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Fatty Acid Synthesis by the Liver Perfused with Deuterated and Tritiated Water†

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ABSTRACT: Fatty acid synthesis has been studied in livers of rats perfused with 10% D_2O and with >90% D_2O. The number of deuterium atoms incorporated in 100% D_2O is 22.3 and 24.9 per molecule of newly synthesized palmitate and stearate respectively. The result for palmitate agrees with the result obtained by Jungas with rat adipose tissue under quite different conditions ((1968), *Biochemistry 10*, 3717). Mass spectrometric measurements of deuterium content were used to measure the rate of fatty acid synthesis. The results so obtained agree well with results obtained by measuring tritium incorporation from ^3H_2O. Mass spectrometric examination of fatty acids synthesized in the presence of high concentrations of D_2O provides direct information concerning the extents to which a fatty acid is formed by *de novo* synthesis and by chain elongation of other fatty acids. Stearate is synthesized at about 40% the rate of palmitate. Of the stearate formed about 97% is made by *de novo* synthesis. (An alternative interpretation of the last result is that the chain elongation mechanism uses only palmitate synthesized *de novo* and little or no preexisting palmitate.)

Deuterium oxide was the first labeled precursor to be used for demonstrating the *de novo* synthesis of fatty acids (Rittenberg and Schoenheimer, 1937; Waelsch *et al.*, 1940; Stetten and Boxer, 1944). It was shown that deuterium from D_2O is incorporated into long-chain fatty acids, but this finding was not developed into a quantitatively reliable method for measuring rates of fatty acid synthesis. This application has come into use only recently, except that the use of tritium oxide is now preferred to deuterium oxide (Clark *et al.*, 1968; Fain *et al.*, 1965; Jungas, 1968; Lowenstein, 1971; Windmueller and

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Spaeth, 1966, 1967). In the interim, most assessments of the rate of fatty acid synthesis *in vivo*, in perfused organs, and in tissue slices, were made by measuring the amount of [^{14}C]-acetate incorporated into long-chain fatty acids. The unreliability of this method has been discussed elsewhere (Lowenstein, 1972); suffice it to say here that in nonruminant mammals the pathway of fatty acid synthesis utilizes little or no free acetate, and that the size and the rate of turnover of the free acetate pool varies depending on the nutritional and hormonal status of the animal. Similar problems are encountered when [^{14}C]glucose is used as precursor. For example, in the perfused liver added glucose contributes a maximum of one-third of the carbon used for fatty acid synthesis. The fraction utilized depends on the concentration of added glucose and on other conditions (Brunengraber *et al.*, 1973). In adipose tissue the fraction utilized may be considerably greater (Jungas, 1968).

The use of hydrogen-labeled water does not suffer from such disadvantages. The conversion of carbohydrate precursors into fatty acids can be expected to yield a constant ratio of hydrogen incorporated to fatty acid synthesized. The conversion of certain other precursors, such as acetate and ethanol, into fatty acids can be expected to yield a somewhat different ratio of labeled hydrogen incorporated to fatty acid synthesized, but this can be allowed for by making appropriate corrections.

Several reasons have prompted the work reported here. In studies of the control mechanisms that affect the rate of fatty acid synthesis it is necessary to measure rates before and after imposing a change. The variability in the rate of fatty acid synthesis in individual animals kept on carefully controlled, schedule-fed diets is such that five or six animals must be used for each rate measurement. Similar considerations apply to liver perfusion experiments. However, each individual animal or preparation shows a reproducible percentage change in response to an identical change in conditions. Therefore if rates of fatty acid synthesis can be measured on the same animal or liver, before and after imposing a change, the number of experiments that needs to be performed is greatly reduced. The availability of D_2O and ^3H_2O makes possible two such consecutive measurements. The methodology for counting ^{3}H-labeled fatty acids is simple and straightforward. On the other hand the assay of the deuterium content of deuterium-labeled fatty acids, in the presence of large amounts of unlabeled fatty acids, has not been used on a routine basis. The present paper demonstrates that mass spectrometry of fatty acid methyl esters can be used for routine assays of deuterium content, and hence for determining the rate of fatty acid synthesis.

