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Research Article

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Retinol Esterification by Microsomes from the Mucosa of Human Small Intestine

EVIDENCE FOR ACYL-COENZYME A RETINOL ACYLTRANSFERASE ACTIVITY

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ABSTRACT The mechanism of the intestinal esterification of retinol has been obscure. Recently, an acyl-Coenzyme A (CoA):retinol acyltransferase (ARAT) was found in rat intestinal microsomes, and experiments were therefore conducted to determine whether a corresponding enzyme exists in human small intestine. When microsomes were incubated with [^{3}H]retinol and palmitoyl-CoA, or retinol and [1-^{14}C]palmitoyl-CoA, radioactive retinyl palmitate was formed as identified by alumina column chromatography and reverse-phase high-pressure liquid chromatography. Heating the microsomes for 30 min at 60°C resulted in loss of activity. The esterification was negligible without exogenous acyl-CoA and markedly stimulated by palmitoyl-, oleoyl-, and stearoyl-CoA in concentrations up to 20 μM. The acyl-CoA was successfully replaced by an acyl-CoA generating system, but not by unactivated palmitate (2.5–200 μM). The assay was dependent on the presence of albumin with optimum activity at 2–10 mg/ml. The optimal retinol concentration was 20–30 μM and pH ~7.4. The esterifying activity was completely inhibited by 8 mM of taurocholate and to 90% by 1 mM of 5,5′-dithiobis(2-nitrobenzoic acid). Activity was found throughout the small intestine. In jejunum the rate of retinol esterification was: 3.44±2.24 nmol [^{3}H]retinyl ester formed · mg microsomal protein^{-1} · min^{-1} (mean±SD, n = 12). The corresponding activity in whole homogenates of biopsies were 1.17±0.28 (n = 8). It is concluded that human small intestine contains a microsomal acyl-CoA:retinol acyltransferase. Due to its high activity in vitro this enzyme is likely to be responsible for the intestinal esterification of retinol.

INTRODUCTION

Vitamin A derives from two major sources; retinyl esters of animal origin that are hydrolyzed in the intestinal lumen prior to absorption, and β-carotene from plant tissues that is largely or completely converted to retinol within the mucosal cell. Whatever the source of intramucosal retinol, it is subsequently absorbed into the body via the lymphatic route almost entirely in the form of retinyl esters associated with chylomicrons (1–5). The intestinal esterification of retinol therefore represents an important step in vitamin A metabolism, but nevertheless only limited information exists about its enzymology (1, 2). The esterifications leading to the synthesis of triglycerides and cholesteryl esters for the chylomicron core and to phospholipids for the chylomicron "membrane" are all catalyzed by microsomal acyl-coenzyme A (CoA) transferases using acyl-CoA formed by the intestinal long-chain fatty acid:CoA ligase (EC 6.2.1.3) in the same subcellular fraction (6, 7). By analogy it is likely that the formation of retinyl ester might be due to a corresponding mechanism. Thus, we have recently published evidence for an active acyl-CoA:retinol acyltransferase (ARAT)[1] in microsomes from rat intestinal mucosa (8). Independently, a similar enzyme has been demonstrated by

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[1] *Abbreviations used in this paper*: ACAT, acyl-CoA:cholesterol acyltransferase; ARAT, acyl-CoA:retinol acyltransferase; BSA, bovine serum albumin; CPT, carnitine palmitoyltransferase; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol; HPLC, high-pressure liquid chromatography.

Ross in lactating rat mammary gland (9) and in cat and rat liver (10).

The studies reported here were designed to test whether ARAT activity exists also in human small intestine. If so, this would certainly increase the probability that this enzyme is of physiological importance.

