Acyl CoA:retinol acyltransferase in rat small intestine: its activity and some properties of the enzymic reaction

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Abstract The present study was conducted to examine whether the intestinal esterification of retinol could be due to a microsomal acyl-CoA transferase. When the 'microsomal fraction' of rat mucosa was incubated with [^{3}H]retinol and palmitoyl-CoA or oleoyl-CoA, [^{3}H]retinyl esters were formed as identified by alumina column chromatography and reverse phase high-pressure liquid chromatography (HPLC). Unlabeled retinol and [1-^{14}C]palmitoyl-CoA yielded retinyl [1-^{14}C]palmitate. The esterifying activity was lost when microsomes were heated at 60°C for 30 min. Only negligible activity was observed without exogenous acyl-CoA while 10–20 μM gave optimum activity provided that 2–5 mg/ml of albumin was present. Replacement of acyl-CoA by palmitate gave no esterification, indicating that the activity was not a reversed hydrolase reaction. Optimum pH was 7.1–7.6 and optimal concentration of retinol was 15 μM. With palmitoyl-CoA, the formation of retinyl ester was 1.00 ± 0.26 nmol · mg protein^{-1} · min^{-1} ($\bar{x} \pm$ SD, n = 4) in rats killed postprandially versus 2.06 ± 0.66 (n = 5) after 36 hr of fasting. Oleoyl-CoA gave lower activity: 0.52 ± 0.14 and 1.41 ± 0.36, respectively. The variation with feeding and fasting was significant ($P < 0.05$) and corresponded to that of the intestinal acyl-CoA:cholesterol acyltransferase (ACAT). Inhibition of retinol esterification was observed with taurocholate and the thiol-blocking agent 5,5′-dithiobis (2-nitrobenzoic acid). The data show that rat intestinal microsomes catalyze the formation of retinyl esters by an acyl-CoA:retinol acyltransferase with several properties in common with ACAT located in the same subcellular fraction.—**Helgerud, P., L. B. Petersen, and K. R. Norum.** Acyl-CoA:retinol acyltransferase in rat small intestine: its activity and some properties of the enzymic reaction. *J. Lipid Res.* 1982. **23:** 609–618.

Supplementary key words retinol esterification • retinol absorption • vitamin A • retinyl esters • intestinal microsomes

It is well established that vitamin A alcohol or retinol[2] absorbed by the intestine is transported via the lymphatic route as retinyl esters, mainly in association with chylomicrons (1–7). The provitamin β-carotene also appears as retinyl esters in human thoracic duct lymph (6) and thoracic or intestinal duct lymph or different animal species (1, 5). The complete conversion of β-carotene into retinyl esters takes place in the wall of the small intestine (1, 8, 9). Catalyzed by a soluble dioxygenase, the molecule is split at the central double bond yielding two molecules of retinal which, like dietary retinal (7), is reduced to retinol by a soluble retinaldehyde reductase (10–12). Furthermore, it is well documented that dietary retinyl esters are hydrolyzed in the intestinal lumen prior to absorption (2, 13, 14). Whether rats were given retinol or different retinyl esters, Mahadevan, Seshadri Sastry, and Ganguly (13) found that retinyl palmitate appeared in the intestinal mucosa. Present knowledge on different aspects of vitamin A absorption therefore strongly suggests that the esterification of retinol, whether absorbed or formed from β-carotene, takes place within the mucosal cell (15). The apolarity of retinyl esters and the negligible exchange between chylomicrons and other lipoproteins (16) also suggest that they are incorporated into the core of the chylomicrons prior to their secretion from the mucosal cell. However, few data exist on this matter. Preparations of human pancreatic carboxyl ester hydrolase (17), acetone powders of rat pancreas (18), and preparations of rat small intestine (19, 20) have retinol esterifying activities that are independent of CoA. The intestinal formation of retinyl esters have, therefore, been interpreted as a reversed esterase or hydrolase reaction (21) similar to that described for intestinal cholesteryl ester formation (22). The physiological significance of this reaction has, however, been questioned (5, 17). No fatty acid specificity could be observed whereas the composition of lymph retinyl esters is remarkably constant with predominantly saturated esters (5, 6). Furthermore, intestinal esterifications are usually catalyzed by acyl-CoA transferases (23).

Abbreviations: HPLC, high-pressure liquid chromatography; DTNB, 5,5′-dithiobis (2-nitrobenzoic acid); DTT, dithiothreitol; DPPD, N,N′-diphenyl-p-phenylenediamine; CPT, carnitine palmitoyltransferase (EC 2.3.1.21); ACAT, acyl-CoA:cholesterol acyltransferase (EC 2.3.1.26); ARAT, acyl-CoA:retinol acyltransferase (Ec 2.3.1. . . .).

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[2] The terms retinol, retinal, and retinyl ester refer, respectively, to vitamin A alcohol, aldehyde, and ester.

