Sex Differences in Long Chain Fatty Acid Utilization and Fatty Acid Binding Protein Concentration in Rat Liver

ROBERT K. OCKNER, DAVID A. BURNETT, NINA LYSENKO, and JOAN A. MANNING,
Department of Medicine and Liver Center, University of California School of Medicine, San Francisco, California 94143

ABSTRACT Female sex and estrogen administration are associated with increased hepatic production of triglyceride-rich lipoproteins; the basis for this has not been fully elucidated. Inasmuch as hepatic lipoprotein production is also influenced by FFA availability and triglyceride biosynthesis, we investigated sex differences in FFA utilization in rat hepatocyte suspensions and in the components of the triglyceride biosynthetic pathway.

Isolated adult rat hepatocyte suspensions were incubated with albumin-bound [^{14}C]oleate for up to 15 min. At physiological and low oleate concentrations, cells from females incorporated significantly more ^{14}C into glycerolipids, especially triglycerides, and into oxidation products than did male cells, per milligram cell protein. At 0.44 mM oleate, incorporation into triglycerides in female cells was approximately twice that in male cells. Comparable sex differences were observed in cells from fasted animals and when [^{14}C]-glycerol incorporation was measured. At higher oleate concentrations, i.e., fatty acid:albumin mole ratios in excess of 2:1, these sex differences were no longer demonstrable, suggesting that maximal rates of fatty acid esterification and oxidation were similar in female and male cells.

In female and male hepatic microsomes, specific activities of long chain acyl coenzyme A synthetase, phosphatidate phosphohydrolase, and diglyceride acyltransferase were similar, but glycerol-3-phosphate acyltransferase activity was slightly greater in females at certain substrate concentrations. Microsomal incorporation of [^{14}C]oleate into total glycerolipids was not significantly greater in females. In further contrast to intact cells, microsomal incorporation of [^{14}C]oleate into triglycerides, although significantly greater in female microsomes, accounted for only a small fraction of the fatty acid esterified.

The binding affinity and stoichiometry of partially purified female hepatic fatty acid binding protein (FABP) were similar to those of male FABP. In contrast, the concentration of FABP, per milligram cytosolic protein, was 44% greater in female liver than in male, as indicated by measurement of [^{14}C]oleate binding and of 280 nm OD in the FABP fraction of 105,000 *g* supernate after gel filtration chromatography.

These experiments demonstrate profound sex differences in hepatocyte utilization of long chain fatty acids at concentrations within and below the physiological range, and suggest that these are attributable at least in part to corresponding differences in cytosolic FABP concentration. At higher FFA concentrations, sex differences in hepatocyte FFA utilization are virtually eliminated, suggesting that under these conditions, differences in FABP concentration are not rate determining. Sex differences in hepatic lipoprotein production may largely reflect these important differences in the initial stages of hepatocyte FFA utilization.

INTRODUCTION

Estrogen administration and pregnancy are associated with increased plasma triglyceride concentrations in human subjects and experimental animals; androgens and certain anabolic steroids may have the opposite effect. Available information suggests that estrogens do not impair very low density lipoprotein (VLDL)[1] removal from plasma but rather accelerate this process (1–10). On this basis, it can be inferred that entry of VLDL-triglyceride into plasma must be increased, a conclusion that is also supported directly (4, 5, 7–10). However, the mechanism of this increase has not been fully defined. Recent studies of Luskey et al. (11) and Chan et al. (12, 13) show that estrogens increase the synthesis of VLDL apoproteins in the rooster and

Received for publication 8 August 1978 and in revised form 18 January 1979.

[1] *Abbreviations used in this paper*: CoA, coenzyme A; FABP, fatty acid binding protein; VLDL, very low density lipoprotein.

cockerel. However, it is conceivable that increased apolipoprotein synthesis may reflect other changes in cellular lipid metabolism, and even if this mechanism pertains to mammals, an increased supply of lipid would nonetheless be required to sustain any increase in lipoprotein production rate.

The possible origins of this lipid therefore warrant consideration. In this connection, Mandour et al. (14) showed that in the estrogenized rat, portal venous insulin:glucagon ratio and the activity of hepatic acetyl coenzyme A (CoA) carboxylase and fatty acid synthetase were increased, suggesting that augmented lipogenesis may contribute. Regarding plasma FFA in estrogen-induced hypertriglyceridemia, studies of human subjects and rats indicate no consistent changes in either FFA concentration or turnover (5, 7). In the estrogenized chick, FFA flux is increased, but this appears to follow rather than precede the hypertriglyceridemia (15) and therefore may reflect recycling of VLDL triglyceride fatty acids.

Although total plasma FFA flux does not appear to be increased by estrogens, published evidence does suggest that entry of plasma FFA into perfused female rat livers may be greater than in male livers (1, 10). However, these studies were conducted over several hours, under conditions that precluded definitive examination of the initial events in hepatic uptake and utilization of plasma FFA. Other evidence from studies of women taking oral contraceptives (5) and the estrogenized chick (8) also suggests that hepatic uptake and utilization of plasma FFA may be increased under these conditions. However, these processes have not been directly investigated, and the possibility that sex or sex steroids influence the initial events in hepatocyte FFA utilization and triglyceride biosynthesis is neither established nor excluded by previously published evidence.

The present experiments were designed to explore this important question in detail, by studying isolated hepatocyte suspensions as well as the components of the triglyceride biosynthetic pathway, including microsomal enzymes and cytosolic fatty acid binding protein (FABP). The results establish the existence of profound sex differences in the utilization of albumin-bound fatty acid by adult rat hepatocytes and suggest that corresponding differences in FABP concentration account for these differences at least in part. Portions of these studies have appeared in a published abstract (16).

METHODS

Materials. [1-^{14}C]Oleic acid and L-[U-^{14}C]glycerol-3-phosphate, disodium salt, were obtained from New England Nuclear (Boston, Mass.), unlabeled oleic acid from Calbiochem (San Diego, Calif.), and [^{14}C]diolein from Dhom Products, Ltd. (North Hollywood, Calif.). Fatty acid-free albumin, S-palmitoyl CoA, oleoyl CoA, and unlabeled DL-α-glycerophosphate (disodium salt) were purchased from Sigma Chemical Co. (St. Louis, Mo.). The albumin contained <0.02 nmol fatty acid/nmol protein.[2]

Animals. Sprague-Dawley rats of specified weights or ages were obtained from Simonsen Laboratories (Gilroy, Calif.), and maintained on standard laboratory chow ad lib. In all experiments except those specifically designed to investigate the effect of fasting, animals were allowed access to food until the morning of the day of the experiment.

