Intestinal vitamin A metabolism: coordinate distribution of enzymes and CRBP(II)

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Abstract In the mucosal layer of the small intestine, we found nearly identical gradients of CRBP(II), retinal reductase, and LRAT levels down the duodenal-ileal axis, suggesting coordinate regulation of these three proteins. In all cases the level of binding protein or enzyme activity was greatest in the proximal intestine and then decreased sharply in the distal half. This pattern fits with the known capacity of the intestine to absorb vitamin A. In addition, the retinal reductase activity was found predominantly in the intestinal mucosa, while LRAT activity was found in both the intestinal mucosa and muscle. An even distribution of LRAT activity along the longitudinal axis of the intestinal muscle was consistent with an even distribution of CRBP in that tissue. In conjunction with LRAT activity and CRBP, we found endogenous retinyl ester stores in the intestinal muscle layer. The patterns of retinyl ester produced by LRAT in vitro and found in vivo were similar, with retinyl palmitate predominating and a high percentage comprised of retinyl stearate. We also observed a bile salt-independent retinyl ester hydrolase activity in intestinal muscle whose distribution paralleled the retinyl ester stores and LRAT levels. This hydrolase appears to be distinct from retinyl ester hydrolases described from other organs as its activity was insensitive to retinyl ester chain length, the presence of bile salts, or the addition of apo-CRBP. This activity was inhibited by diethyl-*p*-nitrophenylphosphate (IC_{50} 100 μM) and diethylpyrocarbonate (IC_{50} 10 μM), demonstrating a requirement for active serine and histidine residues. In addition, we describe an activity present in some intestinal microsomal preparations that can perturb determinations of reductase and LRAT activity and must be avoided. **—Herr, F. M., S. A. Wardlaw, B. Kakkad, A. Albrecht, T. C. Quick, and D. E. Ong.** Intestinal vitamin A metabolism: coordinate distribution of enzymes and CRBP(II). *J. Lipid Res.* 1993. **34:** 1545–1554.

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Vitamin A is obtained from the diet either as long-chain retinyl esters in animal tissue or as plant carotenoid pigments, primarily β-carotene. Both undergo metabolism during absorption. The long-chain retinyl esters are hydrolyzed to retinol in the lumen prior to absorption by pancreatic lipases and, perhaps, by a recently described bile salt-dependent retinyl ester hydrolase intrinsic to the brush border membrane of the intestinal villi (1). After entry into the absorptive cell, the retinol is re-esterified with long chain fatty acids. β-Carotene undergoes oxidative cleavage to retinal after entry. The retinal is then reduced to retinol, which is then esterified (2). Both retinol and retinal, essentially water-insoluble, are bound in the enterocyte to a member of the family of retinoid-binding proteins, cellular retinol-binding protein type II [CRBP(II)] (3). Intestinal CRBP(II) comprises about 1% of the total soluble protein of the jejunal mucosa and is found only in the villus-associated enterocytes (4, 5). CRBP(II) directs the metabolism of vitamin A by permitting the reduction of retinal by a microsomal reductase able to utilize either NADH or NADPH as its cofactor (6) and then presenting the retinol to a microsomal esterifying enzyme, lecithin:retinol acyltransferase (LRAT) (3, 7). The retinol is sequestered from a second intestinal activity that is capable of esterifying free retinol in vitro, acyl CoA:retinol acyltransferase (ARAT) (7, 8).

LRAT transfers the fatty acid from position 1 of phosphatidylcholine to retinol, and the product esters are subsequently incorporated into chylomicrons for export from the gut (9). LRAT is also found in other organs where it esterifies retinol when bound to a different intracellular binding protein, cellular retinol-binding protein (CRBP), with the resulting retinyl esters serving varied roles (10–14). In the retinal pigment epithelium, LRAT-catalyzed esterification is an essential step in the isomeri-

Abbreviations: CRBP, cellular retinol-binding protein; CRBP(II), cellular retinol-binding protein type II; LRAT, lecithin:retinol acyltransferase; DFP, diisopropyl fluorophosphate; DTT, dithiothreitol; DMSO, dimethylsulfoxide; BHT, buytlated hydroxytoluene; BSA, bovine serum albumin; DENP, diethyl-*p*-nitrophenylphosphate.

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zation of all-*trans*-retinoid to 11-*cis*-retinoid (15). Retinyl esters produced by LRAT in the liver are believed to represent the primary site of whole body vitamin A storage (2). In addition, endogenous retinyl ester stores have been detected in other tissues including lung, kidney, testis, and whole intestine (16). Bile salt-independent ester hydrolase activities, necessary for the mobilization of those esters, have also been detected in nonhepatic tissues such as lung, kidney, testis, and intestinal mucosa (17).

