Radiohomogeneity of H^3- and C^{14}-labeled linoleic acid in vivo

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SUMMARY A mixture of linoleic acid-1-C^{14} and linoleic acid labeled with H^3 in the chain was administered to rats, and the H^3/C^{14} ratio in the linoleic acid recovered from liver and adipose tissue of the rat was compared with that in the administered mixture. Agreement in the H^3/C^{14} ratios was taken as evidence that the H^3-labeled linoleic acid was biologically indistinguishable from the C^{14}-labeled.

These findings establish the in vivo stability of the H^3 label in the fatty acid chain and the inability of the rat to discriminate between fatty acids labeled with H^3 in the side chain and with C^{14} in the carboxyl group.

KEY WORDS linoleic acid-9,10,12,13-H^3 · linoleic acid-1-C^{14} · H^3/C^{14} isotope ratio · double-label isotope ratio method · rat · liver · lipids · adipose tissue · radiohomogeneity · stability of H^3

SEVERAL INVESTIGATORS working with H^3-labeled fatty acids have considered the possibilities of physical exchange of tritium atoms in the fatty acid chain with protons of other molecules, and of an isotope dilution mediated by enzymatic hydrogen transfer in animal tissues. Palmitic and stearic acids labeled with H^3 and C^{14} have been employed (1–3) to test the stability of H^3 as a label and to examine the possibility that metabolizing systems could be capable of discriminating between H^3- and C^{14}-labeled fatty acids. The H^3/C^{14} isotope ratio in the administered compound was compared to that of the total lipid or of lipid classes isolated from biological samples. Variations in the isotope ratios were taken as evidence of an isotopic segregation of the two differently labeled fatty acids by the metabolizing system.

However, the method of comparing isotope ratios, known as the double-label isotope ratio technique (4), must meet several requirements when it is used to demonstrate the radiotracer stability of H^3-labeled compounds. One of these required conditions is that the H^3/C^{14} ratio of the administered substrate must be compared to that of the same substrate isolated from the metabolizing system. Another important requirement in many cases is that the substrate must be an exogenous compound.

In the present study a mixture of linoleic acid-9,10,12,-13-H^3 and linoleic acid-1-C^{14} was administered to rats, and the H^3/C^{14} ratio in the injected mixture was compared to that found in linoleic acid after isolation from liver and adipose tissue of the rat. In addition, the H^3/C^{14} ratio in total lipid and lipid fractions was determined and the differential incorporation of the two labels into different lipids was evaluated.

MATERIALS AND METHODS

Radioactive Methyl Linoleate

All-*cis* methyl linoleate-9,10,12,13-H^3 with a specific activity of 35.7 mc/mmole was prepared as previously reported (5). On GLC it was found to be almost 99% radiochemically pure.

Linoleic acid-1-C^{14} with a specific activity of 7.8 mc/-mmole was obtained from the California Corporation of Biochemical Research, Los Angeles, Calif. The methyl ester was prepared using diazomethane in ether, and radioassay by GLC showed that 96% of the total radioactivity was recovered with the methyl linoleate peak. The ester was purified, and the *trans* isomers were removed by chromatography on a column of silicic acid–silver nitrate (6, 7).

Liquid scintillation counting was performed on a Packard Tricarb, Model 314 EX, instrument. Samples were routinely counted in 15 ml of toluene containing 3 g of 2,5-diphenyloxazole and 0.1 g of 1,4-di-[2-(5-phenyl-

Abbreviations: GLC, gas–liquid chromatography; TLC, thin-layer chromatography.

oxazolyl)]-benzene per liter. Samples containing the radioisotopes H^3 and C^{14} were counted with split channel settings, the lower channel registering C^{14} counts only (8). Internal standards were used to correct for H^3 and C^{14} quenching. The discriminator-ratio method was used (9) for calculating H^3/C^{14} ratio in the same sample. Sufficient counts were accumulated so that the errors of the ratios were less than 6%.

