In vitro esterification of cholesterol by pancreatic juice and by acetone powder extracts of small intestine: the effect of unsaturated fatty acids upon esterification with saturated fatty acids

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SUMMARY The esterification of cholesterol by rat pancreatic juice and by extracts of acetone powders of rat small intestine was studied with palmitic, stearic, oleic, linoleic, and linolenic acids. When each fatty acid was tested individually, the extent of esterification was highest with oleic acid and lowest with the two saturated fatty acids. The esterification of cholesterol with palmitic and stearic acids by the intestinal extracts and with palmitic acid by pancreatic juice was greatly increased by the addition of either oleic or linoleic acid to the incubation mixture. It is suggested that the difference between esterification of cholesterol with saturated fatty acids and esterification with unsaturated fatty acids, as well as the enhancement of the esterification with saturated fatty acids by the addition of an unsaturated fatty acid, is accounted for by micellar solubilization of cholesterol and of saturated fatty acid in the presence of an unsaturated fatty acid and bile salts.

KEY WORDS cholesterol esters · biosynthesis · specificity · saturated · unsaturated · pancreatic juice · small intestine · rat · micellar solubilization · bile salt

MURTHY ET AL. (1) reported that esterification of cholesterol with the unsaturated fatty acids oleic, linoleic, and linolenic by acetone powder extracts of rat pancreas and small intestine greatly exceeds that with the saturated fatty acids palmitic, stearic, and myristic acids. In their recent study of absorption of fats from the intestine into the thoracic duct lymph of the rat, Karmen et al. (2) observed a distinct specificity for esterification of cholesterol with oleic acid compared to that with linoleic, palmitic, and stearic acids, but did not find that the esterification was uniformly higher with unsaturated than with saturated fatty acids. In the in vitro study the fatty acids were tested individually, whereas in the in vivo experiments mixtures containing both unsaturated and saturated fatty acids were fed.

It has been proposed that dietary lipids are absorbed from the intestines in the form of a micellar solution (3), the formation of micelles apparently requiring, in addition to a bile salt, an amphiphile such as an unsaturated monoglyceride or unsaturated fatty acid (4, 5). It is possible, therefore, that the very low values observed for esterification of cholesterol with saturated as compared to unsaturated fatty acids by pancreas and small intestine preparations may have been due to poor solubilization of the substrates, cholesterol and saturated fatty acid. The possibilility that unsaturated acids enhance solubilization of saturated fatty acids is also suggested by the observation that absorption of saturated fatty acid from the intestines is increased by the simultaneous feeding of an unsaturated fatty acid (6).

These considerations led us to study the esterification of cholesterol by pancreatic juice and by acetone powder extracts of the small intestines of rats in reaction mixtures containing either individual fatty acids or combinations of saturated and unsaturated fatty acids.

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EXPERIMENTAL METHODS

Preparation of Acetone Powders of Intestinal Mucosa

Male Long-Evans rats that had been fed an adequate stock diet (Diablo Labration) and weighed 150–200 g were exsanguinated by heart puncture while under ether anesthesia. Their small intestines (from just below the point where bile enters to approximately 6 cm above the cecum) were excised and divided into two sections to facilitate washing. Each section was rinsed twice with 30 ml of 0.9% NaCl and then placed in ice-cold 0.9% NaCl. The sections were then slit longitudinally, and the mucosa and muscle were separated from the serosa with the aid of a blunt spatula. Acetone powders were prepared by the procedure of Mahadevan et al. (7) from the scrapings containing mucosa and muscle (10–12 rats per batch). The powders were stored at 4°. As previously reported by Murthy and Ganguly (8), the enzyme activities of these powders remained undiminished for at least 2 months.

Collection of Pancreatic Juice

The procedure for collecting pancreatic juice has been described previously (9). The juice was stored at −15°. Before use, only the required amount of pancreatic juice was thawed. A single freezing, as reported earlier (9), resulted in no significant loss of enzyme activity.

