Effect of chain length on rates of uptake of free fatty acids during in vitro incubations of rat adipose tissue

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SUMMARY The incorporation into triglycerides of 1-^{14}C-labeled saturated free fatty acids (FFA) of different chain lengths (C_2–C_{16}) was studied using incubation of rat epididymal fat pads in vitro. The results indicate that the rate at which adipose tissue incorporates FFA is dependent on chain length. With very short-chain acids a small amount is directly esterified into triglycerides, but the major portion is elongated and esterified. Analyses by means of Factice chromatography showed that acids with a chain length of 10 or longer were esterified without elongation.

KEY WORDS uptake · esterification · free fatty acids · adipose tissue · short-chain · long-chain · rat · isotope ratio method

THE STORAGE OF INGESTED and synthesized fatty acids is a major function of adipose tissue (1). The predominant acids in this tissue contain 16 and 18 carbon atoms (2); this remains true even when large amounts of acids of shorter-chain length are ingested (3–8). In one study, for example, a diet containing 19% sodium butyrate was fed to rats but no butyrate was found in adipose tissue (9).

It is well established that short-chain fatty acids are absorbed via the portal circulation and are not directly esterified to form chylomicrons, as is the case with long-chain fatty acids (10, 11). This portal mechanism of absorption permits elongation and oxidation of acids in the liver before they enter the systemic circulation and are presented to adipose tissue. However, it remains uncertain whether the relative absence of short-chain acids in adipose tissue, when animals are fed diets rich in these acids, is due to hepatic alteration of the acids or whether the adipose cell mechanisms that permit entry and esterification of fatty acids also discriminate against the storage of short-chain acids.

In the present communication, we have studied one feature of adipose tissue metabolism which is probably of key importance in the deposition and storage of fat, namely, the relative rate of esterification of free fatty acids presented to the tissue. Rat epididymal fat pads were incubated in vitro and the relative rates of esterification of even-numbered, straight-chain saturated fatty acids containing from 2 to 16 carbon atoms were measured. The acid under investigation was labeled with ^{14}C and its rate of uptake was compared to that of ^{3}H-labeled palmitic acid. The results indicate that the ability of adipose tissue to esterify free fatty acids is greatly affected by the chain lengths of the acids but that the tissue is nevertheless capable of esterifying short-chain acids.

MATERIALS AND METHODS

Fatty Acids

Radioactive 1-^{14}C-labeled palmitic (C_{16}), myristic (C_{14}), lauric (C_{12}), decanoic (C_{10}), octanoic (C_8), hexanoic (C_6), and butyric (C_4) acids, acetate-1-^{14}C (C_2), and palmitic acid-9,10-^{3}H (C_{16}) were obtained from the New England Nuclear Corp., Boston, Mass. These acids were tested for radioactive purity by Factice chromatography (12). In all cases more than 96% of the label was recovered at the appropriate retention volume. Nonradioactive palmitic acid was purchased from the Hormel Institute, Austin, Minn. Methyl ester standards of C_2 through C_{16} were obtained from Distillation Products Industries, Rochester, N. Y. Analysis by gas–liquid chro-

Abbreviation: TLC, thin-layer chromatography.

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matography showed that these compounds were at least 96% pure.

Glycerides

1,3-Dipalmitoyl glycerol was purchased from the Hormel Institute, Austin, Minn. Radioactive 1,3-dipalmitoyl 2-monoacetyl-(1′-^{14}C) glycerol was kindly supplied by Dr. F. J. Mattson of Procter & Gamble, Cincinnati, Ohio. 2-Monoacetyl glycerol was purchased from the Eastman Kodak Company. TLC showed that the compound contained small amounts of di- and triacetin. It was purified by stepwise gradient elution from Florisil columns prepared according to the method of Carroll (13). The purified monoacetyl glycerol gave a single spot on TLC as described below.

Nonradioactive 1,3-dipalmitoyl 2-monoacetyl glycerol was prepared by the esterification of 2-monoacetyl glycerol with palmitoyl chloride in anhydrous pyridine as described by Swell and Treadwell (14).

