The Effect of Fatty Acid Perfusion on Intestinal Alkaline Phosphatase

II. Studies on the Rat

JUAN-R. MALAGELADA, MD, WILLEM G. LINSCHEER, MD, and WILLIAM H. FISHMAN, PhD

The effect of intestinal fatty acid perfusions on translocation of the brush-border enzyme, intestinal alkaline phosphatase, was assessed in the rat. Each of two consecutive loops of proximal small bowel were perfused simultaneously in anesthetized rats with three solutions in random order. The solutions consisted of a control solvent solution containing sodium taurocholate, a reference fatty acid solution containing C18 : 1 in taurocholate, and a test solution containing either C18 : 1, C16 : 1, C12 : 0, C8 : 0 or C6 : 0 in taurocholate. When fatty acid was present in the perfusate more enzyme was released into the intestinal lumen than by taurocholate alone. Long-chain fatty acids were 3.4 times more effective than medium-chain fatty acids in releasing intestinal alkaline phosphatase ($P < 0.001$). An exponential relationship was found between fatty acid chain length and release of enzyme. The effect of each fatty acid on the release of enzyme was similar in the two loops and reversible. Another brush-border enzyme, sucrase, used as a reference enzyme, was also released by the fatty acid perfusions. The amount released was also related to the carbon-chain length of the fatty acid but less sucrase than intestinal alkaline phosphatase was released in response to fatty acid perfusion. Histochemical studies on the activity of alkaline phosphatase confirmed the outflow of intestinal alkaline phosphatase from the brush border into the bowel lumen. Simultaneously, intestinal alkaline phosphatase also moved in the opposite direction into the mucosal lymphatics. These changes were most pronounced following long-chain fatty acid perfusions, and less apparent after medium-chain fatty acid perfusion. The data demonstrated: (1) a bidirectional flow of brush-border intestinal alkaline phosphatase, and (2) that luminal enzyme activity is exponentially related to the carbon-chain length of the fatty acid being absorbed. The results suggest participation of intestinal alkaline phosphatase in fat absorption.

Previous studies in man (Part I) demonstrated that perfusion of long-chain fatty acids (LCFA) released more intestinal alkaline phosphatase (IAP) from the jejunal mucosa into the bowel lumen than a medium-chain fatty acid. Simultaneously, increased activity of IAP was observed in the lamina propria and in the intestinal lymphatic system during absorption of LCFA.

The present study was designed to confirm the human observations in an animal model and to establish the correlation between carbon-chain length of absorbed fatty acid and the translocation of muco-

From the Lemuel Shattuck Hospital and New England Medical Center Hospitals, Tufts University School of Medicine, Boston, Massachusetts, and the Veterans Administration Hospital and State University of New York Upstate Medical Center, Syracuse, New York.

This work was supported in part by the John A. Hartford, Jr., Foundation, New York, and by Research Grant AM 11566, the Medical Foundation, Inc. Grant (Boston) (to Dr. Willem G. Linscheer) and CA 07538, 5-K6CA18, 453 (to Dr. William H. Fishman), from the National Institutes of Health, U.S. Public Health Service.

Dr. Juan-R. Malagelada was a recipient of a Juan March Foundation Fellowship. His present address is Department of Gastroenterology and Internal Medicine, Mayo Clinic, Rochester, Minesota 55901.

Address for reprint requests: Dr. Willem G. Linscheer, Veterans Administration Hospital, 800 Irving Avenue, Syracuse, New York 13210.

sal IAP by testing a wide range of fatty acids of different carbon-chain lengths. As in the human experiments, the effect of fatty acids on the release of another brush-border enzyme, sucrase, was established.

The rat has been selected for these studies because: (1) Fishman and Ghosh (1) showed that the mucosal IAP in the rat had approximately the same characteristics as the human intestinal isoenzyme. (2) An increase of IAP in the intestinal brush border was observed by Madsen and Tuba (2) following fat feeding.

MATERIALS AND METHODS

Male Wistar rats (250–260 g), whose pancreatic ducts drain only into the common duct, were chosen for study (3). All animals were fasted for 18 hr prior to the procedure. They had free access to tap water.

Rats were anesthetized by pentobarbital (40 mg/kg) injected intraperitoneally. Two sequential loops of small bowel (20 cm in length) were isolated for perfusion studies. The proximal loop was prepared just distal to the papilla of Vater. A 2-mm (OD) catheter was inserted and fixed by a ligature in the proximal ends of the loops. A 4-mm (OD) catheter was used for collection from the distal end. Special care was taken during the surgical procedure to minimize manipulation and injury of the intestine or mesenterium. The abdominal wall was closed with sutures prior to the perfusion procedure. The length of each loop was measured following completion of the study.