Administration of Radioactive Methyl Esters

Two different H^3/C^{14} ratios of radioactive methyl esters were administered. In the first study, 28 mature male rats which had been maintained on a stock grain diet were divided into seven groups of 4 rats each. Approximately 7.35 μc of methyl linoleate-H^3 and 4.2 μc of methyl linoleate-C^{14} (H^3/C^{14} ratio of 1.75) were mixed with 0.5 ml of corn oil and administered to each rat by means of a stomach tube. The animals were decapitated at intervals of 20, 60, 120, 240, 360, 480, and 600 min after the injection of the radioactive material.

In the second study, doses of 200 and 25 μc respectively (H^3/C^{14} ratio of 8.00) were given to each of six rats; four rats were killed after 4 hr and the remaining two after 6 hr. In these rats the isotope ratios in both linoleic and arachidonic acid from the tissues were determined.

Analytical and Chromatographic Procedures

Livers, hearts, and abdominal depot fat were pooled and frozen at once. The total lipid was extracted from these tissues by the procedure of Folch, Lees, and Sloane Stanley (10). Solvent was removed from the extracts under reduced pressure and the residue was taken up in benzene. A small aliquot of the benzene solution was counted in order to determine the total radioactivity recovered.

Neutral lipids were separated from phospholipids by chloroform–methanol elution on a silicic acid column using not less than 1 g of silicic acid per 10 mg of lipid. Neutral lipids were fractionated into their components by the method of Fillerup and Mead (11), and phospholipids by the method of Hanahan, Dittmer, and Warashina (12). As each fraction came off the column, aliquots were taken for radioactivity measurements. Phosphorus analyses using the Bartlett (13) modification of the method of Fiske and Subbarow revealed the relative distribution of the major types of phospholipids. Thin-layer chromatography was used for the tentative identification of components of neutral lipids (14) and phospholipids (15).

Methyl esters were routinely prepared from (*a*) total lipid material, (*b*) neutral lipids and phospholipids after preliminary separation, and (*c*) chromatographed components (appropriately pooled fractions) of neutral lipids and phospholipids, using anhydrous methanol containing 2% concd sulfuric acid.

The methyl esters were subjected to GLC using a 20% diethylene glycol succinate polyester on 60–80 mesh firebrick support. Copper columns 3.5 mm × 6 ft were used in an Aerograph, Model A-90-C (Wilkens Instrument and Research, Inc.) equipped with a thermal conductivity detector. The column effluents were collected in vials containing 15 ml of scintillation solution. The "background" radioactivity of the column was checked prior to analyses, and whenever it was found to be higher than normal the column was replaced after the injector and collector had been thoroughly decontaminated.

Alternatively, the bromoderivatives of the methyl ester mixture were prepared and chromatographed on silicic acid plates (16). The spots were made visible by spraying with dichlorofluorescein and those which migrated the same distance as a standard methyl 9,10,12,13-tetrabromostearate were scraped off and extracted with diethyl ether. The recovered material was further purified by chromatography on silica gel plates and the final extract was counted.

The livers, hearts, and kidneys from the two rats that had been fed the higher dosage of doubly labeled methyl linoleate and killed after 6 hr were pooled and extracted with chloroform–methanol 2:1. The lipids were freed from solvent at room temperature under nitrogen, and saponified with 10% ethanolic KOH. The fatty acids obtained from the saponification mixture were separated by low temperature crystallization from acetone.

The fraction soluble at −70° was found by GLC to contain 14.5% of arachidonic acid. A small portion of this fraction was methylated with diazomethane in ether, and the resulting methyl esters were brominated in ether. The crude ether-insoluble bromoderivatives were dissolved in chloroform and chromatographed on silica gel plates with petroleum ether (bp 40–60°)–ethyl ether–acetic acid 90:10:0.2 (v/v/v). The spots which migrated the same distance as a standard methyl octabromoeicosanoate were scraped off. The scrapings were extracted with ether, and the recovered material upon debromination and hydrogenation (17) gave methyl arachidate as identified by GLC.

