

## Fatty acid binding protein. Role in esterification of absorbed long chain fatty acid in rat intestine.

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### Research Article

Fatty acid binding protein (FABP) is a protein of 12,000 mol wt found in cytosol of intestinal mucosa and other tissues, which exhibits high affinity for long chain fatty acids. It has been suggested that FABP (which may comprise a group of closely related proteins of 12,000 mol wt) participates in cellular fatty acid transport and metabolism. Although earlier findings were consistent with this concept, the present studies were designed to examine its physiological function more directly. Everted jejunal sacs were incubated in mixed fatty acid-monoglyceride-bile acid micelles, in the presence or absence of equimolar concentrations of either of two compounds which inhibit oleate binding to FABP: flavaspidic acid-N-methyl-glucaminate and alpha-bromopalmitate. Oleate uptake, mucosal morphology, and oxidation of [<sup>14</sup>C]acetate remained unaffected by these agents, but oleate incorporation into triglyceride was inhibited by 62-64% after 4 min. The inhibition by flavaspidic acid was reversible with higher oleate concentrations. The effect of these compounds on enzymes of triglyceride biosynthesis was examined in intestinal microsomes. Neither flavaspidic acid nor alpha-bromopalmitate inhibited acyl CoA:monoglyceride acyl-transferase. Fatty acid:coenzyme A ligase activity was significantly enhanced in the presence of partially purified FABP, probably reflecting a physical effect on the fatty acid substrate or on the formation of the enzyme-substrate complex. Activity of the enzyme in the presence of 0.1 mM oleate was only modestly inhibited by equimolar [...]

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# Fatty Acid Binding Protein

## ROLE IN ESTERIFICATION OF ABSORBED LONG CHAIN FATTY ACID IN RAT INTESTINE

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**ABSTRACT** Fatty acid binding protein (FABP) is a protein of 12,000 mol wt found in cytosol of intestinal mucosa and other tissues, which exhibits high affinity for long chain fatty acids. It has been suggested that FABP (which may comprise a group of closely related proteins of 12,000 mol wt) participates in cellular fatty acid transport and metabolism. Although earlier findings were consistent with this concept, the present studies were designed to examine its physiological function more directly.

Everted jejunal sacs were incubated in mixed fatty acid-monoglyceride-bile acid micelles, in the presence or absence of equimolar concentrations of either of two compounds which inhibit oleate binding to FABP: flavaspidic acid-*N*-methyl-glucamate and  $\alpha$ -bromopalmitate. Oleate uptake, mucosal morphology, and oxidation of [<sup>14</sup>C]acetate remained unaffected by these agents, but oleate incorporation into triglyceride was inhibited by 62–64% after 4 min. The inhibition by flavaspidic acid was reversible with higher oleate concentrations.

The effect of these compounds on enzymes of triglyceride biosynthesis was examined in intestinal microsomes. Neither flavaspidic acid nor  $\alpha$ -bromopalmitate inhibited acyl CoA:monoglyceride acyl-transferase. Fatty acid:coenzyme A ligase activity was significantly enhanced in the presence of partially purified FABP, probably reflecting a physical effect on the fatty acid substrate or on the formation of the enzyme-substrate complex. Activity of the enzyme in the presence of 0.1 mM oleate was only modestly inhibited by equimolar

flavaspidic acid and  $\alpha$ -bromopalmitate, and this effect was blunted or prevented by FABP.

We conclude that in everted gut sacs, inhibition of triglyceride synthesis by flavaspidic acid and  $\alpha$ -bromopalmitate could not be explained as an effect on fatty acid uptake or on esterifying enzymes in the endoplasmic reticulum, but rather can be interpreted as reflecting inhibition of fatty acid binding to FABP. These findings lend further support to the concept that FABP participates in cellular fatty acid transport and metabolism. It is also possible that FABP, by effecting an intracellular compartmentalization of fatty acids and acyl CoA, may play a broader role in cellular lipid metabolism.

### INTRODUCTION

The cytosol of rat and human intestinal mucosa, liver, myocardium, kidney, and adipose tissue contains one or more proteins of 12,000-mol wt designated fatty acid binding protein (FABP)<sup>1</sup> which exhibit high affinity for long chain fatty acids (1–3). Hepatic FABP appears closely related to the Z protein of Levi et al. (4), also shown to bind long chain fatty acids (5). We proposed that intestinal FABP participates in the intracellular transport of fatty acid, and that its counterparts in other tissues play a similar role (1).

Recently, the isolation of FABP from rat intestine was described (2), as was the preparation of a specific rabbit antiserum. FABP was quantified in intestinal mucosa by radial immunodiffusion, and was localized chiefly in the cytosol. Its tissue concentration, expressed per milligram of soluble protein, per gram of tissue, and per gram of DNA, was significantly greater in villi than

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<sup>1</sup>Abbreviations used in this paper: FABP, fatty acid binding protein; *R<sub>f</sub>*, retardation factor.

in crypts, and in mucosa from proximal and mid-jejunoileum than from distal. Mucosal FABP concentrations were significantly higher in animals maintained on high fat diets than in those on low fat diets. After intraluminal administration, [<sup>3</sup>H]palmitate was recovered in the FABP fraction of mucosal cytosol.

These findings were consistent with a role for FABP in cellular fatty acid transport, but because the evidence was indirect, additional experimental approaches were required.

Fatty acid uptake by the intestine and other cells is passive; fatty acids appear to interact with plasma membrane lipids, and no specific receptors have been demonstrated (6–10). It was postulated that FABP facilitates the desorption of fatty acid from the inner aspect of the membrane, and that the fatty acid-FABP complex diffuses to the endoplasmic reticulum where the fatty acid is activated to its coenzyme A thioester. If this concept is correct, agents which inhibit the binding of fatty acids to FABP should, thereby, inhibit activation and esterification in everted gut sacs, but should have a lesser effect on initial uptake.

Two compounds which inhibit binding of fatty acid to hepatic Z protein are flavaspidic acid-*N*-methyl-glucamate<sup>3</sup> and  $\alpha$ -bromopalmitate (11). The latter was shown by earlier investigators to inhibit fatty acid oxidation in perfused heart muscle (12) and isolated hepatocytes (13); the oxidation of medium chain fatty acid was not affected (13), while that of glucose was enhanced (12). In 1971, Mehadevan and Sauer concluded that  $\alpha$ -bromopalmitate exerted its effect by competing for an as yet unrecognized cellular transport system for long chain fatty acids (13).