1,3-Dipalmitoyl 2-butyryl glycerol was prepared by refluxing 1,3-dipalmitoyl glycerol with butyric anhydride for 2 hr. The mixture was washed with distilled water to remove the excess butyric anhydride. The di- and triglycerides were then dissolved in petroleum ether and dried with sodium sulfate. 1,3-Dipalmitoyl 2-monobutyryl glycerol was then separated from the unesterified 1,3-dipalmitoyl glycerol by Florisil chromatography. The purification was found to be complete in that a single spot was obtained on TLC which had an R_F value different from those of either long-chain triglycerides or of diglycerides.

Epididymal fat pads were obtained from Sprague-Dawley rats (210–250 g) maintained ad lib. on Purina chow diets.

Incubation Medium

The incubation medium was prepared before each experiment. An aqueous solution of nonradioactive sodium palmitate containing 6 μeq/ml was heated until optically clear, and a 1 ml sample pipetted into a 50 ml round bottom flask. The sample was then dried in a rotary evaporator with successive additions and evaporations of absolute ethanol to insure complete drying. A tracer dose of sodium palmitate-9,10-^{3}H was then added to each flask together with a tracer dose of the sodium salt of the acid (1-^{14}C) under investigation. Labeled fatty acids were stored at −20° as the sodium salt in 0.015 N NaOH. The flask was then dried completely and 6 mg of glucose (Nat. Bur. Stand.) was added.

Defatted bovine albumin (Gallard-Schlessinger Chemical Manufacturing Corp.) was dialyzed for 12 hr before use. Three milliliters of a 10% solution of the albumin adjusted to a pH of 7.4 was then added to each flask as well as 3 ml of double-strength, calcium-free, Krebs-Ringer phosphate buffer. The mixture was heated to 37°, slowly swirled for 5 min to dissolve all the sodium salts present, and filtered through ether-treated sharkskin filter paper. A 3 ml sample of the filtrate was placed in a siliconized incubation flask and a 0.1 ml aliquot of medium taken for a determination of radioactivity as described below.

All media, therefore, consisted of 3 ml of phosphate buffer containing 50 mg of albumin, 1 μeq of sodium palmitate and 1 mg of glucose per ml. The acid (1-^{14}C) under investigation was present in tracer amount. In addition, all media contained tracer amounts of palmitate-9,10-^{3}H. Results obtained during the uptake and esterification of ^{14}C-fatty acids could therefore be related to the uptake of tritium labeled palmitate.

Incubations

All incubations were performed in the morning with the epididymal pads of animals killed by a blow on the head followed by immediate decapitation. Each pad was placed in a siliconized 25 ml erlenmeyer flask. Incubations were carried out for 30 min in an Eberbach water bath–shaker set at 37° and 72 cycle/min.

Extraction and Fractionation of Lipids

Immediately after the incubation the pads were removed and washed in water three times to remove adherent isotope. They were then homogenized in a glass–teflon homogenizer in 250 ml of a mixture containing isopropanol, heptane, and sulfuric acid in the proportions described by Dole (15). Heptane and water were added and the upper phase was removed and washed with pre-equilibrated lower phase. The upper heptane phase was then removed and dried with sodium sulfate. The upper phase contained triglycerides, traces of partial glycerides, free fatty acids, and phospholipids. The heptane was removed in a rotary evaporator. The samples were dissolved in petroleum ether (bp 30–60°) and the glycerides were separated from the free fatty acids and phospholipids by Florisil chromatography.

Methyl esters were prepared from the glyceride fraction with super-dry HCl–methanol and dried at 4°. Methyl esters were separated into fractions of the same chain length by liquid–gel chromatography on "Factice" as described below.

Chromatographic Methods

Various chromatographic methods were used for the purification of reference materials as well as for the detailed analysis of the lipid extracts obtained from the epididymal fat pads.

For the separation of methyl esters and of triglycerides from each other a system of partition chromatography

TABLE 1 COMPARISON OF SIMULTANEOUS INCORPORATION OF ^{3}H-LABELED PALMITIC ACID AND 1-^{14}C-LABELED C_2–C_{16} ACIDS BY THE SAME EPIDIDYMAL FAT PAD IN VITRO

Labeled (1-^{14}C) Acid in Incubation Medium (besides Palmitate-9,10-^{3}H)	^{14}C/^{3}H (dpm ratio) in Incubation Medium (*A*)	^{14}C/^{3}H (dpm ratio) in Triglyceride Extract (*B*)	*B*/*A*
Acetate	0.436*	0.065*	0.149* (0.129–0.163)
Butyrate	0.309	0.052	0.168 (0.137–0.202)
Hexanoate	0.249	0.127	0.510 (0.488–0.530)
Octanoate	0.305	0.153	0.500 (0.461–0.535)
Decanoate	0.290	0.145	0.500 (0.467–0.550)
Laurate	0.269	0.219	0.819 (0.806–0.828)
Myristate	0.250	0.232	0.928 (0.920–0.936)
Palmitate	0.344	0.341	0.990 (0.958–1.039)