It has been demonstrated that the proximal intestine has a higher capacity to absorb vitamin A (18, 19). While some information has been obtained indicating that CRBP(II), retinal reductase, and LRAT are present at higher levels in the proximal small intestine (4, 6, 7, 20), no systematic comparison of the distribution of these three components has been undertaken. In the present study we compared the distribution of these proteins along the longitudinal axis of the small intestine for both the mucosal and muscular layers. Activities necessary for the processing of dietary sources of vitamin A would be expected to be present in the mucosal layer, while activities present in the muscle would be involved only in the processing of blood-borne forms. A similar distribution of these three components in the mucosal layer would be expected if they indeed are involved in vitamin A processing. Because we believe ARAT is not normally involved in esterifying vitamin A, based on its inability to esterify retinol sequestered by binding protein (7, 21, 22) and its apparent inability to esterify retinol in cultured cells (14, 23), we chose not to examine its distribution.

Here we report that the gradient of expression of CRBP(II), retinal reductase, and LRAT in the mucosa down the length of the small intestine was coordinate and consistent with the known ability of the intestine to process vitamin A. We also observed significant LRAT activity, endogenous ester stores, and a bile salt-independent retinyl ester hydrolase activity within the intestinal muscular layer, which demonstrates the capability for storage and mobilization of vitamin A in that tissue. In addition, we describe necessary precautions in the preparation of intestinal samples.

EXPERIMENTAL PROCEDURES

Materials

Intestine obtained from adult Sprague-Dawley rats (Sasco, St. Louis, MO) was used for the bulk of the experimental work presented here. Intestine from Sprague-Dawley rats obtained from Harlan Industries was used for comparison only. All animals were fed normal rat chow except when fasting is indicated.

Apo-CRBP(II) was purified from an *Escherichia coli* strain (JM103) transfected with the pMON-CRBP(II) plasmid (24). The cells were grown overnight at 37°C in Luria broth containing 100 μg/ml ampicillin. The cells were diluted and cultured at 37°C until an A_{600} of 0.5–0.6 was obtained, at which time naladixic acid was added in 0.1 N NaOH to a final concentration of 50 μg/ml. The cells were incubated 3 h, then harvested by centrifugation at 5000 *g* for 30 min, and resuspended in 10 mM Tris, 1 mM EDTA, 100 mM KCl, pH 8.3. The suspension was sonicated and centrifuged at 32000 *g* for 30 min. The supernatant liquid was filtered through Whatman #4 paper and concentrated by ultrafiltration with an Amicon YM 3 membrane to approximately 40 ml. The concentrate was applied to an 87 × 5 cm Sephadex G-75 column equilibrated in 0.008 M imidazole acetate, pH 6.6, with 1 mM β-mercaptoethanol at a flow rate of 1.5 ml/min. The fractions were monitored for their ability to bind retinol. Fractions containing CRBP(II) were pooled and concentrated by ultrafiltration to less than 5 ml. The concentrated material was applied to an LKB HPLC system equipped with a Tosohaas DEAE 5PW column (15 × 20 cm) with guard column, equilibrated in 0.008 M imidazole acetate, pH 6.6, with 1 mM β-mercaptoethanol, at a flow rate of 3 ml/min and was eluted by a gradient from 0.008 M imidazole acetate to 0.054 M imidazole acetate with 1 mM β-mercaptoethanol. This gradient resolved two forms of CRBP(II) differing only in their N-terminal processing. The first form, eluting at 21 min, retained the initial N-terminal methionine, while the second, eluting at 29 min, had been cleaved to N-terminal threonine as has been described previously (25). As the first form was more abundant and behaved identically to the second in enzyme assays, it was used for the preparation of the [^{3}H]retinol-CRBP(II) and [^{3}H]retinal-CRBP(II) used in this study. ApoCRBP was purified from a transfected *Escherichia coli* strain as previously described (26).

Retinol was labeled at the C-15 position as previously described (27). Typical specific activity of the [^{3}H]retinol was 11–13 Ci/mmol. [^{3}H]retinal was prepared from [^{3}H]retinol by oxidation with MnO_2 as described (28), with a specific activity of approximately 10 Ci/mmol. [^{3}H]retinol-CRBP(II) and [^{3}H]retinol-CRBP were prepared as described previously (7), and [^{3}H]retinal-CRBP(II) was prepared immediately prior to each assay by the addition of [^{3}H]retinal in DMSO to a 20% molar excess of apoCRBP(II).

The retinyl esters were synthesized from the acyl chloride (29). The purity and identity of the esters were confirmed as previously described (1). Retinyl palmitate was either synthesized from the acyl chloride for comparison with other synthesized esters or was purchased from Sigma.

DMSO (spectrophotometric grade), aluminum oxide (activated, neutral, Brockman I), and *N,N'*-dimethylformamide (HPLC grade) were from Aldrich. Dithiothrietol, diisopropyl fluorophosphate, n-ethylmaleimide, iodoacetamide, *p*-chloromercuriphenylsulfonic acid, diethyl-

p-nitrophenylphosphate, diethylpyrocarbonate, NADH, and NADPH were from Sigma. All solvents used in the extraction of retinoids were HPLC grade and were obtained from Baxter.