Acyl-CoA:cholesterol acyltransferase (EC 2.3.1.26, ACAT. For review, see ref. 24) plays a major role in the intracellular formation of cholesteryl ester. Attempts to detect intestinal ACAT activity were long unsuccessful (25, 26). After its demonstration in our laboratory (27), the physiological importance of intestinal ACAT has been strengthened by its high activity in different species including humans (28–32).

With this background it was tempting to assume that the intestinal esterification of retinol could be due to the same or a similar enzyme. The current report describes the experiments performed in order to detect the presence of acyl-CoA:retinol acyltransferase (ARAT) activity in the microsomal fraction of mucosa from rat small intestine. The optimal assay conditions and some properties of the enzyme have been studied.

MATERIALS AND METHODS

Chemicals

[1-^{3}H(N)]Vitamin A_1 (all *trans*) with specific radioactivity 5.0 Ci/mmol was purchased from New England Nuclear, Boston, MA. It was stored in ethanol under a nitrogen atmosphere and purified, if necessary, by high-pressure liquid chromatography (HPLC) prior to use (see below). The [^{3}H]retinol was then recovered in methanol and was stored in the same solvent until used. Normally only minor impurities were found, with usually less than 0.5% of the radioactivity recovered in the retinyl ester fraction. The same level of radioactivity in the retinyl ester fraction was found using alumina column chromatography or thin-layer chromatography on silica gel as described below.

During the first part of this study, retinol was prepared from crystalline vitamin A acetate (Hoffmann-La Roche & Co. Ltd., Basel, Switzerland) by saponification in alkaline ethanol and extraction with diethyl ether free of peroxides (33). The extract was taken to dryness in vacuo and redissolved in ethanol. The concentration was calculated on the basis of the absorbance measured (Cary 219 Spectrophotometer, Varian Associates, Inc., Palo Alto, CA) at 328 nm using $E^{1\%}_{1\,cm}$ 1780 (21). The absorption spectrum was characteristic of retinol with maximum about 325 nm (3, 34) and the purity was high when tested by HPLC where one peak was eluted with retention time corresponding to that of [^{3}H]retinol. Crystalline retinol (synthetic, *trans*) was later purchased from Sigma Chemical Co., St. Louis, MO, dissolved in ethanol, and tested correspondingly. Labeled and unlabeled retinol were stored cold in the dark under an atmosphere of nitrogen.

Vitamin A palmitate from Hoffmann-La Roche & Co., Ltd. was dissolved in methanol when used as standard in chromatography.

[1-^{14}C]Palmitoyl-CoA (55 mCi/mmol) and [1-^{14}C]oleoyl-CoA (60 mCi/mmol) were from New England Nuclear, Boston, MA. Palmitoyl-CoA and oleoyl-CoA, Li-CoA, bovine serum albumin (essentially fatty acid-free), DTT, and DTNB were from Sigma Chemical Co. The solutions of acyl-CoA and albumin were prepared and stored as previously described (31). DPPD was purchased from Eastman Kodak Co., Rochester, NY and dissolved in ethanol (0.4 mg/ml) before use as antioxidant. Sodium taurocholate was from Koch-Light Laboratories Ltd., Colnbrook, England. Palmitoyl-carnitine was kindly donated by Prof. Jon Bremer, University of Oslo, Norway. CPT (Ec 2.3.1.21) with a specific activity of about 0.2 U/mg of protein was prepared from calf liver mitochondria (35). All other chemicals used were standard commercial high purity materials.

High-pressure liquid chromatography (HPLC)

All HPLC was performed on a Waters M-45 high pressure liquid instrument (Waters Associates, Inc., Milford, MA) fitted with a Rheodyne injector (loop 100 μl), a Spherisorb 5 μm ODS column (4.6 × 250 mm), and an ultraviolet detector at 280 nm (Dual path monitor UV-2, Pharmacia Fine Chemicals AB, Uppsala, Sweden). The samples to be injected were dissolved in 100% methanol and eluted with the same solvent at ambient temperature and a flow rate of 1 ml/min at a pressure of about 900 psi. Fractions of 1 ml were collected in small counting vials and, after addition of 4.5 ml liquid scintillator (Insta-Gel II), the radioactivity was quantitated as described below.

When [^{3}H]retinol was injected, the radioactivity was recovered in fractions 5 and 6 corresponding to the absorption peak of unlabeled retinol with retention time about 4.6 min. Unlabeled retinyl palmitate was eluted in fractions 14 to 16 corresponding to a single absorption peak with retention time about 13.6 min (see Figs. 1 and 2).

Animals

Male Wistar rats, 200 to 350 g, were used. The commercial pelleted diet (Moellesentralen i/s, Oslo, Norway) contained about 5% of the calories as fat, 21% as protein, and the rest as carbohydrates with adequate amounts of minerals and vitamins (vitamin A, 2.7 mg/kg). If not otherwise stated, the animals were killed in the morning (between 8:30 and 9:30) by a blow on the head and exsanguination. The proximal one-third of jejunum was removed, its mucosa was scraped off, and a microsomal fraction was prepared and stored as previously described (28).