In separate perfusion experiments performed for histochemical studies, a biopsy of the intestinal wall, 5 mm long and involving all layers, was taken just proximal to each loop prior to perfusion and a second biopsy was obtained from the proximal portion of each loop at the conclusion of the perfusion procedure.

Perfusion Technique

A screw-driven syringe pump (Harvard Apparatus Co., Dover, Massachusetts, Model #901) utilizing two 50-ml syringes was used for maintaining a constant rate of perfusion. Each perfusion was preceded and followed by a 15-min washing period with isotonic saline (perfusion rate during washing: 7.64 ml/min). Three solutions (one control, one standard oleic acid solution, and one of the five different test solutions) were perfused in random order for 45 min each (perfusion rate of control and test solutions: 0.392 ml/min). The number of rats studied in each series of perfusions is given in Table 1.

Three-hour perfusions were also performed comparing oleic acid (3 rats), octanoic acid (3 rats), and the control solution (3 rats), in order to study kinetics of enzyme release during long-term perfusions and in an attempt to deplete the brush border of enzymes.

The perfusates were collected from the distal end of each loop in containers maintained at 0° C and then stored at −20° C. There were three 15-min collections during each 45-min perfusion of each test solution and two collections during the 15-min washing procedure (at 12 and 15 min, respectively).

Composition of Perfusates

The control solution consisted of sodium taurocholate (55.7 mM) dissolved in lactose–Ringer solution. The reference standard solution consisted of control solution in which ^{3}H-labeled oleic acid (34.7 mM) was dissolved (New England Nuclear, Boston, Massachusetts, specific activity ^{3}H-labeled fatty acids 1 μCi/0.72 mM; ^{14}C-labeled fatty acids 1 μCi/0.72 mM; palmitoleic acid (C16 : 1) was not labeled). The test solutions contained equimolar concentrations of a ^{14}C-labeled fatty acid of increasing carbon chain length (C6 : 0, C8 : 0, C10 : 0, C12 : 0, C16 : 1, or C18 : 1) (Eastman Organic Chemicals, Rochester, New York; the purity of the fatty acids was tested by gas liquid chromatography; only trace amounts of contaminating fatty acids were present). All solutions contained polyethylene glycol (PEG), 5 g/liter.

The taurocholate concentration in the perfusates (55.7 mM) is similar to that in human gallbladder bile and within the physiologic range for the mouse, but it is higher than normal for the rat. However, biopsies taken after the perfusions, as examined by light microscopy, did not differ from control perfusion studies utilizing lower sodium taurocholate concentrations (see later). Furthermore, experiments in 3 rats with C8 : 0 and C18 : 1 in 30 mM sodium taurocholate solutions released IAP in similar ratios as observed for the higher taurocholate concentration. The presence of fatty acids had no effect on enzyme activity.

Perfusions for Histochemical Studies

Single 3-hr perfusions of the oleic acid reference solution were performed for histochemical studies of the intestinal mucosa (6 rats). In addition, perfusates with lower taurocholate concentrations were studied (5, 10, 20, 30, and 40 mM in groups of 6 rats for each concentration) to assess histologically the effects of taurocholate on the intestinal mucosa. The oleic acid concentration was constant (20 mM). Octanoic acid solutions in sodium taurocholate (20 and 55.7 mM) were perfused in 12 rats. Biopsies were taken before and after each perfusion as described under surgical procedures.

Histochemical Methods

Two methods were used for demonstration of alkaline phosphatase activity. The first method was described in detail in the preceding paper. For the second method, biopsies of the small intestine taken before and after the perfusions were fixed immediately in formol calcium solution for 24 hr (4° C). They were embedded in gelatin and cryostat-sectioned. Sections were stained in 0.05 M propanediol-buffered medium (2-amino-2 methol-1,3 propanediol) containing the alkaline phosphatase substrate, sodium-α-naphthyl acid phosphate (4.4 mM), and Fast Blue BNN for 5 min at pH 9.6. The sections were rinsed briefly in distilled water and mounted with glycerine jelly.

