Synthesis of Fatty Acids by Rat Intestine *in vitro*.* (30769)

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During fat absorption the fatty acid composition of the lymph triglycerides to a large extent reflects the fatty acids present in the diet(1,2). However, in the process of absorption a considerable quantity of endogenous fatty acid is also found in the lymph (3,4) especially in the cholesterol ester and phospholipid fractions. While the origin of these fatty acids is not known, they could be derived from circulating free fatty acids or more likely from direct synthesis within the mucosa.

Only limited studies are available in the literature on intestinal fatty acid synthesis. Coniglio and Cate(5) found labeled palmitic and stearic acid in the intestine of rats injected with C^{14}-acetate. However, these acids could have been synthesized elsewhere and subsequently transported to the intestine. Using *in vitro* methods we have now been able to demonstrate directly that the intestinal mucosa is capable of *de novo* fatty acid synthesis.

Materials and methods. Sodium acetate-1-C^{14}, specific activity 53 mc/mM, was obtained from New England Nuclear Corp., Boston, Mass. Palmitic acid-1-C^{14}, specific activity 4-8 mc/mM, was obtained from Applied Science Laboratories, College Station, Pa. Albino rats (Charles River Laboratories, Brookline, Mass.), weighing 130-350 g, were fasted overnight and killed by a blow on the head. The small intestine was excised, rinsed with cold saline and inverted on a chilled glass rod by the technique of Wilson and Wiseman(6). The duodenum was discarded and consecutive, cylindrical slices of the proximal jejunum were prepared. Three or, more usually, 4 slices weighing a total of 0.126-0.427 g were added to each incubation flask. The incubation solution was Krebs Ringer bicarbonate buffer with a glucose concentration of 100 mg% and half the usual concentration of calcium. The complete ionic composition of the medium was: Na^+, 143; K^+, 5.9; Ca^{++}, 1.3; Mg^{++}, 1.2; Cl^-, 125; HCO_3^-, 25; $HPO_4^=$, 1.2; and $SO_4^=$, 1.2 mM. Flasks, in which palmitic acid was the substrate, contained 2.5% bovine albumin and either 0.8 or 1.4 μmoles of palmitic acid-1-C^{14}. Flasks, in which acetate was the substrate, contained 0.46 μmoles of Na acetate-1-C^{14}. In certain experiments, 40 μmoles of carnitine (Calbiochem, Los Angeles, Calif.) were added to the incubation medium. The total volume of the medium was 3.0 ml.

The flasks were gassed continuously with a mixture of 95% oxygen and 5% carbon dioxide in a Dubnoff shaking incubator at 37°C. When measurement of $C^{14}O_2$ was required, flasks with center wells were used and the CO_2 trapped in hyamine, as described previously(7). In experiments in which hyamine was not used, the incubation was terminated by immersing the flasks in an ice bath. The slices were then removed, rinsed with saline, blotted and weighed. They were homogenized and the lipid extracted with chloroform:methanol, as described previously(8).

Thin layer silicic acid chromatography was carried out in a solvent system containing petroleum ether, diethyl ether and glacial acetic acid (by volume 90:15:1.5). The diglyceride, fatty acid, triglyceride, cholesterol ester and phospholipid-monoglyceride‡ spots

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‡ Silicic acid chromatography using a chloroform: hexane: ethyl acetate: glacial acetic acid solvent system (150: 50: 25: 4) was carried out on samples from representative experiments. This system sep-

were localized with iodine vapor. When preparative studies were carried out, the phospholipid at the origin was covered, the plates sprayed with Rhodamine 6 G and the spots detected under ultraviolet light. Each fraction was recovered with the aspiration technique of Goldrick and Hirsch(9) and eluted from the silicic acid with diethyl ether or methanol.

