^{14}C -effect in amino acids, as demonstrated on the ion-exchange resin Dowex 50. An isotope effect has earlier been noticed for tritium-labelled organic acids with partition chromatography²¹.

band contained 7 520 cpm (0.42 % of the absorbed amount) while 4 720 cpm (0.26 %) were found in the first band.

The first day urine from Rat No. 12 was extracted continuously with ether. The extract containing 51 000 cpm, *i. e.*, 1.9 % of the absorbed activity, was chromatographed on a 9 g column with phase system C. The two main peaks (23 000 cpm each) appeared as in Fig. 3, but in addition there was a front peak (4 000 cpm). This active material, which we have not yet identified, was found to be present also in hydrolyzed urine. After the continuous ether extraction of the first day urine, 5 600 cpm remained in the water phase. After alkaline hydrolysis, however, one half of this activity could be extracted with ether.

When the first band of the unhydrolyzed urine was rechromatographed according to system D, it was found to be heterogeneous, as it was split into three active peaks (Fig. 4). After hydrolysis, however, only two active bands remained (*cf.* Fig. 5). One of them (I) was found at the position of the titration peak of added, unlabelled 2-methylsuccinic acid (5.3 mg), the other one (II) was moving with the front.

When the material of this and a parallel chromatogram was rechromatographed with the reversed phase system C together with 2.4 mg of synthetic unlabelled 2-methyladipic acid, the titration peaks of 2-methylsuccinic acid (I) and 2-methyladipic acid (II) were found at the same positions as the two main active peaks (Fig. 6).

The ether extract of the hydrolyzed urine from Rat No. 10 was chromatographed on a 9 g column with phase system C together with 4 mg of unlabelled 2-methyladipic acid. The titration peak of this acid corresponded to the active peak II, (Fig. 7). Also in a chromatogram with phase system B the titration curve of synthetic 2-methyladipic acid (2.9 mg) was found at the same position as the active band II from the urine of Rat No. 7 (Fig. 8).

The ether extracts of the hydrolyzed urine from Rats No. 8 and 9 were chromatographed separately according to system B. The material from the second active bands (*cf.* Fig. 3) was rechromatographed together with 19.8 mg of inactive 2-methyladipic acid on a 9 g column of hydrophobic kieselguhr with phase system C. After addition of 132.7 mg of inactive synthetic 2-methyladipic acid, the material of the single active band was crystallized twice from water and twice from benzene-light petroleum alternately (Table 5). The specific activity was shown to remain constant. Consequently one of the metabolites of 2-methylstearic acid was 2-methyladipic acid.

Analogously the first active bands from two chromatograms with phase system C were rechromatographed according to system D before crystallization together with unlabelled 2-methylsuccinic acid (125.0 mg). The specific activity remained constant during four crystallizations from hydrochloric acid and from ether-benzene alternately

Table 5. Identification of 2-methyladipic acid in the urine of rats fed 2-methylstearic acid. Specific activity of material from the active band II of chromatogram of urinary extracts with phase system B, after rechromatography in system C recrystallized with inactive 2-methyladipic acid.

Recrystallization from	Crystal weight, mg	Counts/min/mg
water	101.2	390
benzene-light petroleum	76.0	375
water	50.3	355
benzene-light petroleum	35.4	361

Table 6. Identification of 2-methylsuccinic acid in the urine of rats fed 2-methylstearic acid. Specific activity of material from the active band I of chromatograms of urinary extracts with phase system C, after rechromatography in system D recrystallized with inactive 2-methylsuccinic acid.

Recrystallization from	Crystal weight, mg	Counts/min/mg
hydrochloric acid	98.4	154
ethyl ether-benzene	60.0	145
hydrochloric acid	41.0	150
ethyl ether-benzene	20.3	150

(Table 6). Thus 2-methylsuccinic acid was another urinary metabolite of 2-methylstearic acid. The amounts of activity which were extracted from the urine of the different rats and chromatographically identified as 2-methylsuccinic acid or 2-methyladipic acid are given in Table 4 in per cent of the absorbed activity.

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