Methods

Liver perfusions were performed as described previously (Brunengraber *et al.*, 1973). Deuterium oxide was purchased from New England Nuclear Corp. Samples of perfusate were taken half way through the perfusion. The water was purified by sublimation *in vacuo*. Deuterium analyses of the water were performed by J. Nemeth, Urbana, Ill. 61801. At the end of a perfusion the liver was frozen rapidly by being pressed between blocks of aluminum which had been cooled in liquid nitrogen. The livers were stored under liquid nitrogen. The livers were powdered in the frozen state, and lipids were extracted with chloroform–methanol (2:1 v/v), saponified, and extracted into petroleum ether. A detailed description of these procedures is given elsewhere (Brunengraber *et al.*, 1973).

An aliquot of the petroleum ether extract containing the free fatty acids (20–25 mg of fatty acids) was evaporated to dryness under N_2 at 40°. The fatty acids were then esterified by heating at 60° under reflux for 4 hr with 25 ml of methanol containing 1% by volume concentrated sulfuric acid. The resulting solution was poured into an equal volume of water and the methyl esters were extracted with three 10-ml portions of petroleum ether (reagent grade, bp 40–60°) (Jamieson and Reid, 1965). The combined petroleum ether extracts were washed with an aqueous solution of sodium bicarbonate and finally with water. The petroleum ether extract containing the methyl esters was then dried over anhydrous sodium sulfate for a minimum of 1 hr. The petroleum ether layer was removed and evaporated under N_2 at 40°. The methyl esters of the fatty acids were dissolved in a small volume of petroleum ether and were stored under nitrogen.

The mixture of fatty acid methyl esters was separated on a Hewlett-Packard Model 720 gas chromatography apparatus, equipped with a thermal conductivity detector. Stainless steel columns (10 ft × $^3/_{16}$ in. i.d.), packed with 10% EGSS-X as the liquid phase on Gas-Chrom P (100–120 mesh) as the supporting phase, were obtained from Applied Science Laboratories. The columns were operated at 190–200° with helium gas at a flow rate of 40 cm^3/min and an inlet pressure of 18 psi. Identification of the peaks was achieved by comparing the retention time with that of standard fatty acid methyl esters.

Individual peaks, corresponding to the fatty acids 16:0 and 18:0 were collected in U-shaped Pyrex glass tubes which were attached directly to the outlet of the heated exit port of the thermal conductivity detector. Collection of the peaks was started a little before the peaks emerged from the column to avoid possible loss of polydeuterated peaks, which tend to elute ahead of nondeuterated peaks. However, under the operating conditions of our semipreparative columns, fractionation of deuterated from nondeuterated fatty acid methyl esters was not observed. (Such fractionation *was* observed on the more efficient analytical columns.) The collection tubes were packed loosely with Pyrex glass wool and were immersed in a mixture of Dry Ice and ethanol to facilitate sample collection. Recovery was 60–75%. The methyl esters were rinsed from the tubes with 15 ml of petroleum ether. The solvent was evaporated to dryness in a conical tube under N_2 at 30°. The residue was dissolved in a known amount of solvent and was divided into three equal parts (A–C).

Portion A was used for mass spectrometric analyses as described below. Portion B was used for radioactivity determinations at 12° in a Nuclear-Chicago liquid scintillation counter. The scintillating solution consisted of 0.4% 2,5-diphenyloxazole and 0.005% 1,4-bis[2-(5-phenyloxazolyl)]-benzene in toluene. Counting efficiences were determined by the use of appropriate internal standards. Portion C was used for the quantitative determination of fatty acid methyl esters. A known amount of internal standard (methyl pentadecanoate) was added to the sample, the contents were mixed, and an aliquot was analyzed on Packard Model 834 gas chromatography apparatus, equipped with a flame ionization detector. Glass columns (6 ft × $^1/_{16}$ in. (2 mm) i.d.) packed with 10% EGSS-X on Gas Chrom P (100–120 mesh) were operated at 190°. Nitrogen as used was a carrier gas at a flow rate of 40 cm^3/min and an inlet pressure of 25 psi. The area under each peak was determined gravimetrically. It was found to be directly proportional to the weight of the methyl esters represented by peak. The amount in each sample peak (x) was calculated by comparing the area of the sample peaks (A_x)

with the area of the internal standard (A_{st}) as follows: $x = (A_x/A_{st}) \times$ amount of internal standard. Specific radioactivity (dpm/μmol) was calculated by dividing total disintegrations per minute by the amount in micromoles in the peak.

