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A Retinyl Ester Hydrolase Activity Intrinsic to the Brush Border Membrane of Rat Small Intestine†

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ABSTRACT: Retinol esterified with long-chain fatty acids is a common dietary source of vitamin A. Hydrolysis of these esters in the lumen of the small intestine is required prior to absorption. Bile salt-stimulated retinyl esterase activity was present with purified rat intestinal brush border membrane, with the maximum rate of ester hydrolysis at approximately pH 8, the physiological luminal pH. Taurocholate, a trihydroxy bile salt, stimulated hydrolysis of short-chain fatty acyl retinyl esters more than hydrolysis of long-chain fatty acyl esters. Deoxycholate, a dihydroxy bile salt, primarily stimulated hydrolysis of long-chain esters. Calculated K_ms of 0.74 μM for retinyl palmitate (16:0) hydrolysis and 9.6 μM for retinyl caproate (6:0) hydrolysis suggested the presence of two separate activities. Consistent with that, the activity responsible for retinyl caproate hydrolysis could be inactivated to a greater degree than retinyl palmitate hydrolysis by preincubation of the brush border membrane at 37 °C for extended times. Brush border membrane from animals who had undergone common duct ligation 48 h prior to tissue collection showed little ability to hydrolyze retinyl caproate but retained 70% of retinyl palmitate hydrolytic activity, compared to sham-operated controls. Thus, two distinguishable retinyl esterase activities were recovered with purified brush border membranes. One apparently originated from the pancreas, was stimulated by trihydroxy bile salts, and preferentially hydrolyzed short-chain retinyl esters, properties similar to cholesterol ester hydrolase, known to bind to the brush border. The other was intrinsic to the brush border, stimulated by both trihydroxy and dihydroxy bile salts, and preferentially hydrolyzed long-chain retinyl esters, providing the majority of activity of the brush border against dietary retinyl esters.

Vitamin A is a fat-soluble vitamin required for normal vision (Morton, 1972), spermatogenesis (Mason, 1933), and differentiation of epithelial cells (Wolbach & Howe, 1925). Vitamin A is found in the diet either as provitamin carotenes from plants or as fatty acyl esters of retinol (vitamin A alcohol) from animal tissue. β-Carotene appears to be absorbed intact by passive diffusion at the intestinal brush border (Hollander & Ruble, 1978), but retinyl esters cannot be absorbed as such. In vitro and in vivo studies with rats have shown that hydrolysis of retinyl esters to fatty acid and retinol is necessary in order for absorption to occur (Mahadevan et al., 1963a,b). Retinol then crosses the brush border membrane of the absorptive cell (enterocyte) by what is thought to be carrier-mediated passive diffusion (Hollander & Muraldihara, 1977; Said et al., 1988).

This necessary hydrolysis of retinyl esters is generally assumed to be carried out in the lumen of the small intestine by pancreatic enzymes [reviewed in Goodman and Blaner (1984)]. Two enzymes that can hydrolyze retinyl esters have been identified in rat pancreatic juice (Erlanson & Borgstrom, 1968). One of these activities was bile salt stimulated and was

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identified as cholesterol ester hydrolase (CEH,[1] EC 3.1.1.13). The other enzyme was bile salt inhibited and was shown to be pancreatic lipase (EC 3.1.1.3).

Preparations of enterocyte brush border membrane from rat have also been demonstrated to have the ability to hydrolyze retinyl esters (David & Ganguly, 1967). This raises the possibility that some hydrolysis of retinyl esters could also occur at the lumenal surface of the brush border. Intrinsic brush border proteins anchored to face the lumen of the intestine include enzymes such as sucrase, leucine aminopeptidase, and folate conjugase that process dietary carbohydrates, proteins, and a vitamin, respectively [reviewed in Alpers (1987)]. However, the brush border activity for retinyl ester hydrolysis has not yet been well characterized. Recent studies revealing the ability of the brush border to bind, via heparin, both CEH (Bosner et al., 1988) and pancreatic lipase (Bosner et al., 1989) raised the possibility that the brush border retinyl ester hydrolase activity previously described might actually originate from the pancreas.

Here, we have examined the ability of purified rat intestinal brush border to hydrolyze retinyl esters. Two distinguishable activities were found to be present. One appeared to be of pancreatic origin (possibly CEH) and had greatest activity for retinyl esters whose fatty acyl moieties were 10 carbons or less in length. The second activity appeared to be intrinsic to the brush border and constituted the majority of brush border activity toward the long-chain retinyl esters that are typical of those that would be found in the diet.

Experimental Procedures

Materials. Phosphate-buffered saline (PBS) was made from FTA hemagglutination buffer purchased from Becton Dickinson. Triton X-100 was from Pharmacia. Zwittergent 2-12 was from Boehringer Mannheim. All bile salts, purified bovine CEH, retinol, and retinyl acetate were purchased from Sigma. Retinyl propionate and butyrate were synthesized from the acid anhydrides according to the method of Lentz (Lentz et al., 1975) for cholesteryl esters. All other retinyl esters were synthesized from the acyl chloride by published methods (Huang & Goodman, 1965). Purity and identity of all synthesized retinyl esters were confirmed by UV spectra (in ethanol) and reverse-phase (C18) HPLC, monitored at 325 nm. Retinyl palmitate was synthesized from the acyl chloride or purchased from Sigma. Adult Sprague-Dawley rats (>250 g) were purchased from Harlan.