The isolation, debromination, and hydrogenation of larger amounts of methyl octabromoeicosanoate yielded 20 mg of arachidic acid which was diluted with 292 mg of carrier arachidic acid. After recrystallization, it was degraded by the method of Dauben, Hoerger, and Petersen (18) to stearic acid and to two successive samples of benzoic acid, the carboxyl carbon of which represented carbon 1 and 2, respectively, of the original arachidic acid.

The rest of the fraction which was soluble at −70° was methylated, and methyl arachidonate was isolated by column chromatography on silicic acid–silver nitrate

TABLE 1 COMPARATIVE INCORPORATION OF H^3 AND C^{14} INTO LIPIDS OF RAT LIVER AND ADIPOSE TISSUE AFTER ADMINISTRATION OF A MIXTURE OF H^3- AND C^{14}-LABELED METHYL LINOLEATE

Time after Injection	Liver					Adipose Tissue		
	Dpm/mg of Total Lipid			% Incorporation in Total Tissue		Dpm/mg of Total Lipid		
	H^3	C^{14}	H^3/C^{14} Ratio	H^3	C^{14}	H^3	C^{14}	H^3/C^{14} Ratio
min								
20	—*	—	—	—	—	—	—	—
60	574 ± 37†	94 ± 13	6.07 ± 0.5	2.16 ± 0.40	0.62 ± 0.03	19 ± 11	9 ± 7	2.10 ± 0.90
120	572 ± 95	165 ± 28	3.50 ± 0.4	2.15 ± 0.36	1.09 ± 0.18	41 ± 12	17 ± 10	2.40 ± 0.72
240	1098 ± 192	694 ± 77	1.61 ± 0.3	4.13 ± 0.72	4.57 ± 0.51	140 ± 24	70 ± 12	2.00 ± 0.60
360	1076 ± 331	650 ± 77	1.78 ± 0.6	4.05 ± 1.25	4.31 ± 0.49	82 ± 21	69 ± 20	1.19 ± 0.75
480	706 ± 329	468 ± 70	1.56 ± 0.7	2.65 ± 1.24	3.08 ± 0.46	59 ± 13	56 ± 8	1.05 ± 0.68
600	533 ± 139	319 ± 41	1.70 ± 0.4	2.82 ± 0.91	2.10 ± 0.27	53 ± 15	50 ± 11	1.06 ± 0.73

H^3/C^{14} ratio in administered linoleate, 1.75.
* Radioactivity too low for accurate determinations.
† Mean values ± SD. Data from four experimental animals.

according to the method of De Vries (7) as employed by Craig and Bhatty (19). The isolated methylarachidonate was saponified with 5% ethanolic KOH overnight under nitrogen and the recovered arachidonic acid (12 mg) was diluted with 60 mg of carrier arachidonic acid and oxidatively degraded with permanganate periodate (19). Glutaric acid was recovered (29 mg); aliquots were purified by TLC (20) and by GLC, in the latter case using the dimethyl ester, and the radioactivity of the glutaric acid was determined.

RESULTS

A maximum specific activity for both labels appeared 4 hr after administration, with a rather rapid decrease up to 10 hr (Table 1). At all time periods, liver lipid had a higher specific activity than adipose tissue lipid. It is of interest to note that the incorporation of H^3 into liver and adipose lipid was more rapid than that of C^{14}, 1 and 2 hr after administration of the mixed radioactive methyl esters.

Differences in the H^3/C^{14} ratio in different tissues and at different times are shown in Table 1. They reflect differences in the deposition of the two labels as the result of recycling processes, such as degradation of the administered fatty acid and re-use of its fragments for lipid synthesis. Tritium and C^{14} were present not only in linoleic acid but in all lipid components.