Substrates

Cholesterol-4-C^{14} was purchased from the Nuclear Chicago Corp., Chicago, Ill., and purified on silicic acid columns (10). Unlabeled, commercial grade cholesterol was purified by bromination by the method of Fieser (11) before use as carrier. Palmitic acid-1-C^{14}, stearic acid-1-C^{14}, oleic acid-1-C^{14}, and linoleic acid-1-C^{14} were purchased from the New England Nuclear Corp., Boston, Mass. Linolenic acid-U-C^{14} was purchased from Applied Science Laboratories Inc., State College, Pa., and purified on silicic acid columns (10). Unlabeled fatty acids were obtained from Calbiochem, Los Angeles, Calif., and their purity was established by gas–liquid chromatography.

Incubation Procedure

The intestinal acetone powder was extracted with 50 volumes of distilled water in a cold room (4°) for 1 hr, and the extract was centrifuged at 5000 × *g* for 25 min in a model L Spinco ultracentrifuge. The clear supernatant fraction was used as the enzyme source. Each substrate, dissolved in 0.2 ml of acetone, was placed in a 25 ml Erlenmeyer flask containing 10 mg of sodium taurocholate dissolved in 0.1 ml of 0.1 M potassium phosphate buffer (pH 6.2) plus either (*a*) 1.0 ml of intestinal extract (protein, 8–9 mg) and 2.7 ml of 0.1 M potassium phosphate buffer (pH 6.2) or (*b*) 0.2 ml of pancreatic juice (protein, 1.6–1.8 mg) and 3.5 ml of 0.1 M potassium phosphate buffer (pH 6.2). The mixtures were incubated for 3 hr at 37° with continuous shaking. At the end of this period, approximately 15–20 ml of ethanol–ether 3:1 (v/v) was added to each flask to inactivate the enzyme and extract lipids.

Analytical Procedures

Lipids were extracted as described previously (12). Cholesterol esters were separated from free cholesterol and free fatty acids on silicic acid columns (10). The lipid samples eluted from the columns were dried and dissolved in 15 ml of toluene containing 45 mg of 2,5-diphenyloxazole and 1.5 mg of 1,4-bis-[2-(5-phenyloxazolyl)]-benzene and were assayed for C^{14} in a Tri-Carb Scintillation Spectrometer (Packard Instrument Co., La Grange, Ill.).

The protein concentration of the intestinal extracts and pancreatic juice was determined by the biuret reaction (13).

RESULTS

Preliminary experiments with extracts of intestine and pancreatic juice confirmed the bile acid requirement for esterification of cholesterol (14), a pH optimum of 6.2 for the reaction (8), and the lack of requirement for an exogenous source of ATP, Coenzyme A, and Mg^{++} as cofactors (8).

Tables 1 and 2 show the percentages of labeled free cholesterol and fatty acids incorporated into cholesterol esters and the micromoles of each substrate converted to cholesterol esters by pancreatic juice and intestinal extracts. In these experiments a single fatty acid, in amounts of 3.5, 7, and 10 μmoles, was added to the incubation mixture. Esterification by both pancreatic juice and intestinal extracts was much higher with the unsaturated than with the saturated fatty acids at all three concentrations. The differences in esterification observed between saturated and unsaturated fatty acids were more pronounced in the experiments with intestinal extracts than in those with pancreatic juice. In the reaction mixtures containing intestinal extracts, esterification with saturated fatty acids was negligible.

The extent of esterification of cholesterol by both pancreatic and intestinal preparations was greater with oleic acid than with either linoleic or linolenic acids. The observed preference for the esterification with oleic acid agrees with the observation of Murthy et al. (1) and Swell et al. (15) for rat pancreas. With respect to the intestinal enzyme, however, our findings are not in

TABLE 1 FATTY ACID SPECIFICITY FOR ESTERIFICATION OF CHOLESTEROL BY PANCREATIC JUICE AND BY EXTRACTS OF ACETONE POWDERS OF THE SMALL INTESTINES OF RATS