METHODS

Isotopes and chemicals. [1-^{3}H(N)] Vitamin A_1 (all trans) with specific radioactivity 5.0 Ci/mmol (New England Nuclear, Boston, MA) was used without further purification. When tested by high-pressure liquid chromatography (HPLC) <0.5% of the radioactivity comigrated with authentic retinyl ester (see below and ref. 8). Crystalline retinol (synthetic, all trans from Sigma Chemical Co., St. Louis, MO) was dissolved in ethanol and its final concentration was calculated on the basis of the absorbance measured (Cary 219 Spectrophotometer, Varian Associates, Inc., Palo Alto, CA) at 325 nm using $E^{1\%}_{1cm}$ 1780 (2). Both labeled and unlabeled retinol were stored in ethanol at −20°C in the dark under an atmosphere of nitrogen, and all handling was in dim light. The absorption spectrum of the unlabeled retinol (11, 12) was measured at regular intervals during the study and no change was observed. It was also stable when tested by HPLC where one peak was eluted corresponding to that of [^{3}H]retinol (see below).

Vitamin A palmitate (F. Hoffmann-La Roche & Co. Ltd., Basel, Switzerland) was dissolved in methanol and used as standard in HPLC work. [1-^{14}C]Palmitoyl-CoA (55.2 mCi/mmol) was purchased from New England Nuclear, palmitoyl-CoA, oleoyl-CoA, stearoyl-CoA, CoA (lithium salt), bovine serum albumin (BSA, essentially fatty acid free), dithiothreitol (DTT), and 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) from Sigma Chemical Co. The acyl-CoA and albumin were prepared as described (7). *N,N'*-diphenyl-*p*-phenylenediamine (DPPD) from Eastman Kodak Co., Rochester, NY was dissolved in ethanol (0.4 mg/ml) prior to use an antioxidant. Sodium taurocholate was from Koch-Light Laboratories Ltd., Colnbrook, England.

Palmitoyl-carnitine was kindly donated by professor Jon Bremer, University of Oslo, Norway. Carnitine palmitoyltransferase (CPT, EC 2.3.1.21.) with a specific activity of 0.2 U/mg of protein was prepared from calf liver mitochondria (13). All other chemicals were standard commercial high purity materials.

Human material. Some human material used in this study was obtained earlier during a study on intestinal acyl-CoA:cholesterol acyltransferase activity (7). The biopsies and microsomal preparations from proximal duodenum, proximal jejunum, and distal ileum were stored in potassium phosphate buffer (0.2 M, pH 7.4) at −70°C. The oldest preparations used were nearly 3 yr old, but all critical experiments concerning optimization of the ARAT assay and identification procedures were based on microsomes that had been stored for <1 yr. New material from different parts of the small intestine was obtained for this study by resections made at the time of nephrectomy in patients suffering from brain death. Small resectates also derived from jejunum of patients subjected to gastric bypass surgery. From the specimens of proximal jejunum "biopsies" were taken and stored as described above. Whole homogenates of the biopsies were prepared on the day of enzyme assay (14). Microsomes were prepared from "scraped-off" mucosa as previously described (7).

The assay for ARAT activity. All procedures were conducted in dim light and largely as previously described with the standard assay based on production of [^{3}H]retinyl ester from [^{3}H]retinol and preformed acyl-CoA. The [^{3}H]retinol substrate (10 nmol) was a mixture of labeled and unlabeled retinol prepared directly in the glass incubation tubes in the presence of DPPD (0.8 μg) as antioxidant, all constituents added in a total of 17 μl of ethanol. Then 473 μl of potassium phosphate buffer (0.2 M, pH 7.4) with 1.25 mg of BSA and 50 μg of microsomal protein as enzyme source was added. The tubes were then vortexed and transferred to a shaking waterbath (37°C) to warm for 5 min. The incubations were started by the addition of 10 nmol of acyl-CoA giving a final incubation volume of 0.5 ml. Reactions were stopped after 10 min by adding 10 ml of chloroform-methanol 2:1 (vol/vol) and lipids extracted according to Folch et al. (15). The chloroform extract was taken to dryness under nitrogen at room temperature, redissolved in 0.5 ml hexane with vortexing, and applied with two rinses of hexane (1.0 and 0.5 ml) to columns of 1.25 g aluminum oxide (Woelm N-Super 1, Woelm Pharma, Eschwege, West Germany) deactivated with 10% water. The [^{3}H]retinyl esters were then eluted directly into counting vials with 10 ml of hexane-diethyl ether 98:2 (vol/vol), and subsequently the remaining radioactivity from [^{3}H]retinol was eluted with 20 ml of hexane-diethyl ether 50:50 (vol/vol). With this alumina column chromatography leakage of authentic [^{3}H]retinol in the retinyl ester fraction was <0.5% and the recovery of radioactivity was essentially 100%. Following evaporation of the hexane-ether the residues were dissolved in Insta-Gel II (Packard Instrument Co., Inc., Downers Grove, IL) and the radioactivity quantitated in a Packard Tri-Carb liquid scintillation spectrometer, model 3385. Enzyme activity was then calculated as nmol retinyl ester formed · milligram/dl microsomal protein^{-1} · minute^{-1}. All incubations were performed in duplicate with closely agreeing results and the values were corrected for those of control incubations run with heat-inactivated (60°C for 30 min) microsomes. The recovery of radioactivity following the complete assay procedure was consistently >80%.

