4. The results indicate clearly that there is no detectable difference between the routes followed during absorption of fed free oleic or palmitic acids, or of glycerides containing these acids.

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Intestinal Absorption and Metabolism of 2:2-Dimethylstearic Acid in the Rat

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The intestinal absorption of different straight-chain carboxylic acids has been extensively studied during recent years (for a summary see Bergström & Borgström, 1954).

In order to investigate the influence of steric hindrance at the carboxyl group we have prepared [*carboxy-¹⁴C]2:2-dimethylstearic acid and studied its absorption and metabolism in the rat.*

EXPERIMENTAL AND RESULTS

A survey of the methods previously used for the preparation of 2:2-dimethylstearic acid is given by Cason, Sumrell & Mitchell (1950). In the present work it has been prepared by the following reactions.

2:2-Dimethylsuccinic acid. 2:2-Dimethylsuccinic acid was prepared by a slight modification of the method of Vogel (1928). Solutions of ethyl 2-cyano-3:3-dimethylacrylate (20 g.) in ethanol (100 ml.) and KCN (11 g.) in water (25 ml.) were mixed and kept at room temperature for 24 hr. The ethanol was removed by distillation. After cooling, a very large excess of concentrated HCl was added and the solution refluxed for 3 hr. After dilution with water and addition of $(\text{NH}_4)_2\text{SO}_4$, it was extracted five times with ether. The ether solution was dried with Na_2SO_4 and evaporated to dryness. Impurities were removed by boiling the product twice with small amounts of benzene, cooling to room temperature and collecting the crystals by filtration. Yield 13.2 g. (69%); m.p. 142°. (Found: equiv. 73.1. Calc. for $\text{C}_8\text{H}_{10}\text{O}_4$, 73.1.)

2:2-Dimethyl [1- ^{14}C]succinic acid was prepared analogously with [^{14}C]cyanide. One-twentieth of the above quantities was used, except that the ethyl 2-cyano-3:3-dimethylacrylate was here in slight excess. The yield was diminished to 63%, and the melting point of the labelled product was 143°.

Methyl hydrogen 2:2-dimethylsuccinate (3-methoxycarbonyl-3-methylbutyric acid) (cf. Bone, Sudborough & Sprankling, 1904). 2:2-Dimethylsuccinic acid (11.0 g.) was dissolved in methanol and treated with diazomethane in ether solution. The solvents were removed by distillation. The diester formed was converted into the half-ester

by hydrolysis with 1.67N methanolic KOH solution (45.1 ml.) and water (5 ml.). The alkaline solution was kept at room temperature overnight and was then refluxed for 3–4 hr. After addition of water and acidification with HCl, the ester was extracted with ether. The ether solution was dried with Na_2SO_4 . After evaporation of the ether the product was dried *in vacuo*. The yield of crude product was 11.5 g.

Methyl hydrogen 2:2-dimethyl [1- ^{14}C]succinate was prepared analogously.

2:2-Dimethylstearic acid. Methyl hydrogen 2:2-dimethylsuccinate (3-carbomethoxy-3-methylbutyric acid) (5.7 g. of crude product), palmitic acid (20 g.) and sodium (0.45 g.) were dissolved in methanol (300 ml.) and electrolysed (Pt electrodes) with a current density of about 0.3 A/cm.² (cf. Stållberg-Stenhagen, 1950, 1951). During the electrolysis external cooling with ice was necessary and also addition of methanol to replace loss due to evaporation. When the reaction was alkaline to litmus, another 5.7 g. of methyl ester and 20 g. of palmitic acid were added, and the electrolysis was continued until the reaction was slightly alkaline. After cooling to room temperature, the triacontane which had precipitated was filtered off, extracted once with boiling methanol and again filtered after cooling.

The combined methanol solutions were evaporated to dryness and the solid residue was extracted with ether 3 times. The excess palmitic acid was removed by washing the ether solution with portions of Na_2CO_3 solution until no more palmitate precipitated. The precipitate was removed by filtration, and the solution was washed with water until the reaction was neutral. After evaporation of the ether the product was hydrolysed by heating it with KOH (25 g.), water (10 ml.) and ethanol (170 ml.) for 6 hr. at 120° in an autoclave. The hydrolysate was diluted with water (150 ml.) and extracted twice with petroleum ether. The water-ethanol layer was acidified with dil. HCl and extracted with ether. The ether solution was dried with Na_2SO_4 . Evaporation to dryness yielded 2 g. of crude acid. Another 7 g. of acid were obtained by extraction of the light petroleum solution with 50% (w/v) ethanol, acidification of the

ethanol-water solution and extraction with ether. After crystallization from methanol of the combined acid products, 6.8 g. of 2:2-dimethylstearic acid were obtained (29% of the theoretical amount, calculated on 2:2-dimethylsuccinic acid) m.p. 57°. After recrystallization from ligroin the m.p. was 58.5°. (Found: C, 76.8; H, 12.8. Calc. for $\text{C}_{20}\text{H}_{40}\text{O}_2$: C, 76.9; H, 12.9.)