Preparation of Rat BBM. Intestinal brush border membranes (BBM) were prepared from the proximal two-thirds of the small intestine of adult (>250 g) rats. The intestines were removed, rinsed with cold saline, everted, and placed on a glass plate resting on ice. The mucosa was then scraped from the muscularis using glass microscope slides. One to six intestines were used in a given preparation. The mucosa was homogenized for 3 min at the high setting in a Waring blender. Brush border membranes were purified from the homogenized mucosa by a modification of Kessler's divalent cation precipitation technique (Said et al., 1987). Briefly, mucosal scrapings were placed in 15 mL/intestine of 300 mM mannitol, 1 mM EGTA, 2.4 mM Tris-HCl (pH 7.1). After addition of H_2O (60 mL/intestine) and homogenization, 0.75 mL/intestine of 1 M $MgCl_2$ was added, and the homogenate was incubated on ice for 15 min prior to centrifugation at 3000*g* for 15 min. This and subsequent centrifugations were carried out with a Sorvall Superspeed RC2-B centrifuge and GSA or SS34 rotors. The supernatant liquid was collected and centrifuged at 27000*g* for 30 min. The pellet from the second centrifugation was resuspended in 60 mM mannitol, 5 mM EGTA, 12 mM Tris-HCl (pH 7.1) at a ratio of 15 mL/intestine. After addition of 1 M $MgCl_2$ (0.15 mL/intestine), the solution was incubated on ice for 15 min, followed by the two centrifugations described above. The pellet was resuspended in 250 mM mannitol, 20 mM Tris-HCl, pH 8.0 (7.5 mL/intestine) and then centrifuged at 34000*g* for 30 min. The pellet from this final centrifugation was resuspended in 250 mM mannitol and 20 mM Tris-HCl (pH 8.0) using a syringe with a 25-gauge needle (0.5 mL/intestine). This purified BBM preparation was then quick-frozen in a methanol/dry ice bath at −70 °C and stored at −20 °C. Protein determinations were made using Pierce BCA reagents, with bovine serum albumin as a standard.

Purity of BBM preparations was determined using measurement of marker enzyme activities, as previously described (Beaufay et al., 1974; Scharschmidt et al., 1979), except that leucine aminopeptidase was assayed using a kit purchased from Sigma (catalog no. 251-AW). A 12-fold increase in the specific activity of leucine aminopeptidase, a brush border enzyme, was observed on comparison of total mucosal homogenate to BBM preparation. This enrichment is similar to that previously seen (Said et al., 1987). In contrast, the specific activities of cytochrome *c* reductase (endoplasmic reticulum), cytochrome *c* oxidase (mitochondria), and Na^+/K^+-ATPase (basolateral membrane) decreased 5-fold, 1.2-fold, and 3-fold, respectively.

Assay of Brush Border Hydrolase Activity. All PBS used was first adjusted to pH 8 using 0.1 M NaOH, unless otherwise noted. After sequential addition of detergent and retinyl ester substrate (delivered in 5 μL of ethanol) into PBS, the reaction was initiated by the addition of BBM (1.5 μg of protein unless otherwise noted). Total assay volume was 0.5 mL. In inactivation studies, BBM was added to the detergent solution first for the preincubation period and the assays were then initiated by addition of the retinyl ester substrate. Assays were incubated for 10 min on a shaking water bath at 37 °C unless otherwise noted. Reactions were terminated by the addition of 2 mL of ice-cold ethanol containing 100 μg/mL butylated hydroxytoluene. Retinol product was extracted by sequential addition of 8 mL of *n*-hexane containing 100 μg/mL butylated hydroxytoluene and 2 mL of deionized water, each addition followed by vigorous mixing for about 40 s. Seven of the eight milliliters of the organic phase were then removed and evaporated to dryness under N_2. The dried material was resuspended in 100 μL of 90% *n*-hexane, 10% dioxane (vol %), and 85 μL was injected on a Whatman silica gel HPLC column. The eluted retinol was quantitated by absorbance at 325 nm. All assays were done in triplicate. Nonenzymatic hydrolysis and any presence of retinol in the ester substrate stock were determined from incubations with BBM that had been inactivated by heating for 5 min in a boiling water bath prior to the assay. Values from these controls were used for correction (typically less than 5% of enzymatic hydrolysis).

Ligation of the Common Duct. The ligation was performed on three adult rats. Three rats were used as sham-operated controls. All rats were anesthetized using a 2–4% halothane/oxygen mixture. An abdominal incision was made, and the common duct was identified in both experimental and

[1] Abbreviations: CEH, cholesterol ester hydrolase; BBM, brush border membrane; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Tris; tris(hydroxymethyl)aminomethane; PBS, phosphate-buffered saline; HPLC, high-performance liquid chromatography; CHAPS, 3-[(cholamidopropyl)dimethylammonio]-1-propanesulfonate.