In the present studies, flavaspidic acid and  $\alpha$ -bromopalmitate, structurally dissimilar compounds which are not known to be metabolized in the intestine, were shown to inhibit the binding of oleate to partially purified intestinal FABP, and were employed with preparations of rat intestine in experiments designed to test the proposed function of this protein. The results provide additional support for the hypothesis, and also suggest that FABP may play a broader role in cellular fatty acid metabolism. Portions of these studies were included in a preliminary communication (3).

## METHODS

**Materials.** [<sup>14</sup>C]Oleic acid, [<sup>14</sup>C]sodium acetate, and [<sup>14</sup>C]palmitoyl coenzyme A were purchased from New England Nuclear, Boston, Mass. Sephadex G-25 was obtained from Pharmacia Fine Chemicals, Inc., Piscataway, N. J., and  $\alpha$ -bromopalmitic acid from ICN K&K Laboratories Inc., Plainview, N. Y. Flavaspidic acid-*N*-methyl-glucamate was generously provided by Dr. A. Aho, Turku, Fin-

land. Unlabeled oleic acid, glycerol-1-monooleate, and sodium taurocholate were purchased from Calbiochem, San Diego, Calif. Egg lecithin and defatted bovine serum albumin were purchased from Schwarz/Mann, Div., Becton, Dickinson & Co., Orangeburg, N. Y.; the albumin was delipidated further by the method of Chen (14). Egg lecithin and glycerol monooleate were purified by silicic acid chromatography (15). Tris buffer, unlabeled palmitoyl coenzyme A, ATP, coenzyme A, and dithiothreitol were obtained from Sigma Chemical Co., St. Louis, Mo. Hydroxylamine hydrochloride was purchased from Matheson Coleman & Bell, East Rutherford, N. J. Male Sprague-Dawley rats, 300–350 g, maintained on standard laboratory chow (Feedstuffs Processing Company, San Francisco, Calif.) were used in all experiments.

**Partial purification of intestinal FABP and in vitro binding studies.** Intestinal FABP was partially purified as the fraction of 12,000 mol wt from intestinal mucosal 105,000-g supernate on Sephadex G-50, as previously described (1). Three batches of this material, the "FABP fraction," contained 23.1±2.6% (SEM) FABP, as determined by quantitative radial immunodiffusion (2). For semiquantitative estimates of binding of [<sup>14</sup>C]oleate and flavaspidic acid, ligands dissolved in 8  $\mu$ l methyl ethyl ketone or in 50  $\mu$ l 50% propylene glycol were added to 1.0 ml FABP fraction (0.5 mg/ml) and were subjected to nonequilibrium Sephadex G-25 column gel filtration (1).

**Studies of everted jejunal sacs.** Studies were performed as described and standardized previously (16). Sacs were incubated for up to 4 min in mixed micellar solutions of oleic acid, glycerol monooleate, and sodium taurocholate, with or without flavaspidic acid or  $\alpha$ -bromopalmitate, in Krebs-Ringer bicarbonate buffer containing 5 mM glucose, with no calcium or magnesium. Values for "uptake" were not corrected for adsorbed incubation medium. Although this could overestimate uptake at 1 and 2 min, Sallee et al. (17) showed that at 4 min, correction for adherent fluid in studies with long chain fatty acid in mixed micelles was not essential. In the experiments in which conversion of [<sup>14</sup>C]oleate, [<sup>14</sup>C]acetate, and [<sup>14</sup>C]glucose to <sup>14</sup>CO<sub>2</sub> was measured, sacs were incubated under identical conditions in flasks fitted with a center well. At the conclusion of the incubation, 0.4 ml Hydroxide of Hyamine (Packard Instrument Co., Inc., Downers Grove, Ill.) was added to the center well, and the contents of the flasks were acidified with 0.2 ml of 8 M sulfuric acid. <sup>14</sup>CO<sub>2</sub> was collected during an additional 30-min incubation, and the contents of the center well were assayed for radioactivity. In some experiments, incorporation of [<sup>14</sup>C]glucose into the glycerol and fatty acid moieties of mucosal phospholipids and triglycerides was measured.

Sacs incubated for 4 min in mixed micellar solutions, with or without flavaspidic acid or  $\alpha$ -bromopalmitate, were examined histologically. No significant differences were observed, consistent with the earlier observation that flavaspidic acid was not toxic to liver tissue (18).

Jejunal microsomes were prepared by a modification of the method of Rodgers et al. (19). Intestinal mucosa was homogenized in a teflon-glass Potter-Elvehjem homogenizer in 10 vol of 0.28 M mannitol and 50 mM EDTA in 0.01 M Na<sub>2</sub>HPO<sub>4</sub>, pH 7.4, which contained 0.75% defatted bovine albumin. Addition of albumin to the homogenizing medium was found to diminish the microsomal FFA concentration (ordinarily approximately 0.3  $\mu$ mol/mg microsomal protein) by 80–90% (unpublished observations).

**Microsomal enzyme assays.** Fatty acyl coenzyme A: monoglyceride acyltransferase activity was measured by a modification of the method of Rodgers (20). Incubations

<sup>3</sup>Used interchangeably throughout the text with flavaspidic acid.

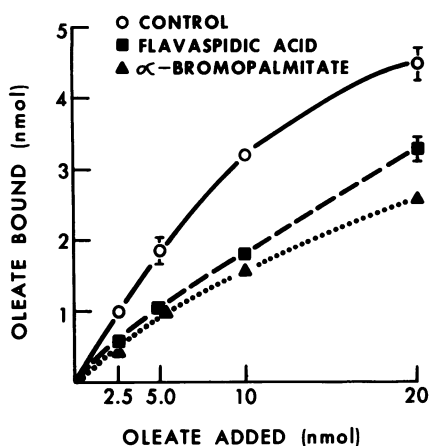


FIGURE 1 Effect of flavaspidic acid and  $\alpha$ -bromopalmitate on [ $^{14}\text{C}$ ]oleate binding to jejunal FABP. [ $^{14}\text{C}$ ]Oleate in 8  $\mu\text{l}$  methyl ethyl ketone was added to 0.52 mg FABP fraction (approximately 6.4 nmol FABP in this preparation) in 1.0 ml 0.154 M KCl in 0.01 M  $\text{KH}_2\text{PO}_4$ , pH 7.4, with or without 10 nmol flavaspidic acid or  $\alpha$ -bromopalmitate. The mixture was applied to Sephadex G-25 columns, 0.9  $\times$  21 cm, 4°C. Oleate binding was calculated from the radioactivity eluting with the void volume.

contained 0.02 mM [ $^{14}\text{C}$ ]palmitoyl coenzyme A, 0.08 mM glycerol monooleate and 0.08 mg microsomal protein in 66.7 mM Tris buffer, and 0.67% albumin; final volume was 0.3 ml. Incubations were for 2 min at 30°C, at which time the reaction was terminated by the addition of methanol and chloroform. Enzyme activity, approximately linear to 2 min and from 0.02 to 0.06 mM palmitoyl coenzyme A, was expressed as nanomoles of palmitate incorporated into diglycerides and triglycerides, determined by thin-layer chromatography of the lipid extract.