Removal and treatment of intestine

Male animals weighing approximately 375 g were killed by decapitation, and the small intestine was removed. All pancreatic tissue adhering to the small intestine was carefully removed. The intestine was then flushed with at least 100 ml of isotonic NaCl (0.9%) and cut longitudinally to expose the mucosal layer. The open intestine was vigorously agitated in isotonic NaCl containing 1 mM diisopropyl fluorophosphate (DFP), and then in isotonic NaCl alone. After each agitation the intestine was blotted dry. The opened intestine, muscle side down, was then placed on a glass plate resting on ice, and divided into either three or six segments of equal length. The mucosa from each segment was scraped free using the edge of a clean, glass microscope slide. The remaining muscle layer was rinsed well with isotonic NaCl and blotted dry.

Preparation of microsomal and cytosolic samples

After separation of each of the six sections into mucosal and muscle samples, microsomes were prepared as before with the following slight modifications (7). A Polytron PT-3000 (Brinkmann Instruments, Inc.) was used for the initial homogenization at 12000 rpm for 40 s, and the microsomal pellets were resuspended in 50 mM potassium phosphate (pH 7.2), 1 mM DTT, 0.1 mM NADH, 0.1 mM EDTA. The supernatant liquid from the 100,000 *g* centrifugation was measured for CRBP(II) by the radioimmunoassay as described previously (20).

Radiometric assay of LRAT and retinal reductase

LRAT was assayed radiometrically by adding 5 μg microsomal protein to 0.2 M potassium phosphate, 1 mM DTT, in a total volume of 100 μl. When tested, 4 nmol BSA was added in 0.2 M potassium phosphate buffer (final concentration of 40 μM). The reaction was initiated by addition of 200 pmol of either [^{3}H]retinol-CRBP(II) (final concentration of 2 μM) for the mucosal samples, or [^{3}H]retinol-CRBP for the muscle samples, and incubated at 37°C for 10 min. The retinyl esters were extracted and separated from retinol by alumina chromatography as described (21).

Retinal reductase was assayed by adding 5 μg microsomal protein to 0.1 M imidazole acetate buffer (pH 6.0) containing 6 nmol NADPH (final concentration of 60 μM) in a final volume of 100 μl. The reaction was initiated by addition of 300 pmol [^{3}H]retinal-CRBP(II) (final concentration of 3 μM), prepared immediately prior to the assay, and incubated for 10 min at 37°C. The reaction was terminated by the addition of 4 volumes of ice-cold ethanol containing 100 μg/ml BHT. Retinoids were extracted by addition of 500 μl of H_2O and 2 ml hexane containing 100 μg/ml BHT. After vigorous mixing, 1 ml of the hexane layer was applied to a column of 1.2 g of alumina deactivated with H_2O (10% by weight). [^{3}H]retinal was eluted with hexane containing 15% ethyl ether. The [^{3}H]retinol product was eluted with a 50:50 hexane–ether mixture directly into scintillation vials, dried under nitrogen, and quantitated by scintillation counting. All incubations were done in duplicate under subdued yellow light.

Determination of esterification products by HPLC

Esterification reactions were carried out with approximately 50 μg microsomal protein in 0.2 M potassium phosphate buffer in a final volume of 0.5 ml. All assays contained 1 mM DTT except those including iodoacetamide. When tested, BSA was added in 0.2 M potassium phosphate to a final concentration of 40 μM (7). The reactions were initiated by addition of either 1 nmol retinol or retinol-CRBP(II) (final concentration of 2 μM) and the retinyl esters were extracted and analyzed as before (7). Inhibitors were preincubated with the microsomes for 10 min at 37°C prior to addition of substrate. DFP and N-ethylmaleimide (NEM) were added in DMSO to 1 mM and 10 μM final concentration, respectively, while iodoacetamide was added in 0.2 M potassium phosphate buffer to 1 mM final concentration. All incubations were done in duplicate under subdued yellow light.

An Isco HPLC system was equipped with a V_4 variable wavelength detector, a Spectra Physics SP4270 integrator, and a 15-cm LC-18 column (3 μm) with a guard column from Supelco. The mobile phase was 100% N,N'-dimethylformamide with a flow rate of 1 ml/min (30). The elution of retinyl esters was monitored by absorbance at 325 nm.

Determination of endogenous retinyl esters

Endogenous retinyl esters were measured after incubation of 50 μg of microsomal protein in 0.2 M potassium phosphate buffer in a total volume of 0.5 ml. The reaction mixture was shaken at 37°C for 20 min. The reaction was stopped by the addition of 2 ml of ice-cold ethanol containing 100 μg/ml BHT. After addition of 2 ml H_2O, the esters were extracted into 8 ml hexane containing 100 μg/ml BHT. Seven ml of the hexane layer was taken to dryness under N_2 and redissolved in 100% N,N'-dimethylformamide for HPLC analysis as above. The esters were quantitated from a calibration curve of the amount of pure retinyl palmitate injected onto the column versus integrated peak areas.

