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The question of biohydrogenation of fatty acids*

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SUMMARY

Rats were fed methyl palmitoleate-1-C^{14} and the palmitic acid was isolated from their organs and depot fat. Degradation studies revealed that very little activity found its way into the palmitic acid by hydrogenation of the palmitoleate. The major route of carbon from the unsaturated to the saturated acids is through acetate.

In 1937, Rittenberg and Schoenheimer (1) carried out an experiment designed to assess the amount of hydrogenation of unsaturated fatty acids occurring in mammalian tissues. Unsaturated fatty acids labeled with deuterium were obtained from mice that had been fed highly labeled saturated acids. The ethyl esters of these acids were fed to two mice, and the concentration of deuterium in their saturated and unsaturated fatty acids and body water were determined. It was found in both cases that the per cent excess deuterium in the saturated acids (0.047 and 0.025) was about 20 per cent of that in the unsaturated acids (0.25 and 0.10) and about equal to that in the body water. The conclusion drawn was that since previous experiments had shown that the saturated fatty acids synthesized in the presence of deuterium-enriched water ultimately achieve a deuterium content one-quarter to one-third that of body water, the excess deuterium in the saturated acids in this experiment could have been derived only by hydrogenation of the body unsaturated acids. This conclusion may not have been justified since the fed unsaturated fatty acids, which had an excess deuterium content of 1.02 atom per cent, were undoubtedly partially broken down following absorption and prior to complete equilibration with the total body unsaturated acids. There was thus a richer source of deuterium-containing substrate available for synthesis of the saturated fatty acids.

There has been some indirect evidence, based on analysis of animal depot and tissue fats, that hydrogenation of unsaturated fatty acids may occur (2 to 5). It is possible, however, to explain these data as well on other bases.

Recently considerable evidence has accumulated indicating that in the mammal fatty acid hydrogenation is not an important reaction. Steinberg *et al.* (6) fed carboxy-labeled linolenic acid to rats and isolated the linoleic acid from their depot and organ fats. Although the fed acid had an activity of 2.16×10^7 disintegrations per second per mmole, that of the linoleic acid was only 92, an activity which might be expected from the small contribution by partial equilibration of the first two carbons with the active acetate derived by oxidation of the linolenic acid. Mead and Howton (7), in a similar study with γ-linolenic acid, found that although the activity of the fed acid was 3.84×10^7 disintegrations per second per mmole, that of the isolated linoleic acid was only 176, while that of oleic acid was only 141, both of these activities being considerably smaller than those of the saturated acids. There is thus little evidence that hydrogenation in this sense (conversion of polyunsaturated to di- and monounsaturated acids) occurs.

Bernhard *et al.* (8) investigated this problem by feeding randomly labeled oleic, linoleic, and linolenic acids to rats and comparing the activities of the saturated acids isolated from their liver and carcass fats. In each case the activity of the stearic acid was about the same as that of the palmitic, and somewhat less than that of the cholesterol isolated from the same source. The authors reasoned that if appreciable hy-

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drogenation had occurred, the stearic acid should be considerably more active than the palmitic, which must obtain its activity largely by total synthesis. The cholesterol activity was taken as a measure of the acetate pool, from which the palmitate would be synthesized. However, these experiments were not designed to detect a low order of hydrogenation and consequently could not prove or disprove its existence. Moreover, the use of the cholesterol activity as a measure of the activity of the acetate available for fatty acid synthesis may not have been justified.

It was thus evident that there was a need for an experiment which would give a definite answer to this question. Such an answer could be obtained by the use of carboxy-labeled palmitoleic acid, since hydrogenation of this acid in the animal body would give palmitic acid with an excess of activity in the carboxy group. It has been shown in several laboratories (9, 10, 11) that in palmitic acid synthesized from acetate-1-C^{14} by well-nourished animals, the activity in all the odd-carbon atoms (including the carboxyl) is approximately equal. If the palmitic acid derived from such an experiment contained an excess of activity on the carboxy group, hydrogenation would have been demonstrated and its quantitative significance could be assessed.

Consequently, palmitoleic acid was isolated from macadamia nuts, which contain approximately 20 per cent of this acid. This was converted to the carboxy-labeled acid by known methods and was fed to normal rats. The results of these experiments permit the evaluation of the hydrogenation process in these animals under the stated conditions.