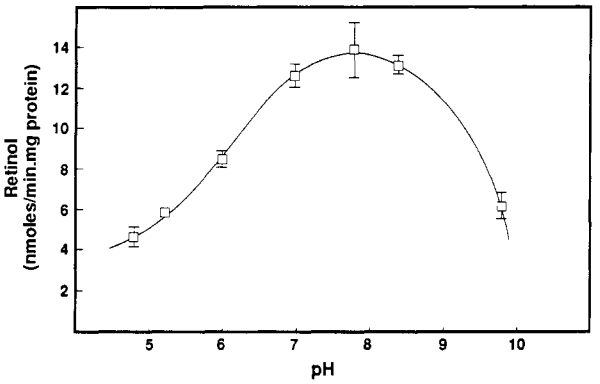

FIGURE 1: pH profile of retinyl palmitate hydrolysis. PBS was titrated to the desired pH using 0.1 M NaOH or 0.1 M HCl. Reactions were conducted with 30 μM retinyl palmitate, 1.5 μg of BBM protein, and 1% (w/v) taurocholate. Reaction conditions and retinol extraction and quantification are given in Experimental Procedures. All points were done in triplicate and are presented as mean ± SD.

control rats. The duct was then ligated as close as possible to the outer surface of the intestine in the experimental animals only. All animals were given ampicillin (intramuscular) immediately following surgery and again 12–24 h later. All animals were restricted from food for 48 h following surgery, at which time the animals were decapitated and the intestines were collected. The above protocol was approved by the Vanderbilt University Animal Care Committee.

Results and Discussion

Presence of Retinyl Esterase Activity in Brush Border Preparations. Our preparations of purified rat intestinal brush border were observed to have a potent enzymatic activity that catalyzed the hydrolysis of retinyl palmitate to retinol and palmitic acid. No activity was observed with heat-inactivated BBM. When assayed in the presence of 1% (w/v) taurocholate (19.6 mM), the rate of hydrolysis of retinyl palmitate substrate increased linearly with brush border protein concentration (up to 15 μg of protein/mL) and was constant with assay time for up to 30 min (data not shown). The pH profile for the hydrolysis of retinyl palmitate is shown in Figure 1. A maximum rate of 13.9 nmol/(min·mg of BBM protein) was observed at a pH of about 8; this is within the physiological pH range found in the duodenal lumen (Powell, 1987).

Effect of Detergents on Brush Border Retinyl Esterase Activity. The activity of many enzymes that function in the lumen of the small intestine are known to be markedly affected by bile salts and other detergents (Vahouny & Brecher, 1968). A number of detergents stimulated the hydrolysis of retinyl palmitate by the brush border preparations under study here (Table I). All detergents containing a steroidal nucleus, including dihydroxy (e.g., deoxycholate) and trihydroxy (e.g., cholate) bile salts and CHAPS, increased activity from 2- to 16-fold, compared to the activity in the absence of detergent. In contrast, other ionic (Zwittergent 2-12) and nonionic (Triton X-100) detergents actually decreased activity. The strongest stimulation was observed with 0.5% deoxycholate. Higher rates of hydrolysis were seen with unconjugated bile salts (e.g., cholate and deoxycholate) than with their taurine- or glycine-conjugated analogues. As shown in Figure 2, the greatest degree of stimulation by dihydroxy bile salts was observed at a concentration of 0.125% for conjugated and 0.5% for unconjugated bile salts, with a decrease in hydrolysis rates occurring at higher concentrations. Increasing concentrations of conjugated and unconjugated trihydroxy bile salts resulted in increased hydrolysis rates, reaching a maximum rate at 2%.

Table I: Relative Hydrolysis[a] of Retinyl Palmitate at the Indicated Detergent Concentration

detergent	0.5%[b]	1%[b]
Triton X-100	2.1 ± 0.3	2.3 ± 0.3
Zwittergent 2-12	2.2 ± 0.4	2.1 ± 0.6
CHAPS	10.0 ± 1.0	63.0 ± 3.0
cholate	81.0 ± 10.0	100.0 ± 7.0
taurocholate	32.0 ± 4.0	38.0 ± 1.0
glycocholate	28.0 ± 1.0	47.0 ± 4.0
deoxycholate	133.0 ± 11.0	92.0 ± 6.0
taurodeoxycholate	16.0 ± 1.0	11.0 ± 2.0
glycodeoxycholate	25.0 ± 4.0	17.0 ± 1.0
chenodeoxycholate	101.0 ± 4.0	76.0 ± 10.0
none	8.5 ± 3.1	

[a] All reactions contained 30 μM retinyl palmitate and 1.5 μg of BBM protein. Retinol extraction and quantification were done as outlined in Experimental Procedures. All determinants were done in triplicate and are expressed as mean ± SD. Relative hydrolysis is presented as the percent of hydrolytic activity seen with 1% cholate [18.8 nmol/(min·mg of BBM protein)]. [b] Weight per volume percent (grams per 100 mL).