* Each value represents the average of three experiments and the range of values is shown. Incubation time, 30 min. Pads were from fed rats, 3 ml of medium contained 1 mg/ml of glucose.

using the synthetic rubber Factice as stationary phase was employed (12). For the separation of methyl esters, 15% water in acetone (v/v) was used as the eluent, but for the separation of triglycerides, 5% water in acetone (v/v) was used. Samples of effluent were collected in a Technicon fraction collector with automatic detection of samples by differential refractometry (16).

Gas–liquid chromatography was used to determine the purity of reference compounds as well as the fatty acid composition of either unfractionated or fractionated triglycerides from the fat pads. The column used depended upon the chain length of the acids present. Six-foot glass U-tubes of methyl silicone gum rubber (SE-30), 12.5% on 80/100 Gas Chrom P were used for the identification of short- and medium-chain acids, with temperature programmed from 60° to 250°. Long-chain acids were identified on 4-ft ethylene glycol adipate polyester columns (30% on 100–120 mesh Celite 545) at a temperature of 185°. Quantitative reliability was assured by testing for detector linearity with National Heart Institute Fatty Acid Standards A–F with a relative error less than 5% for major components and less than 10% for minor components.

TLC was found to be useful in assuring the purity of reference compounds. A 0.25 mm thick layer of Silica Gel G on 6 × 6 cm or 20 × 20 cm plates was developed in acetone–benzene 3:7 to assess the purity of the monobutyryl and monoacetyl glycerol; and in 25, 28, and 38% ethyl ether in petroleum ether for di- and triglycerides containing long- and short-chain fatty acids. Spots were identified by charring with 50% sulfuric acid and heating in an oven at 120°.

Radioactivity Determinations

Radioactivity was determined with a Packard Tri-Carb Scintillation Spectrometer, using the scintillation mixture described by Gordon and Wolfe (17) for 0.1-ml aliquots of the incubation medium and Liquiflor (100 g of 2,5-diphenyloxazole and 1.2 g of 1,4-bis[2-(5-phenyloxazolyl)] benzene per liter of toluene) for all other radioactive samples.

The setting of the scintillation counter channels was such that all tritium was screened from the carbon channel. ^{14}C efficiency at this setting was 55% and that of tritium 12% in the Gordon and Wolfe scintillation mixture, while the Liquiflor mixture had an efficiency of 66% for ^{14}C and 28% for ^{3}H. The calculation of ^{14}C and tritium content of each sample was carried out by the use of simultaneous equations as described by Okita, Kabara, Richardson, and LeRoy (18).

RESULTS

Relative Rates of Incorporation of Label from Different Fatty Acids

The purpose of these experiments was to determine the relative rates of incorporation into adipose triglycerides of C_2–C_{16} saturated fatty acids. The fatty acid under investigation was labeled with ^{14}C and its rate of uptake was compared with that of palmitic acid-^{3}H by dividing the ratio ^{14}C/^{3}H (each expressed as disintegrations per minute) in the medium by the ratio of ^{14}C/^{3}H in the tissue triglyceride after incubation. With such an experimental design, the effect of the metabolic variation usually encountered among fat pads is minimized since each acid is compared to palmitic acid in the same incubation flask and with the same fat pad.

As a methodologic control, incubations were performed in which palmitate was present as both ^{14}C and ^{3}H. These incubations should yield a final ratio of 1.0 since the rates of uptake for ^{14}C- and ^{3}H-labeled palmitate should be identical. That this condition was achieved is seen in the last line of Table 1, which also shows that there is a high degree of discrimination according to chain length in the uptake and esterification process. The results are depicted graphically in Fig. 1.