Fatty acid: coenzyme A ligase was measured by a modification of the method of Rodgers et al. (21), employing the hydroxamate trapping technique of Kornberg and Pricer (22). Approximately 1.0 mg microsomal protein was incubated with substrate and inhibitors in a final 2-ml volume of 0.1 M Tris buffer, pH 7.4, containing 10.0 mM ATP, 10 mM  $\text{MgCl}_2$ , 5 mM KF, 0.2 mM coenzyme A, 1.04 mM dithiothreitol, 0.75 M hydroxylamine hydrochloride, and up to 2% (vol/vol) ethanol. Incubations were carried out for 15 min (during which time the reaction was approximately linear), at 37°C, and were terminated by the addition of 2 ml 95% ethanol followed by 0.5 ml 6 N HCl. Hydroxamates were extracted from the precipitate with petroleum ether and dried under  $\text{N}_2$ ; color was developed with the Hill reagent (23) and read at 520 nm. Hydroxamate formation was calculated on the basis of molar absorptancy,  $E = 1,000$  (22).

**Analytical procedures.** Lipids were extracted by the method of Folch et al. (24). Lipid classes were separated by thin-layer chromatography on 0.25-mm silica gel H, and identified as described previously (16). Appropriate zones were scraped directly into counting vials and assayed for radioactivity, or were eluted for subsequent quantification.

Flavaspidic acid-*N*-methyl-glucaminatate, readily identified by its bright yellow color, was chromatographically pure in the above thin-layer chromatography system, in which it moved with a retardation factor ( $R_f$ ) of 0.11. This mate-

rial was completely extracted into the chloroform phase of the extraction system of Folch et al. (24), and analysis of everted sacs incubated in flavaspidic acid solutions indicated that all pigmented material so extracted moved with an  $R_f$  identical to that of the native compound, suggesting that it was not metabolized. No flavaspidic acid was detected in the aqueous phase. Advantage was taken of these properties of the compound, and its absorbancy peak at 350 nm to develop an assay for tissue flavaspidic acid concentration. Gut sacs were extracted by the method of Folch et al., and the lipid extract analyzed by thin-layer chromatography. The flavaspidic acid spot was identified, scraped, and eluted with chloroform:methanol (2:1). Absorbancy was compared with a standard curve (flavaspidic acid in chloroform:methanol) and was corrected for a silica gel blank. The assay was linear over a wide range (0.001–0.1  $\mu\text{mol/ml}$ ); recovery from the silica gel was approximately 90%.

Radioactivity was assayed in a Beckman LS-250 liquid scintillation system (Beckman Instruments, Inc., Fullerton, Calif.). Aqueous samples were solubilized in a 10% solution of Biosolv BBS-3 (Beckman Instruments, Inc.) in Liquifluor (New England Nuclear) and toluene.

**Statistical methods.** Significance of differences among experimental groups was determined by paired or unpaired *t* tests (25).

## RESULTS

**Effect of flavaspidic acid and  $\alpha$ -bromopalmitate on binding of oleate to FABP in vitro.** Binding of [ $^{14}\text{C}$ ]oleate to partially purified intestinal FABP fraction was estimated by gel chromatography (Methods). The significant and reversible inhibition of binding by flavaspidic acid and  $\alpha$ -bromopalmitate are apparent (Fig. 1). In other experiments, binding of flavaspidic acid to FABP was inhibited to a similar extent by equimolar oleate. These observations are consistent with the findings of Mishkin et al. (11) in studies of hepatic Z protein fraction.

TABLE I

*Reversibility of Oleate Binding to FABP: Effect of Albumin*

Protein(s) applied to G-50 column	[ $^{14}\text{C}$ ]Oleate recovered in G-50 eluate		
	Applied cpm eluting with		Total percent Recovery
	FABP	Albumin	
	%		
FABP fraction (0.26 mg) +buffer	13.4 (12.8, 13.9)	—	12.8, 13.9
FABP fraction (0.26 mg) +albumin (0.26 mg)	10.1 (10.1, 10.1)	70.0 (71.2, 68.8)	81.3, 78.9

[ $^{14}\text{C}$ ]Oleate was combined with FABP fraction and passed through Sephadex G-25 as described in the legend to Fig. 2. The FABP-oleate complex appearing in the void volume (0.17 mg protein/ml) was mixed with an equal volume (1.5 ml) of buffer or defatted albumin in buffer (0.17 mg/ml) and allowed to stand at 20°C for 15 min. The mixture (containing 3.37 nmol oleate) was then applied to a Sephadex G-50 column (2.5  $\times$  37 cm, 4°C, 3.6-ml fractions) and counts per minute eluting with albumin and FABP were determined. Results are expressed as percent of applied counts per minute recovered. (Approximate molar quantities applied to G-50: oleate, 3.4 nmol; FABP  $\sim$ 4 nmol; albumin  $\sim$ 4 nmol.)

Reversibility of [ $^{14}$ C]oleate to FABP is shown in Table I. [ $^{14}$ C]Oleate-FABP complex was prepared by gel chromatography, mixed with an equal volume of buffer or with defatted albumin in buffer, and re-chromatographed on Sephadex G-50, thus permitting separation of FABP from albumin. It can be seen that most of the [ $^{14}$ C]oleate-FABP complex dissociated during G-50 chromatography, whether or not albumin was present. Significantly, although total recovery of [ $^{14}$ C]oleate was greatly increased in the presence of albumin, this protein had little effect on the retention of [ $^{14}$ C]oleate by FABP (13.4 vs. 10.1%), despite the fact that there were almost certainly many more unoccupied binding sites on albumin than on FABP under these conditions.