2:2-Dimethyl [1- ^{14}C]stearic acid was prepared analogously on a small scale.

Preparation of 2:2-dimethyladipic acid. Sodium (0.4 g.) was dissolved in methanol (300 ml.), and methyl hydrogen 2:2-dimethylsuccinate (3.6 g., 0.0225 mole) and methyl hydrogen succinate (12 g., 0.09 mole) were added. The electrolysis was carried out as described above. The reaction mixture was taken to dryness and extracted with several portions of boiling ether.

The combined ether extract was washed with a 5% (w/v) solution of Na_2CO_3 and with water. The ether was distilled off and the residue (5.7 g.) was partially hydrolysed by means of 1.67N methanolic KOH (82 ml.) and water (4.5 ml.) at room temperature overnight. After hydrolysis, water (100 ml.) was added and the alkaline solution was extracted twice with ether to remove methyl tetramethyladipate. The solution was acidified with HCl and extracted 5 times with ether. The combined ether fractions were then dried with Na_2SO_4 and the ether was removed. The residue (4.3 g.) was partitioned between equal volumes (80 ml.) of CHCl_3 and water. The CHCl_3 phase was evaporated to dryness and the residue (1.2 g.) was hydrolysed in an autoclave at 110° for 5 hr. with KOH (5 g.) in water (2 ml.) and ethanol (45 ml.). After saponification the solution was acidified and extracted 3 times with ether. Crystallization of the crude acid from water yielded 520 mg. of 2:2-dimethyladipic acid, m.p. 81°. (Found: C, 55.1; H, 8.1. Calc. for $\text{C}_8\text{H}_{14}\text{O}_4$: C, 55.2; H, 8.1.)

Administration of 2:2-dimethyl [1- ^{14}C]stearic acid to rats. The animals used were adult white male rats weighing about 250 g. and were kept in metabolism cages during the experiments. White bread and water were fed *ad lib*. For further experimental details see the preceding paper (Bergström, Blomstrand & Borgström, 1954). For these experiments a stock solution of 103.5 mg. of 2:2-dimethyl [1- ^{14}C]stearic acid in 5.1 g. of olive oil was used.

Measurement of radioactivity

The isotope content of the synthetic compounds was determined with thin end-window Geiger-Müller tubes after evaporating samples of an ethanolic solution on aluminum planchets to leave less than 0.2 mg./cm.² The urine and faecal extracts were also counted after evaporation on planchets. The self absorption was determined by evaporating samples to which known amounts of highly active dimethyladipic or dimethylstearic acid respectively

had been added. Expired CO_2 was absorbed in alkali, precipitated as BaCO_3 and counted in 'infinite' thickness.

A. Recovery of [^{14}C] in excreta and expired carbon dioxide after administration of [^{14}C]dimethylstearic acid

A rat was fed 0.65 ml. of the stock solution (532 000 counts/min.) by gastric intubation under light ether anaesthesia. It was placed in a 4 l. desiccator at 25–28° through which a constant stream of CO_2 -free air was passed at a constant rate of about 500 ml./min. The CO_2 in the expired air of the rat was collected in NaOH solution and the amount of CO_2 determined by titration of a sample. To another sample NH_4Cl and BaCl_2 were added. The precipitate of BaCO_3 was collected and the isotope content determined on infinitely thick samples. However, the activity was so small that it could not be determined with this method.

The urine was collected on the bottom of the desiccator. From the first 24 hr. the total urine activity was 368 000 counts/min., i.e. 69 % of the amount administered. The urine from 24–72 hr. after administration contained 42 500 counts/min., i.e. 8 % of the given amount. Consequently, a total of 77 % was recovered in the urine.

Faeces were collected on a metal net in the desiccator. After collection, the faeces from the first day were digested in 30 % (w/v) KOH on a steam bath for 12 hr. After acidification with HCl they were extracted with ether.

The total activity was 48 000 counts/min., i.e. 9 % of the administered activity. During the second and the third day 5 and 3 % respectively was recovered, i.e. a total of 17 % was found in the faeces.

B. Administration of [^{14}C]dimethylstearic acid to a rat with a lymph fistula

A rat with a cannula in the thoracic duct was fed 0.5 ml. of the olive oil–dimethylstearic acid mixture (410 000 counts/min.) through a stomach tube. It was placed in a special cage similar to that described by Bollman, Cain & Grindlay (1948), and the lymph was collected for 24 hr. The total lymph fat was separated on a column of silicic acid (Borgström, 1952*a*). The lipids eluted with chloroform (416 mg.) contained a total of 147 000 counts/min. The phospholipids were then eluted with methanol and had a total activity of 5500 counts/min. (3.6 % of the total fat). The chloroform eluate was passed through the ion-exchanger Amberlite IRA-400 as described by Borgström (1952*b*) to ensure that no trace of free fatty acids was present. The neutral fat (390 mg.) had a total activity of 127 500 counts/min., i.e. 87 % of the total lymph-fat activity or 31 % of the total amount administered.