Studies of isolated liver cell suspensions. Suspensions of rat hepatocytes were prepared by modification of the method of Berry and Friend (17). Livers were perfused with Ca^{++}-free Hanks' medium gassed with 95% oxygen, 5% CO_2, containing 0.05% collagenase (type I, Sigma Chemical Co.). Cells were suspended in Ca^{++}-free, bicarbonate-free, glucose-free Hanks' with 10 mM sodium phosphate buffer, pH 7.4, and were incubated at 37°C in a metabolic shaker in 25-ml Erlenmeyer flasks, final volume 2 ml. Incubations, carried out in triplicate for each liver, contained 3–14 mg cell protein (over which range incorporation was linear) and albumin at a final concentration of 1.5 g/ml (0.22 mM). Incubations were initiated by the addition of 1 ml of fatty acid-albumin complex to 1 ml of liver cell suspension. Under all conditions >85% of cells excluded trypan blue at the end of the incubation, and were shown in other experiments to consume O_2 and use oxidizable substrates normally.[2]

[^{14}C]O_2 production was determined in flasks containing a center well. At the end of the incubation, 0.2 ml Hydroxide of Hyamine (Packard Instrument Co. Inc., Downers Grove, Ill.) was added to the center well, and 0.2 ml of 70% perchloric acid to the contents of the flask. [^{14}C]O_2 was collected during an additional 60-min incubation after which the contents of the center well were assayed for radioactivity (see below).

Incorporation of [^{14}C]oleate into lipids and water-soluble products was measured by extraction of cells and medium by the method of Folch et al. (18). Lipids were separated by thin-layer chromatography on 0.25 mm of silica gel 60 (EM Laboratories, Inc., Elmsford, N. Y.) in a solvent system consisting of petroleum ether:diethyl ether:acetic acid, 90:15:1.5, and identified as described (19). Zones were scraped directly into counting vials and were assayed for radioactivity (see below). Water-soluble radioactivity, consisting of ketone bodies, citric acid cycle intermediates, and acetyl CoA (20), was measured directly by radioassay of aliquots of the acidified upper (water-methanol) phase of the extraction system. In some experiments, incorporation of [^{14}C]glycerol-3-phosphate into glycerolipids was measured in the presence of unlabeled oleate and otherwise identical conditions.

Assay of microsomal enzymes. Microsomes were prepared from whole rat liver and assayed for long chain acyl CoA synthetase by the method of Bar-Tana et al. (21) and for acyl CoA:glycerol-3-phosphate acyltransferase by the method of Jamdar and Fallon (22), as described.[2] Incubation media for these reactions contained albumin at concentrations of 0.15 and 0.5 mg/ml, respectively,[2] and substrate concentrations were varied as indicated. Phosphatidate phosphohydrolase activity was measured by the method of Lamb et al. (23). Diglyceride acyltransferase activity was assayed by method I of Coleman and Bell (24), modified in that oleoyl CoA was substituted for palmitoyl CoA, diolein concentration was reduced to 5 μM, and the reaction was carried out at 37°C.

---

[2] Burnett, D. A., N. Lysenko, J. A. Manning, and R. K. Ockner. 1979. Utilization of long chain fatty acids by rat liver: studies of the role of fatty acid binding protein. *Gastroenterology.* In press.

In other studies, microsomal incorporation of [^{14}C]oleate into glycerolipids was measured by the method of Scheig and Isselbacher (25), modified in that [^{14}C]oleate was substituted for [^{14}C]palmitate and dithiothreitol for neutral cysteine, and that the reaction mixture was incubated for 10 min. Products were extracted by the method of Folch et al. (18), isolated by thin-layer chromatography, and assayed for radioactivity.

Studies of FABP. FABP was partially purified as the 12,000 mol wt fraction from the 105,000 *g* supernate of liver homogenate by preparative gel filtration on Sephadex G-50 (Pharmacia Fine Chemicals, Piscataway, N. J.) as described (26). This material was used for studies of [^{14}C]oleate binding by means of column Sephadex G-25 chromatography (26, 27), under which conditions fatty acid eluting with protein in the void volume was bound to FABP. In other experiments, binding of [^{14}C]oleate to whole liver 105,000 *g* supernate was determined by means of Sephadex G-50 chromatography, analogous to earlier similar studies with Sephadex G-75 (26, 28). In these experiments, 40 or 160 nmol of [^{14}C]oleate in 50 μl methylethylketone was added to 40 mg of 105,000 *g* supernatant protein and applied in a volume of 2.0 ml of 0.154 M KCl in 0.01 M phosphate buffer, pH 7.4, to a Sephadex G-50 column, 2.5 × 30 cm, 4°C, 20 ml/h. Fractions of 3.6 ml were collected and assayed for radioactivity and for OD at 280 nm. Previous experiments showed that virtually all radioactivity eluting from the column under these conditions was recoverable as fatty acid, noncovalently bound (25). To permit comparison of the results of chromatographic analyses, the OD and [^{14}C]oleate content of each fraction were expressed in terms of the ratio of the elution volume of that fraction to the void volume, i.e. $V_e:V_0$ (26).

Radioassays. Samples were assayed for radioactivity in Liquifluor-toluene (New England Nuclear) containing 10% Biosolv in a Beckman Liquid Scintillation System model LS-250 (Beckman Instruments, Inc., Fullerton, Calif.). For lipid soluble extracts, Biosolv was not added. Quenching was corrected for by an automatic external standard.

Statistical methods. Significance of differences among experimental groups was determined by the unpaired *t* test (29).