To distinguish the isoenzymes as either intestinal or liver types of alkaline phosphatase, sections were stained following incubation with L-phenylalanine (an inhibitor of

TABLE 1. ALKALINE PHOSPHATASE RELEASE IN BOWEL JUICE (KING-ARMSTRONG UNITS/15 MIN)

	Control solution (taurocholate)				*Taurocholate*				*Taurocholate*			
Perfusion time (min)	0*	15	30	45	0	15	30	45	0	15	30	45
						+ C6 : 0				+ C18 : 1		
Mean (n = 2)	20	314	802	532	27	654	978	1119	63	2825	3744	2287
						+ C8 : 0				+ C18 : 1		
Mean (n = 6)	44	174	443	395	45	246	301	703	20	148	2523	4122
SE	11	53	153	113	14	63	63	396	11	31	870	980
						+ C10 : 0				+ C18 : 1		
Mean (n = 5)	22	141	432	515	14	102	603	909	11	920	2214	3212
SE	10	62	103	107	4	24	246	207	2	421	991	314
						+ C12 : 0				+ C18 : 1		
Mean (n = 7)	22	262	395	384	12	367	1231	602	39	682	2050	3068
SE	8	109	144	121	6	138	374	312	18	463	643	747
						+ C16 : 1				+ C18 : 1		
Mean (n = 4)	42	81	281	268	6	781	1779	1214	23	544	1562	2041
SE	13	26	99	80	3	379	756	538	7	293	535	776

*Time 0 represents alkaline phosphatase activity in the last 3 min. collection of the 15-min saline perfusion.

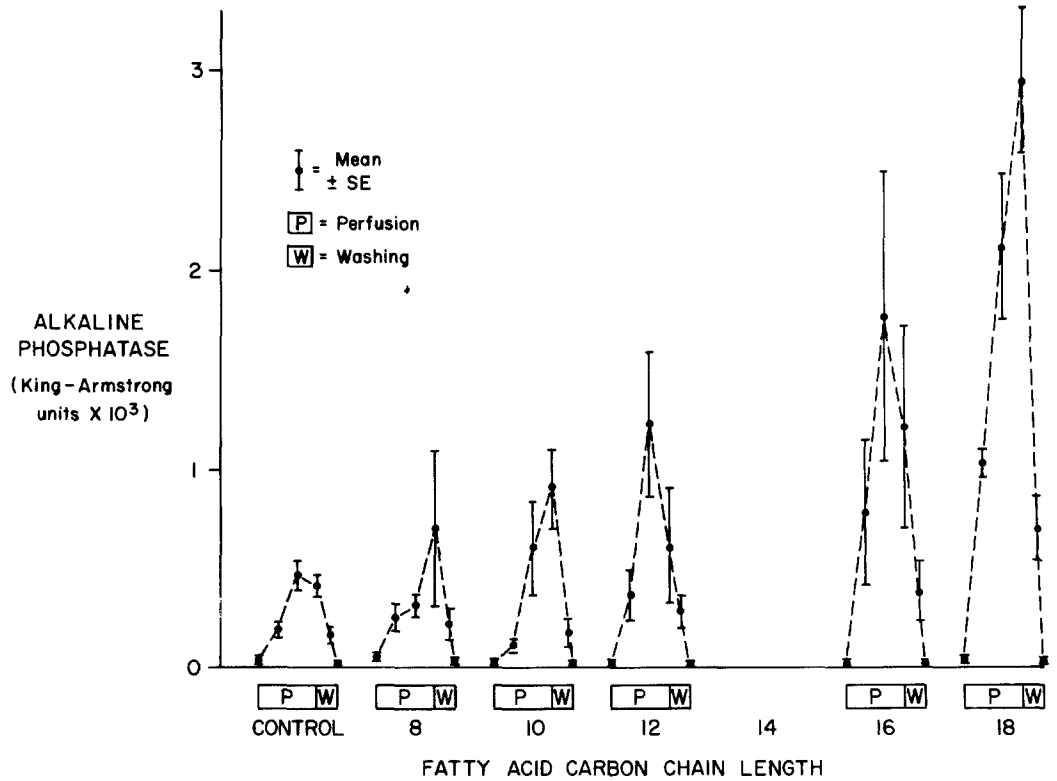

Fig 1. Alkaline phosphatase released from the small-bowel mucosa in perfusates.