METHODS

All melting points are corrected. Gas chromatography was performed with either (*a*) an Aerograph Model A-100-C (Wilkens Instrument and Research Co., Inc.), using a 10-foot, ¼-inch column for analytical results or a 5-foot, ½-inch column for the preparative experiments, both packed with Craig Polyester-succinate (1,4-butanediol-succinate polymer); or (*b*) a Wheelco Model 10 (Barber-Colman Company) with a 6-foot, 6-mm. (I.D.) column of 80 to 100 mesh chromosorb siliconized by treatment with dimethyldichlorosilane (12). Reported *trans*-contents of olefinic substances, based on infrared absorption at 10.3 μ., are considered to be within 5 per cent of actual values.

Ultimate analyses were performed by Dr. A. Elek (Elek Micro Analytical Laboratories, Los Angeles) and the infrared absorption analyses by Mr. G. V. Alexander and Mr. Paul Kratz of this laboratory.

Preparation of Palmitoleic Acid. Shelled macadamia nuts (500 g.)[1] were alternately ground in a mortar and extracted with pentane, evaporation of which gave 376 g. (75 per cent) of a light yellow oil. This was saponified with 2 l. of 8 per cent alocholic KOH under nitrogen and the fatty acids (351 g.) were isolated in the usual manner and converted to the methyl esters. A sample analyzed on the gas chromatograph revealed a composition closely approximating that found in the literature (13). The remainder was fractionally distilled through a 2.5 x 100 cm. heated Vigreaux column at 0.9 mm. pressure and 135°-145°C. Three 50-ml. fractions were collected. The first showed approximately 87 per cent palmitoleate with 6 and 7 per cent, respectively, of myristate and a C_{18} acid and a trace of palmitate. Crystallization of this fraction (43.2 g.) from 2.5 l. of methanol at —60°C gave 12.6 g. of a soluble fraction containing approximately 93 per cent methyl palmitoleate and no detectable amounts of saturated esters. This material was saponified with 9 per cent KOH in methanol-water at room temperature overnight.

A sample purified further by distillation at 80 μ. and a bath temperature of 165°-170°C gave material with m.p. —0.9° to + 0.3°C and $n_D^{25.0}$ 1.4565. These values are in excellent agreement with the reported m.p. of 0°-0.5°C (14) and a linear plot of refractive index versus temperature made from published data (14, 15). The *p*-phenylphenacyl ester melted at 55.7°-57.1°C (56.5°-57°C [14]).[2]

threo-9,10-Dibromohexadecanoic Acid. Ten g. of palmitoleic acid in CCl_4 was brominated at 0°C as detailed for oleic acid (17) to give (without a low temperature crystallization) a quantitative yield of the dibromo acid as a viscous brownish oil. A sample of this crude material was purified by chromatography on a silicic acid column to obtain a colorless viscous oil of $n_D^{25.0}$ 1.4937. The *p*-phenylphenacyl ester was obtained after silicic acid chromatography and five crystallizations from 95 per cent ethanol as clusters of very thin colorless blades melting at 54.5°-55.1°C. Analysis calculated for $C_{30}H_{40}Br_2O_3$: C, 59.2; H, 6.6; Br, 26.3 per cent. Found: C, 59.0; H, 6.8; Br, 26.2 per cent.

threo-1,8,9-Tribromopentadecane. From 11.55 g.

[1] The macadamia nuts were obtained as a generous gift through R. H. Purdy, of the Pacific Vegetable Oil Co., Richmond, Calif.

[2] Baudart (16) prepared the *p*-bromophenacyl ester, m.p. 40°-40.5°C, which Boughton *et al.* (14) mistakenly compared with their *p*-phenylphenacyl ester.

(0.0237 mole) of silver dibromohexadecanoate (prepared in 93.3 per cent yield from the acid) reaction with bromine in dry CCl_4 as described previously (17) produced 5.35 g. (0.0119 mole or 50.2 per cent) tribromopentadecane; 0.75 g. (0.00107 mole or 9.0 per cent) presumably of 8,9-dibromopentadecyl 9,10-dibromohexadecanoate, the Simonini-type ester expected as a by-product of the Borodin reaction (it was not further characterized); 2.52 g. (0.00609 mole or 25.7 per cent) of recovered dibromohexadecanoic acid; and 0.80 g. of unidentified material, together accounting for 95 per cent of the starting dibromohexadecanoate.