HPLC. A detailed description of the HPLC system used in this study has been given elsewhere (8). Briefly, the HPLC instrument (Waters M-45, Waters Associates Inc., Milford MA) with a Rheodyne injection system (loop 100 μl, Rheodyne Inc., Cotati, CA) was equipped with a Spherisorb 5 μm ODS column (4.6 mm i.d. × 250 cm) and a spectrophotometer (Dual path monitor UV-2, Pharmacia Fine Chemicals AB, Uppsala, Sweden) working at 280 nm. All samples to be injected were dissolved in 100% methanol and elution (1 ml/min) was by the same solvent at room temperature and a pressure of ~1,000 psi. Fractions of 1 ml were collected directly in small counting vials, and following the addition of 4.5 ml of liquid scintillator (Insta-Gel II) the radioactivity was quantitated.

Chemicals analyses. The protein determination was according to Lowry et al. (16) using BSA as standard.

RESULTS

Demonstration of microsomal ARAT activity

Effects of variation in incubation conditions. The search for ARAT activity was based on microsomal preparations from jejunum and the initial incubations

were performed with palmitoyl-CoA and [^{3}H]retinol as substrates under conditions optimal for rat intestinal ARAT activity (8). From the lipid extracts of these incubations a substantial amount of radioactivity was recovered corresponding to the retinyl ester fraction isolated by alumina column chromatography. From control incubations with heat-inactivated microsomes, however, usually <0.5% was found in the same fraction. This strongly suggested that human small intestine, like the small intestine of rats, contained ARAT activity and our first step aimed at optimization of the assay system. As shown in Fig. 1 the rate of retinyl ester formation was linear with time for 10 min (panel A) and with the amount of microsomal protein in the incubation mixture up to 60 μg (panel B). Maximal rate of esterification was obtained when the retinol concentration was 20–30 μM, whereas 40 μM was slightly inhibitory (panel C). The enzyme activity was highly dependent on the pressure of bovine serum albumin (panel D). Very little esterification was observed when albumin was omitted from the incubation mixture while concentrations from 2 to 10 mg/ml was optimal. When the concentration of retinol was reduced from 20 to 15 μM we found that 10 mg/ml of albumin was inhibitory corresponding to that shown for rat intestinal ARAT activity (8) (data not shown). The highest rate of retinol esterification was at pH 7.4. At pH 6.6 and 8.0 the activity was reduced to ~75 and 50% of maximum, respectively (data not shown).

Without exogenous acyl-CoA insignificant amounts of retinyl esters were formed (Fig. 2), but the rate of esterification increased substantially with increasing concentrations of oleoyl- or palmitoyl-CoA up to 20 μM. No further change in ARAT activity was found when the acyl-CoA concentrations were increased from 20 to 50 μM. The rate of retinol esterification was somewhat higher with palmitoyl-CoA compared with oleoyl-CoA. The effect of increasing stearoyl-CoA concentrations was also tested, but with another microsomal preparation (data not shown). The curve obtained was qualitatively the same as those in Fig. 2 with an optimal rate of esterification using 20–50 μM of stearoyl-CoA. At 20 μM of stearoyl-CoA the ARAT activity was ~10% lower than that obtained with the same microsomes using palmitoyl-CoA.