Relative Rates of Direct Esterification of Different Fatty Acids

Only the total radioactivity in tissue triglyceride was measured in the above experiments, hence one cannot determine to what extent an individual fatty acid was esterified as such, as opposed to being converted to other

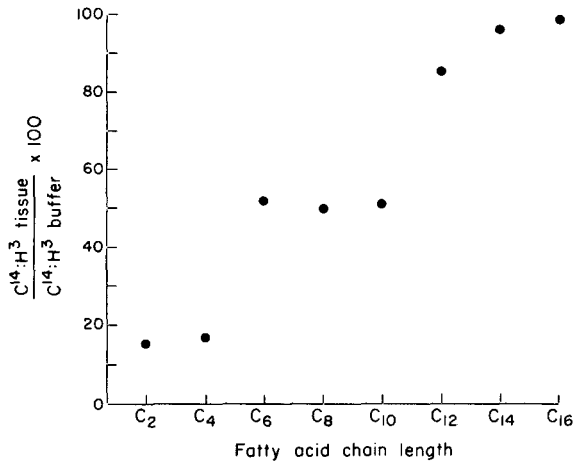

FIG. 1. Incorporation into triglycerides of ^{14}C-labeled fatty acids (C_2–C_{16}) relative to ^{3}H-labeled C_{16} during incubation with rat epididymal fat pads for 30 min in vitro. Each result depicted represents the average of three experiments.

fatty acids with different chain lengths before being incorporated into triglyceride.

In order to clarify this point, triglycerides obtained from the tissue extracts of each incubation were hydrolyzed and the methyl esters of the fatty acids separated by Factice chromatography. Pure methyl ester standards of C_2 to C_{16} were added to the methyl esters derived from each incubation in order to identify each peak. Figure 2 shows a typical chromatogram. Since all peaks were collected and the radioactivity in each was determined, it was possible to determine the relative rate of direct esterification by dividing the ^{14}C (in disintegrations per minute) found in the peak of the methyl ester under investigation by the ^{3}H found in palmitate from the same triglyceride mixture. This ratio divided by the original ^{14}C/^{3}H ratio found in the buffer was used as an index of direct esterification. Table 2 gives the results for each of the incubations. C_{16}, as expected, maintained a final ratio of approximately 1.0, indicating equal rates of esterification of ^{3}H- and ^{14}C-labeled C_{16}; shorter-chain acids showed ratios that decreased in a stepwise manner (emphasized by the representation in Fig. 3) to 0.219 for C_6, while the values for butyric and acetic acids were less than 0.01. These results show, then, that some degree of direct esterification occurs with fatty acids having as few as 6 carbon atoms.

TABLE 2 DIRECT ESTERIFICATION OF ^{14}C-LABELED C_2–C_{16} ACIDS IN ADIPOSE TISSUE COMPARED WITH THAT OF PALMITIC ACID-^{3}H

^{14}C-Labeled Acid in Incubation Medium	^{14}C/^{3}H in Incubation Medium (*A*)	^{14}C/^{3}H Methyl Esters* (*B*)	*B/A*
Acetate	0.436	0.00361	0.0083 (0.010–0.007)
Butyrate	0.309	0.00266	0.0086 (0.011–0.005)
Hexanoate	0.249	0.055	0.220 (0.183–0.282)
Octanoate	0.305	0.128	0.419 (0.389–0.442)
Decanoate	0.290	0.151	0.521 (0.513–0.530)
Laurate	0.269	0.226	0.840 (0.788–0.872)
Myristate	0.250	0.236	0.944 (0.934–0.956)
Palmitate	0.344	0.350	1.020 (0.962–1.063)

* After hydrolysis of the triglycerides, methylation of the liberated fatty acids and Factice chromatography of the methyl esters. The ^{14}C (dpm) in the peak of methyl acetate, butyrate, etc., corresponding to the acid added to incubation medium, is divided by the ^{3}H (dpm) in the methyl palmitate peak. ( ) = range.

Distribution of Label

The percentage of ^{14}C present in each of the peaks eluted after Factice chromatography was also determined in order to find out what degree of transformation each acid had undergone during the 30 min incubation. The results (Table 3) are given as per cent of total radioactivity found in each triglyceride mixture. In fatty acids with 10 or more carbon atoms 94% or more of the radioactive carbon appeared in the unaltered acid while 17% of C_8 and 52% of C_6 had been elongated before incorporation. All the ^{14}C from butyrate and acetate appeared in longer-chain acids.