Assay for acyl-CoA:retinol acyltransferase (ARAT)

The assay used was based on preformed acyl-CoA to react with exogenous [^{3}H]retinol. All procedures were conducted in subdued lighting. When the [^{3}H]retinol has been purified before use by HPLC, the methanol was evaporated under nitrogen and a small volume of ethanol was used for redissolving. The [^{3}H]retinol was mixed directly in the incubation tubes with unlabeled retinol and DPPD as antioxidant, normally resulting in a total volume of 22 μl of ethanol.

The glass extraction tubes used for incubation were shaken continuously in a water bath at 37°C. The standard incubation volume was 0.5 ml containing potassium phosphate buffer (0.2 M, pH 7.4), 5 to 7.5 nmol of [^{3}H]retinol (50,000 to 100,000 cpm), 0.8 μg of DPPD, 1.25 mg of bovine serum albumin, about 25 μg of microsomal protein as enzyme source, and 10 to 15 nmol of acyl-CoA. The addition of microsomes and acyl-CoA started the reaction after a 5-min preincubation and it was terminated after 15 min by the addition of 10 ml of chloroform–methanol 2:1 (v/v). After extraction of lipids according to Folch, Lees, and Sloane Stanley (36), the upper phase was discarded and the chloroform was evaporated under N_2. The residue was dissolved in 0.5 ml of hexane and applied to columns (1.2 g) of alumina (Woelm N-Super 1, Woelm Pharma, Eschwege, Germany) deactivated with 10% water. This was followed by two 0.5-ml rinses with hexane before the retinyl ester was eluted directly into counting vials with 10 ml of 2% diethyl ether in hexane; whereafter unreacted retinol was eluted with 10 ml of 50% ether in hexane. The procedure was a modification of the methods described by Harrison, Smith, and Goodman (37) and Ross and Zilversmit (16). When the efficiency of the separation was tested in preliminary experiments based on [^{3}H]retinol (7.5 nmol), less than 0.5% was eluted in the retinyl ester fraction and about 96% in the retinol fraction, giving total recovery of more than 96%. After evaporation of the hexane–ether under N_2, the residue in each counting vial was dissolved in 5 ml of Insta-Gel II (Packard Instrument Co., Inc., Downers Grove, IL) and the radioactivity was quantitated in a Packard Tri-Carb liquid scintillation spectrometer, model 3385 (Packard Instrument Co.). The immediate result obtained was percentage retinyl ester formed during the incubation, but the enzyme activity was then calculated as nmol retinyl ester formed · mg microsomal protein^{-1} · min^{-1}.

The overall recovery of radioactivity was about 80% of the initial amount applied. Controls were initially run without enzyme, but later and in all essential experiments, heat-inactivated microsomes (60°C for 30 min) were used and the values observed for activity were subtracted from those of the test samples. In both types of controls the radioactivity recovered in the retinyl ester fraction was consistently low and usually less than 1% of the total radioactivity recovered.

Thin-layer chromatography

Lipid extracts of incubation mixtures dissolved in hexane were, in some experiments, subjected to thin-layer chromatography (TLC) on silica gel G (type 60 according to Stahl, E. Merck, Darmstadt, Germany) using light petroleum (b.p. 60–70°C)–diethylether 85:15 (by vol) as ascending solvent (38). Each lane was divided into nine fractions, scraped into counting vials and assayed for radioactivity as described. In this chromatography system retinyl palmitate was recovered in the front fraction and retinol in fractions 8 and 9 as visualized by spraying with $SbCl_3$-reagent according to Carr-Price (3).

Assay for acyl-CoA:cholesterol acyltransferase (ACAT)

The activity was tested as previously described using endogenous cholesterol of the microsomal fraction and exogenous [1-^{14}C]oleoyl-CoA as reactants (28). To reduce procedural losses, the incubations were performed in the glass extraction tubes afterwards used for lipid extraction. The incubation mixture of 0.5 ml potassium phosphate buffer (0.2 M, pH 7.4) contained 120 μg microsomal protein, 5 mg bovine serum albumin, and 16 to 17 nmol of [1-^{14}C]oleoyl-CoA. Incubation time was 2 min and the enzyme activity was calculated as nmol cholesteryl ester formed · mg microsomal protein^{-1} · min^{-1}.

Chemical analyses

The protein determination was according to Lowry et al. (39) using bovine serum albumin as standard.

Statistics

Statistical significance between groups was determined by Wilcoxon's rank sum test (40).

RESULTS

Demonstration of ARAT activity

The initial experiments were performed with trace amounts of [^{3}H]retinol in incubation mixtures otherwise optimal for the assay of intestinal ACAT (28). When the lipid extracts of these incubations were separated by alumina column chromatography (see Methods), significant amounts of radioactivity was eluted in the fraction corresponding to retinyl ester, whereas control incubations without microsomes or with heat-inactivated microsomes gave only about 0.5% radioactivity in the same fraction.