Mass spectrometry was used to determine the deuterium content of the samples. The instrument was a Dupont 21-491 mass spectrometer equipped with a direct introduction probe. The accelerating voltage was 1500 V and the electron energy 70 eV. The mass spectra were recorded on a recording oscillograph (Bell and Howell Datagraph 5-134) equipped with three galvanometers with relative sensitivity ratios of 1:10:100. A 50-Hz response filter was inserted between the electron multiplier of the mass spectrometer and the recording oscillograph to smooth out the signal. The isotope ratios were measured from the recorded mass spectra with a 12-in. Gerber variable scale, Model TP 007100B (The Gerber Scientific Instrument Co., Hartford, Conn.).

For each sample the isotope ratios were measured on ten partial mass spectra and then averaged. An outline of the calculation is described in this paragraph for a sample of methyl palmitate. The same calculation was used for fatty acid methyl esters isolated from experiments in which 10 or 100% D_2O was used. Typical isotope ratios obtained for authentic, unlabeled methyl palmitate follow: *m/e* 270, relative intensity 100.0; 271, 19.3; 272, 2.40; 273 to 725 inclusive, $\leqslant 0.2$. The peaks from the unlabeled controls with *m/e* 273–275 were neglected in the calculations. For a perfusion experiment with 10% D_2O a typical average would be: *m/e* 270, relative intensity 100.0; 271, 24.6; 272, 7.25; 273, 3.00; 274, 1.27; 275, 0.57. From these relative intensities the distribution in mole per cent was calculated following an established procedure (Biemann, 1962). It afforded the following answer when the numerical values for the 10% D_2O perfusion given above were used in the calculation: 88.97 mol % unlabeled species, 4.71 mol % monodeuterated species, 3.41 mol % dideuterated species, 1.89 mol % trideuterated species, 0.69 mol % tetradeuterated species, and 0.33 mol % pentadeuterated species. The percentage deuterium in various molecules was calculated by dividing the actual number of deuterium atoms in a molecule by the number of deuterium atoms in the hypothetical, fully deuterated molecule, times 100, and amounts to 0% D for undeuterated species, 2.94% D for monodeuterated species, 5.88% D for dideuterated species, 8.82% D for trideuterated species, 11.8% D for tetradeuterated species, and 14.7% D for pentadeuterated species. The per cent deuterium content of the sample was obtained by multiplying the per cent deuterium in a species by the mole fraction of that species in the sample and summing the products obtained for all deuterated species. This amounted to 0.64% deuterium for the specific case discussed as an example.

To calculate the percentage of deuterium in a sample incorporated in 90–95% D_2O experiments as a result of chain elongation rather than *de novo* synthesis, the total percentage of deuterium incorporated was first calculated as described in the previous paragraph. This amounted to 7.3% deuterium for methyl stearate. The sum of the contributions to this percentage caused by the presence of mono- through tetradeuterated species in the sample of fatty acid methyl ester was then calculated. In all cases there was no contribution to the total percentage deuterium incorporated in the fatty acid methyl ester from species with more than four deuterium atoms and fewer than fourteen. The sum of the contributions of mono- through tetradeuterated species which amounted to 0.25% for methyl stearate was then divided by the total deuterium content of the sample. The resulting quotient times 100 gave the per cent deuterium incorporated during the experiment as a result of chain elongation and amounted to 0.1% for methyl palmitate and 3% for methyl stearate.

TABLE I: Average Amount of Deuterium Incorporated during Synthesis of Long-Chain Fatty Acids by Liver Perfused with 100% D_2O.