Added Substrate*		Intestinal Extract		Pancreatic Juice	
C^{14}-Labeled	Unlabeled	% C^{14} Recovered as Cholesterol Esters†	μmoles of Labeled Substrate Converted to Cholesterol Esters	% C^{14} Recovered as Cholesterol Esters†	μmoles of Labeled Substrate Converted to Cholesterol Esters
Cholesterol	Palmitic acid	0.7	0.03	16.2	0.81
Cholesterol	Stearic acid	0.7	0.03	31.2	1.56
Cholesterol	Oleic acid	18.5	0.92	67.7	3.38
Cholesterol	Linoleic acid	15.8	0.79	60.5	3.02
Cholesterol	Linolenic acid	12.0	0.60	61.3	3.06
Palmitic acid	Cholesterol	0.5	0.05	8.7	0.87
Stearic acid	Cholesterol	0.7	0.07	16.7	1.67
Oleic acid	Cholesterol	9.4	0.94	32.4	3.24
Linoleic acid	Cholesterol	8.1	0.81	29.7	2.97
Linolenic acid	Cholesterol	5.4	0.54	30.1	3.01

* Each mixture contained 5 μmoles of cholesterol and 10 μmoles of fatty acid.
† Each value is the average of duplicate observations of three experiments.

accord with those of Murthy et al. (1), who reported that the fatty acid specificity for the esterification by intestinal extracts increased with increasing unsaturation of the 18-carbon fatty acids.

Table 1 also shows that, with the exception of those instances in which the esterification was extremely low (with palmitic and stearic acid by the intestinal extract), the values calculated for the number of micromoles of cholesterol esters formed from the labeled cholesterol in the presence of a given unlabeled fatty acid were in reasonable agreement with the values calculated for unlabeled cholesterol in the presence of the same labeled fatty acid. This stoichiometric relation indicates that the concentrations of endogenous cholesterol and fatty acid in both pancreatic juice and intestinal extracts were negligible compared to the amounts that were added to the incubation mixture.

The effect of the addition of an unlabeled unsaturated fatty acid upon the esterification of cholesterol with labeled saturated fatty acids is shown in Table 2. Increasing the amount of labeled palmitic acid from 3.5 to 7.0 μmoles in the reaction mixture raised by about 30% the amount of labeled cholesterol palmitate formed by intestinal extracts. When 3.5 μmoles of unlabeled oleic acid was added to a reaction mixture containing 3.5 μmoles of labeled palmitic acid, about $4^1/_2$ times as much labeled cholesterol palmitate was formed by intestinal extracts as in a reaction mixture containing only the labeled palmitic acid. In parallel experiments the addition of unlabeled linoleic acid to intestinal extracts resulted in increases of about 300% in the amounts of labeled palmitic acid incorporated into cholesterol esters. Similar increases in the incorporation of labeled stearic acid into cholesterol esters by intestinal extract and of labeled palmitic acid into cholesterol esters by pancreatic juice were observed when either unlabeled oleic or linoleic acid was added to the incubation mixture. The extent of esterification of cholesterol with labeled stearic acid by pancreatic juice was greater than with labeled palmitic acid, and the addition of the unlabeled unsaturated fatty acids had no effect on the esterification with labeled stearic acid. Stoichiometric combination of the added cholesterol and fatty acids was again observed. The values calculated for micromoles of cholesterol esters formed from labeled cholesterol and the two unlabeled fatty acids agreed closely with the sum of values calculated for each of the same two labeled fatty acids and unlabeled cholesterol. For example, in Table 2 the sum of the values for mixtures designated *9* and *10* agreed closely with that for *11*.

Table 3 shows the extent of esterification of cholesterol by pancreatic juice and intestinal powder extracts in incubation mixtures containing equimolar amounts of palmitic, oleic, and linoleic acids (only one of which was labeled). In the experiments with intestinal extracts, the extent of esterification with oleic and linoleic acids was about the same, and the esterification values for these two fatty acids were about twice those for palmitic acid. In the case of pancreatic juice, esterification was about 25% higher with oleic than with palmitic or linoleic acid.