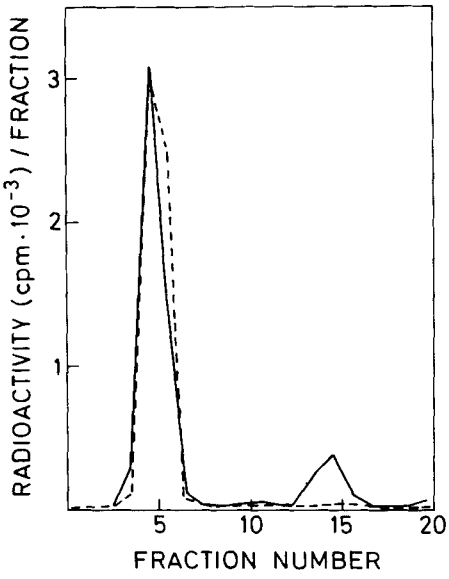

Fig. 1. Enzymatic formation of [^{3}H]retinyl ester from [^{3}H]retinol and palmitoyl-CoA by rat intestinal microsomes. The incubation mixtures contained 120 μg of normal (——) or heat-inactivated (-----) microsomal protein, about 1 nmol of [^{3}H]retinol, and 15 nmol of palmitoyl-CoA. The incubation conditions and procedure were otherwise as described under Methods except that the incubation time was 4 min. The lipid extracts of the incubation mixtures were dissolved in 200 μl of methanol (100%) and aliquots of 20 μl were subjected to high-pressure liquid chromatography on a Spherisorb ODS column and identified as described under Methods. The radioactivity in fractions 5 and 6 (the main peaks) corresponded to retinol, and in fractions 14 and 15 (the small peak) to retinyl palmitate.

In preliminary experiments the lipid extracts of incubation mixtures were also separated by thin-layer chromatography. From incubations performed with active microsomes, radioactivity was recovered corresponding to authentic retinyl ester, whereas very little radioactivity was found in the same band when controls (without microsomes) or [^{3}H]retinol itself was subjected to the same chromatography. When the retinyl ester (front) fraction from test samples was saponified in alkaline ethanol followed by a second thin-layer chromatography, the radioactivity migrated as [^{3}H]retinol.

Fig. 1 illustrates the distribution of radioactivity when the lipid extracts of the incubation mixtures were subjected to HPLC. With heat-inactivated microsomes, all radioactivity was recovered in fractions 5 and 6, corresponding to [^{3}H]retinol. With intact microsomes, however, radioactivity also appeared in fractions 14 and 15 corresponding to retinyl palmitate. When palmitoyl-CoA was omitted from the incubation mixture (not shown), no peak of radioactivity was recovered corresponding to retinyl ester, suggesting that the esterification was due to an acyl-CoA:retinol acyltransferase, and that little endogenous acyl-CoA was present.

Fig. 2 shows the result after HPLC when [1-^{14}C]palmitoyl-CoA was used as the labeled substrate. With microsomes in the incubation mixture, radioactivity was again recovered in fractions 14 and 15 corresponding to retinyl palmitate (panel A), while no radioactivity was found in these fractions when the microsomes were heat-inactivated (panel B) or when retinol was omitted from the incubation mixture (panel C). This showed that the product comigrating with authentic retinyl palmitate was indeed retinyl [1-^{14}C]palmitate since it was formed from unlabeled retinol and [1-^{14}C]palmitoyl-CoA.

Taken together the data so far proved the enzymatic nature of the retinyl ester formation and also indicated that the activity was completely dependent on exogenous acyl-CoA. However, since the microsomal fraction of rat small intestinal mucosa contains an active acyl-CoA hydrolase (28), it could be that the palmitate formed during incubation was the actual substrate. No effort was made to measure the acyl-CoA hydrolase activity during our ARAT assay but, due to its similarity with the ACAT assay, it was likely that the hydrolase was active. Thus the possibility existed that most of the polar material eluted by HPLC in Fig. 2 was [1-^{14}C]palmitate.

In order to evaluate the nature of the retinol esterifying enzyme, it was therefore necessary to test whether the substrate was free fatty acid itself or the CoA derivative. As shown in **Table 1,** a replacement of palmitoyl-CoA by approximately equimolar (2.5–20 μM) or substan-