RESULTS

Utilization of albumin-bound [^{14}C]*oleate by isolated hepatocyte suspensions from adult male and female rats.* Hepatocyte suspensions prepared from fed, sexually mature female and male rats, 240–260 g, were incubated with albumin-bound [^{14}C]oleate. Total utilization, i.e., the sum of all measured products of fatty acid esterification and oxidation, is plotted as a function of time in Fig. 1. It can be seen that fatty acid utilization was linear over 15 min for both females and males and was ≅75% greater in female cells. As shown in Fig. 2, there was no significant difference between female and male cells in total fatty acid oxidation, representing the sum of ^{14}C incorporated into CO_2 and water-soluble metabolites. In contrast, [^{14}C]oleate esterification was 95% greater in female cells. Of the total fatty acid esters represented by the data plotted in Fig. 2, >98% were accounted for by glycerolipids (phospholipids, diglycerides, and triglycerides), whereas cholesterol esters accounted for <2% in all experiments. In Fig. 3, it can be seen that these striking differences in esterification were largely the result of increased incorporation of [^{14}C]oleate into triglyceride in female cells. Incorporation into diglycerides reached a plateau after 5 min in both female and male cells, suggesting that this intermediate had become labeled to constant specific activity. Incorporation of [^{14}C]oleate into

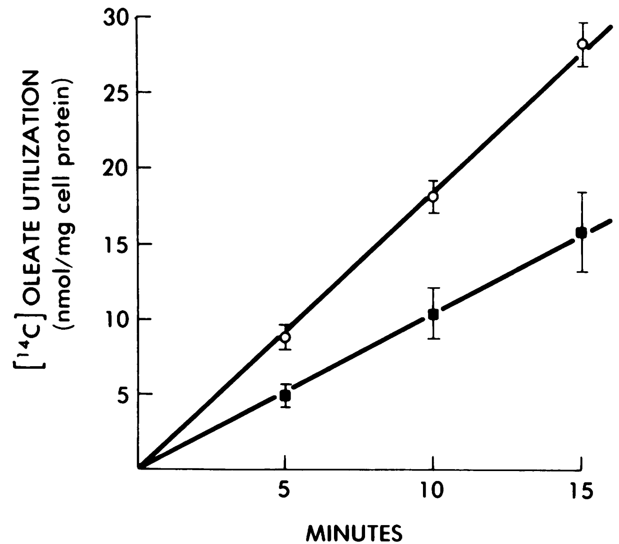

FIGURE 1 Utilization of albumin-bound [^{14}C]oleate by rat hepatocyte suspensions. Hepatocyte suspensions prepared from adult female (○) and male (■) rats were incubated with 0.44 mM [^{14}C]oleate bound to 0.22 mM fatty acid-free albumin. At the indicated intervals, incorporation of ^{14}C into oxidation and esterification products in the entire incubation mixture (cells and medium) was determined as described in Methods. "Utilization" in this figure represents the sum of all measured products of fatty acid oxidation and esterification. Mean±SE; n = 5 for all groups.

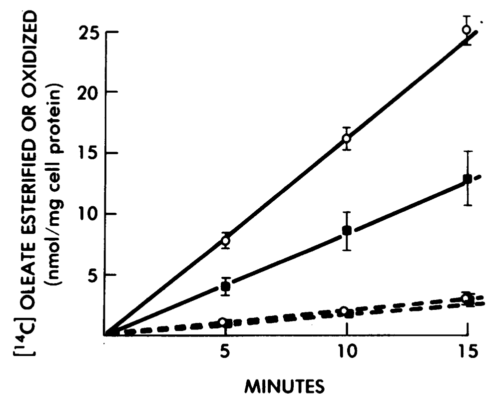

FIGURE 2 Esterification and oxidation of [^{14}C]oleate by rat hepatocyte suspensions. Incorporation of [^{14}C]oleate into total products of esterification (——) and oxidation (– – –) by hepatocyte suspensions from adult female (○) and male (■) rats was determined as in Fig. 1 and Methods. Mean±SE; n = 5 for all groups.

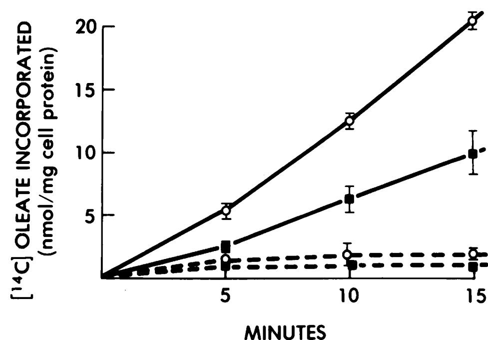

FIGURE 3 Incorporation of [14C]oleate into triglycerides (——) and diglycerides (- - -) by hepatocyte suspensions from adult female (○) and male (■) rats was determined as described in Fig. 1 and Methods. Mean±SE; $n = 5$ for all groups.

phospholipids (Table 1) was also significantly greater, statistically, in female cells than in male cells, but in both groups phospholipids represented only a small fraction of total esterified ^{14}C (11–12% in females; 15–16% in males). Of the 95% greater incorporation of fatty acid into glycerolipids by female cells >75% was accounted for by triglycerides. Similar patterns were observed in 2-min incubations (unpublished data).

As shown in Table II, differences of a comparable nature also characterized cells prepared from fasted animals. Thus, although for both sexes overall utilization of [14C]oleate was substantially reduced when compared with cells from fed rats, both the oxidation of [14C]oleate to [^{14}C]O_2 and its incorporation into triglycerides were significantly greater in cells from fasted females than males. The incorporation of [14C]glycerol into glycerolipids was measured under otherwise similar conditions. As shown in Table III, glycerol incorporation into triglycerides was also much greater in female cells, suggesting that sex differences in cellular fatty acid pools alone could not explain the increased rate of triglyceride biosynthesis. In contrast to the studies with [14C]oleate, however, differences in incorporation into phospholipids and diglycerides were not significant.

To determine the effect of substrate (oleate) concentration on these sex differences, hepatocytes prepared from 60-d-old fed adult female and male rats were incubated for 10 min with concentrations of [14C]oleate ranging from 0.11 to 1.32 mM. Albumin concentration was constant at 0.22 mM, thereby resulting in a range of oleate:albumin of 0.5–6.0. Hepatocytes from 60-d-old rats are very similar to those from 240- to 260-g rats in regard to [14C]oleate utilization (cf. values for 0.44 mM oleate in Figs. 1–5). The results of these studies are

TABLE I
Incorporation of [14C]Oleate into Phospholipids by Adult Rat Hepatocytes

	[14C]Oleate incorporated		
	5 min	10 min	15 min
	nmol/mg cell protein		
Female	1.00±0.08	1.89±0.17	2.71±0.24
Male	0.63±0.06*	1.25±0.14‡	1.86±0.24§

Hepatocytes from fed adult rats, 240–260 g, were incubated with 0.44 mM [14C]oleate, and incorporation into phospholipids was measured as described in Methods. Mean±SE; $n = 5$ for all groups.
* $P < 0.01$ vs. female.
‡ $P < 0.02$ vs. female.
§ $P < 0.05$ vs. female.