Retinyl ester hydrolase assay

Muscle microsomal protein (2 μg) was added to 0.5 ml

PBS, pH 8.2, and the reaction was initiated by the addition 50 nmol of retinyl ester (delivered in 5 μl of ethanol) (final concentration of 100 μM). When used, inhibitors were added in DMSO (with the final DMSO level kept constant at 1%) and allowed to pre-incubate at 25°C for 10 min prior to addition of substrate. The reaction mixtures were incubated in a shaking water bath at 37°C for 10 min. The reactions were terminated by addition of 2 ml of ice-cold ethanol containing 100 μg/ml butylated hydroxytoluene and 2 ml of deionized water. The retinol product was extracted twice by addition of 6 ml hexane containing 100 μg/ml butylated hydroxytoluene and vigorous mixing for about 40 s. Five of the 6 ml of the organic phase was then transferred, and the combined extract was evaporated to dryness under N_2. Hydrolysis of endogenous retinyl ester was examined by incubating 50 μg of intestinal microsomal protein for 30 min at 37°C and extracting the retinol as above. The dried material was resuspended in 100 μl of 82% n-hexane, 10% acetic acid, and 8% dioxane (vol%) and 85 μl was injected on a Whatman silica gel HPLC column with a flow rate of 2 ml/min. The eluted retinol was quantitated by absorbance at 325 nm using a V_4 variable wavelength detector. The appropriate controls for nonenzymatic hydrolysis of retinyl ester (typically less than 10% of product formed) and the presence of retinol in the microsomal fraction (typically less than 1% of product formed) were used for correction of all assays.

RESULTS AND DISCUSSION

Preparation of intestinal samples

In the course of this work, we occasionally observed a retinol esterifying activity that had confounding effects on determination of LRAT activity. It was revealed because the pattern of retinyl esters produced was quite distinct from the pattern produced by LRAT, with a considerable amount of unsaturated acyl moieties present, particularly 20:4, 18:2, and 18:1 (**Fig. 1A**). In addition, while intestinal LRAT activity has been shown to be stimulated two- to three-fold by the addition of BSA (7), this second activity was markedly suppressed by BSA (Fig. 1B). The retinyl ester pattern observed in the presence of BSA was typical for LRAT, although at a level of ester which was approximately 40% of that measured in preparations lacking this additional activity.

It was noted that the amount of BSA-suppressable activity was greater when delays occurred during the preparation of intestinal microsomes. We suspected that this might be due to the action of enzymes, perhaps pancreatic in origin, causing the release of fatty acids from endogenous lipids, which might then serve as substrates for retinyl ester synthesis. Such activities have been noted previously (2). To confirm that free fatty acids could serve as acyl donor for this activity, we presented intestinal microsomes with heptadecanoic acid and observed production of retinyl heptadecanoate (Fig. 1C) in addition to retinyl esters produced from the endogenous donor(s). Addition of BSA to this mixture suppressed the production of retinyl heptadecanoate, in addition to the other unexpected unsaturated esters, presumably due to the ability of BSA to bind free fatty acids (Fig. 1D).

In an effort to suppress this reaction, we examined certain inhibitors. We found that the production of esters from free fatty acids was inhibited by PMSF, which also inactivates LRAT (31), but also by 1 mM DFP, which

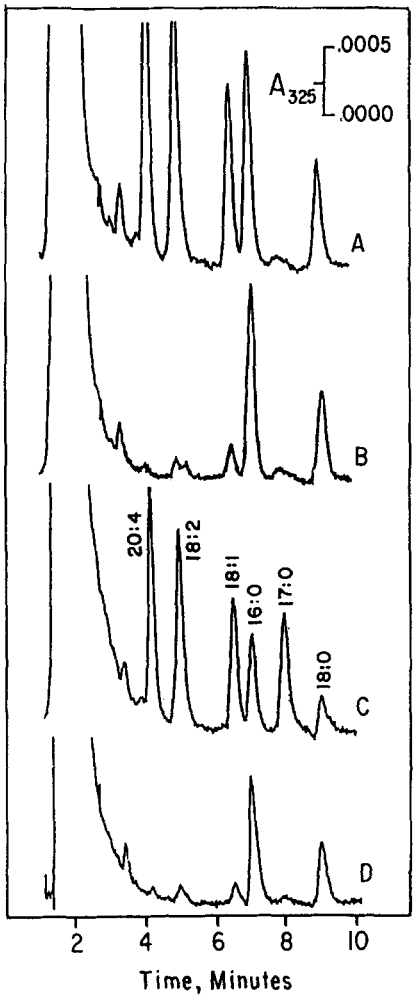

Fig. 1. HPLC profile of retinyl esters synthesized in vitro by activities within intestinal microsomes. Retinyl esters synthesized by both LRAT and an anomalous activity were resolved by HPLC. The assay contained either buffer with 1 mM DTT only (tracing A), with 40 μM BSA (tracing B), 40 μM heptadecanoic acid (tracing C), or with both 40 μM BSA and 40 μM heptadecanoic acid. The reactions were initiated and analyzed as in Experimental Procedures. Identification of the esters, indicated in tracing C, was by reference to the elution position of standards (retinyl esters: 20:4, arachidonate; 18:2, linoleate; 18:1, oleate; 16:0, stearate; 17:0, heptadecanoate; 18:0, stearate).

does not inactivate LRAT (31). It was unaffected by 20 μM n-ethylmaleimide or 1 mM iodoacetamide, which do inactivate LRAT (10) (data not shown). The anomalous activity, therefore, appears to perhaps utilize a serine, in contrast to the presumed active site cysteine(s) utilized by LRAT (31). The effect of these inhibitors on the production of free fatty acids was not examined. The non-LRAT esterifying activity preferred free retinol over retinol-CRBP(II) by about threefold (data not shown) in contrast to LRAT, which esterifies free and bound retinol equally well (26).