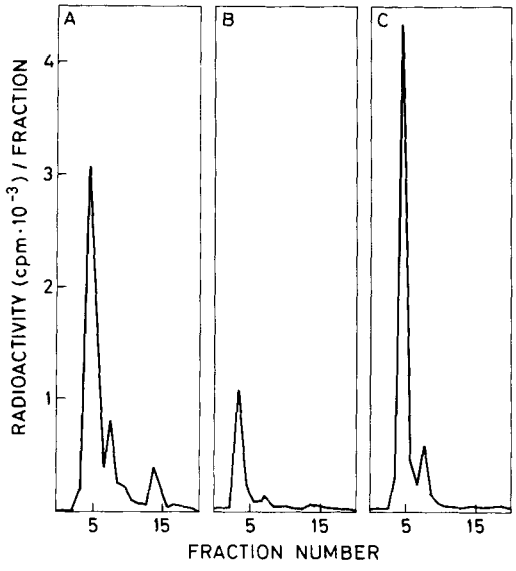

Fig. 2. A, Formation of retinyl [1-^{14}C]palmitate from retinol and [1-^{14}C]palmitoyl-CoA by rat intestinal microsomes. The incubation mixture contained 50 μg of microsomal protein, 3 nmol of retinol, and 5.4 nmol of [1-^{14}C]palmitoyl-CoA (55 mCi/mmol). Conditions and procedure were otherwise as described under Methods except that the incubation time was 10 min. The lipid extract was dissolved in 400 μl of 100% methanol and an aliquot of 25 μl was subjected to HPLC as described under Methods. As in Fig. 1, fractions 14 and 15 corresponded to authentic retinyl palmitate detected by absorption at 280 nm. B, Control with heat-inactivated microsomes. C, Control without retinol.

TABLE 1. [^{3}H]Retinol esterification with rat intestinal microsomes and the dependency on activated fatty acid

Incubation System	Omissions	Additions	[^{3}H]Retinyl Ester Formed[a]
Complete[b]	None	None	1.07
Complete	Palmitoyl-CoA	None	0
Complete	Palmitoyl-CoA	Palmitate (2.5, 5, 10, 20 or 200 μM, respectively)	0.03–0.07
Complete	Palmitoyl-CoA	Palmitoyl-carnitine (20 or 50 μM), CPT (9 mU), DTT (5 mM), and CoA (0.4 mM)	1.14–1.08

[a] Values given (nmol · mg microsomal protein^{-1} · min^{-1}) are based on closely agreeing duplicate determinations.

[b] The complete incubation system contained 7.6 nmol of [^{3}H]retinol, 56.7 μg of microsomal protein, 10 nmol of palmitoyl-CoA (that is 20 μM), 1.25 mg of bovine serum albumin, and 0.8 μg of DPPD. When palmitoyl-CoA was replaced by palmitate, the addition of palmitate coated on albumin marked the starting point of the incubation. When the preformed palmitoyl-CoA was replaced by a palmitoyl-CoA generating system the incubations were started by CoA. The final volume of all incubations was 0.5 ml; the "preincubation" and incubation times were 5 and 10 min, respectively. The assay conditions and procedure were otherwise as described under Methods, with alumina column chromatography for the isolation of retinyl ester.

tially higher (200 μM) concentration of palmitate resulted in practically no retinyl ester formation. However, when microsomes were incubated with an acyl-CoA generating system based on palmitoyl-carnitine, CoA and carnitine palmitoyltransferase (EC. 2.3.1.21)(27, 28, 30), retinyl ester formation took place at a rate equivalent to that observed with preformed palmitoyl-CoA. This conclusively proved that the enzyme was an acyl-CoA:retinol acyltransferase and not a retinyl ester hydrolase.

Optimal conditions for the acyl-CoA:retinol acyltransferase (ARAT) assay

Due to its simplicity and reliability when controlled by HPLC, the alumina column chromatography for separation of retinyl ester and retinol was used in the experiments designed to find optimal conditions for the enzyme assay. As shown in **Fig. 3A,** the rate of retinyl ester formation was linear with time for the 30 min tested. When incubations were carried out for 15 min, a linear relationship was found between the esterifying activity and microsomal protein up to 120 μg per incubation (Fig. 3B).

The assay for intestinal ACAT depends strongly on the concentration of bovine serum albumin (28, 31). Fig. 3C shows that the same is true for ARAT. Without albumin present in the incubation mixture, no esterification took place, while the activity was maximal with concentrations from 2 to 5 mg/ml. Higher concentrations of albumin reduced the enzyme activity. The shape of the curve was similar also when the concentration of oleoyl-CoA used was 18 and 54 μM (data not shown).

The optimal pH for esterification of retinol ranged from 7.1 to 7.6 (data not shown), which was comparable to the pH optimum found for intestinal ACAT activity (28, 31).

Fig. 4 (upper curve) shows that the enzyme activity increased linearly with increasing concentrations of [^{3}H]retinol up to about 10 μM (5 nmol per incubation).

Since retinol was added in ethanol, the inhibition observed at higher concentrations of retinol might have been due to the concomitant increase in ethanol concentration. To test this a similar experiment was performed with the maximal concentration of ethanol from the preceeding experiment (7.4%) in all incubations (Fig. 4, lower curve). The rate of esterification was then lower at all concentrations tested suggesting that 7.4% of ethanol was inhibiting. However, during all standard incubations, the final concentration of ethanol was 4.4%.

Fig. 5 illustrates the importance of oleoyl-CoA and palmitoyl-CoA for the enzyme activity. Both gave pronounced increase in the rate of retinol esterification with optimum reached at the concentrations of 10 and 20 μM, respectively. More than 30 μM of palmitoyl-CoA gave a marked inhibition. The maximal rate of retinyl ester formation was higher with palmitoyl-CoA than with oleoyl-CoA as acyl donor (see below). Without exogenous acyl-CoA small amounts of retinyl esters were still formed, but at a very low rate. Such "basal activity" was a consistent finding in most microsomal preparations, and was easily detected when trace amounts of [^{3}H]retinol with high specific radioactivity was used. This retinol esterification was probably due to endogenous acyl-CoA in the microsomal preparations.