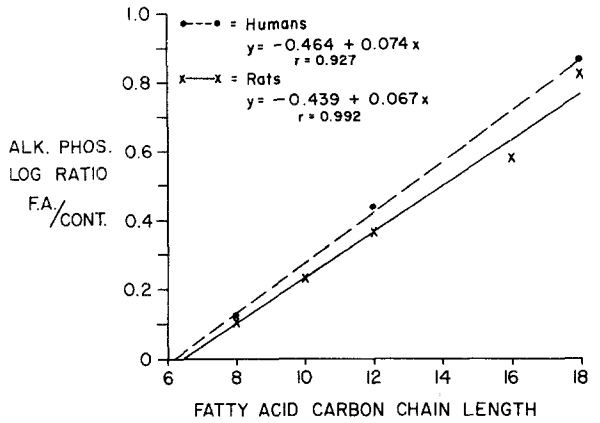

Fig 2. Relation between carbon-chain length of perfused fatty acids and alkaline phosphatase in perfusates in human and rat experiments. The alkaline phosphatase was expressed as the logarithm of ratios between the amounts of alkaline phosphatase released during fatty acid perfusions and during perfusion of taurocholate control solutions.

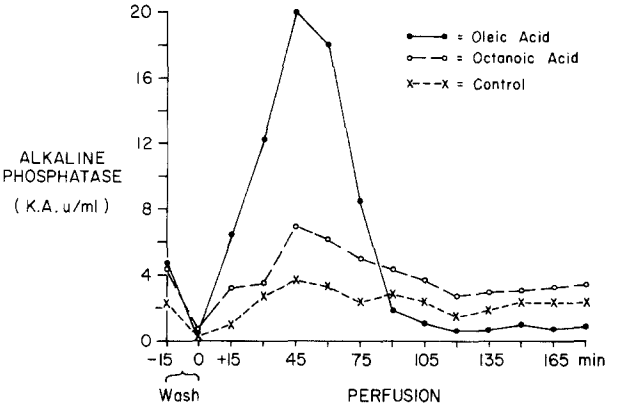

Fig 3. The effect of 3-hr perfusions on the release of alkaline phosphatase in perfusates.

IAP) and L-homoarginine (an inhibitor of hepatic alkaline phosphatase). Electron microscopy was performed in biopsies obtained before and after 3-hr perfusion with the oleic acid solution (3 rats) in order to assess the possibility of anatomical damage of the brush border.

RESULTS

Release of IAP and Sucrase into the Bowel Lumen

The presence of fatty acid in the perfusate significantly increased the activity of IAP over the control taurocholate solution (Table 1). The difference was comparatively small when the perfusate contained medium-chain fatty acids (MCFA) (Figure 1). The mean of the total amounts of IAP released by the three MCFA (C6 : 0, C8 : 0, C10 : 0) over a period of 45 min was 1.5 times higher than that released by the control solution ($P < 0.02$). The LCFA (C12 : 0, C16 : 1, C18 : 1) were much more effective in releasing the isoenzyme. The mean of the total amounts of IAP released into the perfusate was 5.0 times higher than that measured in the control solutions ($P < 0.01$). Thus, LCFA released 3.4 times more IAP than MCFA ($P < 0.01$). In addition, enzyme release was observed to increase expo-

TABLE 2. SUCRASE RELEASE IN BOWEL JUICE (DAHLQVIST UNITS/15 MIN)

	Control solution (taurocholate)				*Taurocholate*				*Taurocholate*			
Perfusion time (min)	0*	15	30	45	0	15	30	45	0	15	30	45
						+ C6 : 0				+ C18 : 1		
Mean (n = 2)	0.25	2.22	2.81	4.22	0.12	3.01	4.83	5.36	0.19	3.39	5.76	16.19
						+ C8 : 0				+ C18 : 1		
Mean (n = 6)	0.32	2.94	4.59	4.71	0.21	2.24	3.45	6.95	0.20	5.49	12.78	16.00
SE	0.03	0.57	1.44	1.37	0.03	0.21	0.81	2.20	0.05	2.43	2.89	3.51
						+ C10 : 0				+ C18 : 1		
Mean (n = 6)	0.21	2.41	3.99	5.03	0.17	4.09	6.56	8.42	0.20	4.30	7.27	16.52
SE	0.04	0.28	0.41	0.58	0.06	0.40	0.81	1.50	0.04	0.65	0.87	1.07
						+ C12 : 0				+ C18 : 1		
Mean (n = 8)	0.13	2.83	3.58	3.92	0.19	3.28	5.17	8.65	0.12	5.71	10.62	14.31
SE	0.02	0.63	0.53	0.71	0.04	0.46	0.75	1.23	0.02	1.29	2.10	2.95
						+ C16 : 1				+ C18 : 1		
Mean (n = 4)	0.14	1.95	3.54	4.69	0.08	3.80	6.25	12.34	0.17	4.79	9.36	15.48
SE	0.05	0.46	0.46	0.35	0.01	0.43	0.91	1.80	0.02	0.69	1.04	1.20

*Time 0 represents sucrase activity in the last 3 min collection of the 15-min saline perfusion.