TABLE II
Utilization of [14C]Oleate in Suspensions of Hepatocytes from Fasted Adult Rats

	Female	Male
	nmol incorporated/mg cell protein/10 min	
CO_2	0.66±0.06	0.36±0.04*
H_2O-soluble	1.33±0.17	1.10±0.30
Total oxidation	1.99±0.15	1.47±0.31
Phospholipids	1.63±0.19	1.36±0.20
Diglycerides	0.66±0.15	0.75±0.33
Triglycerides	7.35±0.37	3.68±0.54*
Cholesterol esters	0.05±0.00	0.13±0.03‡
Total esters	9.69±0.25	5.92±0.69*
Total utilization	11.68±0.29	7.39±0.95*

Hepatocyte suspensions were prepared from 60-d-old female and male rats, deprived of food for 16–20 h. Incorporation of 0.44 mM [14C]oleate into oxidation products and lipids in 10-min incubations was measured as described in Methods. Mean ±SE; $n = 4$ for all groups; each observation represents the mean of triplicate incubations for each rat.
* $P < 0.01$ vs. females.
‡ $P < 0.05$ vs. females.

TABLE III
Incorporation of [14C]Glycerol into Glycerolipids by Rat Hepatocyte Suspensions

	Female	Male
	nmol incorporated/ mg cell protein/10 min	
Phospholipids	1.02±0.16	1.02±0.04
Diglycerides	1.12±0.30	0.46±0.06
Triglycerides	4.54±0.27	1.64±0.08*

Hepatocytes from fed 60-d-old rats were incubated with unlabeled 0.44 mM oleate and 0.5 mM [U-14C]glycerol, and incorporation was measured as described in Methods. Mean±SE; $n = 3$ for all groups.
* $P < 0.001$ vs. female; other differences not significant.

shown in Figs. 4 and 5. In Fig. 4, it can be seen that total incorporation of [^{14}C]oleate into glycerolipids was greater in female cells than in male cells throughout the range of substrate concentrations studied, although the apparent difference at 1.32 mM oleate did not achieve statistical significance. Incorporation of [^{14}C]-oleate into phospholipids was significantly greater in female cells only at the lower concentrations (0.11 and 0.22 mM); in contrast, differences for diglycerides were significant throughout the concentration range. In all of these studies, cholesterol esters accounted for ≅1% of esterified oleate.

In Fig. 5, incorporation of [^{14}C]oleate into triglyceride and total products of oxidation is shown. Oleate oxidation was significantly greater in female cells at 0.11 mM but not at higher concentrations. Similarly, incorporation into triglycerides in female cells was significantly greater than in male cells ($P < 0.001$) at the three lowest oleate concentrations, whereas at 0.88 and 1.32 mM, there was no significant difference.

These experiments indicate that the striking differences in the use of [^{14}C]oleate between female and male hepatocytes depend for their demonstration on substrate concentration. The fact that there are no differences at the highest concentrations suggests that the maximal rates of the oxidative and triglyceride pathways are similar in males and females. At fatty acid concentrations and fatty acid:albumin molar ratios within and

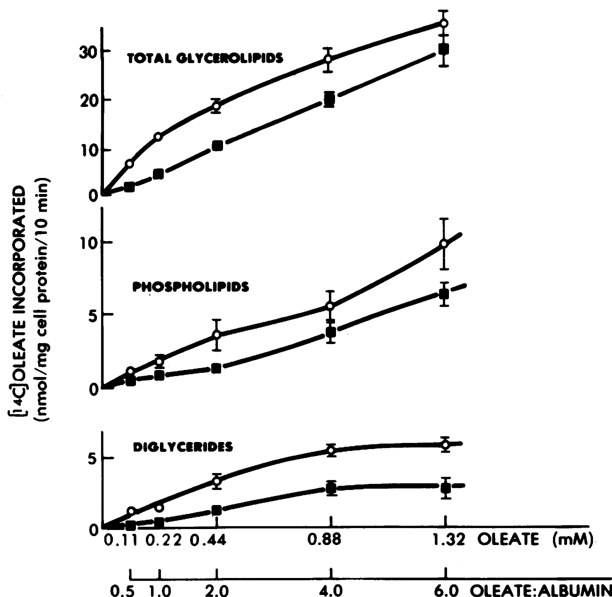

FIGURE 4 Effect of substrate concentration on esterification of [^{14}C]oleate by rat hepatocyte suspensions. Hepatocyte suspensions from adult female (○) and male (■) rats were incubated with [^{14}C]oleate bound to 0.22 mM albumin, and incorporation into esterification products was determined as described in Methods. Mean±SE; $n = 3$ for all groups.

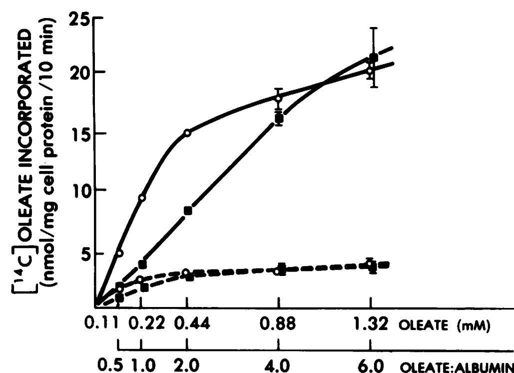

FIGURE 5 Effect of substrate concentration on incorporation of [^{14}C]oleate into triglycerides and products of oxidation by rat hepatocyte suspensions. Hepatocyte suspensions from adult female (○) and male (■) rats were incubated with albumin-bound [^{14}C]oleate as in Fig. 4, and incorporation into triglycerides (——) and total oxidation products (- - -) was determined as described in Methods. Mean±SE; $n = 3$ for all groups.

below the physiological range, however, the rate at which fatty acid enters these pathways is much greater in female cells. To explore possible mechanisms for this difference, components of the triglyceride biosynthetic pathway were examined in liver cell fractions prepared from female and male rats.

Studies of microsomal enzymes of triglyceride biosynthesis. Microsomes were assayed for specific activity of acyl CoA synthetase and glycerol-3-phosphate acyltransferase. As shown in Fig. 6, specific activity of acyl CoA synthetase was similar in females and males over a wide range of oleate concentrations. Specific activity of glycerol-3-phosphate acyltransferase was generally similar in the two sexes, but was significantly greater in female microsomes at 5 and 10 μM palmitoyl CoA. As shown in Table IV, no significant differences in the specific activities of phosphatidate phosphohydrolase and diglyceride acyltransferase were observed.