	Expt 1	Expt 2
A. Deuterium content of perfusate (atom %)	90.9	96.0
B. Average number of deuterium atoms found in (see Figure 1)		
Palmitate	20.0	21.6
Stearate	22.5	24.0
C. Average number of deuterium atoms expected after 100% D_2O perfusion (B × 100)/A		
Palmitate	22.1	22.5
Stearate	24.8	25.0
D. Enrichment expected for *de novo* synthesis starting with 100% D_2O (atoms % D)[a]		
Palmitate	64.8	66.1
Stearate	68.9	69.4

[a] (C × 100)/34 for palmitate; (C × 100)/36 for stearate.

The sources of error in determining the isotopic distribution in labeled molecules has been discussed at length by Biemann (1962) and need not be mentioned here. Suffice it to say that the experimental determination of the mass spectra was carried out in a manner designed to minimize these errors. The presence of $(M - 1)^+$ species in the fatty acid methyl esters was ignored in calculating the deuterium content of labeled molecules because it is very small and amounts to only 0.8 and 0.7% of the intensity of the respective molecular ion peak in authentic methyl palmitate and methyl stearate.

Results

The mass spectrum of authentic, unlabeled methyl palmitate in the *m/e* range of 250–320 consists almost entirely of the molecular ion at *m/e* 270 and the corresponding isotope peaks at *m/e* 271 and 272. Similarly, the mass spectrum of authentic, unlabeled methyl stearate in the *m/e* range of 280–340 consists almost entirely of the molecular ion at *m/e* 298 and the corresponding isotope peaks at *m/e* 299 and 300. This is in sharp contrast with the mass spectra of methyl palmitate and methyl stearate isolated from perfusion experiments in 96% D_2O (Figure 1). These show in addition to the peaks mentioned above, other peaks at higher *m/e* which are mainly molecular ions of polydeuterated fatty acid methyl esters. The new peaks result from deuterium incorporation during the biosynthesis of palmitate and stearate. Graphical averaging of the peak heights shows that the centers of the new peaks are at 291.6 and 322.0 for palmitate and stearate, respectively. In other words, the average number of deuterium atoms found in these substances is 21.6 and 24.0, respectively (Table I). It can then be calculated that the average number of deuterium atoms expected after the *de novo* synthesis of palmitate and stearate in 100% D_2O is 22.3 and 24.9, respectively (Table I, average for expt 1 and 2). These calculations assume that

TABLE II: Rates of Palmitate and Stearate Synthesis in Perfused Livers: Comparison of Results Calculated from D_2O and ^3H_2O Incorporation.[a]

	Expt 1	Expt 2	Expt 3	Expt 4	Expt 5
	Deuterium Results				
A. Deuterium content of perfusate (atom %)	90.9	96.0	9.05	10.10	9.50
B. Enrichment expected for *de novo* synthesis starting with D_2O shown in A ($D^b \times A$)/100					
Palmitate	58.9	63.5	5.93	6.62	6.22
Stearate	62.6	66.6	6.26	6.99	6.57
C. Enrichment actually found (atom %)					
Palmitate	22.99	19.81	0.64	0.74	0.66
Stearate	15.34	8.16	0.26	0.26	0.23
D. Fatty acid synthesis/y hr (mol fraction) (C/B)					
Palmitate	0.390	0.312	0.108	0.112	0.106
Stearate	0.245	0.123	0.0415	0.0372	0.0350
E. Acetyl group incorporated (mol/mol of LCFA[c] per y hr)					
Palmitate (D × 8)	3.12	2.50	0.864	0.896	0.848
Stearate (D × 9)	2.21	1.11	0.374	0.335	0.315
F. Total fatty acid content of liver (μmol/g dry weight)[d]					
Palmitate	113.2	112.0	113.8	103.4	116.5
Stearate	63.4	68.6	80.4	61.0	69.3
G. Acetyl group incorporated (μmol/g dry weight/y hr) (E × F)					
Palmitate	353	280	98	93	99
Stearate	140	76	30.1	20.4	21.8
	Tritium Results				
H. Tritium incorporated from ^3H_2O (dpm/μmol of LCFA)					
Palmitate	33,368	60,522	508	640	659
Stearate	22,731	29,123	256	252	227
I. Specific activity of ^3H_2O (dpm/μmole)	13,525	27,780	759	843	834
J. Water incorporated (mol/mol of LCFA per y hr) (H/I)					
Palmitate	2.47	2.18	0.67	0.76	0.79
Stearate	1.68	1.05	0.34	0.30	0.27
K. Acetyl group incorporated (mol/mol of LCFA per y hr) (J × 1.15)					
Palmitate	2.84	2.51	0.77	0.87	0.91
Stearate	1.93	1.20	0.39	0.35	0.31
L. Acetyl group incorporated (μmol/g dry weight per y hr) (K × F)					
Palmitate	321	281	88	90	106
Stearate	122	82	31.4	21.4	21.5
M. Rate E/rate K (= G/L)					
Palmitate	1.10	1.00	1.12	1.03	0.93
Stearate	1.15	0.93	0.96	0.96	1.02