DISCUSSION

Dietary lipids have been shown to be present in the small intestine in the form of micellar solution and are presumed to be absorbed from the intestines in that form (3). Micellar solubilization apparently requires, in addition to a bile salt, an amphiphile such as an unsaturated monoglyceride or an unsaturated fatty acid

(4, 15). Thus, in the experiments with individually tested fatty acids, our observation that esterification of cholesterol was far greater in the case of the unsaturated than the saturated fatty acids can be accounted for by micellar solubilization of cholesterol in the presence of the unsaturated fatty acid and sodium taurocholate. Likewise, the enhancement of esterification of cholesterol with saturated fatty acids upon addition of an unsaturated fatty acid to the incubation mixture can be explained by solubilization of the saturated fatty acids. Our findings with intestinal extract also show that the number of micromoles of cholesterol esters formed is lower when 5.0 μmoles of cholesterol are incubated with 3.5 μmoles of an unsaturated fatty acid and 3.5 μmoles of a saturated fatty acid than when the same amount of cholesterol is incubated with 3.5 μmoles of an unsaturated fatty acid alone. For example, the value for mixture *11* (Table 2) is lower than the value for mixture *5*. This reduction in the amount of cholesterol esterified suggests that solubilization of the saturated fatty acid by the unsaturated fatty acid limits the amount of cholesterol that can be simultaneously solubilized.

TABLE 2 Effect of the Presence of Unsaturated Fatty Acids on the Esterification of Cholesterol with Saturated Fatty Acids

Mixture No.	Added Substrates		Intestinal Extract		Pancreatic Juice	
	C^{14}-Labeled	Unlabeled	% C^{14} Recovered as Cholesterol Esters*	μmoles of Labeled Substrate Converted to Cholesterol Esters	% C^{14} Recovered as Cholesterol Esters*	μmoles of Labeled Substrate Converted to Cholesterol Esters
1	Palmitic acid, 3.5 μmoles	Cholesterol, 5 μmoles	0.8	0.03	0.9	0.03
2	Palmitic acid, 7.0 μmoles	Cholesterol, 5 μmoles	0.5	0.04	3.7	0.26
3	Stearic acid, 3.5 μmoles	Cholesterol, 5 μmoles	0.8	0.03	17.0	0.59
4	Stearic acid, 7.0 μmoles	Cholesterol, 5 μmoles	0.9	0.07	23.6	1.65
5	Oleic acid, 3.5 μmoles	Cholesterol, 5 μmoles	18.3	0.64	54.5	1.91
6	Oleic acid, 7.0 μmoles	Cholesterol, 5 μmoles	12.1	0.89	42.9	3.00
7	Linoleic acid, 3.5 μmoles	Cholesterol, 5 μmoles	16.1	0.56	44.8	1.57
8	Linoleic acid, 7.0 μmoles	Cholesterol, 5 μmoles	10.4	0.73	35.4	2.47
9	Palmitic acid, 3.5 μmoles	Cholesterol, 5 μmoles + oleic acid, 3.5 μmoles	3.6	0.13	12.3	0.43
10	Oleic acid, 3.5 μmoles	Cholesterol, 5 μmoles + palmitic acid, 3.5 μmoles	8.2	0.29	27.8	0.97
11	Cholesterol, 5 μmoles	Oleic acid, 3.5 μmoles + palmitic acid, 3.5 μmoles	8.4	0.42	26.7	1.33
12	Stearic acid, 3.5 μmoles	Cholesterol, 5 μmoles + oleic acid, 3.5 μmoles	4.2	0.15	20.7	0.72
13	Oleic acid, 3.5 μmoles	Cholesterol, 5 μmoles + stearic acid, 3.5 μmoles	14.0	0.49	49.1	1.71
14	Cholesterol, 5 μmoles	Oleic acid, 3.5 μmoles + stearic acid, 3.5 μmoles	12.1	0.61	51.7	2.58
15	Palmitic acid, 3.5 μmoles	Cholesterol, 5 μmoles + linoleic acid, 3.5 μmoles	3.2	0.11	15.1	0.53
16	Linoleic acid, 3.5 μmoles	Cholesterol, 5 μmoles + palmitic acid, 3.5 μmoles	6.0	0.21	22.8	0.80
17	Cholesterol, 5 μmoles	Linoleic acid, 3.5 μmoles + palmitic acid, 3.5 μmoles	6.4	0.32	26.5	1.32
18	Stearic acid, 3.5 μmoles	Cholesterol, 5 μmoles + linoleic acid, 3.5 μmoles	4.6	0.16	18.9	0.66
19	Linoleic acid, 3.5 μmoles	Cholesterol, 5 μmoles + stearic acid, 3.5 μmoles	8.1	0.27	27.8	0.97
20	Cholesterol, 5 μmoles	Linoleic acid, 3.5 μmoles + stearic acid, 3.5 μmoles	9.2	0.46	34.7	1.73