In other experiments, the incorporation of [^{14}C]oleate into glycerolipids and cholesterol esters was compared in female and male microsomes. The results, shown in Table V, differ in several important respects from the incorporation of [^{14}C]oleate by intact cells. First, total glycerolipid synthesis, i.e., the sum of phospholipids, diglycerides, and triglycerides, is generally similar in female and male microsomes, the former exceeding the latter by a statistically insignificant 14%. Second, the distribution of ^{14}C among glycerolipid classes differs strikingly from that observed with intact cells in that phospholipid and diglyceride account for 88% or more, and triglycerides for only 8–12%. In intact cells, in contrast, at least 55% of glycerolipid ^{14}C was accounted for by triglycerides under all conditions, and >70% at the lowest substrate concentrations. Despite this very modest incorporation of [^{14}C]oleate into triglycerides,

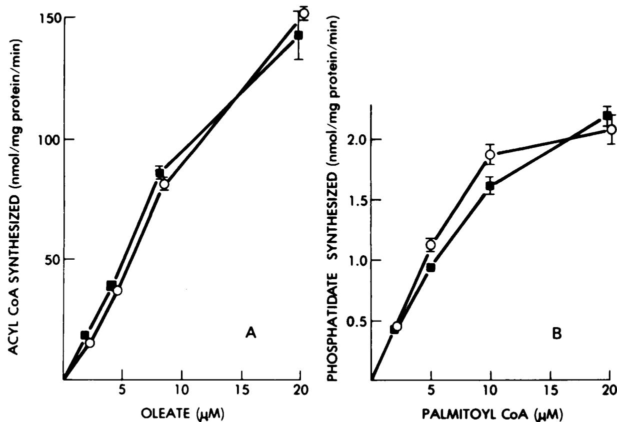

FIGURE 6 Specific activity of rat liver microsomal acyl CoA synthetase (A) and glycerol-3-phosphate acyltransferase (B). Enzyme assays were performed over the indicated range of substrate concentrations, as described in Methods. Each point represents the mean±SE of results of triplicate incubations of microsomes from each of three rats. For acyl CoA synthetase, no differences are significant. For glycerol-3-phosphate acyltransferase, differences are significant at 5 μM ($P \cong 0.02$) and 10 μM ($P < 0.05$). ○, female; ■, male.

however, it was significantly greater in female than in male microsomes. (In preliminary experiments in which incorporation of [^{14}C]glycerol into glycerolipids by whole liver homogenate was measured, a similar distribution of ^{14}C among products was observed, but overall ^{14}C incorporation was 39% greater in female homogenate.)

Thus, although sex differences in microsomal esterification of [^{14}C]oleate are demonstrable, these differ both qualitatively and quantitatively from, and seem insufficient to account for, those that characterize [^{14}C]oleate utilization by the intact hepatocyte.

Studies of FABP. Because the studies of several groups of investigators (28, 30–33)[2] have suggested that FABP plays a role in triglyceride biosynthesis, this protein was compared in female and male rat liver. The binding affinities of partially purified FABP fraction, prepared from the 105,000 g supernate of mature female and male rats, were compared by Sephadex G-25 chromatography as described in Methods. As shown in Fig. 7, results are very similar to previously published values (27) and demonstrate no difference between the sexes. In contrast (Fig. 8), chromatography of equal quantities of 105,000 g supernatant protein with [^{14}C]-oleate on Sephadex G-50 demonstrated consistent female-male differences. These were characterized by

TABLE IV
Specific Activity of Rat Liver Enzymes of Triglyceride Biosynthesis

	Female	Male
	nmol product/ mg microsomal protein/min	
Phosphatidate phosphohydrolase (n = 4)	0.209±0.049	0.218±0.033
Diglyceride acyltransferase (n = 3)	0.250±0.029	0.229±0.013

Assay of enzymes from adult rat livers were performed as described in Methods. Mean±SE. No differences between female and male are statistically significant.

TABLE V
Incorporation of [^{14}C]Oleate into Glycerolipids and Cholesterol Esters by Rat Liver Microsomes

	Female	Male
	pmol incorporated/mg protein	
Phospholipids	232±13	212±15
Diglycerides	27.0±0.5	25.1±1.7
Triglycerides	35.2±2.9	20.0±2.0*
Cholesterol esters	9.6±3.2	8.5±1.3
Total glycerolipids	294±12	257±15‡
Total esters	304±12	265±16

Microsomes were prepared from livers of fed 60-d-old rats and incubated for 10 min with [^{14}C]oleate, and incorporation was measured as described in Methods. Mean±SE of results of triplicate incubations of microsomes from each of three rats.
* $P < 0.02$ vs. female.
‡ $P \cong 0.07$ vs. female.

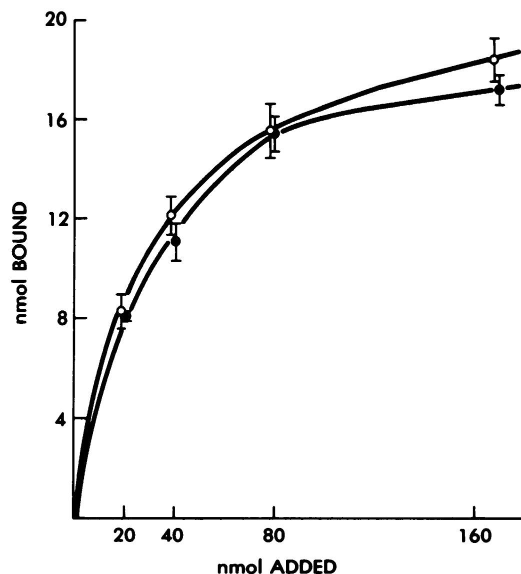

FIGURE 7 Binding of [^{14}C]oleate to the FABP fraction of rat liver 105,000 g supernate. Binding of [^{14}C]oleate to 0.42 mg partially purified rat liver FABP was determined by Sephadex G-25 gel filtration as described in Methods. Mean±SE; n = 4 for each point. ○, female; ■, male.