[a] In expt 1 and 2, D_2O and ^3H_2O were present during the whole perfusion, from zero time to 120 min, and y = 2 hr. In expt 3, 4, and 5, D_2O and ^3H_2O were added after the perfusion had been running for 1 hr, and the perfusion was then continued for 1 additional hr, hence y = 1 hr. In expt 3–5, Krebs–Ringer bicarbonate containing 4% bovine serum albumin in 100% D_2O was added to the perfusion reservoir after the first hour of perfusion, to yield the D_2O concentrations shown. [b] *D* from Table I. [c] LCFA is long-chain fatty acid. [d] Determined by method of Novák (1965).

isotope effects do not change in going from 90.9 or 96% to 100% D_2O. Deuterium incorporation in the presence of 100% D_2O into palmitate by rat epididymal fat pad *in vitro* was studied by Jungas (1968). The mole fraction of palmitate labeled with deuterium in his experiments was only about one-fiftieth of that reported here (see Table II). Jungas reported that the average number of deuterium atoms incorporated during the synthesis of palmitate is 23, in good agreement with our results for liver. This agreement confirms that the mechanisms of the terminal stages of carbohydrate degradation and of the pathway of fatty acid synthesis in liver and adipose tissue are very similar if not identical.

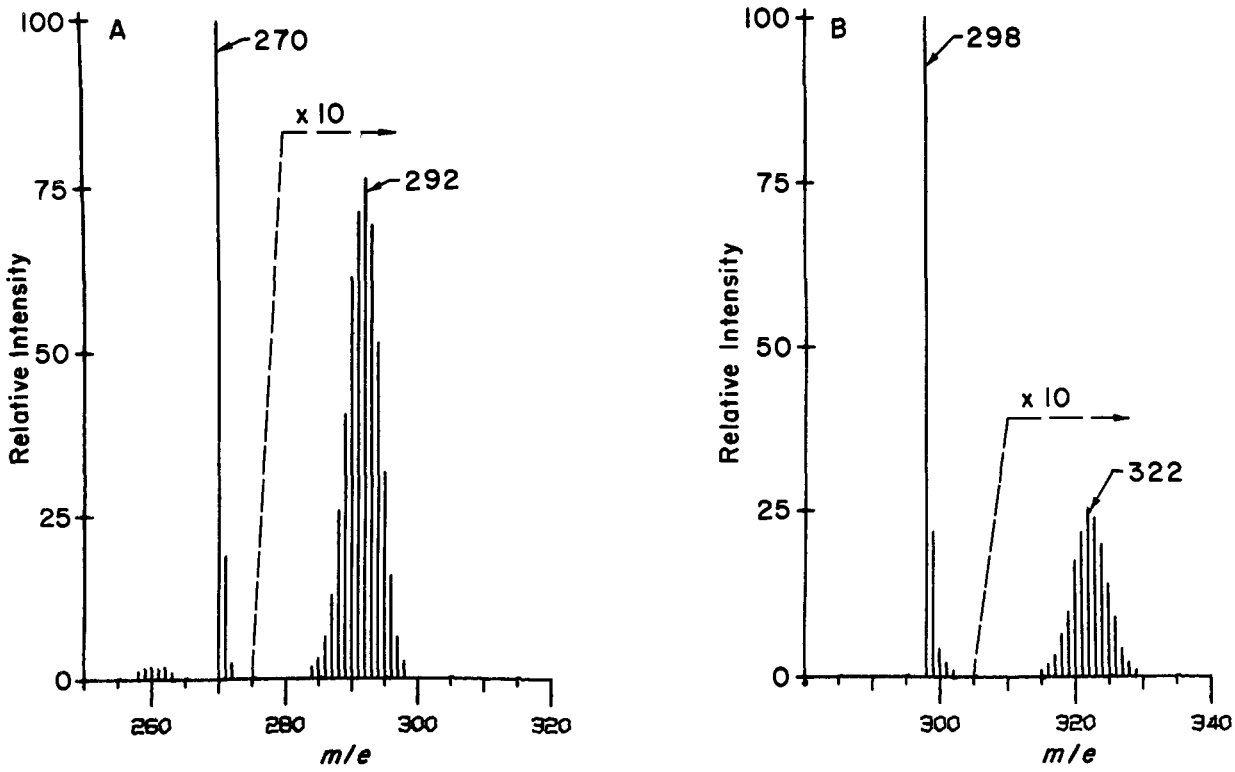

FIGURE 1: Portions of mass spectra of (A) methyl palmitate and (B) methyl stearate isolated from rat liver perfused in 96% D_2O.

Results obtained with two perfusions in >90% D_2O and three perfusions in about 10% D_2O are detailed in Table II. It should be noted that the perfusions with D_2O were for 2 hr in the case of expt 1 and 2, and for 1 hr in the case of exp 3, 4, and 5. No correction for the different times of the perfusions has been made in order to present the results actually obtained. Table II (line D) shows that livers of rats kept on a high carbohydrate diet, and perfused with 25 mM glucose, synthesized between 11 and 20% of their total palmitate per hr; and between 4 and 6% of their total stearate per hr. An exception was the stearate synthesized in expt 1 which amounted to 12% of the total stearate present per hr.

The perfusions also contained ^3H_2O in order to provide a comparison of labeling by the two isotopes, and to validate the double-labeling approach for measuring rates of fatty acid synthesis. Table II shows that there is excellent agreement between the results obtained with deuterium and tritium labeled water.