Earlier studies have described activities from both the pancreas and the small intestine that are capable of esterifying retinol when free fatty acids are provided (32, 33). Here we found that intestinal preparations that contained such an activity had levels of LRAT and retinal reductase that were depressed when compared to comparable preparations without this activity (data not shown). This suggested the concurrent presence of proteases that were degrading the enzymes of interest here. We found we were able to suppress this problem by careful attention to removal of all pancreatic tissue from the intestine and by flushing the intestinal mucosa in saline containing 1 mM DFP, which does not affect either LRAT or retinal reductase. Our criteria for assessing the quality of the intestinal microsomal preparations were the demonstration of a two- to threefold stimulation in esterification activity by BSA and resistance to DFP inhibition. If either of these criteria was not met, or if an anomalous retinyl ester pattern was observed, the microsomal preparation was discarded. As expected, the problem arose most often in the intestinal section containing the duodenum, the section most prone to pancreatic tissue contamination. In general, complications arising from pancreatic protease activities in studying intestinal microvilli preparations have been described for many systems, and the quality of the intestinal preparation must be stressed because the enzymes of interest here were quite sensitive.

The esterification of retinol with free fatty acids appears to have occurred in our study of the levels of retinol esterifying activity in the rat small intestine during perinatal development (34). The retinyl ester acyl pattern obtained from microsomes from suckling animals was unlike that expected for LRAT, but similar to the anomalous pattern observed here. Although the total esterifying activity was attributed to LRAT, we speculated that it was possible that a second activity might also be present. We have re-examined microsomes from that study and found the characteristics typical of the activity noted here, including suppression by BSA, sensitivity to the same inhibitors, and the ability to use free fatty acids. This activity was not noted in preparations obtained prior to birth or after weaning, suggesting the possibility that it is present in milk. Interestingly, the acyl pattern observed was enriched in fatty acids that are abundant in milk (34). Consequently, the very high rates of retinyl ester synthesis observed for microsomes prepared from the intestine during the suckling period were due, in part, to this additional activity, as well as to LRAT. Preliminary results indicate, however, that the overall developmental pattern of LRAT in the small intestine was qualitatively similar to that reported, but that levels of LRAT present in the intestine of suckling rats are about one-third to one-half of the total activity measured in that study (34).

Distribution of LRAT and retinal reductase between the mucosal and muscular layers of the small intestine

Dietary vitamin A processing occurs in the absorptive cells of the intestinal mucosa (2). CRBP(II) has also been localized exclusively in those cells (4, 5). Consequently, we were interested in the relative distribution of the intestinal enzymes believed to be involved in the metabolism that occurs during absorption between the mucosa and the underlying muscle layer of the intestine. To determine the levels of activity in each tissue, we divided the rat small intestine into three equal segments from the pylorus to cecum and scraped the mucosa from the muscle. Levels of retinal reductase and LRAT activity from each sample are presented in **Table 1.** The absolute activity of both enzymes was found to differ with the supplier of rats, but the pattern of distribution remained similar. For example, Sprague-Dawley rats purchased from Harlan Industries and killed after identical treatment consistently resulted in enzyme levels approximately 50% lower than those seen for animals purchased from Sasco and used for the studies presented here. The basis of this supplier difference has not been determined but was unrelated to time of housing in local facilities.

As expected, the specific activity for retinal reductase was greater in the intestinal mucosa than in the muscle. Because rats pass little dietary carotene into the blood

TABLE 1. Muscle and mucosal levels of retinal reductase and LRAT along the small intestine

Intestinal Segment	Specific Activity		Ratio
	Mucosa	Muscle	
	pmol/min/mg		
Retinal reductase			
1	65 ± 6	5.4 ± 0.5	12
2	26 ± 2	5.2 ± 0.2	5.1
3	11 ± 1	6.8 ± 0.1	1.6
LRAT			
1	68 ± 9	38 ± 6	1.8
2	29 ± 3	27 ± 3	1.0
3	10 ± 0	34 ± 1	0.3

A total of six rats was used for the preparation of mucosal and muscle microsomes. The values given are for three determinations of activity ± SEM.

(35), carotene cleavage is accomplished primarily, if not solely, during absorption, and little reductase activity would be expected in the muscle. An aboral gradient of reductase activity in the mucosa contrasted with the muscle levels, which remained at a constant low level down the axis. LRAT, however, was not as restricted to intestinal mucosal tissue as the reductase, being abundant in the smooth muscle and actually exceeding the mucosal level for the terminal third of the small intestine. Indeed, the LRAT level observed for the intestinal muscle segments is almost 50% of the level determined for liver microsomal LRAT (76 pmol/min per mg protein) (26). This suggested a substantial ability of the smooth muscle to esterify vitamin A. The mucosal LRAT activity decreased down the duodenal-ileal axis (as for the reductase), but, in contrast, the LRAT level for the muscular layer remained relatively constant and at a higher level than that observed for the reductase. The esterification activity in the muscle microsomes produced a retinyl ester profile and sensitivity to inhibitors typical of LRAT.