Rechromatography of the crude tribromopentadecane on silicic acid produced a colorless, viscous oil of $n_D^{28.5}$ 1.5076 and $d^{28.5}$ 1.4025 g. per ml. Assay of this material by gas chromatography indicated that it contained only 70 to 80 per cent of tribromopentadecane.

cis-1-Bromo-8-Pentadecene. This compound was prepared from 4.49 g. (0.0100 mole) of once-chromatographed 1,8,9-tribromopentadecane in benzene by reaction with 4.0 g. (2.0061 mole) granular zinc preactivated with HBr under absolute ethanol; after refluxing for a total of 35 minutes, the mixture was worked up as described elsewhere (17) to give, after a preliminary distillation, 2.34 g. (81.0 per cent yield) of a colorless oil. By gas chromatography, this material was 93 per cent bromopentadecene. Using gas chromatography on a preparative scale, followed by silicic acid chromatography to remove any volatile products from the stationary liquid phase and vacuum distillation to remove solvent, there were obtained 127 mg. of a colorless mobile oil of $n_D^{30.0}$ 1.4676 and $d^{30.0}$ 1.0148 g. per ml. The molar refraction calculated from these constants was 79.19 and may be compared with a theoretical value of 79.766. Analysis by gas chromatography showed this product to be better than 99 per cent bromopentadecene, with a small amount of lower homologue and a trace of what is probably the saturated bromopentadecane. The *trans* content was estimated to be 5.0 per cent. Analysis calculated for $C_{15}H_{29}Br$: C, 62.3; H, 10.1; Br, 27.6 per cent. Found: C, 62.2; H, 10.0; Br, 27.5 per cent.

The sample of pentadecenyl bromide used in the next step contained, by gas chromatography, 95 per cent pentadecenyl bromide and approximately 5 per cent of a 17-carbon bromide; no saturated 15-carbon bromide was detectable. The *trans* content was 9.8 per cent.

Methyl Palmitoleate-1-C^{14}. The preparation and carbonation of the Grignard reagent from 0.856 g. (2.96 mmole) of pentadecenyl bromide and 3.10 mmole of carbon dioxide containing 1.927 mc. C^{14} were carried out as described previously for heptadecenyl bromide (17). The 0.2207 g. (0.822 mmole or 27.7 per cent from pentadecenyl bromide) of methyl palmitoleate thus obtained was shown by gas chromatographic analysis to be 97 per cent pure, with 3 per cent C_{18} ester and no trace of palmitate. In the second of the two feeding experiments performed (see below), however, the palmitoleic acid was purified further by reversed phase chromatography with removal of the portion of the fraction which might contain any palmitic acid impurity. The activity of the methyl palmitoleate was 1.04×10^5 d.p.s. per mg.[3] or 1.09×10^5 d.p.s. per mg. for the free acid. For the first feeding experiment, 111 mg. of this acid was diluted to 804 mg. with inactive methyl palmitoleate.

Animal Experiments. Two experiments were performed using slightly different conditions. In experiment I, 755 mg. of the diluted methyl palmitoleate (1.1×10^7 d.p.s. total) was administered orally to three nonfasted male rats weighing a total of 786 g. on a normal commercial diet.

In experiment II, two male rats of similar weight were placed for 10 days on the same diet with additional 30 per cent of corn oil, before administration of 405 mg. of methyl palmitoleate (5.6×10^6 d.p.s. total). The rationale behind this treatment was, first, to inhibit fatty acid synthesis, thus proportionally increasing the possible effect of hydrogenation and, second, to provide an excess of unsaturated fat with the hope that a hydrogenation mechanism might be emphasized. An additional change involved the further purification of the fed palmitoleic acid, as described above.

In both experiments the rats were killed after 4 hours and the organs and depot fat were removed, frozen, and lyophilized. The 4-hour time was chosen since it is sufficient to allow complete absorption of the small amounts of oil administered and represents a compromise between maximum incorporation of activity into the various fatty acids and extensive catabolism to carbon dioxide. In experiment I, from 13.5 g. of dried tissues was obtained, following extraction with 4:1 methylal:methanol, 3.53 g. of fat with an activity of 63.6 d.p.s. per mg., saponification of which gave 2.61 g. of fatty acids with an activity of 85.0 d.p.s. per mg. Similarly, from experiment II was obtained

[3] Counting was performed with the single-channel room temperature liquid scintillation counter described by Hodgson *et al.* (18). At 1150 volts, sensitivity was 77.0 ± 0.2 per cent. An external standard was used for preliminary counts and an internal standard (40.7 d.p.s. of ring-labeled toluene) for all final counts to correct for self-absorption. Samples were counted in 3-dram vials dissolved in 5 ml. of toluene containing 5 g. phenylbiphenyloxadiazole and 0.10 g. 1,4-bis-2-(phenyloxazolyl)-benzene per liter.