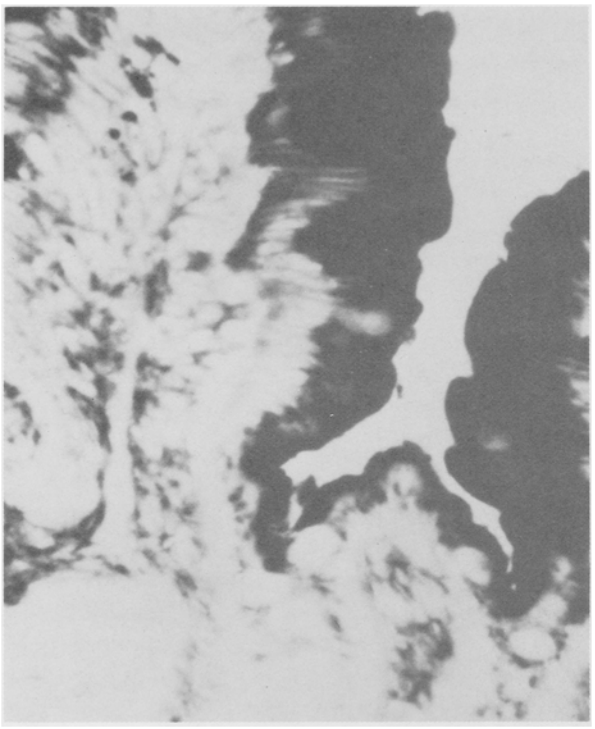

Fig. 4A. Alkaline phosphatase activity (in black) in rat duodenal mucosa before perfusion. The picture represents sections of villi and crypts. Enzyme activity is high in the proximal parts of the epithelial cells and low in the stroma.

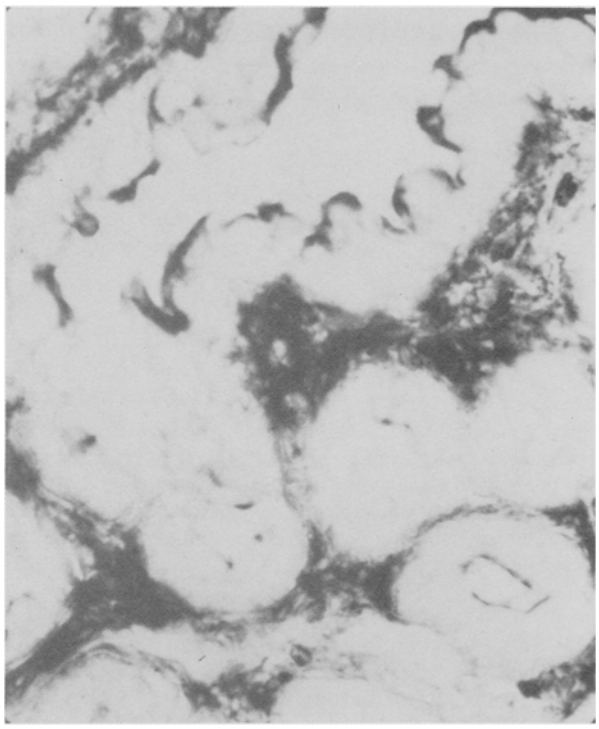

Fig 4B. Alkaline phosphatase activity (in black) in the same rat following a perfusion with oleic acid (3 hr). Enzyme activity decreases in the brush-border area and increases considerably in the stroma. In this preparation enzyme activity became low in the crypts.

nentially with the carbon-chain length of the perfused fatty acids, since a linear correlation was noted between the logarithm of enzyme release (expressed as the ratio of IAP in test and control perfusate) and the carbon-chain length of the fatty acid perfused (Figure 2, $r = 0.992$). In additional studies (oleic acid vs octanoic acid) performed with a lower concentration of sodium taurocholate (30 mM), similar ratios were found between enzyme release and fatty acid carbon-chain length.

Sucrase was released into the bowel lumen during both control and fatty acid perfusions (Table 2). Perfusates contained 1.38 times more sucrase during MCFA perfusions than during the control perfusions ($P < 0.01$). Sucrase released by LCFA increased only by a factor of 1.9 over MCFA as compared to a factor of 3.4 for IAP ($P < 0.01$). The order of infusions had no effect on the release of either enzyme.