The eluate of each fraction was evaporated to dryness, dissolved in 2 ml of acid methanol (2% concentrated H_2SO_4 in absolute methanol), sealed in glass vials and maintained at 65°C for 16 hours(2). Two ml of H_2O were added, and the methylated fatty acids were extracted into 8 ml of heptane, evaporated to dryness and dissolved in 0.5 ml of acetone for gas liquid chromatography. Most gas chromatographic analyses were carried out on a Barber-Coleman model 25C gas chromatograph using a 6 foot coiled glass column with 20% Apiezon L on Anachrom A support (Analytical Engineering Laboratories, Inc., Hamden, Conn.) at 195°C. The argon flow rate was 45 ml per minute with a 1:4 split, respectively, to a tritium ionization detector and to a Packard model 830 Gas Chromatography Fraction Collector. Gas fractions were collected on para-terphenyl crystals in glass cartridges from the start of the chromatogram through arachidonic acid. In addition, a 2-hour post-arachidonic fraction was collected. The amount of radioactivity recovered from each peak area was expressed as a percentage of total radioactivity recovered during the chromatographic run. About 5 to 8% of the radioactivity appeared in the 2-hour post-arachidonic acid fraction, distributed among several peaks and between peak areas which were not identified. A few samples from representative experiments were analyzed on a Barber-Coleman model 15 gas chromatograph using an 8 foot U-shaped column with 15% polyethylene glycol succinate on Anakrom AB support at 173°C. This column separates oleic from linoleic and linolenic acids. These analyses demonstrated that all but a negligible fraction of the radioactivity recovered in the unsaturated 18 carbon fatty acids was in oleic acid.

Radioactivity was measured in a Packard liquid scintillation spectrometer. The solutions prepared for counting substances in organic solvents of hyamine contained 8 parts toluene with 0.01% p-bis-2 (5-phenyloxazolyl)-benzene and 0.3% 2,5-diphenyloxazole and 3 parts absolute ethanol. For counting aqueous materials, a solution of p-dioxane containing 5% naphthalene, 0.05% p-bis-2 (5-phenyloxazolyl)-benzene, 0.7% 2, 5-diphenyloxazole and 13.3% absolute ethanol was used. Corrections for thermal quenching were made using the 2 channels ratio method of Bush(10), with frequent checks using internal standards. Gas chromatographic fractions on para-terphenyl crystals were counted directly using Packard glass cartridge adapters.

Results. Acetate-1-C^{14}. The results obtained clearly demonstrate that intestinal mucosa is capable of incorporating acetate into fatty acids. In experiments in which jejunal slices were incubated with 460 mμmoles of Na acetate-1-C^{14}, 8-60 mμmoles were incorporated into lipid per gram (wet weight) of tissue. This incorporated radioactivity appeared in a characteristic distribution (Table I) in all experiments. Over 80% of the radioactivity was found in 4 fatty acids; namely, stearic (44%,), palmitic (30%), myristic (5%) and oleic (4%) acids. Furthermore, most of the labeled fatty acids were in the phospholipids (Table I) with about 31% of the C^{14} appearing as stearate in the phospholipid fraction.

The effect of variations in glucose concentration, and the addition of insulin or carnitine was also studied. The optimum concentration of glucose was found to be around 100 mg%; lowering the concentration to 50 mg% reduced acetate incorporation by 10-25%, while glucose concentrations up to 200 mg% produced no appreciable change. Addition of 0.3 unit of crystalline zinc insulin to the medium did not influence the amount of acetate incorporated into lipid.

arates the monoglyceride and phospholipid fractions. Only a negligible fraction of phospholipid-monoglyceride radioactivity was found in monoglycerides.

TABLE I. Distribution of Radioactivity in Fatty Acids of Jejunal Slice Lipids After Incubation with Acetate-1-C^{14}.

Lipid fraction	Fatty acid (%) 14:0	16:0	18:0	18:1	Total
Phospholipids	2.0	14.2	30.9	2.0	49.1
Diglycerides	.4	2.7	2.6	.5	6.2
Free fatty acids	.4	2.1	2.1	.3	4.9
Triglycerides	1.7	9.8	7.8	1.0	20.3
Cholesterol esters	.5	.9	.4	.2	2.0
Total	5.0	29.7	43.8	4.0	82.5

Incubation mixture contained 460 mμmoles (40.3×10^6 cpm) of sodium acetate-1-C^{14} and 1.67 μmoles (100 mg %) of glucose. The complete ionic composition of the mixture was Na^+, 143; K^+, 5.9; Ca^{++}, 1.3; Mg^{++}, 1.2; Cl^-, 125; HCO_3^-, 25; $HPO_4^=$, 1.2; and $SO_4^=$, 1.2 mM. The total volume was 3.0 ml. Preparative thin layer chromatography was carried out using the method of Goldrick and Hirsch(9). The fatty acids in each lipid fraction isolated were separated by gas lipid chromatography. The radioactivity in each fatty acid of each lipid fraction is expressed as a percentage of the total radioactivity recovered in all fractions.