Distribution of CRBP(II), retinal reductase, and LRAT along the mucosa of the small intestine

The profile of reductase and LRAT activities within the mucosa along the longitudinal axis of the small intestine was investigated further and compared to levels of CRBP(II) by dividing the intestine lengthwise into six equal sections. The results for each protein were normalized to the value for segment 2 for ease of presentation (**Fig. 2**). Although the height of intestinal villi decreases from the upper duodenum to the terminal ileum, the percentage of enterocytes within the epithelium layer remains relatively constant (36). The comparisons here are based on specific activity and are, therefore, not due to decreased enterocyte number. For all three proteins the levels were greatest in the proximal intestine and then decreased abruptly in the distal half (Fig. 2). The gradients of the three proteins were nearly identical.

A graded distribution of activity along the intestine is not uncommon, reflecting the functional differences found among the duodenum, jejunum, and ileum (37). For example, cholesterol synthesis, as measured by HMG-CoA reductase activity, is fourfold higher in the ileum than in the jejunum (38). In contrast, the level of acyl-CoA:cholesterol acyltransferase activity does not change along the axis of the intestine (38). Sucrase activity and transport mechanisms for the absorption of amino acids are somewhat higher in the proximal small intestine (37, 39). Interestingly, the jejunum has been shown to be the site of greatest fat absorption in the intestine, in addition to being the site of greatest vitamin A absorption (18, 19). Here we observe that the jejunum also contains the highest levels of several of the activities involved in the absorption and metabolism of the lipophilic vitamin A.

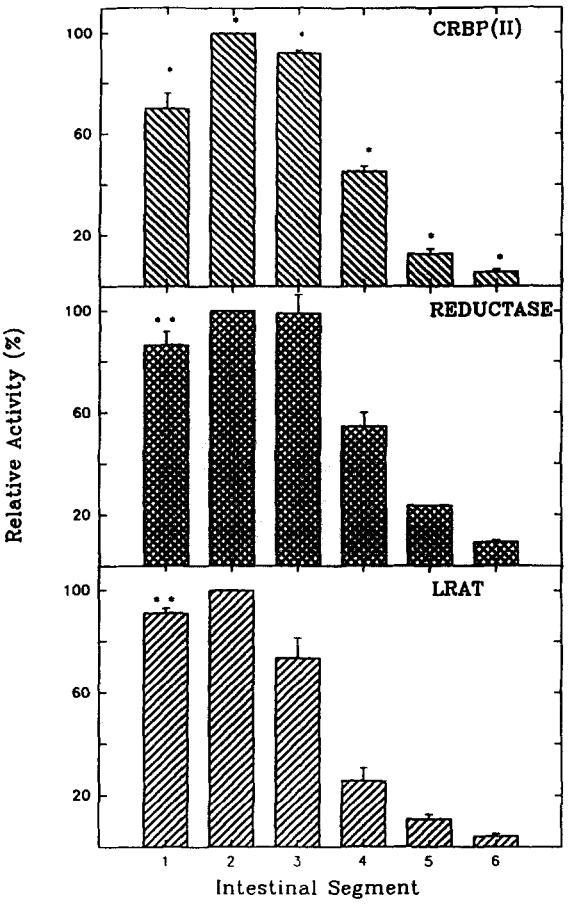

Fig. 2. Distribution of CRBP(II), retinal reductase, and LRAT along the longitudinal axis of the small intestine. Intestines were divided into six equal segments from the pylorus to the cecum (segments 1 to 6), and the mucosal layer was carefully scraped from the muscle. The cytosolic fraction from the mucosa was assayed for CRBP(II) levels using a sensitive radioimmunoassay. The retinal reductase and LRAT activities were assayed radiometrically as in Experimental Procedures. The results for each protein are normalized to the determination for segment 2. The values given are the average from nine rats, six rats (*), or three rats (**) ± the SEM.

The data in Fig. 2 clearly demonstrate a coordinated distribution of the three proteins along the small intestinal mucosal layer. CRBP(II), retinaldehyde reductase, and LRAT, therefore, may be induced and regulated by similar factors. Intestinal LRAT levels are insensitive to the vitamin A status of the animal (40), suggesting regulation must be independent of gross plasma retinol levels but may be regulated by minor metabolites of retinol unaffected by intake levels. One possibility is 9-*cis*-retinoic acid as this retinoid has been implicated in the upregulation of CRBP(II) expression in vitro (41). Further work demonstrating in vivo effects of 9-*cis*-retinoic acid will be necessary to substantiate any involvement of this isomer in the regulation of proteins involved in vitamin A metabolism.

It is important to note that retinal-CRBP(II) is the substrate for the microsomal retinal reductase, which produces retinol, and retinol-CRBP(II) is the substrate for the microsomal LRAT, which produces retinyl esters. The possibility that these two enzymes are physically juxtaposed in the endoplasmic reticulum of the enterocyte allows for the speculation that CRBP(II), through direct contact with both enzymes, might be serving to directly channel the retinoids between the two enzymes. Such metabolic channeling might increase the efficiency of vitamin A absorption. The coordinate distribution of the three proteins along the longitudinal axis of the intestine is consistent with such a possibility.