Identification of the retinyl ester formed. When apparently optimal incubation conditions were established it was necessary to provide evidence that the radioactive product formed during the incubation actually was retinyl ester. Separation of retinyl esters from retinol and its more polar metabolites is best performed by reverse-phase HPLC (8, 17). Subsequent

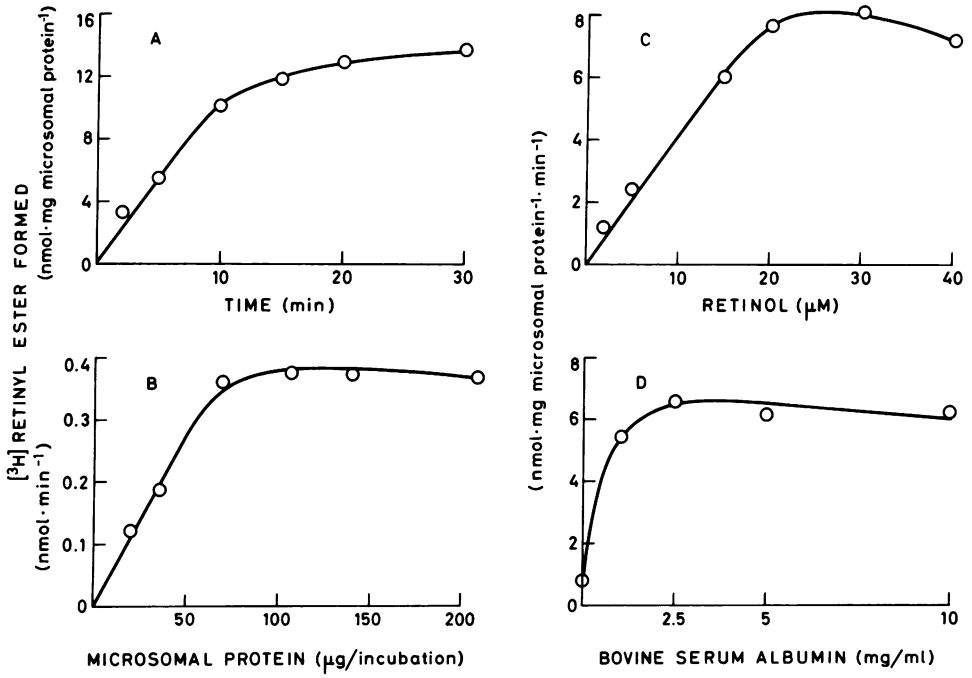

FIGURE 1 The effect of time (A); concentration of microsomal protein (B); retinol (C); and BSA (D) on the [^{3}H]retinyl ester formation by human small intestinal microsomes. Except for the variations shown the incubation conditions and procedures were as described (Methods) with isolation of retinyl ester by alumina column chromatography. Values given are means of duplicate determinations corrected for controls with heat-inactivated microsomes.

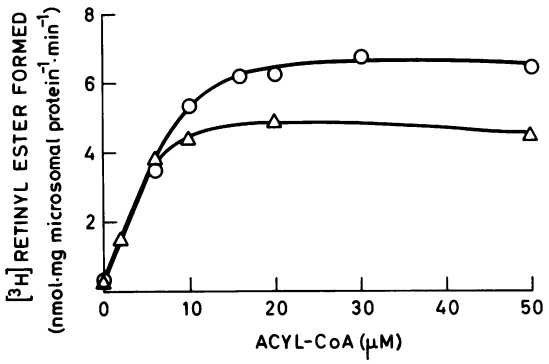

FIGURE 2 The effect of palmitoyl-CoA (O—O) and oleoyl-CoA (Δ—Δ) concentration on the formation of [^{3}H]retinyl ester by the same microsomal preparation. The assay conditions and procedure as described in Methods and in Fig. 1.