20 g. of dried tissues from which was derived 8.8 g. of fatty acids with an activity of 5.4 d.p.s. per mg.

Preparation of Palmitic Acid. The total fatty acids from experiment I were crystallized from acetone at −30°C, giving 845 mg. of saturated acids with an activity of 20.0 d.p.s. per mg. A portion of the saturated acid mixture (331.5 mg.) was separated on the reversed phase column of Howard and Martin (19) as described previously (20), giving 233.5 mg. of palmitic acid, representing 82.5 per cent of the saturated acids and 26.5 per cent of the total fatty acids. Crystallization of this acid from acetonitrile gave 198.4 mg. of palmitic acid, which, when chromatographed as the methyl ester, showed no trace of any other substance. It had an activity of 8.42 d.p.s. per mg. palmitic acid.

Similarly, from experiment II was obtained 1.7 g. of saturated fatty acids with an activity of 0.32 d.p.s. per mg. and, following reversed phase chromatographic separation and crystallization of 319 mg. of this mixture, 233 mg. of pure palmitic acid with an activity of 0.35 d.p.s. per mg.

In an exactly similar manner, palmitic acid was derived from the saturated fatty acids of rats which had been fed carboxy-labeled γ-linolenic acid (7). This acid had an activity of 1.87 d.p.s. per mg. and served as a control for the palmitic acid derived from methyl palmitoleate.

Degradation of Palmitic Acid. Degradation was carried out by the method of Dauben *et al.* (10) with the modifications discussed previously (21). Only the carboxy-carbon, as benzoic acid, and the remaining pentadecanoic acid were counted.[4]

TABLE 1. ACTIVITIES OF FATTY ACIDS AND DEGRADATION PRODUCTS FROM METHYL PALMITOLEATE-1-C^{14}

Fraction	*d.p.s./mg.*	*d.p.s./mmole*
Palmitoleate Fed		
palmitoleic acid (prep'd)	1.09×10^5	2.78×10^7
Experiment I		
palmitoleic (fed)	1.44×10^4	3.66×10^6
total fatty acids	85.0	
saturated fatty acids	20.0	
palmitic acid	8.42	2158.9
benzoic acid (C_1 of palmitic acid)	6.08	742.4*
pentadecanoic acid (C_2 thru C_{16} of palmitic acid)	6.64	1621.8 (231.7)†
Experiment II		
palmitoleic acid (fed)	1.38×10^4	3.50×10^6
total fatty acids	5.42	
saturated fatty acids	0.32	
palmitic acid	0.22	56.9
benzoic acid (C_1 of palmitic acid)	0.066	9.0*
pentadecanoic acid (C_2 thru C_{16} of palmitic acid)	0.13	32.7 (4.7)†
γ-Linolenate Fed		
palmitic acid	1.87	479.5
benzoic acid (C_1 of palmitic acid)	0.58	71.1*
pentadecanoic acid (C_2 thru C_{16} of palmitic acid)	2.01	491.2 (70.2)†

* Since the total activity of the benzoic acid resides in the carboxy carbon, d.p.s./mmole = d.p.s./milliatom carbon.

† In parenthesis, d.p.s./milliatom active carbon.

Since palmitic acid formed from carboxy-labeled acetate has its activity in the odd carbons (even carbons for pentadecanoic acid) the activity per active carbon is $\frac{\text{d.p.s./mmole}}{7}$.

RESULTS AND DISCUSSION

In Table 1 are listed the activities of the various acids and their degradation products. It can be seen that for the palmitic acid formed by condensation of carboxy-labeled acetate derived from γ-linolenic acid, the ratio of the activity of carboxy carbon to the activity of average active carbon is 1.01. For the palmitoleic-fed rats, however, the ratio is 3.21 for experiment I and 1.92 for experiment II.