The H^3/C^{14} ratio in the linoleic acid recovered from the total lipid material and purified by chromatographic procedures was found to be constant for 10 hr and identical with the H^3/C^{14} ratio of the administered mixture (Table 2). When the total lipid was fractionated into neutral lipids and phospholipids by elution from silicic acid columns, variations in the H^3/C^{14} ratio were observed (Table 3). However, when aliquots of the neutral lipid and phospholipid fractions were methylated and the H^3/C^{14} ratio of the methyl linoleate from these fractions was determined, it was found to be the same as the H^3/C^{14} ratio in the original mixture of methyl linoleate which was administered to the rats (Table 4). This was true for any sample of methyl linoleate or its tetrabromide isolated either from the same animal but different tissues, or any lipid component and different animals (Table 4).

The course of the chromatography of neutral lipids from the rat liver is shown in Fig. 1. The data shown are from one rat, but have been confirmed on four rats. Variations of the H^3/C^{14} ratio in the lipid fractions, as separated by silicic acid chromatography, indicated that an increasing incorporation of H^3 into cholesterol, diglycerides, and monoglycerides had occurred. A marked change in the H^3/C^{14} ratio was seen within the triglyceride peak (III), which seemed to be indicative of an accumulation of H^3 in the tail of the peak. This may be artefactual, as the eluted triglycerides (and other neutral lipids) were accompanied by impurities contaminated with tritium. In one instance, where liver cholesterol was

TABLE 2 H^3/C^{14} RATIO IN METHYL LINOLEATE ISOLATED FROM RAT LIVER TOTAL LIPID IN ABSORPTION STUDIES

Time after Injection	Isolated by GLC	Isolated as the Tetrabromide by TLC
min		
20	*	*
60	1.77 ± 0.16†	1.69 ± 0.12
120	1.74 ± 0.21	1.73 ± 0.19
240	1.74 ± 0.24	1.80 ± 0.24
480	1.75 ± 0.16	1.75 ± 0.20
600	1.76 ± 0.14	1.69 ± 0.24

H^3/C^{14} ratio in administered mixture, 1.75.
* Radioactivity too low for accurate determination.
† Mean values ± SD. Data from four experimental animals.

TABLE 3 COMPARATIVE UPTAKE OF H^3 AND C^{14} INTO LIPID CLASSES OF RAT LIVER 4 HR AFTER ADMINISTRATION OF A MIXTURE OF H^3- AND C^{14}-LABELED METHYL LINOLEATE

Lipid Components	H^3			C^{14}		H^3/C^{14} Ratio
	mg	dpm/mg	% Incorporation	dpm/mg	% Incorporation	
Total lipid material	614	25,594	3.9	4,284	4.60	6.79
Neutral lipids	282	28,956	1.83	3,811	1.93	7.60
Cholesterol esters	10.7	3,466	0.083	703	0.014	4.93
Methyl esters	2.8	8,697	0.054	1,450	0.073	5.80
Triglycerides	235.5	32,396	1.718	4,262	1.814	7.61
Cholesterol	31.0	290	0.001	25	0.001	11.6
Di-, monoglycerides	2.8	4,657	0.002	391	0.002	11.9
Phospholipids	332	22,740	1.70	4,385	2.63	5.17
Cardiolipin	20.6	57,061	0.264	6,874	0.25	8.3
Phosphatidyl ethanolamine and serine	98.3	18,731	0.410	4,072	0.724	4.6
Phosphatidyl inositol and serine	31.0	18,395	0.068	2,911	0.16	6.32
Phosphatidyl choline	172.9	23,618	0.962	4,631	1.44	5.10
Sphingomyelin and lysolecithin	11.6	2,093	0.004	227	0.004	9.20

H^3/C^{14} ratio in administered methyl linoleate, 8.0. Data from one rat, but confirmed in four rats.

purified through its digitonide, the H^3/C^{14} ratio dropped from a value of 11.6 to 10.3.