Retinyl ester stores in intestinal muscle

The distribution of LRAT in both intestinal mucosa and muscle, in contrast to the relative lack of reductase in muscle, was not unexpected as LRAT has been found in microsomes from a variety of tissues. In these tissues its product retinyl esters serve varying roles depending on the organ. In the intestinal mucosa, esterification by LRAT allows for the export of retinyl esters; in the liver and other organs, LRAT effects retention of retinol in the form of esters. Here, in intestinal muscle, esterification of retinol by LRAT would not be involved in the export of retinol from the tissue; rather, it would serve to produce stores of retinyl esters. Importantly, cellular retinol-binding protein is found in intestinal muscle, at equal levels in the jejunum and ileum (42). This even distribution of CRBP in intestinal muscle is unlike the CRBP(II) distribution found in intestinal mucosa (Fig. 2) but is consistent with the equal LRAT distribution down the axis of the muscular layer (Table 1). The function of CRBP in intestinal muscle may be, in part, to act as substrate carrier for this LRAT activity.

Consistent with the demonstrated LRAT activity, the microsomes from intestinal muscle contained endogenous retinyl esters, comprised predominantly of retinyl palmitate and retinyl stearate (200 pmol total ester/mg microsomal protein, on average) (**Table 2**). Interestingly, this level of retinyl ester is nearly 60% of the level of total ester found associated with microsomes prepared from rat liver (340 pmol total ester/mg microsomal protein) (21; D. E. Ong, P. N. MacDonald, and A. M. Gubitosi, unpublished data). Total tissue extracts from intestine were not examined. The absolute level of ester stores, however, varied with supplier of rats and correlated with differences in LRAT activity. After fasting the animals for 28 h, the endogenous retinyl esters were found to be largely retained (87% of nonfasted). The composition of the retinyl esters synthesized by LRAT in vitro showed considerable similarity to the composition of retinyl esters present within the microsomes (Table 2). The relative proportion of retinyl esters shown in Table 2 are different from those previously reported for ester synthesis and storage in rat liver (21). The percentage of total ester stores comprised of retinyl stearate in vivo in the intestinal muscular layer (36%) is significantly higher than that found in microsomes from liver (15%) (21) or sertoli cell preparations (6%) (14). The high proportion of retinyl stearate synthesized by LRAT in vitro in the muscular layer of the intestine is similar to levels determined for acylation in rat epididymal tissue and spermatozoa (30). The relative proportion of esters found in vivo or synthesized in vitro is presumably due to the available pool of acyl moieties at the *sn*-1 position of phosphatidylcholine, so the similarities or differences between tissues must also reflect similarities or differences in the proportion of stearate at this position.

TABLE 2. Comparison of the relative proportions of retinyl esters synthesized in vitro from retinol-CRBP to retinyl esters observed in vivo

Retinyl Ester	Formed in Vitro	Present w/Microsomes
	% of total ester recovered	
Retinyl palmitate	50 ± 1	47 ± 1
Retinyl stearate	36 ± 1	40 ± 3
Retinyl oleate	11 ± 1	10 ± 1
Retinyl linoleate	3 ± 2	3 ± 1

Characterization and determination of distribution of a retinyl ester hydrolase activity in rat intestinal muscle

The LRAT activity and retinyl ester stores we observed in the muscular layer of the small intestine led us to examine whether the same preparations contained an activity capable of hydrolyzing retinyl esters. We were able to detect a retinyl ester hydrolase activity that would hydrolyze the endogenous retinyl esters. For further study we presented exogenous retinyl ester to the microsomes from intestinal muscle. Using retinyl palmitate at 100 μM final concentration we observed linear production of retinol for 15 min at 37°C with microsomal protein up to 10 μg/ml (data not shown). The distribution of the retinyl ester hydrolase activity paralleled the distribution of CRBP, LRAT, and endogenous ester stores down the longitudinal axis of the intestinal muscle layer with hydrolase specific activities of 640 ± 80, 560 ± 90, and 630 ± 90 pmol/min per mg protein ± SEM for the three segments (n = 2, 3, and 3 determinations, respectively) and again contrasted with the significant gradient seen in the processing of vitamin A in the intestinal mucosa (Fig. 2).

We determined the rate of hydrolysis for a series of retinyl esters increasing in chain length from retinyl decanoate to retinyl arachidate and found no appreciable difference in the rates. The relative insensitivity to acyl chain length is unlike the preference for retinyl palmitate found for the bile salt-dependent retinyl ester hydrolase

from rat brush border membranes (1). The lack of specificity fits the requirement of the intestinal muscle layer, however, as retinyl stearate and retinyl palmitate were hydrolyzed with equal rates and, as shown in Table 2, both are present as endogenous stores in this tissue.