incubation aliquots of the lipid extracts were therefore subjected to HPLC as shown in Fig. 3. From incubations based on active microsomes with [^{3}H]retinol and palmitoyl-CoA as substrates, absorption (280 nm) was found corresponding to authentic retinyl palmitate as well as authentic retinol and more polar metabolites. When aliquots from control incubations based on heat-inactivated microsomes were analyzed, however, no absorption was found corresponding to retinyl palmitate, whereas that corresponding to authentic retinol was increased (data not shown).

In similar experiments we also tested the distribution of radioactivity after HPLC (8). From incubations performed with active microsomes radioactivity was recovered in two peaks; one corresponding to [^{3}H]retinol, the other corresponding to retinyl palmitate. When microsomes were heat-inactivated the amount of radioactivity corresponding to retinyl palmitate was very small with more recovered as "[^{3}H]retinol." The same was true for incubations performed without palmitoyl-CoA.

The identification work was further extended by incubations run with radioactivity in the acyl moiety; that is [1-^{14}C]palmitoyl-CoA and unlabeled retinol were used as substrates. Again, with active microsomes radioactivity was recovered corresponding to retinyl palmitate, but not when heat-inactivated microsomes were used or when retinol was omitted from the incubation mixture.

Together, HPLC data showed that retinyl ester formation took place during incubations with intact microsomes, but not after their heat-inactivation. Not only did the product comigrate with authentic retinyl palmitate, but its formation was dependent on the presence of retinol as well as palmitoyl-CoA, and radioactivity from both substrates was incorporated into retinyl ester.

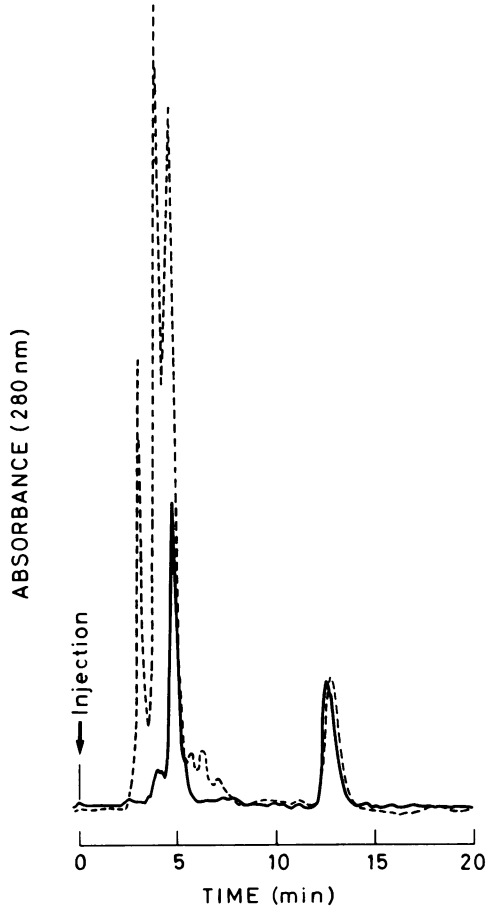

FIGURE 3 Formation of retinyl palmitate from [^{3}H]retinol and palmitoyl-CoA by human small intestinal microsomes. After a standard incubation the lipid extract was dissolved in 400 μl of 100% methanol and an aliquot of 50 μl was subjected to HPLC as described in Methods (- - -). The unbroken line shows the retention times of authentic retinol (4.8 min) and retinyl palmitate (12.6 min).