The activity of the palmitic acid isolated in these experiments is derived from several sources. The first is by direct hydrogenation of the fed palmitoleic acid. Since this activity would be in the carboxy group alone while that of the palmitic acid totally synthesized from the acetyl coenzyme A derived by degradation of the palmitoleic acid would be equally distributed in the odd carbons, its relative magnitude may be obtained by subtraction of eight times the activity per active carbon of the pentadecanoic acid from the activity of the whole (Table 1). In this manner it can be estimated that in experiment I, 21.5 per cent of the palmitic acid activity was derived by hydrogenation of palmitoleic acid while 78.5 per cent was the result of total synthesis. Similarly, from experiment II, 10.5 per cent of the activity was from hydrogenation and 89.5 per cent from total synthesis. Since in the γ-linolenate-fed animals, in which palmitic acid was totally synthesized from acetate, the carboxy carbon activity was equal to that of the average active carbon, this preliminary conclusion appears justified. However, it should be borne in mind that although both types of active palmitic acid (by hydrogenation or total synthesis) are equally diluted by inactive palmitic acid from other sources, the totally synthesized palmitic acid suffers a further dilution of a different sort. The acetyl coenzyme A derived by degradation of the palmitoleic acid is diluted by inactive coenzyme A from

[4] See footnote 3.

other sources and suffers losses to other synthetic processes. This dilution cannot be accurately measured since the pool of coenzyme A actually used for palmitic acid synthesis is probably different from that used for other acetylations. However, its result is to decrease the observed importance of the total synthesis pathway. Thus the estimates of its importance given below are probably minimal.

Although these figures seem to indicate that hydrogenation of the fed palmitoleic acid did indeed occur and that a definite fraction of the palmitic acid of the animal body was formed in this manner, they do not permit a direct calculation of the importance of this process in the general metabolism of fatty acids. This can be roughly estimated from the data obtained above with several approximations. Although the total fatty acids of the bodies of the rats were not isolated, it can be assumed that the sample used was representative of the whole. For experiment I, therefore, 140 g. of fat (18 per cent of body weight) (22) with 63.6 d.p.s. per mg. gives a total of 9×10^6 d.p.s. The same value is obtained if the calculation is based on total fatty acids and fatty acid activity. Therefore some 80 per cent of the total ingested activity is present in the total body fatty acids 4 hours after ingestion. Since in this experiment palmitic acid was found to be about 26 per cent of the total fatty acids, it may be estimated that 3×10^5 d.p.s. are present in the palmitic acid. If 21.5 per cent of this figure derives from hydrogenation, about 6.5×10^4 d.p.s. came from this reaction. Thus a maximum of about 0.6 per cent of the administered activity as palmitoleate appears in palmitate as the result of hydrogenation.

Whether this figure, taken at 4 hours, can be carried any further to give some idea of the total process on a continuous basis is doubtful. It may be instructive, however, to compare it with the conversion of γ-linolenic acid to arachidonic acid at the same time and under the same conditions. From the activities of the fed and isolated acids in this experiment (7), it can be estimated that 15 per cent of the fed γ-linolenate is present in arachidonic acid 4 hours after feeding. Thus it can be seen that in comparison with a process such as this (involving dehydrogenation and addition of acetate), the hydrogenation reaction is relatively minor.

Experiment II suffers from the very low activity recovered in the palmitic acid. It is evident that the dietary manipulations in this experiment were all too successful and that very little palmitic acid was synthesized from the acetate derived by degradation of the fed palmitoleic acid. It is equally evident that very little activity found its way into palmitic acid by any other route. The specific activity of the palmitic acid is very low and the excess activity in the carboxy group is even smaller than in experiment I. It is thus possible that some of the apparent evidence for hydrogenation in experiment I may have been the result of an immeasurably small but very active contamination of the fed palmitoleic acid by palmitic acid. Thus, for experiment II, the proportion of palmitic acid derived by hydrogenation of fed palmitoleate is considerably smaller than for experiment I, and a similar estimate of the amount of palmitic acid formed by hydrogenation of palmitoleate indicates a maximum of 0.01 per cent of the administered activity at 4 hours.

Although these studies serve to minimize the importance of biohydrogenation of unsaturated fatty acids as a major metabolic pathway, they do not disprove its existence. A definite portion of the active palmitic acid obtained from animals fed active palmitoleate was apparently derived by this route. This amount of hydrogenation would probably have escaped detection in the experiments of Bernhard *et al.* (8) and could have come to light only in experiments such as the above.

Another question not answered by the present experiments is whether the hydrogenation observed might have occurred in the intestine as a result of bacterial action. This will have to be answered before any further idea on the importance of biohydrogenation can be gained.

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