*Effect of flavaspidic acid and  $\alpha$ -bromopalmitate on fatty acid uptake and utilization by everted jejunal sacs.*

As shown in Fig. 2, neither flavaspidic acid nor  $\alpha$ -bromopalmitate significantly affected oleate uptake. In contrast, both compounds markedly inhibited incorporation of oleate into triglyceride (Fig. 3). Incorporation into other esterified lipids (phospholipids, diglycerides, and cholesterol ester) also was inhibited, but more variably and to a lesser degree (Table II). Since these other lipids together account only for approximately 10% of the oleate esterified, and only 5–6% of mucosal uptake, the significance of changes in these minor fractions is difficult to interpret. Because of the overall decrease in esterification, however, [ $^{14}$ C]oleate accumulated in the mucosa (Fig. 4), suggesting that little if any fatty acid was diverted into oxidative pathways. In fact, oxidation of [ $^{1-14}$ C]oleate to  $^{14}$ CO $_2$  also was significantly inhibited (Table III), although this minor pathway accounted

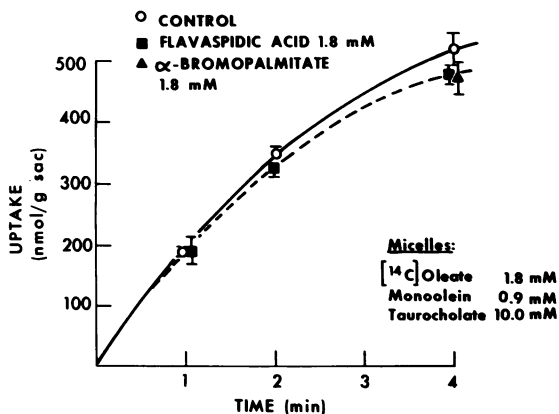


FIGURE 2 Effect of flavaspidic acid and  $\alpha$ -bromopalmitate on uptake of micellar [ $^{14}$ C]oleate by everted gut sacs. Everted jejunal sacs were incubated in Krebs-Ringer bicarbonate medium lacking calcium and magnesium, and containing 5 mM glucose and mixed micelles, with or without flavaspidic acid or  $\alpha$ -bromopalmitate. Fatty acid uptake was calculated from total lipid-extractable radioactivity in the sacs. Mean  $\pm$  SE;  $n = 6$  for controls and flavaspidic acid,  $n = 4$  for  $\alpha$ -bromopalmitate.

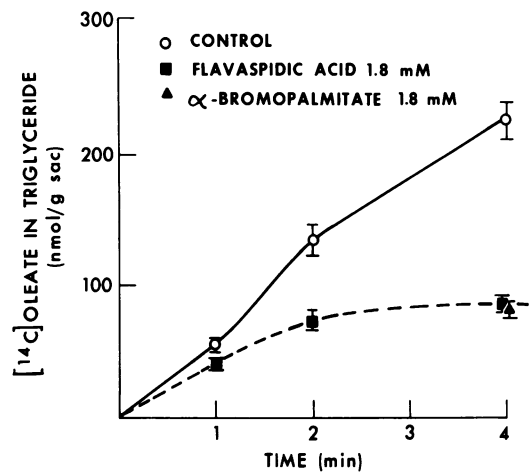


FIGURE 3 Effect of flavaspidic acid and  $\alpha$ -bromopalmitate on gut sac incorporation of [ $^{14}$ C]oleate into triglyceride. Everted jejunal sacs were incubated in mixed micelles, with or without flavaspidic acid or  $\alpha$ -bromopalmitate, as described in Fig. 1 and Methods. Lipids were extracted from sacs at the conclusion of the experiment, and analyzed for  $^{14}$ C after separation by thin-layer chromatography. Mean  $\pm$  SE;  $n = 6$  for controls and flavaspidic acid,  $n = 4$  for  $\alpha$ -bromopalmitate.

for less than 1% of the oleate taken up. Flavaspidic acid was taken up by everted sacs at approximately half the initial rate of [ $^{14}$ C]oleate (Fig. 5). Saturation was nearly reached at 4 min, possibly due to its insignificant biotransformation, so that as tissue concentration gradually increased the medium-tissue diffusion gradient (upon which uptake depends) decreased.

As shown in Table III, neither flavaspidic acid nor  $\alpha$ -bromopalmitate significantly inhibited  $^{14}$ CO $_2$  production from acetate, while both compounds caused in-

TABLE II  
*Effect of Flavaspidic Acid and  $\alpha$ -Bromopalmitate on Gut Sac Incorporation of [ $^{14}$ C]Oleate into Esters other than Triglycerides*

	Phospholipids	Diglycerides	Cholesterol esters
	<i>nmol incorporated/g sac/4 min</i>		
Control	23.2 $\pm$ 1.4	60.0 $\pm$ 3.1	6.2 $\pm$ 1.7
Flavaspidic acid, 1.8 mM	23.1 $\pm$ 1.6	44.3 $\pm$ 1.7*	5.3 $\pm$ 2.0
$\alpha$ -Bromopalmitate, 1.8 mM	11.4 $\pm$ 1.5†	21.9 $\pm$ 3.1§	2.3 $\pm$ 0.7

Incorporation of [ $^{14}$ C]oleate (1.8 mM in mixed micelles) into mucosal esters other than triglyceride was determined at 4 min as described in the legend to Fig. 4. Mean  $\pm$  SE;  $n = 6$  for control and flavaspidic acid;  $n = 4$  for  $\alpha$ -bromopalmitate. \*  $P < 0.005$  vs. controls. †  $P < 0.001$  vs. controls. §  $P < 0.0001$  vs. controls.

TABLE III

Effect of Flavaspidic Acid and  $\alpha$ -Bromopalmitate on  $^{14}\text{CO}_2$  Production in Everted Jejunal Sacs

Substrate	$^{14}\text{CO}_2$ formed, nmol/g sac/4 min		
	Control	Flavaspidic acid, 1.8 mM	$\alpha$ -Bromopalmitate, 1.8 mM
[1- $^{14}\text{C}$ ]Oleate, 1.8 mM (n = 3)	4.5 $\pm$ 0.5	2.0 $\pm$ 0.1*	—
(n = 3)	2.7 $\pm$ 0.2	—	0.73 $\pm$ 0.28*
[2- $^{14}\text{C}$ ]Acetate, 2mM (n = 4 or 2)	16.3 $\pm$ 1.2	15.7 (14.7, 16.6)	17.8 (21.5, 14.1)
[U- $^{14}\text{C}$ ]Glucose, 5 mM (n = 3)	11.5 $\pm$ 0.6	19.3 $\pm$ 0.1‡	—
(n = 3)	19.7 $\pm$ 0.9	—	25.1 $\pm$ 1.5§

Everted sacs were incubated for 4 min in mixed micelles (Methods) containing the indicated labeled compound. Evolved  $\text{CO}_2$  was collected in a center well and assayed for  $^{14}\text{C}$  (Methods).