Pronounced effects on the jejunal metabolism of acetate occurred when carnitine was added to the medium. It was observed that maximal acetate incorporation into lipid occurred when 40 μmoles of carnitine were added to the incubation medium. Under these conditions (Table II) acetate incorporation increased from about 8 to 125%, the mean increase being approximately 50%. Experiments were also carried out with homogenates of jejunal mucosa to which 1 μmole of carnitine had been added so as to provide concentrations similar to those used by Fritz(11) in cell free systems. Under these conditions there was also a 50% stimulation ($p<0.05$) of acetate incorporation by carnitine.

It is noteworthy that while carnitine increased the C^{14}-acetate incorporation into lipid by jejunal mucosa, it did not alter the distribution of the incorporated radioactivity. Thus, the results of a typical experiment shown in Table III demonstrate that the greatest amount of C^{14} was incorporated into phospholipids and again stearic acid was the major labeled fatty acid in this fraction.

Carnitine also increased the oxidation of acetate-1-C^{14} to $C^{14}O_2$ by jejunal slices. In paired incubations, when carnitine was added (40 μmoles), the amount of acetate oxidized to CO_2 increased from 12.7 to 21.2 μmoles per g tissue. This difference was significant at the 1% level.

Palmitic acid-1-C^{14}. A number of experiments using C^{14}-palmitate showed that this acid was metabolized in many respects like acetate. It was taken up by the cells, oxi-

TABLE II. Effect of Carnitine on Incorporation of Acetate-1-C^{14} into Lipid by Jejunal Slices.

Exp	Acetate-1-C^{14} incorporated (mμmoles/g wet tissue) (A) Without carnitine	(B) With carnitine	Ratio (B/A)
1	20.3	38.8	1.91
2	24.3	41.4	1.70
3	29.2	31.5	1.08
4	9.0	12.9	1.43
5	15.1	18.2	1.21
6	29.1	37.9	1.30
7	31.7	42.9	1.35
8	7.9	10.5	1.33
9	21.0	36.0	1.71
10	60.3	76.9	1.28
11	18.5	20.5	1.11
12	45.1	101.7	2.25
13	12.4	23.7	1.91
Mean	24.9	37.9	1.51

Incubation mixture was the same as in Table I except that 40 μmoles of carnitine was added as indicated. The mean incorporation with carnitine was significantly greater ($p < .01$).

TABLE III. Effect of Carnitine on Distribution of Radioactivity in Jejunal Slice Lipids (A) and Fatty Acids (B) After Incubation with Acetate-1-C^{14}.

	Lipid fraction	Distribution of C^{14} (%) Without carnitine	With carnitine
	Phospholipids	64.5	65.2
	Diglycerides	4.5	5.7
A	Free fatty acids	3.6	4.9
	Triglycerides	24.7	21.6
	Cholesterol esters	2.7	2.6
	Myristic	2.9	3.8
B	Palmitic	31.6	24.1
	Stearic	48.9	51.2
	Oleic	1.9	2.0

Incubation conditions were as in Table I. Lipids (A) in aliquots of the chloroform extracts were separated by thin layer chromatography. Radioactivity in each lipid fraction is expressed as a percentage of radioactivity in all fractions. Fatty acids (B) in aliquots of the chloroform extracts were separated by gas liquid chromatography. Radioactivity in each fatty acid is expressed as a percentage of total radioactivity recovered during a 3-hour chromatographic run.

TABLE IV. Effect of Carnitine on Uptake and Oxidation of Palmitic Acid-1-C^{14} by Jejunal Slices.

Exp	Uptake of palmitic acid-1-C^{14} (mμmoles/g wet tissue) (A) Without carnitine	(B) With carnitine	Ratio (B/A)	Oxidation of palmitic acid-1-C^{14} to $C^{14}O_2$ (mμmoles/g wet tissue) (C) Without carnitine	(D) With carnitine	Ratio (D/C)
1	3570	5650	1.58	57.2	78.8	1.38
2	1800	2130	1.18	29.1	44.3	1.52
3	2370	3350	1.51	35.1	87.1	2.48
4	1560	1910	1.22	23.6	36.6	1.54
5	1280	1480	1.15	37.9	34.6	.91
6	1710	1790	1.05	25.7	27.5	1.07
Mean	2050	2720	1.26	34.8	51.5	1.65