Figure 3 depicts the release of IAP in response to the fatty acid perfusions of longer duration (3 hr). A gradual increase of the IAP concentration in the luminal contents was observed during the first 45-min perfusion of oleic acid, remaining constant for the next 15 min and decreasing during the next half hour to approximately 20% of the peak value. It then remained constant for the last 90 min of perfusion. When octanoic acid was perfused, peak values were considerably lower than those observed during oleic acid perfusions. Perfusion of control solution resulted in low levels of IAP, which remained stable throughout the perfusion period. Perfusions with oleic acid and octanoic acid resulted in higher sucrase levels, however, remained constant throughout the 3-hr perfusion period.

Fatty acid absorption rates (fatty acid perfusion rate: 13,600 nmol/min). Rates of absorption of perfused fatty acids expressed in n mol/cm of intestine

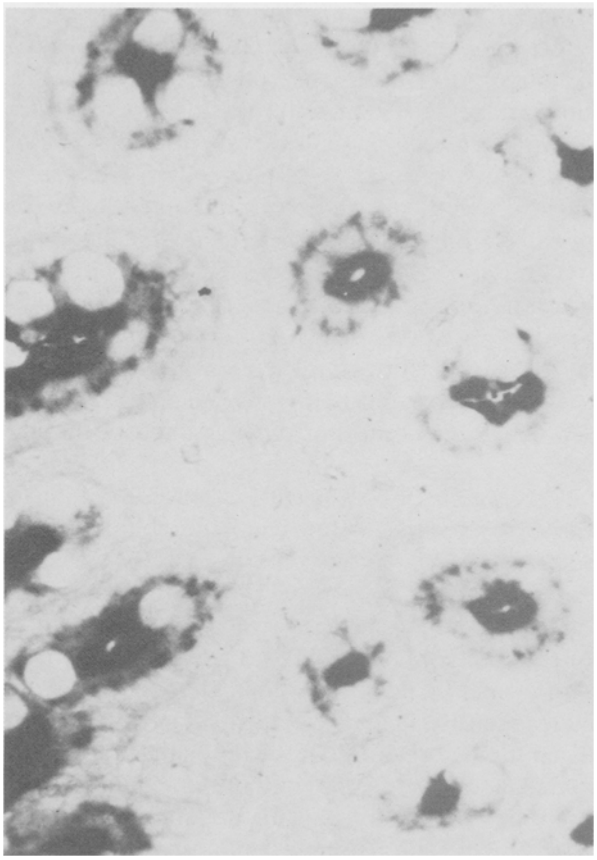

Fig 5A. Alkaline phosphatase activity (in black) in the duodenal crypts before perfusion.

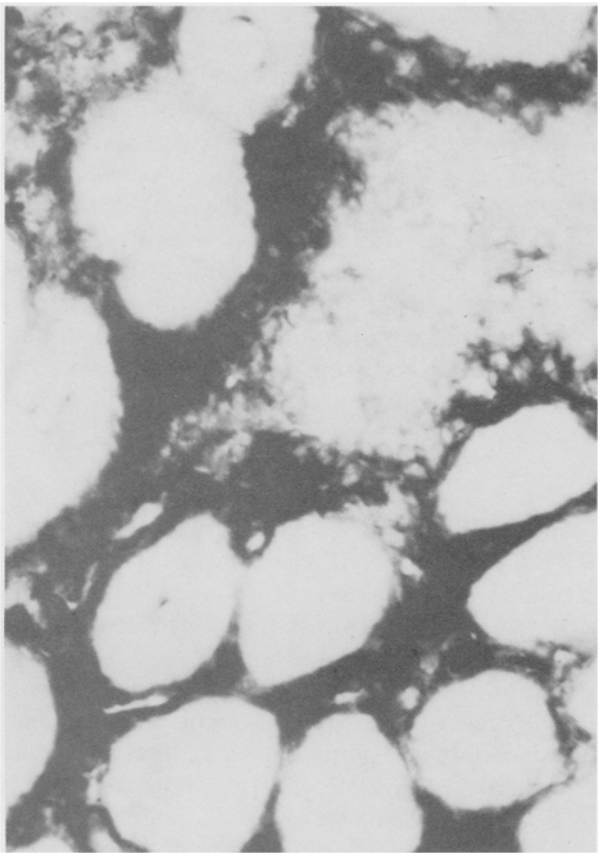

Fig 5B. Alkaline phosphatase activity (in black) after oleic acid perfusion (3 hr). This is a selected preparation showing a strong shift of enzyme activity from the brush border region of the interglandular stroma.

perfused/minute were: C6 : 0, 201.9 ± 12.6 (mean ± SE); C8 : 0, 191.6 ± 7.4; C10 : 0, 177.6 ± 11.9; C12 : 0, 157.5 ± 13.0; and C18 : 1, 113.0 ± 9.0. The rate of absorption decreased linearly with the increase in the fatty acid carbon-chain length (the correlation coefficient $r = 0.997$).