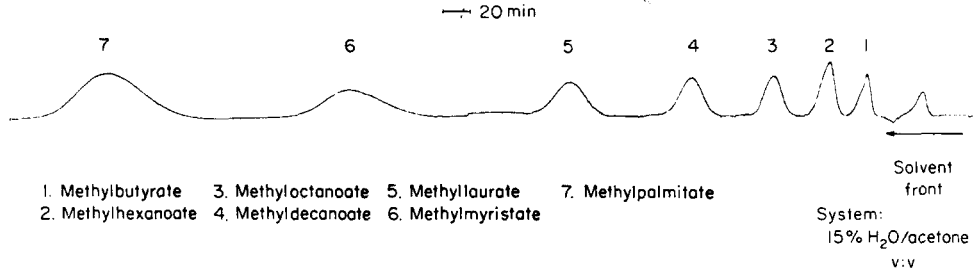

FIG. 2. Separation of methyl esters of fatty acids by Factice chromatography. Each peak represents approximately 5 μl of methyl ester. The identity and purity of each peak was confirmed by fraction collection and gas–liquid chromatographic analysis.

TABLE 3 DISTRIBUTION OF ^{14}C RADIOACTIVITY IN METHYL ESTER PEAKS (C_2–C_{18}) ON FACTICE CHROMATOGRAPHY*

^{14}C Labeled Acid	C_2	C_4	C_6	C_8	C_{10}	C_{12}	C_{14}	C_{16}	C_{18}
Acetate	**tr.**†	2.0	3.3	3.5	4.1	3.1	10.2	62.3	7.3
Butyrate	tr.	**0.1**	0.2	0.4	0.9	5.2	33.0	56.0	4.0
Hexanoate	tr.	2.1	**43.0**	6.5	1.9	4 9	1.9	31.0	7.7
Octanoate	tr.	0.1	0.1	**82.0**	2.9	1.0	4.4	7.7	0.6
Decanoate	tr.	0.3	tr.	0.6	**95.0**	0.4	0.7	1.8	0.3
Laurate	tr.	0.5	0.4	tr.	1.2	**94.0**	1.8	0.7	0.1
Myristate	0	0.2	0.1	tr.	tr.	0.5	**99.0**	0.1	tr.
Palmitate	0	0.2	0.3	0	0	0.5	1.6	**96.0**	1.4

* Values expressed as % of total ^{14}C activity recovered.
† < 0.1%.

It should be noted, however, that although recovery of radioactivity in the methyl esters derived from the hydrolyzed triglycerides was quite complete for acids containing 6 carbon atoms or more, only 90–95% of the radioactivity of triglycerides from the C_2 and C_4 incubations was recovered after Factice separation. The possibility could not be excluded that methyl acetate or methyl butyrate were present but were lost during the necessary drying procedures, since these are very volatile compounds.

It was further reasoned that if triglycerides containing labeled C_2 or C_4 acids were formed, the probability of finding more than one acetate or butyrate residue per triglyceride would be quite remote, since the relative amount of each in the incubation medium was small in comparison to the unlabeled sodium palmitate. Hence the majority, if not all, of the directly esterified acetate and (or) butyrate should appear in a triglyceride with 2 moles of a long-chain fatty acid and either butyrate or acetate in the third position. Factice chromatography offers an ideal method for the detection of such compounds, since one can separate triglycerides of varying chain lengths. With this technique 1,3-dipalmitoyl 2-monoacetyl glycerol can be clearly separated from 1,3-dipalmitoyl 2-monobutyryl glycerol, as seen in Fig. 4. In addition, both compounds can be identified when added as carriers to triglycerides derived from rat epididymal fat pads, as shown in Figs. 5 and 6.

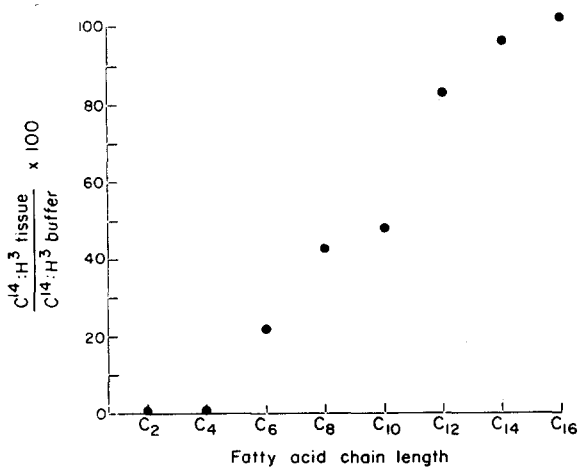

FIG. 3. Direct esterification of ^{14}C-labeled fatty acids (C_2–C_{16}) relative to ^3H-labeled palmitate during incubation with rat epididymal fat pads for 30 min in vitro. Each result depicted represents the average of three experiments.