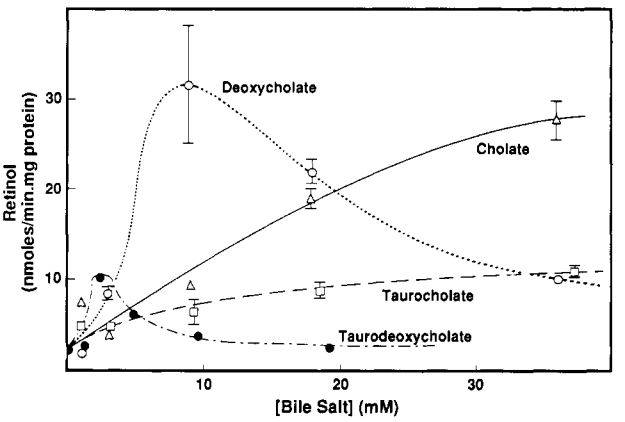

FIGURE 2: Concentration dependence of bile salt stimulation of retinyl palmitate hydrolysis. All reactions were done at pH 7.8 in PBS with 30 μM retinyl palmitate substrate, 1.5 μg of BBM protein, and the indicated concentration of either cholate (△), taurocholate (□), deoxycholate (○), or taurodeoxycholate (●). Reaction conditions and retinol extraction and quantification are given in Experimental Procedures. All points were done in triplicate and are presented as mean ± SD.

Stimulation of the brush border hydrolase activity for retinyl palmitate by either trihydroxy or dihydroxy bile salts made it unlikely that this activity could be accounted for primarily by either pancreatic lipase or CEH. Hydrolysis of retinyl palmitate by pancreatic lipase is actually inhibited by taurocholate (Erlanson & Borgstrom, 1968), and inhibition of pancreatic lipase activity for other substrates has also been observed with a wide variety of bile salts (Erlanson & Borgstrom, 1973). This inhibition can be relieved to some extent by colipase, but no stimulatory effect is observed with any bile salt (Borgstrom, 1977). With regard to CEH, hydrolysis of both retinyl acetate and retinyl palmitate by human CEH has a strict requirement for trihydroxy bile salts (Lombardo & Guy, 1980), and no stimulation is observed with dihydroxy bile salts, specifically those lacking a 7α hydroxyl. Similar results are seen for rat CEH with cholesteryl ester as substrate (Vahouny et al., 1965), in contrast to the results obtained here with purified BBM.

Retinyl Ester Specificity of Brush Border Hydrolase Activity. Preliminary studies suggested that changing the length of the fatty acyl moiety of the ester also affected the rate of hydrolysis by purified brush border. Therefore, the hydrolysis rates for a variety of retinyl esters, from retinyl acetate (2:0) to retinyl arachidate (20:0), were determined. When brush

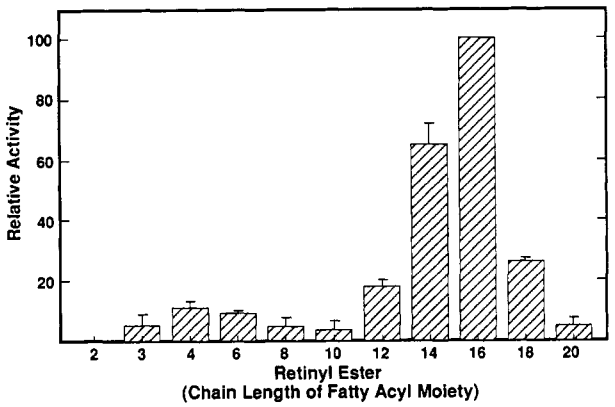

FIGURE 3: Relative hydrolysis of retinyl esters in 0.5% deoxycholate. Relative activity is defined as the percent of hydrolytic activity observed for retinyl palmitate (16:0) substrate in the given detergent [31.6 nmol/(min·mg of BBM protein)]. Chain length refers to the number of carbons in the saturated fatty acyl moiety of the ester. All esters had saturated fatty acyl moieties. All reactions were done at pH 7.8 with 30 μM retinyl ester and 1.5 μg of BBM protein in PBS. Reaction conditions and retinol extraction and quantification are as given in Experimental Procedures. All determinations were done in triplicate on either two or three separate occasions and are reported as the average of the mean ± SEM.

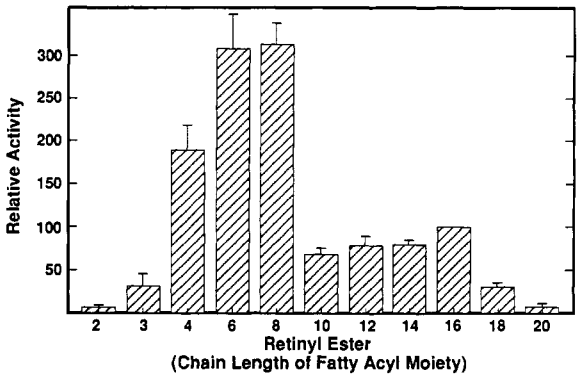

FIGURE 4: Relative hydrolysis of retinyl esters in 1% taurocholate. Relative activity is defined as the percent of hydrolytic activity observed with retinyl palmitate substrate [8.73 nmol/(min·mg of protein)]. All other conditions are as given in the legend to Figure 3 and in Experimental Procedures.

border preparations were assayed with deoxycholate at 0.5% and ester substrate at 30 μM (Figure 3), hydrolysis of esters with fatty acyl moieties shorter than retinyl laurate (12:0) was relatively minimal, but rates then increased considerably as the chain length increased, with a maximum rate observed for retinyl palmitate [16:0, 31.6 nmol/(min·mg of protein)]. A sharp drop in hydrolysis rate was then observed for retinyl stearate (18:0) and retinyl arachidate (20:0).