Incubation conditions were as described in Table I except that 0.8 μmoles of palmitic acid-1-C^{14} (with 2.5 g % bovine albumin as carrier) were added to each incubation mixture instead of acetate-1-C^{14}. Carnitine (40 μmoles) was added to each mixture where indicated. Uptake and oxidation of palmitic acid-1-C^{14} were significantly greater with than without carnitine ($p < 0.05$).

dized to CO_2, esterified, and to a small but significant degree, converted to stearic acid. Carnitine stimulated all of these processes. Table IV shows that the uptake of palmitic acid by jejunal slices was increased from 5 to 58% by the addition of carnitine while oxidation of palmitate to CO_2 was increased in 5 out of 6 experiments, the average increase being 65%. In experiments with chromatographically pure palmitic acid-1-C^{14}, newly labeled stearate was produced and most was found in the phospholipid fraction. As with acetate, carnitine did not alter the amount of label converted or the distribution of label in the various lipid classes (Table V). However, because of the increased uptake of C^{14}-palmitate by the slices, the total amount of radioactivity in all of these fractions was greater than in the acetate studies.

Quite similar results were obtained with *in vivo* experiments. When C^{14}-palmitic acid in corn oil was given to rats by stomach tube, 30 minutes later about 95% of the radioactivity in the intestinal lymph(12) appeared as palmitate in triglyceride (Table VI). However, 1 and 1.3% of the lymph radioactivity was present in stearic and oleic acids, respectively. Equal amounts (around 40%) of the labeled stearate appeared in phospholipid and triglyceride, while labeled oleate was distributed evenly in the free fatty acid, diglyceride and triglyceride fractions.

Discussion. On the basis of present evidence, 3 different pathways for the synthesis of fatty acids from acetyl-CoA have been proposed(13): 1) a non-mitochondrial, and a similar mitochondrial, biotin-dependent pathway with malonyl-CoA as an intermediate and saturated fatty acid as the end product; 2) a mitochondrial system involving some of the enzymes of the β-oxidation system which primarily, if not exclusively, serves

TABLE V. Distribution of Radioactivity in Jejunal Slice Fatty Acids After Incubation with Palmitic Acid-1-C^{14}.

Lipid fraction	Fatty acid (%) Palmitate	Stearate
Phospholipids	4.4	1.8
Free fatty acids	67.8	.9
Triglycerides	20.5	.5
Total	92.7	2.2

TABLE VI. Distribution of Radioactivity in the Fatty Acids of Intestinal Lymph After Feeding Palmitic Acid-1-C^{14}.

Lipid fraction	Fatty acid (%) 16:0	18:0	18:1	Total
Phospholipids	2.0	.4	.1	2.5
Diglycerides	2.4	.1	.4	2.9
Free fatty acids	2.6	.1	.4	3.1
Triglycerides	86.5	.4	.4	87.3
Cholesterol esters	.9	.0	.0	.9
Total	94.4	1.0	1.3	96.7

Palmitic acid-1-C^{14} (50 μc) in 1 ml of corn oil was administered to the rat by stomach tube. Thirty minutes later lymph was collected from intestinal lymphatics. Analysis was carried out as described in Table I.

to elongate existing fatty acids by the addition of 2 carbon units; and 3) a mitochondrial, non-biotin dependent pathway leading to the synthesis of oleic acid. The principal saturated fatty acid produced by the malonyl-CoA pathway in non-mitochondrial systems is palmitic acid(14), but within the mitochondria the same pathway produces mainly stearic acid(13). The predominance of stearic acid among the labeled fatty acids produced by jejunal slices from C^{14}-acetate, along with formation of a small amount of oleic acid, would suggest that most of the intestinal fatty acid synthesis observed in the present study may have occurred in the mitochondria.

