Measurements of Fatty Acid Synthesis by Incorporation of Deuterium from Deuterated Water†

George M. Patton‡ and John M. Lowenstein*

ABSTRACT: Fatty acid synthesis by perfused livers of rat is measured by using D_2O as tracer. The newly synthesized, deuterium-labeled fatty acids are separated from unlabeled fatty acids by gas chromatography using glass capillary columns. The areas of the deuterium-labeled peaks are proportional to the amounts of fatty acids synthesized. The absolute amounts of the individual fatty acids synthesized are obtained by use of an internal standard. The number of deuterium atoms incorporated, as determined by mass spectrometry, is proportional to the D_2O concentration of the perfusate, except at very high concentrations of D_2O. The relative retention times of the newly synthesized, deuterium-labeled fatty acids are proportional to their deuterium content.

Incorporation of D_2O and ^3H_2O during the de novo synthesis of fatty acids affords a convenient tool for measuring rates of fatty acid synthesis, especially under physiological conditions when the relative contributions made by various carbon precursors are not known. Previous publications validated this approach as a quantitatively reliable method (Jungas, 1968; Wadke et al., 1973). In the present paper, we report that fatty acids synthesized de novo in the presence of D_2O can be separated from preexisting unlabeled fatty acids by high-resolution gas chromatography. In the presence of internal standards, the areas of the deuterated peaks give a reliable measure of the amount of fatty acid synthesized. Evidence is presented that the degree of separation that is obtained between labeled and unlabeled fatty acid is a measure of the deuterium content of the fatty acids. Typical results are presented which show that the major products of fatty acid synthesis by perfused rat liver are palmitate, stearate, palmitoleate, oleate, and myristate, all of which are made de novo.

Materials and Methods

Liver perfusions with perfusate containing deuterium oxide, extraction of lipids, hydrolysis, and methylation of fatty acids were performed as described previously (Brunengraber et al., 1973; Wadke et al., 1973).

The mixture of fatty acid methyl esters was separated on a Shimadzu GC Mini 1 gas chromatography apparatus equipped with a flame ionization detector purchased from Shimadzu Scientific Instruments, Columbia, MD 21045. Glass capillary columns were made by using Shimadzu Glass Drawing Machine GDM-1. Soda glass tubing, 2.0–2.5 mm internal diameter (obtained from VWR Scientific), was cleaned with chromic acid, rinsed, and dried. The tube was then drawn into a capillary at an oven temperature of 620 °C by using a drawing ratio of 64:1. Allowing for wastage of the ends of the starting material, this resulted in a capillary coil 60-m long. The capillary was flushed with HCl gas at 300 °C for 3 h and then with N_2 gas to remove the HCl. A solution of EGSS-X (Supelco, Bellefonte, PA) dissolved in chloroform (10%, weight for volume) was then forced through the capillary at a constant velocity of 0.6 m/min by using Shimadzu microcolumn treating stand MCT-1A. The volume of solution used was equal to one-quarter of the volume of the capillary (~0.5 mL). The column was then dried with a stream of N_2 gas, and the last 10–15 m was broken off and discarded. The resulting column was 45–50-m long. Capillary columns 95-m long were also made and tested, but for the purposes of the work described in this paper they did not provide a significant improvement in resolving deuterium-labeled peaks from unlabeled peaks.

The capillary column was operated at 150–165 °C for fatty acid methyl esters up to methyl oleate, and at 180–195 °C for fatty acid methyl esters of longer chain length, with nitrogen gas at an inlet pressure of 25–30 psi. Normally 1–4 μL of petroleum ether containing the mixture of fatty acids was injected into a stream splitter (Shimadzu) which provided a split ratio of between 50:1 and 100:1 depending on the resistance of the column. The linear flow velocity was 10 cm/s (0.25 mL/min).

Results

Proportionality of Deuterium Incorporation with D_2O Concentration. The number of deuterium atoms incorporated into fatty acids during de novo synthesis is proportional to the D_2O concentration up to 70% excess (Figure 1). Between 70 and 90% excess D_2O, a deviation from linearity sets in so that the number of deuterium atoms incorporated is greater than would be expected on the basis of a linear relationship. The deviation from linearity is shown more clearly for palmitate in the inset to Figure 1. Stearate showed similar deviations in these additional perfusions (not included in the inset).

The proportionality is maintained for each of the five fatty acids that were analyzed in the mass spectrometer. The best straight line was drawn through the data for palmitate. The lines drawn through the data for myristate, palmitoleate, and oleate were calculated by assuming that all four fatty acids are synthesized de novo and that the deuterium incorporation is proportional to the number of methylene and methyne hydrogens in the fatty acids. Three of the calculated lines fall on the experimental points, showing that this assumption is correct. The line calculated for myristate shows a deviation of about 4% from the experimental points.