Dependency on activated fatty acid. Human small-intestinal microsomes contain an active acyl-CoA hydrolase (7). In separate experiments the activity of this enzyme was found to be high also under the incubation conditions optimal for retinol esterification (data not shown). This opened up the possibility that the dependency on acyl-CoA (Fig. 2) was only apparent and that the enzyme activity actually was due to a retinol esterase. It was therefore mandatory to test whether the substrate was the free fatty acid or its CoA ester (8). As shown in Table I, practically no retinyl ester formation was observed when the preformed palmitoyl-CoA (20 μM) was replaced by palmitate (2.5–200 μM). When a palmitoyl-CoA generating system based on palmitoyl-carnitine, CoA, and CPT (EC 2.3.1.21., ref. 14) was used, however, the yield of retinyl ester

TABLE I
[^{3}H]Retinol Esterification with Microsomes from Human Jejunum and Its Dependency on Activated Fatty Acid

Incubation system			[^{3}H]Retinyl ester formed
	Omissions	Additions	
			nmol · mg microsomal protein^{-1} · min^{-1}
Complete*	None	None	4.78
Complete	Palmitoyl-CoA	None	0
Complete	Palmitoyl-CoA	Palmitate‡	0–0.09
Complete	Palmitoyl-CoA	Palmitoyl-CoA generating system I§	4.39
Complete	Palmitoyl-CoA	Palmitoyl-CoA generating system II‖	3.76

* The complete incubation system consisted of 9.68 nmol of [^{3}H]retinol, 49.4 μg of microsomal protein, 1.25 mg of BSA, 0.8 μg of DPPD, and 10 nmol (i.e. 20 μM) of palmitoyl-CoA in 0.5 ml of 0.2 M potassium phosphate buffer, pH 7.4. All incubations were performed in duplicate with isolation of retinyl ester by alumina column chromatography as described in Methods. When performed palmitoyl-CoA was replaced by palmitate or palmitoyl-CoA generating systems the reactions were started with palmitate bound to albumin and CoA, respectively.
‡ Final concentrations 2.5, 5, 10, 20, and 200 μM, respectively.
§ Palmitoyl-carnitine 20 μM, DTT 5 mM, CPT 9 mU, CoA 0.4 mM.
‖ Palmitoyl-carnitine 50 μM, DTT 5 mM, CPT 9 mU, CoA 0.4 mM.

was comparable to that observed with the preformed metabolite. Thus, the enzyme studied was dependent on activated fatty acid and, therefore had to be an ARAT.

Activity and some properties of ARAT

The activity in different parts of the small intestine. Absorption of lipids and lipid soluble vitamins normally takes place in the proximal part of the small intestine. Our results described so far were therefore based on microsomes from jejunum. From one experiment to another some variation was observed in the ARAT activity of each preparation, but within each experiment reproducible results were obtained as to the relative activity of the different preparations. Table II shows that ARAT activity was found throughout the small intestine. It appeared that the activity was higher in microsomes prepared from jejunum than in those from duodenum or ileum. This was also found when the ARAT activity in two individuals was tested with microsomes from all three regions. In another individual the activity in microsomes from duodenum versus jejunum was tested with the same result. However, although these data do suggest a higher activity in jejunum any firm conclusion on the relative importance of the different gut regions will have to await the analysis of more sets of samples taken from separate individuals. Table II also shows the ARAT activity in whole homogenates of biopsies from distal duodenum/proximal jejunum. Representing one-third of that obtained with microsomes from the same region, these data indicated that the activity was of microsomal origin. No further work was done, however, to study the subcellular localization of ARAT activity.

Inhibition of ARAT activity by DTNB and taurocholate. The microsomal ARAT activity in rat small intestine has several properties in common with the intestinal acyl-CoA:cholesterol acyltransferase (ACAT) (8). Thus, DTNB and taurocholate are inhibiting to both enzymes as they are to ACAT activity in human small intestine (7, 14). Correspondingly, when the ef-

TABLE II
Activity of ARAT in Human Small Intestine

	Number of patients	· Retinyl ester formed
		nmol · mg protein^{-1} · min^{-1}
Microsomal preparations		
Duodenum	3	0.73 (0.62)
Jejunum	12	3.44 (2.24)
Ileum	5	1.21 (1.00)
Whole homogenates of biopsies from jejunum	8	1.17 (0.28)

The enzyme assay was performed as described under Methods using [^{3}H]retinol and palmitoyl-CoA as reactants and isolation of the [^{3}H]retinyl ester by alumina column chromatography. Each preparation was analyzed in duplicate with closely agreeing results. Values are given as means (SD). Microsomal preparations and biopsies were stored at −70°C until assay within a few days or maximally up to 3 yr later. Whole homogenates of biopsies were prepared on the day of the ARAT assay.