The retinyl ester hydrolase activity was independent of cholate as its activity was unaffected by the addition of either 3 mM or 18 mM cholate and is, therefore, distinct in this property from other reported microsomal hydrolase activities, which are inhibited by bile salts (17, 43). A retinyl ester hydrolase from rat liver microsomes appears to require apoCRBP for activity as it is virtually undetectable in its absence (43). We examined the effect of apoCRBP on the hydrolysis of exogenous retinyl ester in intestinal muscle and saw no stimulation.

We next examined this hydrolase activity for its sensitivity to certain chemical modifiers. Incubation of muscle microsomes with 1 mM of the sulfhydryl reagents iodoacetamide, n-ethylmaleimide, and *p*-chloromercuriphenylsulfonic acid resulted in 65%, 55%, and 55%, respectively, of the activity recovered from untreated microsomes. Increasing the concentration of inhibitor did not increase inhibition, suggesting either the presence of two activities capable of hydrolyzing retinol or the presence of one activity that is affected by sulfhydryl modification but does not directly use a sulfhydryl in the catalytic mechanism. DFP (1 mM), an active-site serine modifier, was only able to decrease retinyl ester hydrolysis levels by 16%. In contrast, diethyl-*p*-nitrophenylphosphate (DENP), a serine-directed reagent with enhanced ability to enter hydrophobic binding pockets of enzymes (44), was able to inhibit the hydrolase with an IC_{50} of 100 μM (**Fig. 3**). For comparison, lecithin:cholesterol acyltransferase (LCAT) utilizes a serine in its phospholipase cleavage of lecithin and is completely inhibited by 100 μM DENP (45). Our inability to inhibit the retinyl ester hydrolase with DFP or low levels of DENP suggests that access to the active-site serine in the hydrolase is restricted. In contrast, an active-site histidine was readily accessible to diethylpyrocarbonate as it inhibited the retinyl ester hydrolase with an IC_{50} of 10 μM (Fig. 3). The sigmoidal curves seen in Fig. 3 are consistent with a single activity capable of hydrolyzing retinyl esters. These inhibition studies suggest that the active site of the retinyl ester hydrolase from rat intestinal muscle contains a histidine and a serine, with sulfhydryl moieties likely involved in some aspect of maintaining the integrity of the protein. The lack of sensitivity to acyl chain length, bile salts, apoCRBP, and the pattern of inhibition seen with various chemical modifiers make this hydrolase from the muscular layer of the intestine unlike other retinyl ester hydrolases thus far examined.

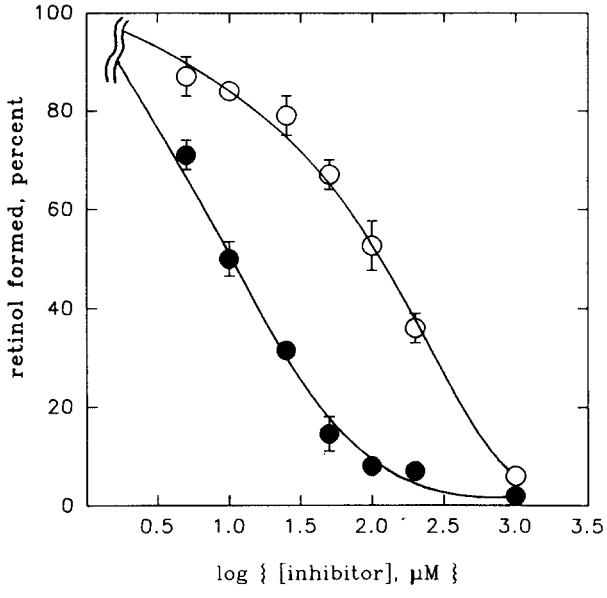

Fig. 3. Effect of serine and histidine-directed reagents on the hydrolysis of retinyl esters in intestinal muscle microsomes. The level of hydrolysis of exogenous retinyl palmitate was determined as detailed in Experimental Procedures in the presence of increasing concentrations of either diethylpyrocarbonate (●) or diethyl-*p*-nitrophenylphosphate (○). The results are expressed as the percentage of activity remaining when compared to untreated protein. The average of four determinations (± SEM) is shown.

Historically, the study of vitamin A storage and the maintenance of constant circulating retinol levels has concentrated on the liver, where a majority of total vitamin A is stored as retinyl esters in cytoplasmic lipid droplets within the stellate cell population of the liver, with these stores being depleted when vitamin A uptake is deficient (2). Related to our observations here, Yamamoto et al. (46) have demonstrated vitamin A-like fluorescence in cells resembling stellate cells in the muscular layer of the small intestine. The existence of these stellate-like cells and the demonstration of significant LRAT activity, endogenous retinyl ester stores, and a retinyl ester hydrolase activity in intestinal muscle emphasizes the potential role of nonhepatic tissues in the storage of vitamin A and their possible involvement in contributing to the maintenance of constant retinol levels either systemically or locally. The parallel and even distribution of vitamin A metabolizing enzymes and CRBP in the intestinal muscle layer is in contrast to the dramatic gradient of CRBP(II), retinaldehyde reductase, and LRAT in the intestinal mucosa. This coordinate distribution suggests similar regulation of the proteins involved.

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