Histochemistry. Before perfusions (Figures 4A and 5A), an intensely positive reaction for alkaline phosphatase was demonstrated in the brush border and Golgi region of the epithelial cells of the villi and crypts. Material giving a moderately positive reaction was observed in the basal region of these cells, in the lamina propria of the villus, and between the crypts. Preincubation of the slides with L-phenylalanine, an inhibitor of IAP, markedly decreased the reaction in the brush border and Golgi region. No substantial change was detected after perfusion with the control solution containing only sodium taurocholate. After octanoic acid perfusion, some granules giving a positive reaction for IAP were seen in the lamina propria, but most of the enzyme remained in the original location. In contrast, after oleic acid perfusion (Figures 4B and 5B), enzyme activity was markedly decreased in the brush border and Golgi region of the columnar epithelium but an intense positive reaction was observed in small granules (approximately 1 μm in diameter) present in the lumen of the intestine. Similar granules appeared in the eosinophilic leukocytes, in the macrophages and matrix of the lamina propria tissue, and in the reticulum of submucosal lymph nodes. Histochemical studies of serial sections demonstrated that particles with a high alkaline phosphatase activity were located in the same area as

the fat particles. Details concerning the location and properties of the isoenzymes have been published elsewhere (4).

Light microscopy studies failed to demonstrate any differences between solutions containing variable concentrations (5–40 mM) of taurocholate. Electron microscopy studies of the intestinal epithelium before and after perfusion with oleic acid in 55.7 mM taurocholate solution were also performed to determine whether fatty acid and bile acid perfusions produced visible damage to the brush border. After 3 hr perfusion with the oleic acid solution, the microvilli remained intact.

DISCUSSION

These experiments were designed to establish rodent brush-border IAP release in response to perfused fatty acids of varying carbon-chain length and to compare the results with the human experiments described in the preceding paper.

As in the human, the control solution containing bile salts released more IAP from the intestinal mucosa than did the isotonic saline solution. This may be explained by the detergent properties of bile salts since other detergents have a similar effect (5). Fatty acids released IAP into the bowel lumen, and a linear relation was found between the logarithm of the ratios of enzyme activity in perfusates of fatty acid test solutions vs control solutions and the carbon chain length of the perfused fatty acid. This is the expression of an exponential function. The similarity of the slopes of the regression lines in the human and rat experiments (Figure 2) indicates that the degree of enzyme release in the lumen is probably identically related to the carbon-chain length of the luminal fatty acids in both species.

Luminal IAP concentrations in the rat decreased to 20% of peak values after 60 min of perfusion in contrast to stable outputs in humans during 3-hr perfusions. This suggests that the release of brush-border IAP in the rat, but not in man, probably exceeded the rate of synthesis. This was supported by the histochemical studies showing loss of IAP activity in the brush border (Figures 4B and 5B). LCFA released only 1.9 times more sucrase than the control solution, in contrast to a factor 5 for IAP. Thus, sucrase is probably released at a slower rate, explaining the more sustained luminal sucrase levels observed during the 3-hr perfusions.

Histochemical methods confirmed the flow of IAP from the brush border into the bowel lumen during LCFA perfusions. During fasting, the epithelial cells are very rich in the intestinal isoenzyme of alkaline phosphatase, while the activity of alkaline phosphatase in the lamina propria is low (6, 7) and consists primarily of the liver isoenzyme (4). After oleic acid perfusion, enzyme activity decreased drastically in the brush borders and practically disappeared from the Golgi region. At the same time, IAP activity strikingly increased in the mucosal lamina propria (Figures 4B and 5B). These changes were less apparent after MCFA perfusions. IAP and lipid studies performed on serial sections suggested that fat particles and IAP were present in the same locations during transport of lipids across the mucosa.