The triglycerides from the incubations containing radioactive butyrate and acetate were separated by Factice chromatography in the same manner in the presence of the appropriate 1,3-dipalmitoyl derivative added as a carrier, and the radioactivity in all the peaks was determined. It was found that 5% of the total radioactivity in the triglycerides derived from the acetate incubation appeared in the 1,3-dipalmitoyl 2-monoacetyl glycerol peak. During methylation and drying most of the radioactivity was lost from these peaks while 100% was recovered from the other peaks. Similarly, 7% of the total radioactivity derived from the butyrate incubation appeared in the 1,3-dipalmitoyl 2-monobutyryl glycerol peak, but was lost during methylation. Thus the triglycerides of pads incubated with labeled C_2 and C_4 contained at least 5% of C_2 label and 7% of C_4 label as a monoacetyl or monobutyryl glyceride.

DISCUSSION

Previous reports concerning fatty acid uptake by rat epididymal fat pads have dealt solely with long-chain fatty acids that contain 16 and 18 carbon atoms, and indicate that these acids are esterified directly, i.e., appear in the adipose tissue triglycerides with unaltered chain length (1). There is, however, no information concerning the metabolic fate and rate of esterification of short-chain fatty acids during in vitro incubation with adipose tissue.

Under the conditions of the present experiments it is evident that the ability of adipose tissue to esterify free fatty acids is dependent upon their chain length. The major portion of fatty acids containing eight or more

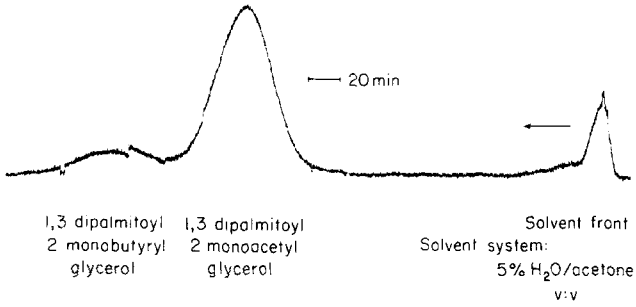

FIG. 4. Separation of 1,3-dipalmitoyl 2-monobutyryl glycerol and 1,3-dipalmitoyl 2-monoacetyl glycerol by Factice chromatography.

carbon atoms is esterified directly, while the extremely short-chain acids (C_2, C_4) are primarily elongated to acids with 16 and 18 carbon atoms, with a negligible amount of direct esterification. Hexanoic acid appears to be equally divided between direct esterification and elongation. The term "elongation" is used here to indicate the finding of label in fatty acids of longer chain length than the labeled substrate. No effort was made to determine whether such elongation was occurring primarily as a result of addition of two-carbon units to the labeled substrate or by complete degradation of the substrate to two-carbon units and subsequent use of these for fatty acid synthesis.

These results are similar to, but not identical with, those reported for the intestinal mucosa and liver in that a marked difference in metabolic activity between short- and long-chain acids has been found (19–23). During intestinal absorption it has been shown that the major portion of fatty acids with less than 12 carbon atoms is not found in the triglyceride fraction of lymph but appears in unesterified form in the portal vein (10, 11, 24–26). In vitro studies have also demonstrated the lack of incorporation of octanoic acid-^{14}C into neutral fat by homogenates of rat small intestinal mucosa, whereas palmitic acid is readily incorporated (19). Since the action of these homogenates is dependent upon CoA, ATP, and magnesium ion, it is believed that the differences in incorporation result from differences in rate of formation of the fatty acid thiol esters which are necessary for the esterification process. Indeed, it has been postulated that this may be the mechanism involved in the differential modes of absorption of long- and short-chain fatty acids (27). The division into two systems is made sharper by the action of pancreatic lipase, which has been shown to have greater activity on triglycerides containing fatty acids of less than 10 carbon atoms (28). Since these acids are therefore more likely to appear as free fatty acids in the intestinal contents, and less likely to be esterified by the intestinal mucosa, the portal route of absorption is strongly favored.