\*  $P < 0.01$ , vs. control.

‡  $P < 0.001$ , vs. control.

§  $P < 0.05$  vs. control.

creased oxidation of glucose. This evidence, together with the findings of other investigators (12, 13, 18) indicates that flavaspidic acid and  $\alpha$ -bromopalmitate do not exert a general depressive effect on cell metabolism under these conditions. The conversion of [ $^{14}\text{C}$ ]glucose to mucosal lipids also was examined (Table IV). Similar to their effects on oleate esterification, both flavaspidic acid and  $\alpha$ -bromopalmitate profoundly inhibited incorporation of glucose into triglycerides (both the

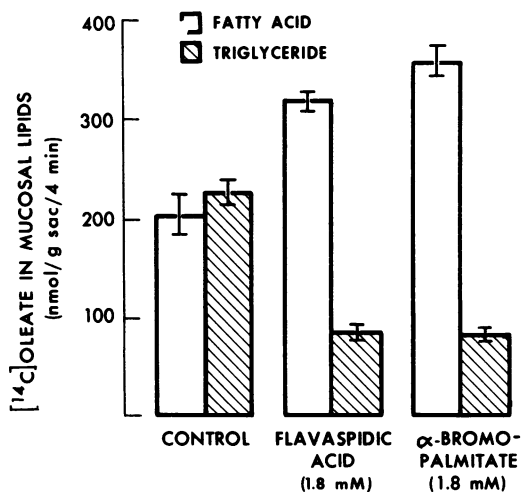


FIGURE 4 Effect of flavaspidic acid and  $\alpha$ -bromopalmitate on incorporation of [ $^{14}\text{C}$ ]oleate into mucosal lipids. Everted jejunal sacs were incubated in mixed micelles, with or without flavaspidic acid or  $\alpha$ -bromopalmitate, as described in Fig. 1 and Methods. Lipids were extracted and analyzed for  $^{14}\text{C}$  in triglycerides and fatty acids by thin-layer chromatography (Fig. 2 and Methods). Mean  $\pm$  SE;  $n = 6$  for controls and flavaspidic acid,  $n = 4$  for  $\alpha$ -bromopalmitate.

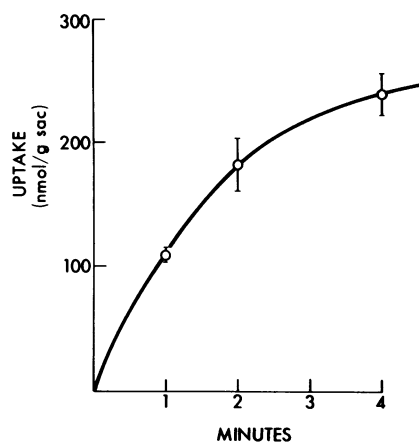


FIGURE 5 Uptake of flavaspidic acid by everted jejunal sacs. Everted jejunal sacs were incubated in mixed micellar solutions (1.8 mM oleate) and 1.8 mM flavaspidic acid. Sacs were extracted and assayed for flavaspidic acid content, as described in Methods. Mean  $\pm$  SE;  $n = 3-6$ .

glycerol and fatty acid moieties), whereas incorporation into phospholipids was virtually unaffected. In Tables III and IV, the effects of flavaspidic acid and  $\alpha$ -bromopalmitate on [U- $^{14}\text{C}$ ]glucose incorporation into  $^{14}\text{CO}_2$  and lipids were studied on different occasions, and each is compared with its own control.

Further evidence that flavaspidic acid did not irreversibly impair mucosal cell function is shown in Fig. 6. In these studies, the effect of 1.8 mM flavaspidic acid on uptake of [ $^{14}\text{C}$ ]oleate and its incorporation into triglyceride by everted sacs was examined as a function of increasing micellar oleate concentration. At all oleate concentrations mucosal uptake was unaffected (Fig. 6A). In the flavaspidic acid experiments (Fig. 6B) increasing oleate led to corresponding increases in incorpora-

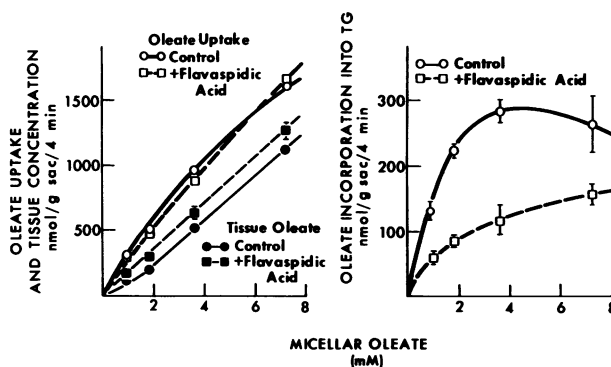


FIGURE 6 Flavaspidic acid inhibition of triglyceride synthesis in everted jejunal sacs: Effect of oleate concentration. Everted jejunal sacs were incubated for 4 min in mixed micelles containing [ $^{14}\text{C}$ ]oleate, monoolein at a concentration one-half that of oleate, and 10 mM taurocholate, with or without flavaspidic acid, 1.8 mM. Sacs were analyzed for  $^{14}\text{C}$ -lipids, as described in Methods. Mean  $\pm$  SE;  $n = 4-6$ . (A). Oleate uptake and mucosal concentration of [ $^{14}\text{C}$ ]FFA. (B). Mucosal [ $^{14}\text{C}$ ]triglyceride.