The combination of perfusion studies and histochemical techniques showed a bidirectional translocation of IAP activity during LCFA perfusion from the brush border into: (1) the intestinal lumen, and (2) the lamina propria, where enzyme activity concentrated in areas where the absorbed lipids were located. Our observations are open to several interpretations. One possibility is that fatty acids affected the permeability of the epithelial membranes producing release of enzymes into the lumen. However, this would not explain the simultaneous increase in IAP activity in the lamina propria. Alternatively, it can be proposed that release and translocation of the enzymes is mediated via the gut hormones like cholecystokinin–pancreozymin (CCK-PZ) or secretin. Others (8–10) supported this hypothesis by demonstrating release of intestinal alkaline phosphatase from the gut mucosa by parenteral administration of CCK-PZ in dogs and in humans. Further, Malagelada et al (11) showed by a human CCK-PZ bioassay that more CCK-PZ was released by LCFA than by MCFA, which may explain the observed greater effect of LCFA over MCFA.

Our rat and human data raise the possibility of a functional role of intestinal alkaline phosphatase in the absorption of LCFA. Glickman et al (12) have observed increasing concentrations of alkaline phosphatase in intestinal lymph of the rat during fatty acid absorption. Lam and Mistilis (13) have correlated IAP activity and fat transport in intestinal lymph and postulated a possible role of the enzyme in reesterification of fatty acid or lipoprotein synthesis in the intestinal mucosa. Further support for this hypothesis is provided by previous experiments from our laboratory showing a significant reduction of the rate of absorption of oleic acid by addition of

L-phenylalanine (a specific inhibitor of IAP) to oleic acid perfusate in normal volunteers (14). The same inhibitor incorporated in the diet of patients with type V hyperlipoproteinemia significantly lowered plasma triglyceride concentrations (15). The above evidence suggests that IAP participates in the process of fat absorption, but further studies are required to define its role and to elucidate its mechanism of action.

ACKNOWLEDGMENTS

The authors are indebted to the late Dr. Hugo Muench for performing the statistical analysis. They also extend grateful thanks to Patricia Burns, Sheila Liberman, Aldona Rajacki, and Willem Timmer for their technical assistance.

REFERENCES

1. Fishman WH, Ghosh NK: Isoenzymes of human alkaline phosphatase. Adv Clin Chem 10:255–370, 1967
2. Madsen NB, Tuba J: One source of alkaline phosphatase in rat serum. J Biol Chem 195:741–750, 1952
3. Lambert R. Surgery of the Digestive System of the Rat. Springfield, Illinois, Charles C Thomas, 1965, p 110
4. Bluestein M, Malagelada JR, Linscheer WG, Fishman WH: Enzymorphology of alkaline phosphatase of rat intestine following fatty acid infusion. J Histochem Cytochem 18:679, 1970(abstract)
5. Heizer WD, Isselbacher KJ: Intestinal peptide hydrolases: Differences between brush border and cytoplasmic enzymes. Clin Res 18:382, 1970(abstract)
6. Hugon J, Borgers M: Submicroscopic localization of the alkaline phosphatase activity in the duodenum of the rat. Exp Cell Res 45:698–702, 1967
7. Nordstrom C, Dahlqvist A, Josefsson L: Quantitative determination of enzymes in different parts of the villi and crypts of rat small intestine. J Histochem Cytochem 15:713–721, 1967
8. Dyck WP, Hall FF, Ratliff CR: Hormonal control of intestinal alkaline phosphatase secretion in the dog. Gastroenterology 65:445–450, 1973
9. Dyck WP, Martin JH, Ratliff CR: Influence of secretion and cholecystokinin on intestinal alkaline phosphatase secretion. Gastroenterology 64:599–602, 1973
10. Warnes TW, Hine P, Kay G: The action of secretion and pancreozymin on small intestinal alkaline phosphatase. Gut 15:39–47, 1974
11. Malagelada JR, Go VLW, DiMagno EP, Summerskill WHJ: Pancreatic and biliary secretion are regulated by rate of fatty acid absorption. Gastroenterology 64:767, 1973(abstract)
12. Glickman RM, Alpers DH, Drummy GD, Isselbacher KJ: Increased lymph alkaline phosphatase after fat feeding: Effects of medium chain triglycerides and inhibition of protein synthesis. Biochem Biophys Acta 201:226–235, 1970
13. Lam KCh, Mistilis SP: Role of intestinal alkaline phosphatase in fat transport. Austr J Exp Biol Med Sci 51:411–416, 1973
14. Linscheer WG, Malagelada JR, Fishman WH: Diminished oleic acid absorption in many by L-phenylalanine inhibition of an intestinal phosphohydrolase. Nature (London), New Biol 231:116–117, 1971
15. Linscheer WG: Reduction of hyperchylomicronemia (type V) by ingestion of an inhibitor of intestinal alkaline phosphatase. Gastroenterology 66:A-78/732, 1974(abstract)