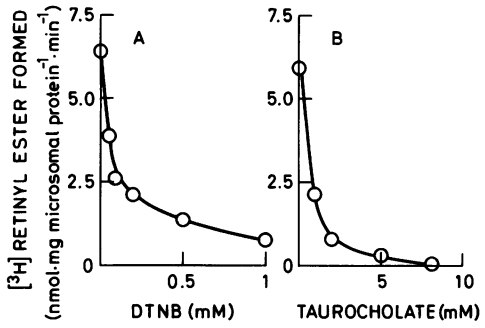

FIGURE 4 Effect of DTNB (panel A) and taurocholate (panel B) on the rate of [^{3}H]retinol esterification. The complete ARAT assay was as described (Methods), except that the inhibitor was added in potassium phosphate buffer before start of preincubation.

fect on human intestinal ARAT activity was tested (Fig. 4) we found 90% inhibition by 1 mM of DTNB (panel A) and complete inhibition by 8 mM of taurocholate (panel B).

DISCUSSION

This study is the first to show retinyl ester synthesis by membrane preparations of human intestinal mucosa. Unequivocal identification of retinyl ester as the reaction product was obtained by the fact that radioactivity from both the retinol and acyl moiety was incorporated and that the product comigrated with authentic retinyl ester by HPLC. The ultimate aim of the study, however, was to determine whether the esterification was catalyzed by an ARAT as recently shown for rat small intestine (8) and thus analogous to the esterification of other lipid alcohols.

The amount of retinyl ester formed with no exogenous sources of acyl-CoA was consistently very low (Fig. 2) and probably due to a small amount of endogenous acyl-CoA as shown by Ross for the corresponding assay with microsomes from rat liver and mammary gland (9, 10). The reaction was highly stimulated by exogenous acyl-CoA whether preformed or generated during the incubation, but not by unactivated fatty acid (Fig. 2, Table I). These data prove that the enzyme studied was in fact an ARAT and not a simple esterase.

Goodman et al. have previously shown that the predominating acyl groups in retinyl ester of rat and human lymph are palmitate, stearate, oleate, and linoleate, in that order (3, 4). This report shows that the in vitro incorporation of activated palmitate, oleate, and stearate into retinyl ester is effectively catalyzed by ARAT. It is not known if the acyl-pattern of lymph retinyl ester in vivo is due to acyl-CoA specificity of the enzyme or simply due to the actual access to acyl-CoA, but our findings in vitro strongly increase the possible physiological role of ARAT. The specific activity of the enzyme was apparently highest in jejunum where most lipid absorption normally takes place (18, 19). Based on the activity in vitro it is evident that this enzyme may account for all retinyl ester formation during absorption. Physiological importance of ARAT is further strengthened by its demonstration in tissues known to be active in retinol esterification; that is until now in rat mammary gland (9), liver (10) and intestine (8) and in this report, human intestine. The enzyme is membrane-bound and the optimal assay conditions are rather similar. In all tissues the activity is strongly inhibited by taurocholate suggesting that micelles may interfere in some way or another. Like other microsomal acyltransferases including the intestinal ACAT (20, 21), ARAT is also inhibited by thiol-blocking agents (Fig. 4A, ref. 8, 10). Since the properties of rat intestinal ARAT correspond closely with those of ACAT, we have previously raised the question whether the intestinal esterification of retinol and cholesterol is due to the same enzyme (8). In human small intestine the activity of ARAT is one order of magnitude higher than that of ACAT (7) but the question is still open and warrants further investigation.

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