The difference in relative retention times between the methyl esters of deuterated and nondeuterated fatty acids is proportional to the average number of deuterium atoms found in the newly synthesized deuterated compound (Figure 2). The difference in relative retention time can be used as a measure of the average deuterium content of the deuterated species. The deuterated peaks possess a bell-shaped distribution of deuterated species. Because of this, the deuterated

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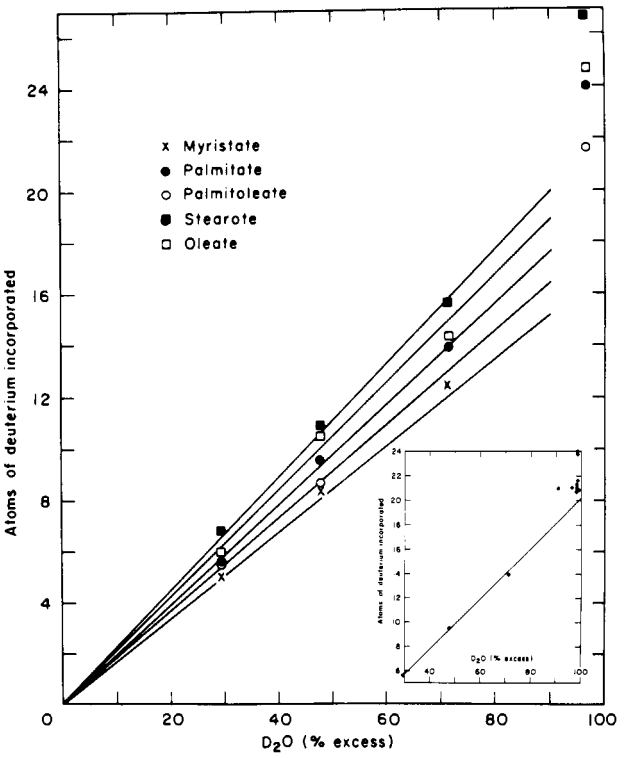

FIGURE 1: Atoms of deuterium incorporated during synthesis of fatty acids by perfused rat liver as a function of the D_2O concentration. Methyl esters of the fatty acids were separated by gas chromatography; individual peaks were collected and subjected to mass spectrometry as described previously (Wadke et al., 1973). The inset, which shows results for palmitate, includes eight additional perfusions at high concentrations of D_2O.

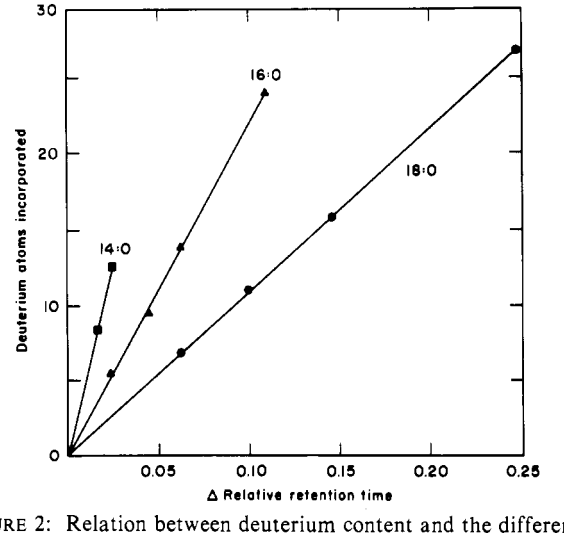

FIGURE 2: Relation between deuterium content and the difference in relative retention times between deuterated and nondeuterated fatty acids. Relative retention time was obtained from the retention time for each peak divided by the retention time for nondeuterated methyl pentadecanoate (15:0). The deuterated fatty acid methyl esters emerge from the column ahead of their unlabeled counterparts.

peaks are always somewhat broader than the nondeuterated peaks. The latter contain only one major mass species and only minor amounts of species 1 and 2 daltons heavier which reflect mainly the natural abundance of ^{13}C (1.11%) and ^{18}O (0.204%) (Wadke et al., 1973).