However, when brush border preparations were assayed in the presence of 1% taurocholate (Figure 4), a different pattern was observed. In addition to considerable hydrolysis of retinyl palmitate [8.73 nmol/(min·mg of protein)], greater hydrolytic rates were seen for short-chain esters, particularly retinyl caproate [6:0, 26.9 nmol/(min·mg of protein)] and retinyl octanoate (8:0).

These results suggested that at least two activities were present with the purified BBM. One was stimulated more strongly by dihydroxy bile salts and preferentially hydrolyzed long-chain retinyl esters. The other was stimulated much more strongly by trihydroxy bile salts and preferentially hydrolyzed short-chain esters.

Kinetic Parameters for Retinyl Caproate and Retinyl Palmitate Hydrolysis. Kinetic parameters for hydrolysis of several retinyl esters were determined in the presence of 1%

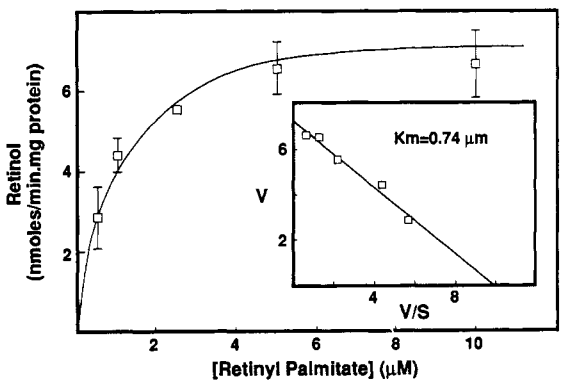

FIGURE 5: Retinyl palmitate concentration dependence of hydrolytic activity. All reactions were done at pH 7.8 with 1.5 μg of BBM protein in 1% taurocholate in PBS solution. Reaction conditions and retinol extraction and quantification were done as outlined in Experimental Procedures. All determinations were done in triplicate and are reported as mean ± SD. The inset is a linear transformation of the data (Eadie–Hofstee) to obtain values for K_m and V_{max} (r^2 = 0.97).

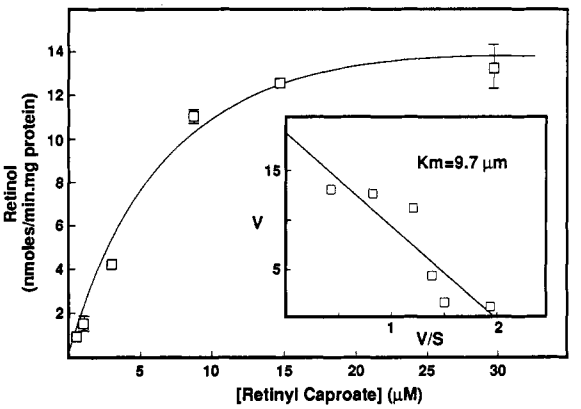

FIGURE 6: Retinyl caproate concentration dependence of hydrolytic activity. All other conditions are as given in the legend to Figure 5. The inset is a linear transformation of the data (Eadie–Hofstee) to obtain values for K_m and V_{max} (r^2 = 0.80).

taurocholate, which was able to stimulate significantly the hydrolysis of both long- and short-chain retinyl esters. Typical Michaelis–Menten kinetics were observed for retinyl palmitate hydrolysis by purified brush border as the concentration of retinyl palmitate substrate was increased (Figure 5). Eadie–Hofstee transformation of the data (Figure 5, inset) provided a K_m of 0.74 μM and a V_{max} of 7.3 nmol/(min·mg of protein). With retinyl stearate as a substrate, the calculated K_m was 0.42 μM (data not shown).

However, K_m values obtained with short-chain retinyl ester substrates differed significantly from those observed with long-chain ester substrates. Kinetic analysis of the hydrolysis of retinyl caproate (Figure 6) resulted in values of 9.6 μM for K_m with V_{max} at 18.9 nmol/(min·mg of protein). When retinyl octanoate was used as a substrate, a K_m of 6 μM was obtained (data not shown). These K_m values are similar to that observed for purified rat CEH and retinyl ester substrate (K_m = 10 μM), when assayed under similar conditions (Harrison & Gad, 1989). The difference in observed K_m for short-chain and long-chain ester substrates was also consistent with the presence of two different activities for these two types of substrates.