The elution pattern of individual phospholipids from rat liver lipids is shown in Fig. 2. The data shown are from one rat, but have been confirmed on four rats. Five clearly defined fractions could be detected and were identified as cardiolipin, phosphatidyl ethanolamine with serine, phosphatidyl inositol with serine, phosphatidyl choline, and sphingomyelin with lysolecithin. The H^3/C^{14} ratio varied with each fraction and in each animal (Table 3).

To determine whether there is any discrimination between linoleic-H^3 and linoleic-C^{14} acid in the conversion of linoleic to arachidonic acid, the latter was isolated as its octabromide. The H^3/C^{14} ratio of the octabromide was 9.85. A ratio different from that of the administered linoleic acid (8.0) was expected in the isolated arachidonic acid since it has been established by Steinberg, Slaton, Howton, and Mead (21) that arachidonic acid is formed in vivo by the addition of acetate to the carboxyl end of linoleic acid. Mead, Steinberg, and Howton (17) have also shown that linoleic acid from an exogenous source is rapidly catabolized and, if this linoleic acid is radioactive, the radioactive fragments will contribute to the chain lengthening process. The H^3/C^{14} ratio in the acetate pool could have any value depending on a number of factors, such as the age, sex, and nutritional state of the animal, the time of sacrifice, and probably other factors.

TABLE 4 H^3/C^{14} RATIO IN METHYL LINOLEATE ISOLATED (GLC) FROM RAT LIVER, HEART, AND ADIPOSE TISSUE

	H^3/C^{14} Ratio		
	Liver	Heart	Adipose Tissue
Neutral Lipids	8.10 ± 0.10*	8.15 ± 0.22	8.2 ± 0.22
Cholesterol esters	7.92 ± 0.20	7.92 ± 0.25	7.93 ± 0.15
Triglycerides	8.05 ± 0.22	7.88 ± 0.13	7.82 ± 0.30
Di-, monoglycerides	†	†	†
Phospholipids	7.91 ± 0.25	8.12 ± 0.15	—
Cardiolipin	8.11 ± 0.10	7.99 ± 0.21	—
Phosphatidyl ethanolamine and serine	8.04 ± 0.12	7.97 ± 0.20	—
Phosphatidyl inositol and serine	8.08 ± 0.25	7.93 ± 0.16	—
Phosphatidyl choline	7.94 ± 0.15	8.10 ± 0.12	—
Sphingomyelin and lysolecithin	†	†	†

H^3/C^{14} ratio in administered methyl linoleate, 8.0.
* Mean values ± SD. Data from four experimental animals.
† Radioactivity too low for accurate determination.

After debromination and hydrogenation, the octabromide of the arachidonic acid, which had a H^3/C^{14} ratio of 9.85, yielded arachidic acid, which after purification by GLC had a H^3/C^{14} ratio of 8.12. The loss in tritium indicated by these results could, in view of a report by Bitner, Selke, Rohwedder, and Dutton (22) and of studies in progress in our laboratory, be due to an evolution of tritium during the in vitro hydrogenation of unsaturated compounds labeled with H^3 at the active centers of unsaturation. Because of this isotopic effect during catalytic hydrogenation, the H^3/C^{14} ratio of the stearic acid derived from the stepwise degradation of arachidic acid was lower than the H^3/C^{14} ratio of the administered linoleic acid. Hence, the H^3/C^{14} ratios could not be correlated with any possible discrimination between H^3- and C^{14}-labeled linoleic acid in its conversion into arachidonic acid, using the arachidic acid approach. The stepwise degradation of the arachidic acid gave the distribution of C^{14} radioactivity shown in Table 5.