Hydrolysis of lymph fat with bile-pancreatic juice. A sample (120 mg.) of neutral fat from the above-mentioned fraction was hydrolysed with 5 ml. rat bile-pancreatic juice according to the procedure described by Borgström

(1952*c*). The pH was adjusted to 6.3, the reaction temperature was 40° and analyses were made after 1, 3, 6, 12 and 24 hr. The results are shown in Table 1.

Hydrolysis of olive oil containing free [^{14}C]dimethylstearic acid with bile-pancreatic juice. When the above experiment was repeated with unlabelled olive oil in which 2 % of free [^{14}C]dimethylstearic acid had been dissolved, the results shown in Table 2 were obtained. The activity of the mixture was 900 counts/min./mg.

The data in the last column should be compared with corresponding experiments in which free labelled palmitic or oleic acid had been added (Borgström, 1954). In these experiments the glyceride fatty acids had reached about 40 % of the activity of the free fatty acids already after 2 hr. incubation.

C. Identification of the main isotopic compound in urine after administration of [^{14}C]dimethylstearic acid

In an experiment similar to A, 1 ml. of the solution (820 000 counts/min.) was fed to a rat. The urine was collected for 48 hr. after the administration. The activity was 517 000 counts/min., i.e. 63 % of the given amount. The acidified urine was extracted 3 times with 3 vol. of ether. The total activity of the ether phase was 350 000 counts/min. The total activity of the aqueous phase after ether extraction was 134 000 counts/min. Consequently, 72 % of the activity was in the ether phase.

Table 1. Distribution of radioactivity during enzymic hydrolysis of lymph fat

For details see text.

Time (hr.)	Amount of hydrolysis (%)	Radioactivity (counts/min./mg.)	
		Neutral fat	Acids
1	22.4	424	—
3	35.8	463	16
6	53.0	616	26
12	72.2	818	20
24	86.2	1502	30

Table 2. Distribution of radioactivity during enzymic hydrolysis of olive oil containing free [^{14}C]dimethylstearic acid

For details see text.

Time (hr.)	Amount of hydrolysis (%)	Radioactivity of glyceride fatty acids (counts/ min./mg.)	Activity of glyceride fatty acids as a percentage of the activity of free fatty acids
1	18.2	3	0.2
2	32.6	9	0.9
4	43.4	12	1.1
6	52.8	16	1.8

Hydrolysis of the aqueous phase. After alkaline hydrolysis 45% of the remaining activity could be extracted with 3 vol. of ether. This indicates that the urine compound was partly in conjugated form.

Chromatography of the ether extract of the urine. About 4% of the residue of this ether extract was mixed with 2:2-dimethylsuccinic acid (7.9 mg.) and 2:2-dimethyladipic acid (6.3 mg.) and subjected to reversed-phase partition chromatography according to the method of Howard & Martin (1950), with phases similar to those used in the separation of bile acids by Bergström & Sjövall (1951) and Norman (1953). Stationary phase: 50% (v/v) isooctanol-chloroform, moving phase: 40% (v/v) aqueous methanol. 4.5 g. of hydrophobic kieselguhr (Hyflo Supercel treated with silane) and 4 ml. stationary phase were used. The rate of flow was regulated to 2.5 ml./5 min. Each fraction was titrated with 0.02N-NaOH (bromthymol blue) and then evaporated to dryness in an oven at 105°. The contents of each tube were dissolved in 50% (v/v) ethanol and transferred to copper planchets. From some tubes samples were taken so that the weight of substance did not exceed 0.5 mg. on each planchet.

The results are shown in Fig. 1. Almost 90% of the activity was contained in the second band which corresponds to dimethyladipic acid.

Recrystallization of 97.8 mg. of 2:2-dimethyladipic acid and about 18% of the ether extract of the urine was performed with water and benzene-light petroleum (b.p. 40–60°) alternately. The specific activity of the crystals stayed constant during six recrystallizations from water and benzene-light petroleum.

Fig. 1. Partition chromatography of urinary extract from experiment C together with 2:2-dimethylsuccinic acid (7.9 mg.) and 2:2-dimethyladipic acid (6.3 mg.); stationary phase: isooctanol- CHCl_3 (1:1, v/v) supported on 4.5 g. hydrophobic Supercel. Moving phase: 40% (v/v) aqueous methanol. Titration values, solid line; counts/fraction, dotted line.