Inhibition of retinol esterification by DTNB and taurocholate

Fig. 6A shows that the esterification of retinol was inhibited by DTNB; a concentration of 1 mM gave almost complete inhibition.

The retinol esterification was also inhibited by taurocholate (Fig. 6B). The reaction was completely inhibited at a concentration of 10 mM and to more than 50% by 1 mM. Both DTNB and taurocholate have previously been found inhibiting to intestinal ACAT from rats in the same concentration ranges (28).

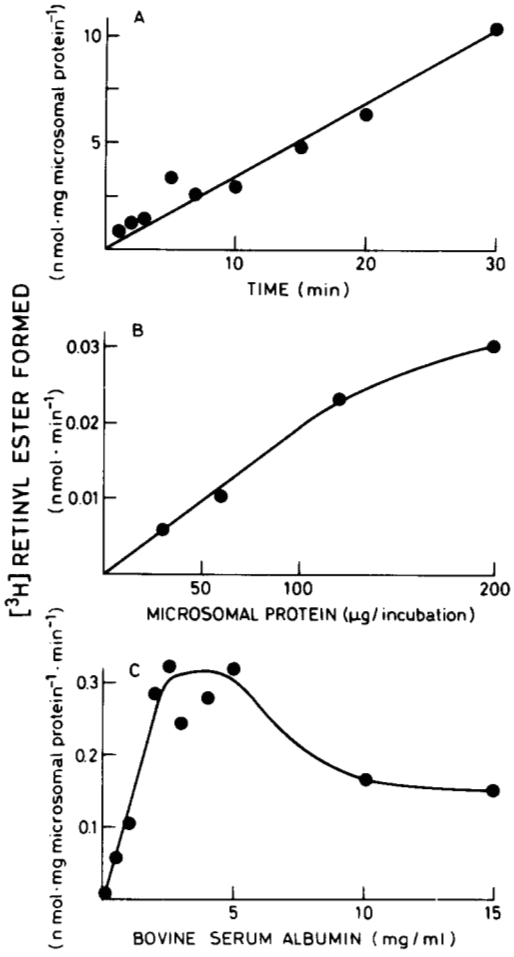

Fig. 3. The effect of incubation time, A; microsomal protein concentration, B; and bovine serum albumin concentration, C; on the formation of [^{3}H]retinyl ester by rat intestinal microsomes. Except for the variations shown, the incubation time was 15 min and the incubation mixtures contained 15 nmol of oleoyl-CoA, 6.5–6.9 nmol of [^{3}H]retinol, 25 µg of microsomal protein, and 1.25 mg of bovine serum albumin. The incubation conditions were otherwise as described under Methods with isolation of retinyl ester by alumina column chromatography. Each point in panels A and C is from a single determination, those of panel B are from closely agreeing duplicates. All values for enzyme activity have been corrected by subtraction of activities found in controls with heat-inactivated microsomes.

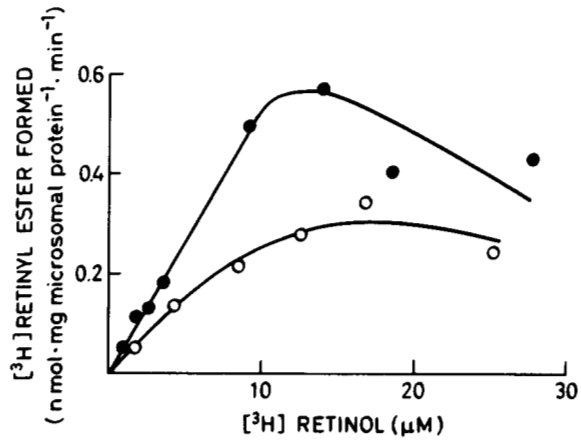

Fig. 4. The effect of [^{3}H]retinol concentration on the formation of [^{3}H]retinyl ester. The increase in total [^{3}H]retinol concentration was obtained by addition of increasing amounts of unlabeled retinol dissolved in ethanol to incubation mixtures containing the same amount of tracer. The result of two separate experiments is shown. In the upper curve (● —— ●) no correction was made for the concomitant increase in total concentration of ethanol from 1.4% at the lowest to 7.4% at the highest concentration of [^{3}H]retinol tested. In the lower curve (○ —— ○) the concentration of ethanol was 7.4% in all incubations. The reactions were started with 15 nmol of oleoyl-CoA and the conditions were otherwise as described in Methods.

Variation in acyl-CoA:retinol acyltransferase (ARAT) activity

After the optimal conditions for the ARAT assay were defined, the level of activity was tested in rats with a normal supply of vitamin A (**Table 2**). The activity of intestinal ACAT activity increases with fasting,[3] and the rate of retinol esterification was therefore tested in one group of animals killed postprandially (A) and another after prolonged fasting (B). Furthermore, since the data from Fig. 5 suggested that the ARAT activity was higher with palmitoyl-CoA instead of oleoyl-CoA, both acyl-CoA's were used.