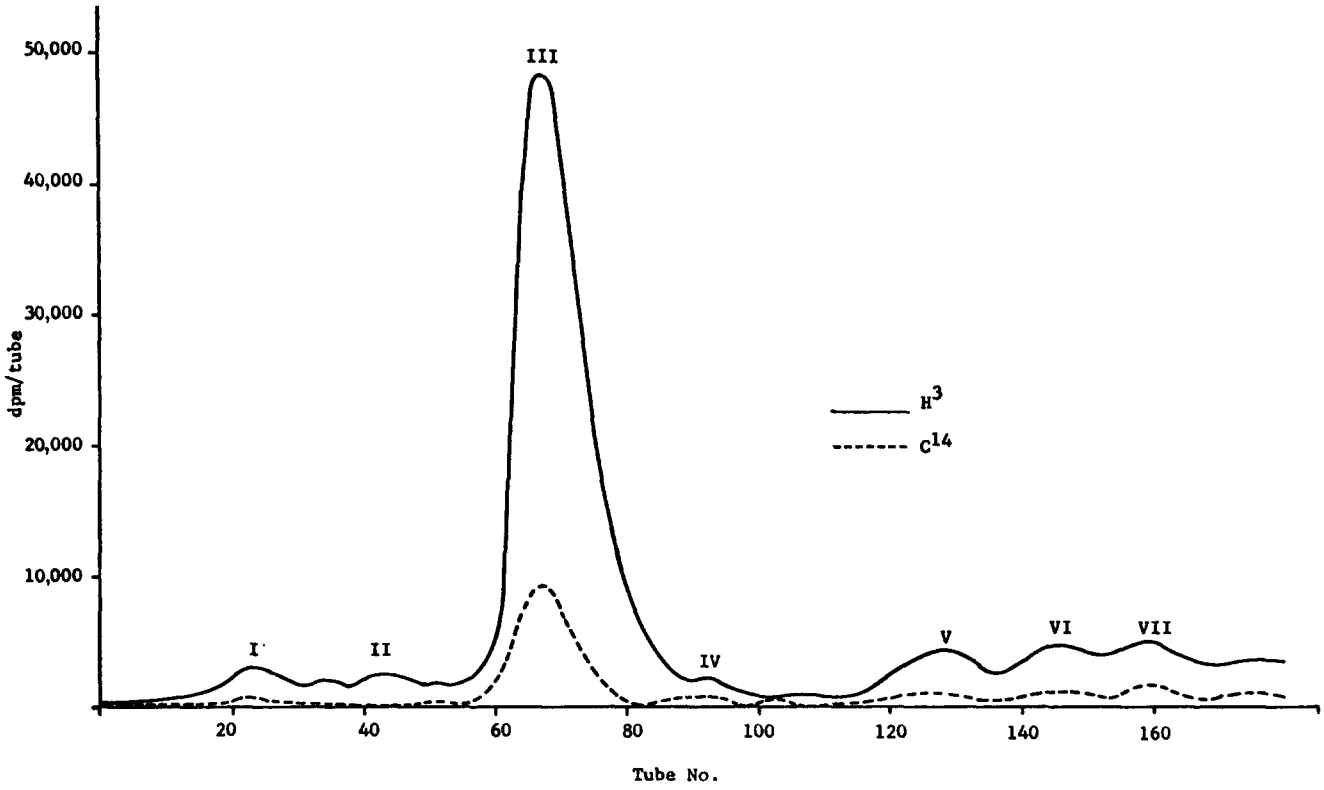

FIG. 1. Curve showing the incorporation of H^3 and C^{14} labels into neutral lipids of rat liver. Rats were administered a mixture of doubly labeled methyl linoleate (H^3/C^{14} = 8.0). I, cholesterol esters; II, methyl esters; III, triglycerides; IV, free fatty acids; V, cholesterol; VI, diglycerides; VII, monoglycerides.

In order to determine the amount of H^3 and C^{14} activity contributed by acetate, arachidonic acid from the fraction soluble at −70° was isolated as its methyl ester by chromatography on a silicic acid–silver nitrate column. It had a H^3/C^{14} ratio of 9.76 (Table 5). Upon permanganate–periodate oxidation, glutaric acid was isolated from the products of oxidation derived from the 1 to 5 carbons of arachidonic acid. After repeated TLC it showed a H^3/C^{14} ratio of 7.63; a similar H^3/C^{14} ratio was found in the dimethyl glutarate isolated by GLC.