A parallel situation is found in the liver. Kornberg and Pricer (22, 23) showed that microsomal preparations from guinea pig liver activate acids with 10 or more car-

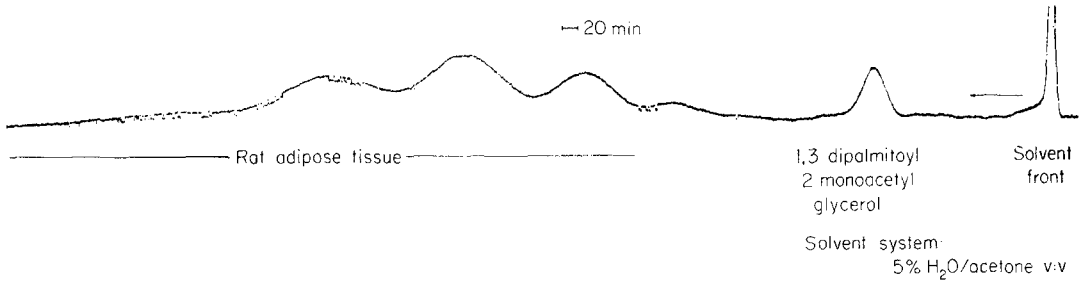

FIG. 5. Factice chromatographic separation of 1,3-dipalmitoyl 2-monoacetyl glycerol from triglycerides of rat adipose tissue.

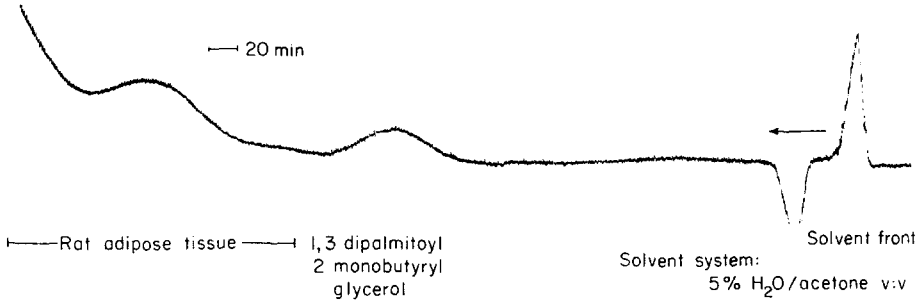

FIG. 6. Factice chromatographic separation of 1,3-dipalmitoyl 2-monobutyryl glycerol from triglycerides of rat adipose tissue.

bon atoms more readily than those with eight carbon atoms. They have shown that octanoic acid forms 50% less CoA derivative than palmitic acid and that negligible esterification of the octanoate results after activation. Thus the short-chain acids tend to remain in their unesterified state. Those medium- and short-chain acids not elongated to 16 or 18 carbon acids are probably oxidized, since it has been shown that CO_2 production from these acids is greater, when they are isocalorically substituted in the diet, than that from their long-chain counterparts (29–31).

It is apparent, then, that the intestinal mucosa and liver do not readily activate and esterify acids with less than 10 carbons, whereas adipose tissue is capable of esterifying large amounts of acids with six or more carbon atoms and some quantities of the shortest acids. Thus, the fatty acid composition of adipose tissue in the intact organism might be different if the modifying effects of the intestinal wall and liver were removed.

The different rates of esterification could presumably be due to the differences in substrate availability, since the shorter-chain acids were present only in tracer amounts and may have been rapidly oxidized. However, time–course studies utilizing tracer doses of acetate-1-^{14}C under conditions identical with those reported above showed linearity of incorporation for at least 1 hr. Thus, there appeared to be no evidence for exhaustion of substrate during 1 hr of incubation. In addition, experiments were performed in which palmitate was present in tracer doses and its incorporation was compared with that of 1-^{14}C-acetate, -butyrate, and -hexanoate present in the medium at a concentration of 1 μeq/ml. These results indicate that the relative rates of incorporation were similar regardless of the concentration of short-chain acids in the medium.

One reasonable explanation for the observed differences in rates of esterification is the differences in ability to form fatty acid thiol esters. Steinberg, Vaughan, and Margolis (32) have shown in tissue homogenates of rat epididymal fat pads that a very active thiokinase system is present for long-chain fatty acids and it is reasonable to assume that, as in the liver and intestines, active CoA derivatives are necessary for final esterification of all fatty acids. This being the case, the results of the experiments reported here indicate a much wider range of activity of thiokinase in adipose tissue than in previously described preparations.

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