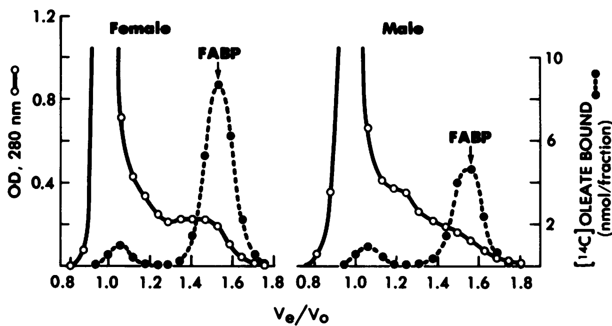

FIGURE 8 Gel filtration chromatography of [^{14}C]oleate with rat liver 105,000 *g* supernate. Binding of 40 nmol [^{14}C]oleate to 40 mg of rat liver 105,000 *g* supernatant protein was determined by Sephadex G-50 gel filtration as described in Methods and is plotted in this representative pair of experiments against 280 nm OD in the corresponding column fractions. For purposes of comparison, the elution volume of each fraction is expressed as a function of the void volume (V_e/V_0).

greater binding of [^{14}C]oleate and the presence of a distinct shoulder on the OD tracing in the FABP region (12,000 mol wt, elution volume:void volume [V_e:V_0] ≅ 1.55) of the female, suggesting the presence of a higher concentration of 12,000 mol wt protein in female 105,000 *g* supernate. The amount of [^{14}C]oleate eluting just after the void volume, presumably in association with albumin, was small and was similar in female and male supernate. Table VI shows the mass of [^{14}C]-oleate associated with the FABP fraction in these experiments, in which two different amounts of ligand were added. At both high and low amounts, binding to the FABP region was ≅40–50% greater in female cytosol, although statistical significance was achieved only with the higher amount.

Together, these experiments suggest that although the binding affinity and capacity of partially purified female and male hepatic FABP are similar, the concentration of FABP in cytosol in female liver is greater than in male liver.

TABLE VI
[^{14}C]Oleate Binding to FABP Fraction of Rat Liver Cytosol

[^{14}C]Oleate added	Female	Male
	nmol bound to FABP/mg cytosol protein	
40 nmol (n = 4)	0.55±0.07	0.36±0.04*
160 nmol (n = 6)	1.43±0.15	0.99±0.11‡

Binding of [^{14}C]oleate to the 12,000 mol wt FABP fraction of the whole 105,000 *g* supernate was determined by means of Sephadex G-50 gel filtration as described in Methods and Fig. 8. The indicated amount of fatty acid was mixed in all cases with 40 mg supernatant protein. Total oleate bound was calculated on the basis of the ^{14}C eluting with the FABP fraction.
* Mean±SE vs. female: $P < 0.06$.
‡ Mean±SE vs. female: $P < 0.05$.

DISCUSSION

These studies document profound differences between hepatocytes from adult female and male rats in the earliest stages of [^{14}C]oleate utilization. The differences are characterized by greater oxidation and esterification at low substrate concentration, and are demonstrable in cells from both fed and fasted animals. At higher concentrations, these differences were largely eliminated. In unpublished experiments, "uptake" of [^{14}C]oleate, defined as cell-associated ^{14}C, was measured directly,[2] did not differ significantly from total [^{14}C]-oleate utilization (i.e., the sum of ^{14}C recovered in all measured oxidation and esterification products), and therefore was also greater in females. This indicates that any cell-associated pool of unmetabolized [^{14}C]-oleate was too small to detect under the experimental conditions, and that the [^{14}C]oleate that did enter the cell was used very rapidly.

The finding that estrogens induce the synthesis of hepatic VLDL apoproteins in avian species (11–13) has been interpreted as evidence for a direct and possibly rate-determining role of sex differences at this later stage of the overall processes of VLDL secretion and, by inference, triglyceride biosynthesis. However, it is well recognized that a primary increase in availability of FFA, with a secondary increase in hepatic triglyceride biosynthesis, also effectively and rapidly stimulates VLDL synthesis. In providing evidence of profound sex differences in the earliest events in hepatocyte FFA utilization, the present studies suggest that corresponding sex differences in hepatic lipoprotein production may be determined to a large extent by factors that precede and are independent of VLDL apoprotein synthesis.

In attempting to understand the basis for these differences, several factors must be considered. First, it is conceivable that physical or structural differences between female and male hepatocytes might be responsible (34). Information in this area is quite limited, and, to our knowledge, adequate morphometric data are not available. Limited evidence, obtained by light microscopy (35), suggests that female hepatocytes may be somewhat smaller, possibly implying an increased cell surface:volume or surface:protein ratio. However, it seems most unlikely that these small differences could account, per se, for the observed differences in FFA utilization. Moreover, in experiments with cells from estradiol- or testosterone-treated animals, cell counts were measured directly and compared with cell protein, and no consistent differences were found in the calculated number of cells per milligram protein

or, by inference, cell surface area per milligram protein.[3] It is theoretically possible that sex differences in the permeability of the liver cell surface membrane to long chain fatty acids could be a factor, but available evidence bearing on this elusive point has been interpreted as suggesting that movement through the membrane is not rate determining for cellular utilization of fatty acids (36).

On the basis of these considerations, therefore, the present observations suggest that intrinsic sex differences exist in the pathways of fatty acid utilization within the cell envelope. Although the most striking differences were seen in esterification, it is noteworthy that total oxidation also was significantly greater in female cells at low concentrations (Fig. 5), and that a plateau was reached at 0.44 mM oleate and above. Fatty acid supplied in excess of this upper limit is diverted to esterification pathways, confirming the earlier studies of Ontko (37). The present data indicate, however, that when availability of fatty acids is low, their entry into the oxidation pathway is greater in female cells. A similar pattern also characterized incorporation of [^{14}C]oleate into esterification products (Fig. 4), especially triglycerides (Fig. 5).

Together, these observations suggest that under the experimental conditions, maximal rates of the fatty acid oxidation and triglyceride biosynthesis pathways are essentially equal in adult female and male cells. At lower concentrations, however, fatty acid enters these pathways more readily in female cells. In other words, even though the maximal reaction velocities in microsomes and mitochondria may be rate determining for overall fatty acid utilization at higher fatty acid concentrations and fatty acid:albumin mole ratios, they do not appear to be so in the lower ranges.

Although these data cannot be subjected to formal kinetic analysis, the sex differences in the concentration dependence of [^{14}C]oleate incorporation into triglyceride and oxidation products by intact hepatocytes (Fig. 5) may be regarded as analogous to enzyme reactions with similar maximal velocities (V_{max}) but differing affinities of enzyme for substrate (reflected in apparent K_m). In the present system, entry of fatty acid into both oxidative and triglyceride pathways in female cells may be regarded as exhibiting a lower K_m (i.e., attainment of half-"maximal" velocity at a lower substrate concentration) than in male cells (Fig. 5). Two possible explanations, which are not mutually exclusive, may account for this difference: (*a*) the K_m of the rate-determining enzymes for each of the pathways involved is lower in female cells; or (*b*) the kinetic properties of these enzymes are similar, but there is more rapid access of fatty acid to, or interaction with, these enzymes in female cells.