Differential Inactivation of Retinyl Esterase Activities. The presence of two activities associated with the BBM was also suggested when it was noted that extended preincubation of the BBM at 37 °C caused a considerably greater loss of hydrolytic activity for retinyl caproate, compared to the loss of retinyl palmitate hydrolytic activity (Figure 7). After 120

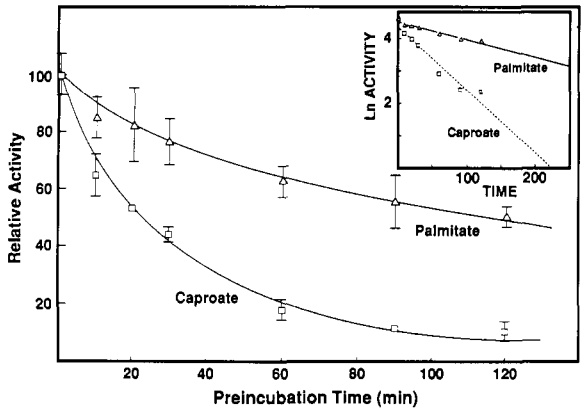

FIGURE 7: Time-dependent inactivation of retinyl ester hydrolytic activities. BBM protein (1.5 μg) was added to 495 μL of a 1% taurocholate solution (in PBS) and preincubated on a 37 °C shaker bath for the indicated time. The reaction was initiated by the addition of 5 μL of 3 mM retinyl palmitate (Δ) or retinyl caproate (□) in ethanol, giving a final substrate concentration of 30 μM. After a 10-min incubation period, the reaction was stopped by the addition of 2 mL of cold ethanol with 100 μg/mL butylated hydroxytoluene. The relative activity is defined as the percent of activity observed with the indicated substrate and no preincubation of the BBM. Retinol extraction and quantification were done as outlined in Experimental Procedures. All points were done in triplicate and are expressed as mean ± SD. The inset is a linear transformation of the data to obtain inactivation rate constant (r^2 = 0.96 with retinyl palmitate and 0.94 with retinyl caproate).

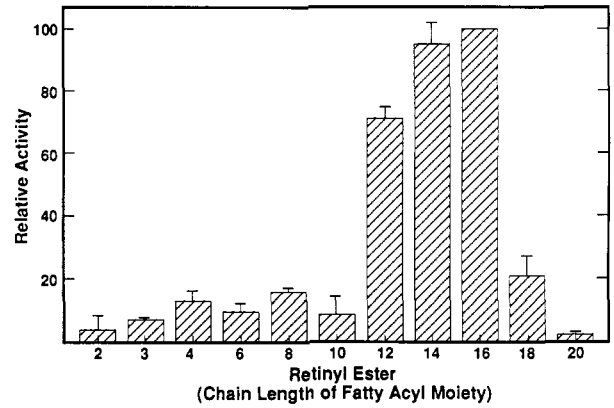

FIGURE 8: Relative hydrolysis of retinyl ester by BBM preparations from common duct ligated animals. Relative activity refers to the percent of hydrolytic activity observed with retinyl palmitate substrate [6.2 nmol/(min·mg of protein)]. Reactions contained 1.5 μg of BBM protein and 30 μM retinyl ester substrate with 1% taurocholate in PBS solution. Common duct ligation, BBM preparation, and retinol extraction and quantification were done as outlined in Experimental Procedures. All determinations were done in triplicate on either two or three separate occasions, and results were expressed as the average of the mean ± SEM.

min of preincubation at 37 °C in 1% taurocholate, 50.3% of the initial activity for retinyl palmitate substrate was retained, in contrast to only 10.5% of the activity for retinyl caproate. A plot of the natural logarithm of specific activity vs preincubation time was linear, as expected for a first-order inactivation (Figure 7, inset). From this linear transformation of the data, the rate constant for inactivation of retinyl caproate hydrolase activity (1.95×10^{-2} min^{-1}) was found to be almost 4-fold greater than the rate constant for inactivation of retinyl palmitate hydrolase activity (5.52×10^{-3} min^{-1}). These constants give a half-life of 36 min for the retinyl caproate hydrolytic activity, as opposed to 126 min for retinyl palmitate hydrolytic activity under these conditions.

Origin of Short-Chain and Long-Chain Retinyl Ester Hydrolase Activities. In order to determine if either or both of these two activities originated from the pancreas, brush border membrane preparations from rats who had undergone common duct ligation were compared to preparations from sham-operated control animals. The small intestines were collected 48 h after surgery. This allowed for preparation of BBM from a population of enterocytes that had arisen in the absence of pancreatic juices, as 1.7- and 1.4-day turnover times have been reported for rat duodenal and jejunal epithelium, respectively [reviewed in Creamer (1967)]. Consequently, for enzymatic activities intrinsic to the brush border, preparations obtained from common duct ligated rats should be similar to preparations obtained from sham-operated controls. In contrast, if an activity originates from the pancreas and later associates with the brush border, the brush border preparations obtained from common duct ligated animals should have substantially reduced activity when compared to sham-operated controls. A similar method has been used to demonstrate that immunohistochemical staining of the absorptive cell with anti-CEH antibodies is due to CEH of pancreatic origin (Gallo et al., 1980).

The preparations from animals with ligated common ducts were found to have only 3.5 ± 1.4% of the specific activity for retinyl caproate hydrolysis observed for the sham-operated controls (on the basis of analysis of three BBM preparations assayed in taurocholate, each prepared from a single animal). In contrast, 71 ± 6% of retinyl palmitate hydrolytic activity was retained, compared to the sham-operated controls. This substantial retention, when compared to the almost complete loss of retinyl caproate hydrolytic activity, indicated that most or all of the long-chain retinyl esterase activity was intrinsic to the brush border, while the short-chain retinyl esterase activity originated from the pancreas. The moderate decrease in retinyl palmitate hydrolytic activity may have been due to several factors. Along with the possibility that some of the activity against retinyl palmitate originated from the pancreas, loss of activity may have resulted from secondary effects from events such as pancreatitis or loss of bile salts in the lumen of the small intestine. Also, individual variation between rats or slight differences in the preparations of the BBM may have contributed to the slightly lower specific activity.