From the activities of the various fractions (Table 5) it can be calculated that the percentage distribution of C^{14} in the different portions of the arachidic acid molecule was as follows:

	$C_{18}H_{36}$—	CH_2—	COOH
Total activity %	28	1.5	70.6

Thus, under the present experimental conditions, 72% of the C^{14} activity was contributed by the acetate moiety. On the other hand, the glutaric acid contained all of the C^{14} radioactivity incorporated into arachidonic acid by both linoleate and acetate portions and all of the tritium radioactivity of acetate. Compilation of the data pre-

TABLE 5 CONVERSION OF H^3- AND C^{14}-LABELED LINOLEATE INTO ARACHIDONIC ACID; ISOTOPE CONCENTRATION IN VARIOUS FRACTIONS

Fraction	Dpm/mg		Total dpm		H^3/C^{14} Ratio
	H^3	C^{14}	H^3	C^{14}	
Arachidic acid (after dilution)	893	110	278,678	34,320	8.12
Benzoic acid (carbon atom 1)	—	225	—	24,190	—
Benzoic acid (carbon atom 2)	—	5.5	—	514	—
Stearic acid	630	98.5	61,500	9,610	6.40
Arachidonate (isolated from silicic acid-silver nitrate column)	15,713	1,610	188,563	19,320	9.76
Glutaric acid (purified by TLC)	4,677	613	—	—	7.63
Glutaric acid (purified by GLC as the dimethyl ester)	4,580	583	—	—	7.85

H^3/C^{14} ratio in administered methyl linoleate, 8.0.