The first of these possibilities is excluded by the present studies in the case of acyl CoA synthetase, but slight differences were demonstrated for glycerol-3-phosphate acyltransferase, and may conceivably apply to later reactions in the triglyceride pathway. The second possibility is relatively simple in concept, in that it requires only that there be a quantitative difference in a single common process, and is consistent with the demonstration that FABP concentration is higher in female cytosol than in male.

Several laboratories have provided substantial evidence for the participation of FABP in the cellular utilization of long chain fatty acids in small intestine and liver (26, 28, 30–33, 38)[2] Enhancement by FABP of mitochondrial and microsomal acyl CoA synthetase (31)[2] and a number of other microsomal glycerolipid-synthesizing enzymes (32)[2] has been demonstrated directly, in vitro. The possibility that this 12,000 mol wt soluble protein may also serve as a cytosolic carrier for long chain fatty acids (26) is consistent with available data[2] but has not been established. In interpreting the present studies, however, sex differences in cytosolic FABP concentration could explain the corresponding differences in [^{14}C]oleate utilization whether it is assumed that FABP facilitates the interaction of fatty acid with mitochondrial and microsomal enzymes, the movement of fatty acid through cytosol, or both.

These studies are of significance in several respects. First, they demonstrate that important sex differences in hepatocyte fatty acid utilization reflect factors early in the pathways of oxidation and triglyceride biosynthesis, apparently independent of lipoprotein synthesis. Second, corresponding sex differences in cytosolic concentration of FABP, and to a lesser extent in the specific activity of microsomal glycerol-3-phosphate acyltransferase, are established, and may entirely or in large part account for the observed sex differences in fatty acid utilization in intact cells. Finally, the data provide additional support for a role of FABP in cellular fatty acid utilization, and suggest that it may be of particular importance when availability of extracellular fatty acid is within or below the physiological range. It is also likely that FABP interacts with, and facilitates the utilization of, fatty acids that originate within the cell via *de novo* synthesis or hydrolysis of fatty acid esters (27).[2]

The data do not suggest that FABP influences the partitioning of fatty acid between oxidation and esterification pathways. Rather, they suggest that FABP facilitates entry of FFA into both pathways, and that the partitioning of fatty acid between the two is determined

[3] Ockner, R. K., N. Lysenko, and D. A. Burnett. 1979. Effects of age, castration, and hormone replacement on fatty acid utilization and triglyceride biosynthesis by rat hepatocyte suspensions. Manuscript in preparation.

by the relative amounts and kinetics of the enzymes involved and by fatty acid supply. This concept is consistent with recent evidence that increased fatty acid uptake and oxidation by livers of clofibrate-treated rats also is associated with increased hepatic FABP concentration (39). It also seems clear that rate determinants of hepatic uptake and utilization of long chain fatty acids will differ under various circumstances, depending on physiological, pharmacological, and developmental factors. As a corollary, no single factor, be it FABP concentration, membrane permeability, or microsomal enzyme kinetic properties, is likely to be rate determining under all conditions.

In other studies, we have found that these sex differences in FFA utilization are not present in hepatocytes from immature animals, can be largely prevented by castration, and can be reproduced by administration of estradiol or testosterone to castrates of either sex.[3] Together, the findings imply that, directly or indirectly, sex steroids modulate early events in hepatocyte FFA utilization, and at least in part through this mechanism, influence synthesis, secretion, and plasma concentrations of triglyceride-rich lipoproteins.

ACKNOWLEDGMENTS

Laura Beausoleil and Gail MacNeil assisted in the preparation of the manuscript.

This work was supported in part by research grant AM-13328, Liver Center research grant P50 AM-18520, and research training grant GM-07546 from the National Institutes of Health.