TABLE IV  
Effect of Flavaspidic Acid and  $\alpha$ -Bromopalmitate on Incorporation of [ $^{14}$ C]Glucose into Phospholipids and Triglycerides by Everted Jejunal Sacs

	Phospholipids			Triglycerides		
	Fatty acids	Glycerol	Total	Fatty acids	Glycerol	Total
	<i>ng atoms incorporated/g sac/4 min</i>					
Control	5.2 $\pm$ 0.2	69.7 $\pm$ 0.7	74.9 $\pm$ 0.9	15.1 $\pm$ 1.6	12.9 $\pm$ 2.3	28.1 $\pm$ 1.5
Flavaspidic acid, 1.8 mM	8.9 $\pm$ 1.3	59.5 $\pm$ 4.5	68.4 $\pm$ 5.7	4.3 $\pm$ 0.6*	3.6 $\pm$ 0.3†	7.8 $\pm$ 0.8§
Control	3.8 $\pm$ 1.2	25.1 $\pm$ 1.0	29.0 $\pm$ 2.2	14.3 $\pm$ 2.3	17.4 $\pm$ 2.8	31.6 $\pm$ 1.3
$\alpha$ -Bromopalmitate, 1.8 mM	5.8 $\pm$ 0.6	19.4 $\pm$ 1.6†	25.2 $\pm$ 2.1	2.7 $\pm$ 0.2*	3.8 $\pm$ 0.1*	6.5 $\pm$ 0.3§

Everted jejunal sacs were incubated for 4 min in mixed oleate-monoolein-taurocholate micelles, as described in Methods and Fig. 4, containing in addition [ $^{14}$ C]glucose, 5 mM. Mucosal lipids were extracted and phospholipids and triglycerides were isolated by thin-layer chromatography. Incorporation into each lipid class and into its lipid-soluble (fatty acids) and water-soluble (glycerol) fractions after saponification is expressed in terms of nanograms atom glucose.

Mean $\pm$ SE,  $n = 3$ .

\*  $P < 0.01$  vs. controls.

†  $P < 0.05$  vs. controls.

§  $P < 0.001$  vs. controls.

tion into triglyceride, so that at 7.2 mM, incorporation was inhibited by only 40%.

It is important to note that differences in oleate-dependent increase in mucosal triglyceride synthesis between control and inhibited sacs could not be explained by differences in mucosal [ $^{14}$ C]oleate. Thus (Fig. 6B), an increase in micellar oleate from 1.8 to 7.2 mM did not significantly affect triglyceride synthesis in controls, whereas synthesis in the sacs incubated with flavaspidic acid nearly doubled. These divergent responses occurred despite (a) similar increments in mucosal [ $^{14}$ C]oleate concentrations under the two conditions (Fig. 6A), and (b) similar mucosal uptakes of flavaspidic acid (237 $\pm$ 41 nmol/g at 1.8 mM oleate; 205 $\pm$ 12 nmol/g at 7.2 mM oleate). These relationships suggest that in control sacs, the overall process of fatty acid esterification is "saturated" at relatively low oleate concentrations in medium and tissue. This and similar previous observations with linoleate and palmitate (16) suggest that one or more post-uptake steps in fatty acid absorption become rate limiting as luminal fatty acid concentrations increases. In contrast, with flavaspidic acid the incorporation of fatty acid into triglyceride is reversibly inhibited, so that "saturation" of this process is approached only at higher substrate concentrations.

Taken together, these experiments indicate that flavaspidic acid and  $\alpha$ -bromopalmitate cause a relatively selective inhibition of fatty acid utilization at a stage after uptake, and at either or both of two sites: (a) in the endoplasmic reticulum, affecting fatty acid activation and(or) esterification; and(or) (b) the intracellular transfer of fatty acid which precedes activation. To resolve this question, the effects of flavaspidic acid and

$\alpha$ -bromopalmitate on the microsomal fatty acid activating and esterifying enzymes were examined.

Effect of flavaspidic acid and  $\alpha$ -bromopalmitate on acyl coenzyme A: monoglyceride acyltransferase activity in jejunal microsomes. Incorporation of [ $^{14}$ C]palmitoyl coenzyme A into diglyceride and triglyceride was examined in intestinal microsomes (Methods). It can be seen (Table V) that neither flavaspidic acid nor  $\alpha$ -bromopalmitate significantly inhibited activity, despite the fact that their concentrations were five times that of the substrate. Therefore, it is most unlikely that inhibition of triglyceride synthesis in everted sacs was due to an effect on monoglyceride acyltransferase. Furthermore, the lack of inhibition of [ $^{14}$ C]palmitoyl CoA incorporation into triglyceride in these experiments suggests that

TABLE V  
Effect of Flavaspidic Acid and  $\alpha$ -Bromopalmitate on Jejunal Monoglyceride Acyltransferase Activity

	Incorporation, nmol/mg protein		
	Diglycerides	Triglycerides	Total activity
Control, $n = 4$	15.0 $\pm$ 0.3	0.8 $\pm$ 0.1	15.8 $\pm$ 0.4
Flavaspidic acid, 0.1 mM	12.6 (12.7, 12.5)	0.6 (0.4, 0.8)	13.2 (13.1, 13.3)
$\alpha$ -Bromopalmitate, 0.1 mM	20.3 (20.8, 19.7)	1.5 (1.6, 1.3)	21.7 (21.0, 22.4)

Jejunal microsomes were prepared and assayed for acyl coenzyme A: monoglyceride acyltransferase activity, as described in Methods. Incubations contained 0.02 mM [ $^{14}$ C]palmitoyl coenzyme A, 0.08 mM glycerol mono-oleate, 0.08 mg microsomal protein, in 66.7 mM Tris buffer containing 0.67% albumin, with or without 0.1 mM flavaspidic acid or  $\alpha$ -bromopalmitate in a final volume of 0.3 ml; 2 min, 30°C.

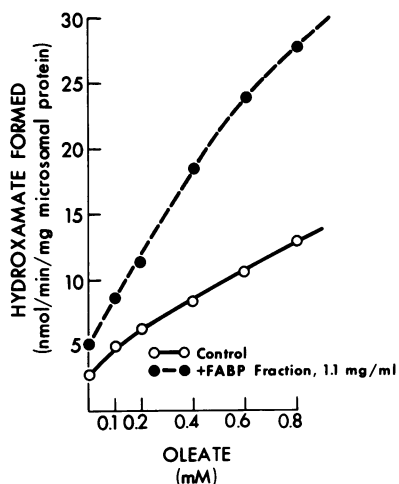


FIGURE 7 Fatty acid: coenzyme A ligase in intestinal microsomes: Effect of substrate and FABP. Jejunal microsomes, 1.1 mg protein, were incubated in 2 ml 0.1 M Tris buffer, pH 7.4, containing oleate, 10.9 mM ATP, 10 mM magnesium chloride, 5 mM KF, 0.2 mM coenzyme A, 1.04 mM dithiothreitol, and 0.75 mM hydroxylamine hydrochloride, for 15 min at 37°C, with or without added FABP fraction (1.1 mg/ml). Fatty acid activation was measured as the amount of hydroxamate formed, as described in Methods. Values shown are means of duplicate incubations.

diglyceride acyltransferase activity also was unaffected.