The effect of common duct ligation was seen clearly when a complete retinyl ester specificity profile was determined with taurocholate from the BBM prepared from those rats (Figure 8). The marked decrease in the ability to hydrolyze esters of fatty acyl moiety chain lengths of 10 or less by preparations from common duct ligated animals was clearly evident, and the pattern was distinctly different from that seen with BBM preparations from unaltered animals assayed with the same detergent (compare to Figure 4). Preparations from common duct ligated animals now hydrolyzed retinyl palmitate at the greatest rate, with significant hydrolysis of retinyl myristate (14:0) and retinyl laurate. This pattern, with taurocholate, closely resembled the pattern seen with 0.5% deoxycholate for preparations from unaltered animals (see Figure 3). The retinyl palmitate hydrolytic activity of BBM from common duct ligated animals was also stimulated by deoxycholate (data not shown) to a degree similar to that seen for BBM from unaltered animals (Figure 2). This established that the long-chain esterase activity intrinsic to the brush border was stimulated by both dihydroxy and trihydroxy bile salts. The possibility that more than one enzyme was responsible for this intrinsic activity cannot yet be eliminated, however.

We also examined the hydrolysis of two unsaturated retinyl esters, which are normally a minor component of dietary esters. With standard BBM preparations, the hydrolysis rates of

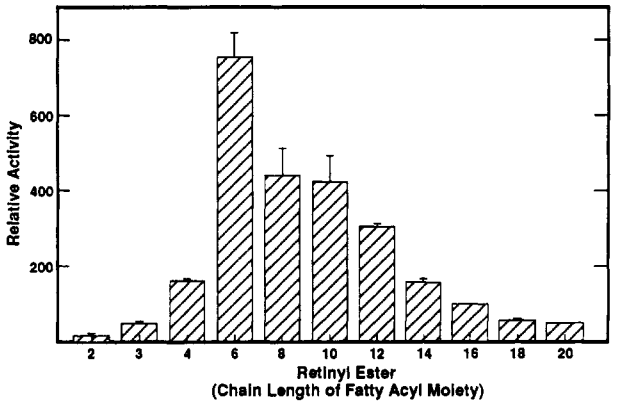

FIGURE 9: Relative hydrolysis of retinyl ester by purified bovine CEH. Relative activity refers to the percent of hydrolytic activity observed with retinyl palmitate [420 nmol/(min·mg of CEH)]. All assays contained 30 ng of CEH and 30 μM retinyl ester substrate with 1% taurocholate in PBS. All other conditions and procedures are given in Experimental Procedures. All determinations were done in triplicate on either two or three occasions, and results were expressed as the average of the mean ± SEM.

retinyl oleate (18:1) and retinyl linoleate (18:2) were 61% and 60% of the rate with retinyl palmitate in 1% taurocholate. However, we observed that only 26% and 31%, respectively, of the activity of standard BBM preparations with retinyl oleate and linoleate substrates were retained in BBM preparations from common duct ligated animals. Thus, the role of the intrinsic long-chain esterase may be less prominent in the hydrolysis of esters with unsaturated fatty acyl moieties.

The brush border short-chain esterase activity demonstrated several properties similar to those known for CEH, in addition to its pancreatic origin. When purified bovine CEH was tested for retinyl ester substrate specificity in 1% taurocholate, maximum rates of hydrolysis were seen with short-chain retinyl esters (Figure 9). This pattern thus was similar to the *difference* between normal and common duct ligated animals in an assay in 1% taurocholate (Figures 4 and 8). This difference would correlate to any brush border retinyl esterase activity that originated from the pancreas. This short-chain retinyl ester hydrolase activity was stimulated only by trihydroxy bile salts (compare Figure 3 to Figure 4), as was reported for human and rat CEH (Lombardo & Guy, 1980; Vahouny et al., 1965). Finally, the K_m for retinyl caproate hydrolysis by purified brush border was similar to that reported for retinyl ester hydrolysis by purified rat CEH (10 μM) in an assay under similar conditions (Harrison & Gad, 1989).

In contrast to the almost complete loss of short-chain retinyl ester hydrolytic activity following common duct ligation, the ability of brush border to hydrolyze retinyl laurate, myristate, palmitate, and stearate was substantially retained. The properties of this intrinsic long-chain esterase indicate that it may have an important physiological role. The calculated K_m for retinyl palmitate and retinyl stearate was less than 1 μM, an appropriately low K_m for an enzyme that would normally encounter only micromolar concentrations of retinyl ester. In an assay in 1% taurocholate, a typical physiological bile salt, a V_{max} of approximately 10 nmol/(min·mg of BBM protein) was observed. Typically, 5 mg of brush border protein was normally obtained from the upper two-thirds of the small intestine of one rat. Therefore, if retinyl palmitate were present at a concentration equal to the calculated K_m for its hydrolysis, that portion of the intestine could hydrolyze 25 nmol/min under these conditions. A growing rat requires 20 IU of vitamin A per day, equivalent to 20 nmol of fatty acyl ester (Altman & Dittmer, 1974). Thus, this esterase could hydrolyze sufficient retinyl palmitate in less than 1 min to meet the daily vitamin A requirement for a rat under these conditions. While the contribution of pancreatic enzymes to luminal retinyl ester hydrolysis may still be significant, it would not appear to be necessary, given the presence of this potent intrinsic activity. The localization of this activity at the brush border, where the liberated retinol is absorbed, suggests that this potent activity may play a significant role in the utilization of dietary fatty acyl retinyl esters.