REFERENCES

1. Applebaum, D., A. P. Goldberg, O. J. Pykälistö, J. D. Brunzell, and W. R. Hazzard. 1977. Effect of estrogen on post-heparin lipolytic activity. Selective decline in hepatic triglyceride lipase. *J. Clin. Invest.* **59:** 601–608.
2. Chait, A., J. D. Brunzell, J. J. Albers, and W. R. Hazzard. 1977. Type-III hyperlipoproteinemia ("remnant removal disease"). Insight into the pathogenetic mechanism. *Lancet.* **I:** 1176–1178.
3. Kushwaha, R. S., W. R. Hazzard, C. Gagne, A. Chait, and J. J. Albers. 1977. Type-III hyperlipoproteinemia: paradoxical hypolipodemic response to estrogen. *Ann. Intern. Med.* **87:** 517–525.
4. Kekki, M., and E. A. Nikkila. 1971. Plasma triglyceride turnover during use of oral contraceptives. Metab. Clin. Exp. **20:** 878–889.
5. Kissebah, A. H., P. Harrigan, and V. Wynn. 1973. Mechanism of hypertriglyceridemia associated with contraceptive steroids. *Horm. Metab. Res.* **5:** 184–190.
6. Hamosh, M., and P. Hamosh. 1975. The effect of estrogen on the lipoprotein lipase activity of rat adipose tissue. *J. Clin. Invest.* **55:** 1132–1135.
7. Kim, H. J., and R. K. Kalkhoff. 1975. Sex steroid influence on triglyceride metabolism. *J. Clin. Invest.* **56:** 888–896.
8. Kudzma, D. J., F. St. Claire, L. DeLallo, and S. J. Freidberg. 1975. Mechanism of avian estrogen-induced hypertriglyceridemia: evidence for overproduction of triglyceride. *J. Lipid Res.* **16:** 123–133.
9. Wilcox, H. G., W. F. Woodside, K. J. Breen, H. R. Knapp, Jr., and M. Heimberg. 1974. The effect of sex of certain properties of the very low density lipoprotein secreted by the liver. *Biochem. Biophys. Res. Commun.* **58:** 919–926.
10. Soler-Argilaga, C., and M. Heimberg. 1976. Comparison of metabolism of free fatty acid by isolated perfused livers from male and female rats. *J. Lipid Res.* **17:** 605–615.
11. Luskey, K. L., M. S. Brown, and J. L. Goldstein. 1974. Stimulation of the synthesis of very low density lipoproteins in rooster liver by estradiol. *J. Biol. Chem.* **249:** 5939–5947.
12. Chan, L., R. L. Jackson, B. W. O'Malley, and A. R. Means. 1976. Synthesis of very low density lipoproteins in the cockerel. Effects of estrogen. *J. Clin. Invest.* **58:** 368–379.
13. Chan, L., and A. R. Means. 1978. Very low density lipoprotein (VLDL) synthesis in the cockerel: purification of a specific mRNA synthesis of its DNA complement and identification of a putative VLDL precursor. *Clin. Res.* **26:** 303A. (Abstr.)
14. Mandour, T., A. H. Kissebah, and V. Wynn. 1977. Mechanism of oestrogen and progesterone effects on lipid and carbohydrate metabolism: alteration in the insulin:glucagon molar ratio and hepatic enzyme activity. *Eur. J. Clin. Invest.* **7:** 181–187.
15. Kudzma, D. J., P. M. Hegsted, and R. E. Stoll. 1973. The chick as a laboratory model for the study of estrogen-induced hyperlipidemia. *Metab. Clin. Exp.* **22:** 423–434.
16. Ockner, R. K., D. A. Burnett, N. Lysenko, and J. A. Manning. 1978. Sex differences in free fatty acid utilization and triglyceride biosynthesis in rat hepatocytes. *Clin. Res.* **26:** 531A. (Abstr.)
17. Berry, M. N., and D. S. Friend. 1969. High-yield preparation of isolated rat liver parenchymal cells. A biochemical and fine structural study. *J. Cell Biol.* **43:** 506–520.
18. Folch, J., M. Lees, and G. M. Sloane Stanley. 1957. A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* **226:** 497–509.
19. Ockner, R. K., J. P. Pittman, and J. L. Yager. 1972. Differences in the intestinal absorption of saturated and unsaturated long-chain fatty acids. *Gastroenterology.* **62:** 981–992.
20. Sauer, F., S. Mahadevan, and J. D. Erfle. 1971. The accumulation of citrate cycle intermediates in rat liver cells oxidizing palmitate. *Biochim. Biophys. Acta.* **239:** 26–32.
21. Bar-Tana, J., G. Rose, and B. Shapiro. 1971. The purification and properties of microsomal palmitoyl-CoA synthetase. *Biochem. J.* **122:** 353–362.
22. Jamdar, S. C., and H. J. Fallon. 1973. Glycerolipid biosynthesis in rat adipose tissue. I. Properties and distribution of glycerophosphate acyltransferase and effect of divalent cations on neutral lipid formation. *J. Lipid Res.* **14:** 509–516.
23. Lamb, R. G., S. D. Wyrick, and C. Piantadosi. 1977. Hypolipidemic activity of in vitro inhibitors of hepatic and intestinal SN-glycerol-3-phosphate acyltransferase and phosphatidase phosphohydrolase. *Atherosclerosis.* **27:** 147–154.
24. Coleman, R., and R. M. Bell. 1976. Triacylglycerol synthesis in isolated fat cells. Studies on the microsomal diacylglycerol acyltransferase activity using ethanol-dispersed diacylglycerols. *J. Biol. Chem.* **251:** 4537–4543.
25. Scheig, R., and K. J. Isselbacher. 1965. Pathogenesis of ethanol-induced fatty liver. III. In vivo and in vitro effects of ethanol on hepatic fatty acid metabolism in rat. *J. Lipid Res.* **6:** 269–277.
26. Ockner, R. K., J. A. Manning, R. B. Poppenhausen, and W. K. L. Ho. 1972. A binding protein for fatty acids in

cytosol of intestinal mucosa, liver, myocardium, and other tissues. *Science (Wash. D. C.).* **177**: 56–58.
27. Lunzer, M. A., J. A. Manning, and R. K. Ockner. 1977. Inhibition of rat liver acetyl coenzyme A carboxylase by long chain acyl coenzyme A and fatty acid. Modulation by fatty acid binding protein. *J. Biol. Chem.* **252**: 5483–5487.
28. Ockner, R. K., and J. A. Manning. 1974. Fatty acid-binding protein in small intestine. Identification, isolation, and evidence for its role in cellular fatty acid transport. *J. Clin. Invest.* **54**: 326–338.
29. Snedecor, G. W., and W. G. Cochran. 1967. Statistical Methods. Iowa State University Press, Ames, Iowa. 6th edition. 593 pp.
30. Mishkin, S., L. Stein, G. Fleischner, Z. Gatmaitan, and I. M. Arias. 1975. Z protein in hepatic uptake and esterification of long-chain fatty acids. *Am. J. Physiol.* **228**: 1634–1640.
31. Ockner, R. K., and J. A. Manning. 1976. Fatty acid binding protein. Role in esterification of absorbed long chain fatty acid in rat intestine. *J. Clin. Invest.* **53**: 632–641.
32. O'Doherty, P. J. A., and A. Kuksis. 1975. Stimulation of triacylglycerol synthesis by a protein in rat liver and intestinal mucosa. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* **60**: 256–258.
33. Mishkin, S., and R. Turcotte. 1974. Stimulation of monoacylglycerophosphate formation by Z protein. *Biochem. Biophys. Res. Commun.* **60**: 376–381.
34. Campbell, R. M., and H. W. Kosterlitz. 1950. The effects of growth and sex on the composition of the liver cells of the rat. *J. Endocrinol.* **6**: 308–318.
35. Korenchevsky, V., K. Hall, R. C. Burbank, and J. Cohen. 1941. Hepatotrophic and cardiotrophic properties of sex hormones. *Br. Med. J.* **1**: 396–399.
36. Dietschy, J. M. 1978. General principals governing movement of lipids across biological membranes. *In* Disturbances in Lipid and Lipoprotein Metabolism. J. M. Dietschy, A. M. Gotto, Jr., and J. A. Ontko, editors. American Physiological Society, Bethesda, Md. 1–28.
37. Ontko, J. A. 1972. Metabolism of free fatty acids in isolated liver cells. Factors affecting the partition between esterification and oxidation. *J. Biol. Chem.* **247**: 1788–1800.
38. Mishkin, S., L. Stein, Z. Gatmaitan, and I. M. Arias. 1972. The binding of fatty acids to cytoplasmic proteins: binding to Z protein in liver and other tissues of the rat. *Biochem. Biophys. Res. Commun.* **47**: 997–1003.
39. Renaud, G., A. Foliot, and R. Infante. 1978. Increased uptake of fatty acid by the isolated rat liver after raising the fatty acid binding protein concentration with clofibrate. *Biochem. Biophys. Res. Commun.* **80**: 327–334.