The difference in ACAT activity between groups A and B (Table 2) was statistically significant and confirmed our previous data. The same was also true for ARAT activity both when oleoyl-CoA and when palmitoyl-CoA were used as substrate ($P < 0.05$). The ratios between the high fasting and low postprandial ARAT activities were about 2.7 and 2.1 for oleoyl-CoA and palmitoyl-CoA, respectively, as compared to 1.8 for ACAT using oleoyl-CoA as substrate.

Based on oleoyl-CoA as acyl-donor, the activity of ACAT and ARAT was almost identical in fasting rats (B) while a small difference was noted in rats killed postprandially (A). Thus, for the nine rats examined,

[3] Helgerud, P., R. Haugen, and K. R. Norum. Unpublished results; submitted for publication.

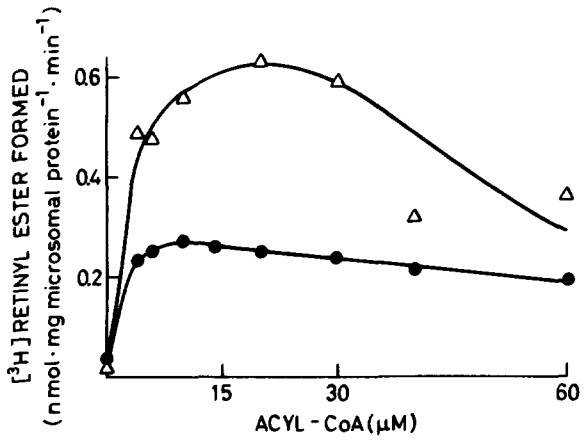

Fig. 5. Effect of acyl-CoA on the rate of [^{3}H]retinyl ester formation. In two different experiments the same microsomal preparation was tested with oleoyl-CoA (● —— ●) and palmitoyl-CoA (△ —— △) in the concentration range shown. The incubation conditions and procedure were otherwise as described in Methods.

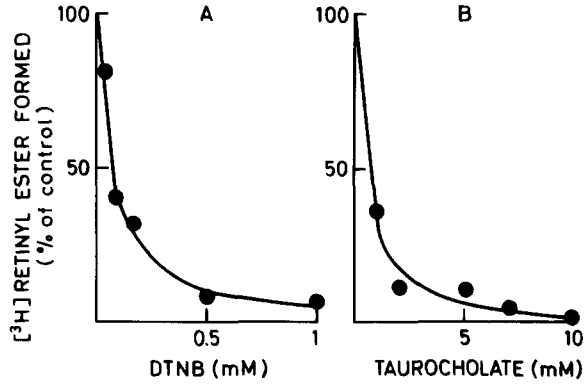

Fig. 6. Effect of 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB), A; and taurocholate, B; on the rate of [^{3}H]retinol esterification. The inhibitors were dissolved in potassium phosphate buffer and added together with the microsomes. After preincubation for 5 min, the reactions were started by 15 nmol oleoyl-CoA. The conditions were otherwise as described in Methods.

a high correlation was found between ACAT and ARAT activity ($r = 0.95$, $P < 0.001$). It was also evident that the rate of retinol esterification was higher with palmitoyl-CoA than with oleoyl-CoA. For the nine rats tested the mean ratio was 1.68 and the values were highly correlated ($r = 0.89$).

DISCUSSION

Numerous investigations have established that the endoplasmic reticulum is the site for intestinal synthesis of triacylglycerols, glycerophospholipids, and cholesteryl esters (23, 27–32). These esterifications are generally catalyzed by acyl-CoA transferases using acyl-CoA's formed by a long-chain fatty acid:CoA ligase (EC 6.2.1.3) in the same subcellular fraction (23). Thus, if intestinal esterification of retinol was due to an acyl-CoA transferase, it would most likely have a microsomal location. This work was, therefore, mainly based on microsomal preparations. Although other subcellular fractions were not tested, the activity found in microsomes compared to whole homogenates (Table 2) suggested that the enzyme studied actually was of microsomal origin.

The data presented prove that the enzymatic product studied was retinyl ester. Not only was radioactivity from both [^{3}H]retinol and [1-^{14}C]palmitoyl-CoA incorporated, but the product also comigrated with authentic retinyl palmitate by HPLC (Figs. 1 and 2). Reverse phase HPLC for separation of natural retinoids has recently been described and it offers major advantages for high resolution and quantitative recoveries with little or no production of artifacts (41). Its adaptation and use in the present study was therefore a valuable supplement to the use of conventional alumina column chromatography for isolation of retinyl ester.

Furthermore, our findings strongly indicate that the enzymic activity studied was due to an acyl-CoA:retinol acyltransferase and not simply the result of a reversed hydrolase reaction. Thus, practically no esterification was produced by palmitate itself, whereas a marked stimulation was observed when an acyl-CoA generating system or preformed acyl-CoA was used (Table 1, Fig. 5). The low "basal" activity observed with no exogenous acyl-CoA added (Fig. 5) might be explained by a small pool of endogenous acyl-CoA in the microsomal fraction.