A bile salt-stimulated, intrinsic brush border phospholipase A_2 has been purified from rat (Pind & Kuksis, 1989) and guinea pig (Gassama-Diagne et al., 1989). Since lipases are often broad in substrate specificity, it is possible that this long-chain retinyl ester hydrolase activity is due (at least partially) to this enzyme. However, the long-chain retinyl esterase activity described here was specific with regard not only to the length of the fatty acyl moiety but also to the nature of the alcohol moiety, as we observed that 13-*cis*-retinyl palmitate was hydrolyzed at only 3% the rate of all-*trans*-retinyl palmitate. In addition, it has been reported that the brush border phospholipase A_2 activity in rat is more strongly stimulated by conjugated bile salts than their unconjugated analogues (Pind & Kuksis, 1988), in contrast to our observed results with retinyl palmitate (Table I). Purification of the enzyme(s) responsible for the long-chain retinyl ester hydrolase activity associated with the brush border should enable us to clarify this point.

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Enzymatic Degradation of Cyclic 2,3-Diphosphoglycerate to 2,3-Diphosphoglycerate in *Methanobacterium thermoautotrophicum*†

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ABSTRACT: 2,3-Diphosphoglycerate (2,3-DPG) has been found to be the product of the enzymatic degradation of cyclic 2,3-diphosphoglycerate (cDPG) in the archaebacterium *Methanobacterium thermoautotrophicum* ΔH. Although 2,3-DPG has not previously been detected as a major soluble component of *M. thermoautotrophicum*, large pools accumulated at an incubation temperature of 50 °C (below the optimum growth temperature of 62 °C). Under these conditions, cellular activity was significantly decreased; a return of the culture to the optimum growth temperature restored the 2,3-DPG pool back to original low levels and caused steady-state cDPG levels to increase again. While $^{13}CO_2$-pulse/$^{12}CO_2$-chase experiments at 50 °C showed that the cDPG turned over, the appearance of 2,3-DPG at NMR-visible concentrations required at least 10 h. Production of 2,3-DPG in vivo was prevented by exposure of the cells to O_2. The enzyme responsible for this hydrolysis of cDPG was purified by affinity chromatography and appears to be a 33-kDa protein. Activity was detected in the presence of oxygen and was enhanced by a solution of 1 M KCl, 25 mM $MgCl_2$, and dithiothreitol. Both K_m and V_{max} have been determined at 37 °C; kinetics also indicate that in vitro the product, 2,3-DPG, is an inhibitor of cDPG hydrolysis. These findings are discussed in view of a proposed role for cDPG in methanogens.

Methanogens are archaebacteria with unusual chemistry (Balch et al., 1979; Daniels et al., 1984; Keltjens & van der Drift, 1986; Jones et al., 1987). Most uniquely, they reduce CO_2 with H_2 and use the electron flow from this reaction to drive ATP synthesis. Methanogens have been shown to have a wide variety of unusual compounds. Some, like methanofuran (Leigh et al., 1984), methanopterin (Van Beelen et al., 1984), and coenzyme M (Taylor & Wolfe, 1971), are involved in methanogenesis. Others like cyclic 2,3-diphosphoglycerate, cDPG[1] (Kanodia & Roberts, 1983; Seeley & Fahrney, 1983; Evans et al., 1985), and β-glutamate (Robertson et al., 1989) have functions that are not well understood. Under standard growth conditions, cDPG is found at very high intracellular concentrations (Seely & Fahrney, 1983) and is the major carbon and phosphorus pool in the thermophilic, autotrophic organism *Methanobacterium thermoautotrophicum* ΔH. cDPG has also been detected (Tolman et al., 1986) in several members of Methanobacteriaceae, a phylogenetically coherent group which have a pseudomurein cell wall. More recently, it has been detected at low intracellular levels in *Methanosarcina frisia* (Rudnick et al., 1990), an organism from a non-pseudomurein-containing family. This compound is under unusual and stringent regulation. Its levels have been correlated with cell growth (Seely & Fahrney, 1984); the lower the rate of growth, the lower the cDPG levels. While its

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[1] Abbreviations: cDPG, cyclic 2,3-diphosphoglycerate; 2,3-DPG, 2,3-diphosphoglycerate; 3-PG, 3-phosphoglycerate; 2-PG, 2-phosphoglycerate; 1,3-DPG, 1,3-diphosphoglycerate; TES, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; P_i, inorganic phosphate; CCCP, carbonylcyanide *m*-chlorophenylhydrazone; TEA, triethanolamine.

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