Isotope discrimination in the experiments under consideration can be expected to be minimum at high concentrations of D_2O since the mass of the tritium atom is only 50% greater than that of the deuterium atom. More discrimination would be expected to occur at low D_2O concentrations since the mass of the tritium atom is 200% greater than that of the hydrogen atom. However Table II, line M, shows that no differences were observed between perfusions at high and low D_2O concentrations. The reasons for this lack of difference are not clear. It may be due to a systematic error in the analyses of the results obtained at high but not at low concentrations of D_2O, or *vice versa*. However, the analyses were repeated several times with essentially the same results.

Differences between the two mass spectra shown in Figure 1A,B are apparent. First, the relative abundance of the polydeuterated species is greater in methyl palmitate (Figure 1A) than in methyl stearate (Figure 1B) by a factor of about 3, although the peak number of deuterium atoms incorporated in methyl stearate is greater than in methyl palmitate by about 2. Secondly, the relatively high abundance of polydeuterated species in methyl palmitate (Figure 1A) leads to the appearance of peaks in the *m/e* range 258–263. These result from the loss of $\cdot OCH_3$ or partly deuterated $\cdot C_2H_5$ from the various polydeuterated methyl palmitate molecular ions. Methyl stearate undergoes the same losses from its molecular ion but this is not apparent from Figure 1B because of the low relative abundance of polydeuterated species in the methyl stearate sample. Finally, the appearance of the mass spectra in the region of the molecular ion of the unlabeled species is clearly different. Methyl palmitate (Figure 1A) shows only peaks at *m/e* 270, 271, and 272 expected for unlabeled methyl palmitate, whereas methyl stearate shows peaks at *m/e* 298–302. The relative abundance of the peaks at *m/e* 301 and 302 is too large to arise from the presence of naturally occurring isotope peaks of the unlabeled methyl stearate molecular ion at *m/e* 298, and provide direct evidence for the presence in the sample of tri- and tetradeuterated methyl stearate species, which are interpreted as having been made by chain elongation in the course of the perfusion experiment.