Further support for intramitochondrial synthesis comes from experiments in which carnitine was employed. This relatively simple, biologically ubiquitous compound serves as a carrier of activated fatty acyl groups across mitochondria and has been shown to increase the *in vitro* oxidation rate, especially by heart muscle, of many fatty acyl-CoA derivatives. Increases of up to 8-fold have been observed with carnitine added to cell-free homogenates(11). We found that carnitine increased the oxidation of palmitate to CO_2 by jejunal slices about 65%, which is comparable to the 25-50% increase in the *in vivo* oxidation of palmitic acid reported by Miller and Krake(15) in mice injected with carnitine. The less dramatic effect of carnitine in the latter 2 instances is due presumably to endogenous carnitine present in the intact animal and slice preparations. We also found that carnitine increased by about 50-70% the incorporation of C^{14}-acetate into lipid and its oxidation to CO_2 by jejunal slices. These results suggest that under the conditions of our experiments, acetate is activated to acetyl-CoA and then transferred across the mitochondrial membranes into the mitochondria where it is either oxidized to CO_2 or incorporated into certain specific fatty acids. Bressler and Katz(16) have recently reported that in the liver carnitine participates in 2 types of acyl-CoA transport: 1) the movement of long chain fatty acyl-CoA derivatives from the microsomes where they are formed to the mitochondria where they are oxidized; and 2) the movement of acetyl-CoA from the mitochondria where it is formed to extra-mitochondrial sites of long chain fatty acid synthesis. Our data suggest that in the intestine the reverse may be true. Acetyl-CoA, with the aid of the carnitine transport system, moves into the mitochondria where it is incorporated into fatty acids by some or all of the intramitochondrial fatty acid synthesizing systems. These newly synthesized fatty acids may then be transported out of the mitochondria to sites where esterifying enzymes are predominantly located(17). Furthermore, in contrast to the findings in liver (16), the principal fatty acid synthesized by intestine is stearate rather than palmitate.

The overall biological importance of intestinal fatty acid synthesis remains to be established. However, since a significant portion of the lipid in intestinal lymph during fat absorption is of endogenous origin, it seems likely that the small bowel mucosa may actively contribute to the phospholipids present in chylomicrons of the lymph. Furthermore, *de novo* fatty acid synthesis may be of importance for the synthesis of the structural lipids of the intestinal epithelial cells.

Summary. Slices of rat intestine have been shown to incorporate acetate-1-C^{14} into fatty acids. Stearic acid was the predominant fatty acid which was synthesized and it was found primarily in the phospholipid fraction. Addition of carnitine *in vitro* stimulated fatty acid synthesis but did not alter the distribution of C^{14} in the fatty acids or lipid classes. The intestine was also able to convert C^{14}-palmitate into labeled stearate.

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Sodium-Potassium Dependent Adenosine Triphosphatase of Mammalian Reticulocytes and Mature Red Blood Cells.* (30770)

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It is known that sodium is required for the active transport of certain aminoacids by intestinal mucosa(1), thymocytes(2), human leukocytes(3) and mammalian reticulocytes(4). In addition wherever sodium dependency is present potassium is required for maximal transport rates(3,4). These observations raise the fundamental question of whether active amino acid transport is linked directly or indirectly to Na-K-dependent strophanthin-inhibited, membrane adenosine triphosphatase (ATPase). Post has postulated a direct role of this enzyme in cationic transport(5). In a recent study in mammalian red blood cells it was found that, in contrast to the reticulocyte, the mature erythrocyte had a markedly reduced or absent capacity to concentrate aminoacids against a chemical gradient(4). Two possible explanations were considered for the altered transport pattern in the mature red cell: 1. The loss of capacity to concentrate aminoacids could be due to cessation of protein synthesis concomitant with red cell maturation. This possibility was examined by studying the effect of puromycin; this drug caused complete inhibition of protein synthesis in the reticulocyte without affecting its concentrative amino-acid uptake indicating that protein synthesis *per se* was not the responsible factor(4). 2. An alternative explanation could be a change in the level of activity or the distribution of Na-K-dependent ATPase in the mature red cell. While it has been demonstrated that the mature erythrocyte retains an active Na-K-dependent membrane ATPase(5) it is not known if the intacellular organelles of reticulocytes (which are lost upon maturation) contain an active Na-K-dependent ATPase which plays a key role in the transport and intracellular distribution of aminoacids. In the present study, this question has been examined by assaying the ATPase activity in mammalian reticulocytes and mature red blood cells. Both total and Na-K-dependent ATPase activities were found to be directly proportional to the percentage of reticulocytes in the various red cell preparations tested.

Materials and methods. Reticulocytosis was induced in New Zealand white rabbits and in Holtzman albino rats by repeated removal of 20-50 ml of blood from rabbits and 3-5 ml from rats by intracardiac puncture at 1-2 day intervals. Blood was collected in EDTA (final EDTA concentration 0.005 M). Each animal was given 10 mg of iron dextran (Imferon®) per kg body weight intramuscularly to compensate for iron loss. To obtain a reticulocyte-rich and reticulocyte-poor preparation from the same blood sample, the sample was centrifuged at 2000 × *g* for 2

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