Typical results obtained with a perfused rat liver are shown in Table I. Myristate exhibits the highest rate of synthesis in terms of the mole fraction synthesized de novo, but palmitate shows the highest rate in terms of absolute amounts. Stearate

Table I: Fatty Acid Synthesis by a Perfused Rat Liver[a]

	% of total C in fatty acids			de novo synthesis	
fatty acid	D[b]	H[b]	D + H	mol fraction of fatty acid	μmol of acetyl/ (g wet wt/ 2 h)
14:0	0.24	0.44	0.68	0.36	0.78
16:0	8.39	26.54	34.93	0.24	27.7
16:1 ($n-9$)[c]		1.25	1.25		
16:1 ($n-7$)	0.60	7.51	8.11	0.074	2.01
18:0	2.52	21.19	23.71	0.11	8.49
18:1 ($n-9$)[d]	0.57	22.60	23.17	0.025	1.95
18:1 ($n-7$)		6.36	6.36		
all others up to 18:1		1.79	1.79		
total	12.32	87.68	100.00		41.0

[a] Rats were deprived of food for 2 days and were then fed a diet high in carbohydrate for 7 days. Results are shown for a liver perfused with 70% D_2O for 2 h as described previously (Brunengraber et al., 1973; Wadke et al., 1973). Internal standard (8 μmol of pentadecanoic acid/g of wet weight of liver) was added at the beginning of the extraction. [b] D and H refer to deuterated and nondeuterated, respectively. [c] Degradation studies show that only one-half of this peak is 16:1 ($n-9$); the other component has not been identified. [d] A small narrow peak which runs in the broad deuterated peak is not 18:1 ($n-9$) and has been subtracted.

is made at one-third the rate of palmitate, but even palmitoleate and oleate are made at about 5% each of the total rate of fatty acid synthesis. No de novo synthesis is observed beyond 18:1 (not shown in Table I). Note that all of the information contained in Table I is obtained from a single chromatogram.

Discussion

The gas chromatographic method for measuring the rate of fatty acid synthesis has the following advantages. The primary measurements are of the areas of deuterated and nondeuterated peaks. The areas of these peaks are independent of the number of deuterium atoms incorporated because the flame ionization detector measures only carbon. Moreover, it is immaterial whether a peak corresponding to a newly synthesized fatty acid contains many deuteriums and is widely separated from the corresponding nondeuterated peak or contains fewer deuteriums and is only slightly separated from the nondeuterated peak, as long as the two peaks are separated sufficiently to permit an accurate measurement of their areas. The method is independent of the source of carbon that the organism or system employs for fatty acid synthesis.

The method provides a profile of which fatty acids have been synthesized and of their amounts, and it does so in a single gas chromatogram. Quantitation is readily achieved by adding an internal standard, for example, pentadecanoate, at the beginning of the extraction procedure. If desired, the actual gas chromatography can be quantitated with another standard, for example, heptadecanoate. (These odd-numbered fatty acids are either absent from higher animal systems or represent only a very small portion of the total fatty acids.)

The differences in retention times of deuterated and nondeuterated species of stearate and oleate show that the deuterated compounds are made de novo. Chain elongation of preexisting palmitate to stearate and of preexisting palmitoleate to oleate would result in the incorporation of only 1 or 2 atoms of deuterium. On the capillary columns described here, the methyl esters of such fatty acids travel at the leading edge of the corresponding unlabeled fatty acid methyl ester peaks. The finding that most of the stearate and oleate is made

de novo is in agreement with results obtained with the mass spectrometer (Wadke et al., 1973). Our results do not imply that stearate cannot be made primarily by chain elongation under some conditions; for example, this might occur when an animal is kept on a diet high in palmitate and low in stearate.

Incorporation of D^+ instead of H^+ from water appears to have a negligible effect on the overall rate of fatty acid synthesis (Wadke et al., 1973). However, discrimination between D^+ and H^+ from water may occur even if the steps during which they are added are not rate limiting. The insertion of fewer deuteriums than would be expected if there were no isotope discrimination makes no difference to the mole fraction of fatty acid synthesized. A reduced number of deuterium atoms per molecule would simply result in a gas chromatogram in which the peak of the newly synthesized deuterated fatty acid runs closer to the preexisting, nondeuterated fatty acid.

We showed previously that, in rat liver perfused with 100% D_2O, about 22 atoms of D are incorporated per molecule of palmitate synthesized de novo. The corresponding value for stearate is 25 atoms of D (Wadke et al., 1973). The values extrapolated from 29, 48, and 71% D_2O yield 19.5 and 22 atoms of D incorporated into palmitate and stearate, respectively, at 100% D_2O (Figure 1). In other words, at very high concentrations of D_2O, the number of D atoms incorporated is actually greater than predicted by extrapolation to 100% D_2O from lower concentrations of D_2O. Using fatty acid synthase from yeast, Seyama et al. (1977) found 16 atoms of D incorporated during the de novo synthesis of stearate in 100% D_2O. The lower value obtained by these workers is probably attributable to their use of the purified enzyme. The additional 6–9 atoms of D incorporated into stearate by the perfused liver are probably introduced via deuterium-labeled reduced pyridine nucleotides. The latter acquire deuterium either from substrates which have become deuterated or by exchange with D_2O catalyzed by NADP-specific flavoprotein enzymes (Drysdale, 1959).

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