In previous reports on retinol esterifying activity in bovine retina (20, 42), bovine and rat intestine (19, 20), and cat liver (43), no requirement could be found for

TABLE 2. Activity of microsomal acyl-CoA transferases from rat jejunum in esterification of cholesterol (ACAT) and retinol (ARAT)

	ACAT	ARAT	
Group	Cholesteryl Oleate	Retinyl Oleate	Retinyl Palmitate
A. After normal night-feeding	0.78 (0.10)	0.52 (0.14)	1.00 (0.26)
B. After fasting for 36 hr	1.43 (0.35)	1.41 (0.36)	2.06 (0.66)
	$P < 0.032$[a]	$P < 0.008$	$P < 0.016$

[a] Statistical significance is based on Wilcoxon's rank sum test.

The ACAT assay with [1-^{14}C]oleoyl-CoA as labeled substrate was as described under Methods. The ARAT assays were performed with 7.5 nmol [^{3}H]retinol as the labeled substrate and 10 nmol of oleoyl-CoA or palmitoyl-CoA. The conditions were otherwise as given under Methods, except that the incubation time was 10 min. The enzyme activities are given as nmol ester formed · mg microsomal protein^{-1} · min^{-1}. Values are mean (SD) of four rats in group A and five rats in group B. The determinations in each rat were closely agreeing duplicates.

either ATP and CoA, palmitoyl-CoA alone, or ATP, CoA and fatty acids. In a recent abstract, however, it was stated that retinol esterification by microsomes from lactating rat mammary gland was stimulated substantially by the addition of ATP plus CoASH or palmitoyl-CoA, suggesting that the reaction was due to a fatty acyl-CoA:retinol acyltransferase (44). In the earlier attempts to detect acyl-CoA dependency, the incubations were apparently performed without albumin present. This might have been one reason for the failure to discover in vitro stimulation by the exogenous acyl-CoA, at least in the intestine as shown in this report (Fig. 3C).

Several unrelated enzymes (45–48) including ACAT (49) are inhibited in vitro by long-chain acyl-CoA's at concentrations above their critical micellar concentration (CMC) (50). This has mostly been ascribed to a non-specific detergent effect (45, 46) which may be prevented (47) or even reversed (48, 49) by albumin due to its acyl-CoA binding capacity. The molar ratio of acyl-CoA to albumin may therefore be critical for the activity of the enzyme as shown for rat liver ACAT (49). Correspondingly, at a given concentration of acyl-CoA above its CMC, albumin apparently stimulates the in vitro activity as shown for intestinal ACAT (28, 31). The most simple explanation for the absolute dependency on albumin for in vitro ARAT activity (Fig. 3C) might therefore be that the 30 μM acyl-CoA used would otherwise have inhibited the enzyme. There is also some evidence that albumin may bind retinol and thereby to some extent increase its stability in aqueous solutions with the hydroxyl group still free and susceptible to metabolic transformation (51).

It is unlikely, however, that albumin plays any physiological role with intracellular enzymes. Most rat tissues including intestinal mucosa contain a cellular retinol-binding protein (CRBP) (52). Although unproved, it is possible that the esterification of retinol occurs while it is still associated with this protein (20).

The esterification of retinol by ARAT was primarily examined with oleoyl-CoA. The predominating acyl group in retinyl esters of rat (5) and human (6) lymph, however, is the palmitate (40 to 60%) with relatively small amounts of oleate (10 to 25%). The activity was therefore also tested with palmitoyl-CoA, and the relative acyl-CoA specificity found for ARAT in vitro (Table 2) corresponds well with the previously published in vivo data. The specific activity of ARAT is about 1 nmol · mg protein^{-1} · min^{-1}, and in the same order of magnitude as that found for rat intestinal ACAT. The rat can absorb 6 mg retinol a day (21), corresponding to about 17 nmol/min, with most of it leaving the intestine as retinyl palmitate (5). The activity of the microsomal ARAT described in the present report can account for all retinyl esters recovered in lymph, suggesting that the enzyme has physiological importance.

In addition to the similarities already discussed, the assays of ARAT and ACAT have several other properties in common. The inhibition of ARAT activity by DTNB (Fig. 6A) suggests that the enzyme is dependent on reduced thiol groups as previously shown for intestinal ACAT (28). Inhibition by the detergent taurocholate is also a common feature of both enzymes (Fig. 6B, and ref. 28, 30, 53), as is the pH optimum. Furthermore, the specific activity of the two enzymes with oleoyl-CoA as the acyl donor was almost the same and highly correlated (r = 0.95). The covariation with feeding and fasting suggests that both enzymes are regulated in the same manner. Taken together, this leads to the possibility that both enzyme activities might be due to the same enzyme. Much more work is needed, however, before one can say whether or not a rat is a cat.

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