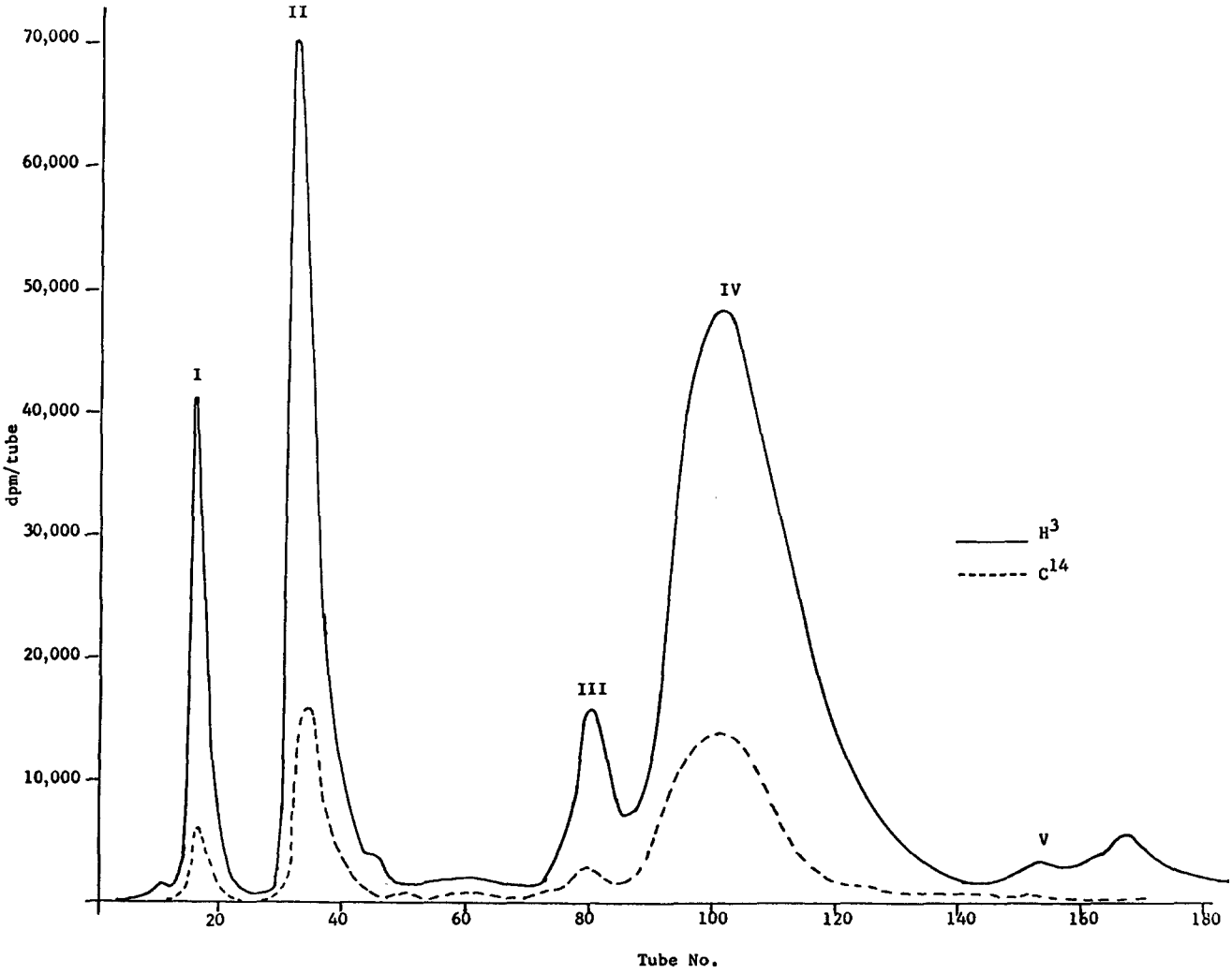

FIG. 2. Curve showing the incorporation of H^3 and C^{14} labels in phospholipids of rat liver. Rats were administered a mixture of doubly labeled methyl linoleate (H^3/C^{14} = 8.0). I, cardiolipin; II, phosphatidyl ethanolamine and serine; III, phosphatidyl inositol and serine; IV, phosphatidyl choline; V, sphingomyelin and lysophosphatidyl choline.

sented shows that the acetate moiety that was incorporated into arachidonic acid had a H^3/C^{14} ratio of 10.6, and that the linoleate incorporated had a H^3/C^{14} ratio of 7.75. This last value was indicative of the equivalence of the conversion of the two differently labeled linoleic acids into arachidonic acid.

DISCUSSION

In previous studies (1–3) the H^3/C^{14} ratio of an administered fatty acid was compared to that in the total lipid or lipid components in the tissue. This introduced an unknown variable into the experiments, for the H^3/C^{14} ratio in the tissue lipid components would be expected to result from the recycling of labels during degradation of the administered fatty acid and utilization of its radioactive fragments for synthesis of the tissue lipids.

In the present study this variable was eliminated by comparing the H^3/C^{14} ratio of the administered linoleic acid with the H^3/C^{14} ratio of linoleic acid recovered from the biological samples and purified chromatographically. Identical isotope ratios demonstrated that the stability of the H^3 label in the fatty acid chain was as great as that of the C^{14} label.

Short-term experiments have indicated the relative stability of H^3 labeled palmitic acid (23). However, in long-term experiments, the administered palmitic acid, which can be formed endogenously, would be diluted with newly synthesized palmitic acid and the isotopic content of the isolated palmitic acid from the biological sample would be changed. For this reason linoleic acid is a more suitable vehicle for long-term observation.

Furthermore, the present results clearly indicated that a variable disposition of the two labels in synthesis of

other lipids had occurred. Perhaps the most striking example was the formation of labeled cholesterol, where the initial transfer of the two isotopes was assumed to be zero.

In conclusion, it became evident that H^3-labeled fatty acids are as stable as C^{14}-labeled fatty acids in vivo.

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