*Studies of fatty acid: coenzyme A ligase activity in jejunal microsomes.* The effect of substrate on microsomal fatty acid: coenzyme A ligase concentration is shown in Fig. 7. Incubations were carried out under two conditions. In the first, a sonicated oleate emulsion in buffer was added to the incubation. In the second, the incubation included FABP fraction, 1.1 mg/ml (approximately 0.25 mg/ml FABP, i.e., 0.021 mM), or about one-third the concentration present in jejunal cytosol (2). Enzyme activity was nearly linear over a wide range of substrate concentration, but was much greater in the presence of FABP. Possible explanations for this stimulatory effect include the following: (a) endogenous FABP-bound fatty acid was added to the incubation with FABP fraction, thereby increasing substrate concentration; (b) the FABP fraction contained activating enzyme; (c) the FABP complexed an inhibitor present in the microsomes; and (d) the FABP solubilized the fatty acid, or in some manner promoted enzyme-substrate interaction. Each of these possibilities was considered.

With regard to the first, the concentration of endogenous fatty acid in the FABP fraction was approximately equal to that of FABP on a mole basis, as noted in Methods. Thus, 1.1 mg/ml of FABP fraction would increase fatty acid concentration in the incubation by only 0.021 mM, an amount too small to account for the observed differences.

The second possibility, i.e., that FABP fraction contained active enzyme, is essentially excluded by the fact that no hydroxamate was formed in the absence of microsomes (unpublished observations).

The third possible explanation is that an inhibitor, present in the microsomes, was bound by the FABP fraction. Direct examination of this possibility is difficult, since the presence of significant quantities of fatty acid in the microsomes and uncertainties regarding actual "substrate" (presumably monomer) concentration preclude formal kinetic analysis of the data. Furthermore, the nature of this possible inhibitor is not apparent; fatty acid itself or acyl coenzyme A seem unlikely, since substrate concentrations as high as 0.8 mM had no detectable inhibitory effect (Fig. 7) and since all acyl CoA was immediately converted to the hydroxamate. Finally, the addition of albumin, which might also be expected to bind any potential inhibitor, failed to enhance ligase activity (Fig. 8).

These considerations suggest that the fourth possibility is the most likely explanation for the effect of FABP on fatty acid: CoA ligase activity, i.e., that FABP favors the formation of the enzyme-substrate complex, probably via a physical effect on the oleate. Consistent with this concept is the finding that an even more pronounced (but presumably less specific) enhancement of ligase activity was observed when oleic acid was added to the system dispersed in a lecithin emulsion (Table VI). The lecithin employed in these experiments was purified by silicic acid chromatography and did not contain detectable free fatty acids when analyzed by thin-layer chromatography. That the lecithin effect also was not attributable to contamination by fatty acid or to *in vitro* release of lecithin fatty acids by microsomal phospholipase is shown by control experi-

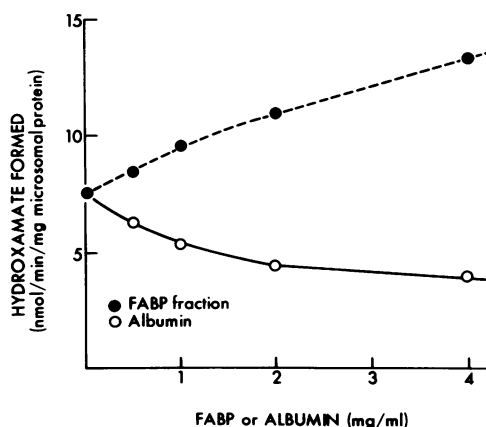


FIGURE 8 Fatty acid: coenzyme A ligase in intestinal microsomes: Effect of FABP and albumin. Jejunal microsomes, 1.1 mg, were incubated with 0.2 mM oleate as described in Fig. 7 and Methods, with varying concentrations of FABP fraction and albumin. Mean of duplicate incubations.



TABLE VI  
Fatty Acid:Coenzyme A Ligase Activity in Intestinal  
Microsomes: Effect of Various Additions

	Hydroxamate formed, nmol/min/mg microsomal protein	
	0.2 mM oleate	No oleate
No additions	5.8	3.9
Lecithin, 0.69 mg/ml	19.7	6.5
FABP fraction, 1.1 mg/ml	12.0	4.4

Jejunal microsomes, 1.2 mg protein, were assayed for fatty acid:coenzyme A ligase activity in the presence or absence of 0.2 mM oleate, as affected by the addition of egg lecithin, 0.69 mg/ml, or FABP fraction, 1.1 mg/ml. Results are expressed as the means of duplicate incubations.

ments in which lecithin (without added fatty acid) was incubated with the microsomal system (Table VI). It can be seen that the enhancement of fatty acid:coenzyme A ligase activity by lecithin was not demonstrable in the absence of the oleic acid substrate. Albumin in contrast to FABP, inhibited the reaction over a wide concentration range (Fig. 8), suggesting that "solubilization" of fatty acid per se does not explain the increased activity observed with FABP.

Taken together, these studies suggest that enhancement of fatty acid:coenzyme A ligase activity in vitro by FABP fraction is best explained by an effect on the interaction of the enzyme with the fatty acid substrate.

TABLE VII  
Effect of Flavaspidic Acid and  $\alpha$ -Bromopalmitate on  
Fatty Acid:Coenzyme A Ligase Activity  
in Intestinal Microsomes

	Hydroxamate formed		Mean inhibition %
	Control	Inhibitor, 0.1 mM	
	nmol/min/mg microsomal protein		
Flavaspidic acid			
No FABP, <i>n</i> = 4	10.7 ± 0.9	8.6 ± 1.1	20
FABP, <i>n</i> = 3	12.5 ± 1.2	12.2 ± 0.7	2
$\alpha$ -Bromopalmitate			
No FABP, <i>n</i> = 4	8.5 ± 0.8	7.0 ± 0.4	18
FABP, <i>n</i> = 3	9.9 ± 0.8	9.1 ± 0.8	8

Jejunal microsomes (0.8–1.3 mg) were assayed for fatty acid:coenzyme A ligase activity in the presence of 0.1 mM oleate, with or without flavaspidic acid or  $\alpha$ -bromopalmitate, 0.1 mM, and FABP fraction, 0.5–1.0 mg/ml. Each individual value is the mean of duplicate incubations. None of the differences between means of control and inhibited incubations was statistically significant (paired *t* test).