Discussion

Mass spectrometry is the only direct analytical method available for detecting and determining the presence of stable isotopes in intact molecules. Indirect methods for determining the extent of deuterium incorporation in molecules exist and require that the material under investigation be combusted to water as a step in the analysis. The deuterium content of the water is then determined in a variety of ways (Robertson, 1949). Mass spectrometry was selected to carry out the deuterium analysis for three reasons. (1) The extent of deuterium incorporation as well as the distribution of deuterium atoms may be determined. For example, for each individual fatty

acid in the case of experiments run in high D_2O concentrations, mass spectrometry yielded information not only on the total amount of deuterium incorporated but also on the amount of fatty acid formed by *de novo* synthesis *vs.* the amount formed by chain elongation, without necessitating tedious degradations. (2) The size of sample required for the mass spectrometric determination of deuterium content is quite small. In this investigation, approximately 10 μg of fatty acid methyl ester was used. This amount of material is well below that required by indirect methods for determining deuterium. (3) The analysis by mass which is carried out with the spectrometer effectively separates the unlabeled fatty acids from the labeled ones. A pool of unlabeled fatty acids is present in the tissue prior to the start of the experiment. Deuterium is incorporated solely into the fatty acids synthesized during the experiment, while sampling of the fatty acids is perforce done on the total fatty acids present at the end of the experiment. Interference with the deuterium analyses by the unlabeled fatty acids increases as the concentration of D_2O used in the experiments is decreased. This interference is minimized by mass spectrometric determinations.

The choice of mass spectrometry as the analytical method for deuterium necessitated that another choice be made namely the method of sample introduction into the mass spectrometer. The heated inlet system, the direct introduction system and the gas chromatograph are three possible contenders for sample introduction (Arsenault, 1973). The heated inlet system was quickly set aside because it would require a larger sample size than the other two methods of sample introduction, and the vapor pressure of some fatty acid methyl esters would be too low to permit the heated inlet system to be used for all compounds. The gas chromatograph–mass spectrometer combination might have been ideal if we had wished to measure only the deuterium content of the sample. However, a measure of tritium incorporation was also sought. It was deemed best to perform deuterium and tritium analyses on the same samples in order to verify the validity of the double-labeling technique by obtaining constant D/T ratios. The separation of mixtures into individual fatty acid methyl esters by gas chromatography, and collection of each methyl ester for analysis of tritium and deuterium content, was therefore decided upon. This in turn sealed the choice of using the indirect introduction probe since relatively pure specimens of fatty acid methyl esters were available for deuterium analyses. Moreover, the direct introduction probe has two distinct advantages over the gas chromatograph introduction in this application. First, manipulation of the position of the probe while the analysis was in progress insured that the signal recorded for all samples within a group was comparable, thus reducing to a minimum the possibility of error caused by ion–molecule reactions (Biemann, 1962). Secondly, the direct introduction probe affords very little isotopic fractionation of the sample over a brief period of time, while partial gas chromatographic separation of isotopic species is known to occur (Biemann, 1962).

It is generally considered that palmitate is the normal end product of fatty acid synthesis in mammalian tissues, and that stearate not derived from the diet is made by chain elongation of palmitate. In four out of the five experiments shown in the Results, stearate was made at 23–30% the rate of palmitate, and in the remaining experiment it was made at 38% the rate of palmitate. The two experiments with greater than 90% D_2O in the perfusate show that 97% of this stearate is made from two carbon fragments and not, for example, by chain elongation of preexisting palmitate. Examination of the mass spectra of methyl palmitate obtained from the same livers shows that not more than 0.1% of the newly synthesized palmitate is made by chain elongation.

Our results do not enable us to distinguish between the following possibilities: (a) palmitate and stearate are both made *de novo*, by the same long-chain fatty acid synthase, some chains being terminated to yield palmitate and others to yield stearate; and (b) palmitate is made *de novo*, and only the *de novo* synthesized palmitate is made available to the chain elongation mechanism to yield stearate, as a result of which the newly formed stearate possesses the deuterium content expected from *de novo* synthesis of stearate. It should be pointed out that chemical degradation methods would also fail to distinguish between these possibilities.

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