DISCUSSION

The results presented indicate that 2:2-dimethylstearic acid was well absorbed from the intestine when fed in free form dissolved in olive oil. The extent of transport via the lymphatic pathway appears from the limited data to be approximately the same as for the ordinary long-chain fatty acids with the same technique and in the same strain of animals (cf. Bergström *et al.* 1954; Blomstrand, 1954). The labelled acid that had been administered in free form was transported via the lymphatics incorporated into glycerides and phospholipids as is the case with the ordinary straight-chain fatty acids.

However, the α -disubstituted acid showed a different behaviour with pancreatic lipase. When a labelled straight-chain fatty acid was present when glycerides were split by pancreatic lipase it was found by Bergström (1952c, 1954) that a rapid incorporation of the labelled fatty acid into glyceride ester bonds occurred simultaneously with the overall hydrolysis. This has been demonstrated *in vitro* as well as *in vivo* in the intestinal contents of rats. In this work it has been found that the 2:2-dimethyl-substituted acid only became incorporated in traces under comparable experimental condition. The reason might be ascribed to the steric influence of the substituents at the α -carbon atom that also causes the well-known effect on the rate of esterification and hydrolysis of acids and esters of this type. The substituents might possibly also hinder an appropriate close packing of the acid with the glycerides and straight-chain fatty acid at the interface where the lipase is acting.

Glyceride ester bonds of the branched chain acid were also found to be almost resistant to the action of pancreatic lipase. When the lymph triglycerides containing the labelled acid were subjected to the action of rat bile-pancreatic juice the usual rapid hydrolysis occurred. However, almost all of the labelled branched-chain fatty acid remained bound in the glyceride residue, indicating that a glyceride ester bond of the substituted acid was virtually resistant to the action of pancreatic lipase. It appears likely that the steric effect of the two methyl groups close to the ester bond hinders the appropriate contact between the lipase molecule and the substrate that is necessary for hydrolysis to occur.

The fact that the substituted acid had been incorporated into glyceride ester bonds during the passage through the intestinal cells shows that the synthesis of ester bonds which is taking place in the intestinal cells as expected is not a simple reversal of the lipase mechanism. Presumably this synthesis is taking place via acyl transfer from energy-rich S-acyl compounds, by analogy with the mechanism shown to occur in liver when phosphatidic acid is acylated (Kornberg & Pricer, 1953).

The results show that most of the absorbed dimethylstearic acid that had been transported via the lymphatics incorporated into neutral fat and phospholipids had thus been rapidly set free and metabolized in the body of the rat. The disubstitution at the α -carbon atom hinders the usual β -oxidation completely and the molecule is instead ω -oxidized. The dicarboxylic acid had then been β -oxidized from the unsubstituted end 6 times leaving the substituted adipic acid with six carbon atoms and this was rapidly excreted in the urine.

These results are in general accordance with earlier extensive work on the metabolism of fatty acids with branched chains. For a summary see Weitzel (1950, 1951). Kuhn, Köhler & Köhler (1936) found that α -methylstearic acid was completely metabolized in the rabbit without any products being found in the urine. As α -methyladipic acid itself when administered appears in the urine to 60–70 %, these experiments indicate that β -oxidation from the carboxyl end had occurred to a considerable extent. Thus, monosubstitution at the α -carbon atom does not appear to hinder β -oxidation but when the substituent becomes larger (ethyl < propyl < butyl) more acid degradation products are excreted with the urine mainly as the corresponding substituted adipic acids. After feeding α -n-butylstearic acid to dogs, about 6.5 moles per cent could be recovered in the urine as α -butyladipic acid (Weitzel, 1951).

The experiments recorded in this work show that two methyl groups at the α -carbon completely hinder any β -oxidation from this end of the molecule. The recovery of 90 % of the absorbed isotope as [^{14}C]dimethyladipic acid also shows that a rapid and complete ω -oxidation followed by β -oxidation had taken place.

SUMMARY

1. [*carboxy*- ^{14}C]2:2-Dimethylstearic acid and 2:2-dimethyladipic acid have been synthesized.
2. The former acid was well absorbed when dissolved in olive oil and administered to rats.
3. The absorbed acid was transported via the lymphatics incorporated into glycerides and phospholipids.

4. No isotope was found in the expired carbon dioxide, but more than 90 % of the absorbed isotope was recovered in the urine as [^{14}C]dimethyladipic acid.

5. Dimethylstearic acid did not take part in the formation of ester bonds occurring during hydrolysis of olive oil with pancreatic lipase. When the lymph glyceride containing this acid was treated with bile-pancreatic juice, this ester bond appeared virtually resistant to the action of lipase.

6. Other types of enzymes which apparently are not hindered by the two α -methyl groups affect the synthesis of glyceride ester bonds in the intestinal cells and the splitting of these bonds in the tissues of the rat.

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