At concentrations equal to that of oleic acid (0.1 mM),  $\alpha$ -bromopalmitate and flavaspidic acid were only modestly inhibitory (Table VII), and their effect was blunted when FABP fraction was added to the system. Neither inhibitor was activated to a significant extent (unpublished observations), a finding consistent with earlier observations of Pande et al. (26) in regard to  $\alpha$ -bromopalmitate. The concentrations of inhibitor (0.1 mM) employed in these experiments is based on the estimated mean tissue concentration of flavaspidic acid present during the 4-min everted sac experiments (Fig. 5); tissue uptake of  $\alpha$ -bromopalmitate could not be determined. In all probability, however, the actual cytosolic concentration of these inhibitors in the gut sac experiments was less than that employed in the microsomal studies, because of binding to cell membranes. It is likely, therefore, that these concentrations would overestimate the inhibitory effect of these compounds on the activating enzyme. On the basis of these experiments, it is reasonable to conclude that in the everted sacs, fatty acid activation was inhibited minimally or not at all by flavaspidic acid and  $\alpha$ -bromopalmitate.

## DISCUSSION

In the present studies, we investigated the role of FABP in intestinal fatty acid absorption, employing compounds which inhibit its reversible binding of long chain fatty acids. The findings clearly demonstrate that while flavaspidic acid and  $\alpha$ -bromopalmitate did not affect uptake of oleate by everted jejunal sacs, they did inhibit fatty acid esterification to diglyceride and triglyceride, and oxidation to CO<sub>2</sub>. The inhibition was not associated with a general depression of mucosal metabolism, and could not be explained by an effect on transacylation. Incorporation into phospholipid and cholesterol esters was affected to a lesser degree. This difference in the observed effect on incorporation into various lipid classes may reflect a change in overall fatty acid utilization, analogous to the observations of Ontko (27) in studies of isolated hepatocytes.

One possible explanation for the observed decrease in fatty acid esterification was that activation by fatty acid:CoA ligase in the endoplasmic reticulum was inhibited. It is of interest that activity of this enzyme was enhanced significantly in the presence of FABP fraction, probably due to an effect on enzyme-substrate interaction, since it could not be explained simply as "solubilization," e.g. by albumin. However, flavaspidic acid and  $\alpha$ -bromopalmitate were only weak inhibitors of the activating enzyme (Table VII), and this effect was blunted by FABP fraction at concentrations within the physiological range.

Thus, it is reasonable to conclude that in the everted sac experiments, flavaspidic acid and  $\alpha$ -bromopalmitate

inhibited fatty acid esterification by intestinal absorptive cells at a stage after uptake, but before activation and transacylation in the endoplasmic reticulum. The only known event between cellular uptake and microsomal activation is the movement of fatty acid from the microvillus membrane to the endoplasmic reticulum. Since the inhibitors employed in these studies would not be expected to interfere with simple diffusion of fatty acid through an aqueous phase, it can be inferred that this translocation process depends on a somewhat more complex mechanism, the simplest model for which is the binding of long chain fatty acid to a soluble acceptor, i.e. FABP. The demonstrated inhibition of fatty acid binding to this protein by flavaspidic acid and  $\alpha$ -bromopalmitate (Fig. 1) is consistent with this concept and can account for the effects of these compounds in the intact cell (everted sacs).

Thus, FABP may be viewed as an intracellular fatty acid transport protein. In addition, its enhancement of microsomal fatty acid: CoA ligase activity (Fig. 5) suggests that it also may promote the interaction of enzyme and substrate in this reaction. This effect appears to be relatively specific, since it is not observed when albumin is substituted for FABP.

A theoretical basis for the role of FABP in cellular fatty acid transport was considered earlier (2), and included the probability that long chain fatty acids were unlikely to desorb from the inner aspect of the microvillus membrane into the aqueous cytosol at a rate sufficiently rapid to account for observed rates of mucosal triglyceride synthesis. It was suggested that a soluble fatty acid acceptor (FABP) in the cytosol would facilitate this desorption. Recently, Sallee has provided evidence concerning the thermodynamics of long chain fatty acid permeation of the rat intestinal surface membrane (28). His findings demonstrate that adsorption of long chain fatty acid monomer from an aqueous phase into the membrane is associated with an unexpectedly large decrease in free energy, implying that the membrane is highly nonpolar (29). These findings imply, as a corollary, that fatty acid desorption at the cytosolic aspect of the membrane would require (in the absence of a soluble acceptor) an increase in free energy of similar magnitude; therefore, this process should indeed be facilitated to a significant extent by an acceptor in the aqueous phase.

Taken together, these different lines of evidence are consistent with, and more directly supportive of, the concept that intestinal FABP participates in the cytoplasmic transport and metabolism of absorbed long chain fatty acids. Recent studies in our laboratory suggest a similar role for hepatic FABP (30). Also implicit in these observations is the possibility that FABP effects a compartmentalization of cytosolic FFA and other substances which may be toxic or regulatory with respect

to various aspects of cell metabolism. Thus, Mishkin and Turcotte have shown that long chain acyl CoA binds to the hepatic Z protein fraction (31), and our own unpublished experiments confirm this in regard to intestinal and hepatic FABP fractions. Our recent studies of intestine (32) support the earlier evidence (33) that FFA (and, by implication, acyl CoA) are compartmentalized within cells and suggest that FABP may participate in this compartmentalization. It is of interest that Jacobs and Majerus (34) and Halestrap and Denton (35) have suggested that FABP may participate in the regulation of acetyl coenzyme A carboxylate, and therefore of fatty acid synthesis, by governing the concentrations of bound and free (i.e. potentially inhibitory) acyl CoA and fatty acid; recent experiments in our laboratory support this concept (36). These broader aspects of the possible physiological function of FABP may be of considerable significance, and warrant further investigation.

*Note added in proof.* Flavaspidic acid has been reported to uncouple oxidative phosphorylation in isolated rat liver mitochondria (37), but did not affect oxygen consumption in perfused rat liver (18). Preliminary experiments (D. Burnett and R. Ockner, unpublished observations) are consistent with a concentration-dependent uncoupling effect of flavaspidic acid in isolated hepatocytes, in that this compound results in increased oxygen consumption and conversion of [1-<sup>14</sup>C]oleate, [<sup>14</sup>C]acetate, and [<sup>14</sup>C]glucose to <sup>14</sup>CO<sub>2</sub>. In everted gut sacs, in contrast (present report), oleate oxidation was inhibited by flavaspidic acid while acetate oxidation was unaffected (Table III). These differences, together with the fact that flavaspidic acid inhibition of oleate esterification was reversed by increasing oleate concentration (Fig. 6) suggest that uncoupling was not of major significance under the present conditions.

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