* Each value is the average of duplicate observations.

TABLE 3 FATTY ACID SPECIFICITY FOR CHOLESTEROL ESTERIFICATION BY PANCREATIC JUICE AND BY EXTRACTS OF ACETONE POWDER OF SMALL INTESTINES OF RATS IN THE PRESENCE OF EQUIMOLAR MIXTURES OF PALMITIC, OLEIC, AND LINOLEIC ACIDS

Added Substrates		Intestinal Extract		Pancreatic Juice	
C^{14}-Labeled	Unlabeled	% C^{14} Recovered as Cholesterol Esters*	μmoles of Labeled Substrate Converted to Cholesterol Esters	% C^{14} Recovered as Cholesterol Esters*	μmoles of Labeled Substrate Converted to Cholesterol Esters
Palmitic acid, 3.5 μmoles	Oleic acid, 3.5 μmoles + linoleic acid, 3.5 μmoles + cholesterol, 5 μmoles	4.2	0.15	20.0	0.70
Oleic acid, 3.5 μmoles	Palmitic acid, 3.5 μmoles + linoleic acid, 3.5 μmoles + cholesterol, 5 μmoles	7.8	0.27	26.0	0.91
Linoleic acid, 3.5 μmoles	Palmitic acid, 3.5 μmoles + oleic acid, 3.5 μmoles + cholesterol, 5 μmoles	7.7	0.27	21.0	0.73
Cholesterol, 5 μmoles	Palmitic acid, 3.5 μmoles + oleic acid, 3.5 μmoles + linoleic acid, 3.5 μmoles	13.1	0.65	47.0	2.35

* Each value is the average of duplicate observations of three experiments.

Cholesterol esterification with either palmitic or stearic acid by pancreatic juice was considerably higher than that by the intestinal extract, and in the case of pancreatic juice the addition of unsaturated fatty acids increased the esterification with palmitic acid but not with stearic acid. This difference between the pancreas and intestines might be the result of a difference between the enzymes of the two tissues concerned with fatty acid specificity or of the presence in pancreatic juice of a substance capable of solubilizing cholesterol and saturated fatty acids.

It is apparent from our studies, particularly those conducted with intestinal extracts, that in order to determine the relative ability of a given saturated or unsaturated fatty acid to undergo esterification with cholesterol, both saturated and unsaturated fatty acids should be included in the reaction mixture. When the specificity of palmitic, oleic, and linoleic acids for esterification of cholesterol by pancreatic juice and extracts of acetone powders of the small intestine was examined in a mixture containing all three fatty acids, a difference between the two preparations in fatty acid specificity was observed. In the experiments with pancreatic juice, the specificity for the esterification in vitro resembled closely the specificity observed in vivo during absorption into the chyle of rats fed a mixture of these same three fatty acids (2). In both cases a distinct preference for oleic acid to the other two fatty acids has now been demonstrated. In the intestines, on the other hand, esterification with each of the two unsaturated fatty acids was about the same and in both cases was higher than esterification with palmitic acid.

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