

# Sex Steroid Modulation of Fatty Acid Utilization and Fatty Acid Binding Protein Concentration in Rat Liver

ROBERT K. OCKNER, NINA LYSENKO, JOAN A. MANNING, SCOTT E. MONROE,  
and DAVID A. BURNETT, *Department of Medicine, Liver Center, and  
Department of Obstetrics, Gynecology and Reproductive Sciences, University  
of California, San Francisco, School of Medicine, San Francisco, California 94143*

**ABSTRACT** The mechanism by which sex steroids influence very low density hepatic lipoprotein triglyceride production has not been fully elucidated. In previous studies we showed that [ $^{14}\text{C}$ ]oleate utilization and incorporation into triglycerides were greater in hepatocyte suspensions from adult female rats than from males. The sex differences were not related to activities of the enzymes of triglyceride biosynthesis, whereas fatty acid binding protein (FABP) concentration in liver cytosol was greater in females. These findings suggested that sex differences in lipoprotein could reflect a sex steroid influence on the availability of fatty acids for hepatocellular triglyceride biosynthesis. In the present studies, sex steroid effects on hepatocyte [ $^{14}\text{C}$ ]oleate utilization and FABP concentration were investigated directly.

Hepatocytes from immature (30-d-old) rats exhibited no sex differences in [ $^{14}\text{C}$ ]oleate utilization. With maturation, total [ $^{14}\text{C}$ ]oleate utilization and triglyceride biosynthesis increased moderately in female cells and decreased markedly in male cells; the profound sex differences in adults were maximal by age 60 d. Fatty acid oxidation was little affected.

Rats were castrated at age 30 d, and received estradiol, testosterone, or no hormone until age 60 d, when hepatocyte [ $^{14}\text{C}$ ]oleate utilization was studied. Castration virtually eliminated maturational changes and blunted the sex differences in adults. Estradiol or testosterone largely reproduced the appropriate adult pattern of [ $^{14}\text{C}$ ]oleate utilization regardless of the genotypic sex of the treated animal.

In immature females and males, total cytosolic FABP concentrations were similar. In 60-d-old animals, there was a striking correlation among all groups (females, males, castrates, and hormone-treated) be-

tween mean cytosolic FABP concentration on the one hand, and mean total [ $^{14}\text{C}$ ]oleate utilization ( $r = 0.91$ ) and incorporation into triglycerides ( $r = 0.94$ ) on the other. In 30-d-old animals rates of [ $^{14}\text{C}$ ]oleate utilization were greater, relative to FABP concentrations, than in 60-d-old animals.

The sex differences that characterize fatty acid utilization in adult rat hepatocytes are not present in cells from immature animals, and reflect in part the influence of sex steroids. It remains to be determined whether the observed relationship of hepatic FABP concentration to [ $^{14}\text{C}$ ]oleate utilization in adult cells is causal or secondary to changes in cellular fatty acid uptake effected through another mechanism. In either case, modulation of triglyceride-rich lipoprotein production by sex steroids appears to be mediated to a significant extent by their effects on hepatic fatty acid utilization.

## INTRODUCTION

Available evidence suggests that sex steroid effects on plasma lipid concentrations reflect in part parallel changes in hepatic production of triglyceride-rich, very low density lipoproteins (VLDL),<sup>1</sup> but the mechanism has not been fully elucidated. Recently, we examined the possibility that sex differences in VLDL production might be secondary to differences in hepatic fatty acid utilization and triglyceride biosynthesis. In suspensions of isolated hepatocytes from adult rats, profound sex differences in the utilization of albumin-bound [ $^{14}\text{C}$ ]oleate were demonstrated, characterized by greater incorporation into triglycerides, other glycerolipids, and oxidation products in female hepatocytes than male (1, 2). These differences were not

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<sup>1</sup> Abbreviations used in this paper: FABP, fatty acid binding protein; VLDL, very low density lipoproteins.

correlated with activities of enzymes in the triglyceride biosynthetic pathway, but the concentration of fatty acid binding protein (FABP) was significantly higher in liver cytosol of females. Sex differences in hepatic uptake and utilization of FFA also have been demonstrated recently in vivo (3) and in the isolated perfused rat liver (4).

To further investigate the mechanism of these striking sex differences in FFA utilization, the present studies were designed to determine: (a) whether they could be attributed to the sex steroids, per se; (b) whether hepatic FABP concentrations were influenced by the sex steroids; and (c) whether FABP concentrations correlated with rates of FFA utilization under conditions in which the latter was varied over a range of hormonal influences.

The results show that the profound sex differences in FFA utilization between adult female and male hepatocytes are not present in cells from immature rats, are largely prevented by castration, and are reproduced by continuous administration of estradiol or testosterone to castrates of either sex. Among all groups of adult rats (female, male, castrated, and hormone-treated) there is a striking correlation between FFA utilization and estimated hepatic FABP concentration. Portions of these studies have been reported in published abstracts (1, 5).

## METHODS

**Materials.** [ $^{14}\text{C}$ ]oleic acid and L-[U- $^{14}\text{C}$ ]glycerol-3-phosphate, disodium salt, were obtained from New England Nuclear (Boston, Mass.), unlabeled oleic acid from Calbiochem Behring Corp., American Hoechst Corp. (San Diego, Calif.), and [ $^{14}\text{C}$ ]diolein (72% 1,3; 28% 1,2) from Dhom Products, Ltd. (North Hollywood, Calif.). Fatty acid-free albumin, S-palmitoyl coenzyme A (CoA), oleoyl CoA and unlabeled DL- $\alpha$ -glycerophosphate (disodium salt), were purchased from Sigma Chemical Co. (St. Louis, Mo.). The albumin contained <0.02 nmol fatty acid per nmol protein (6).

**Animals.** Sprague-Dawley rats were obtained from Simonsen Laboratories (Gilroy, Calif.), and were allowed access to standard laboratory chow ad lib.

**Studies of hepatocyte suspensions.** Hepatocyte suspensions were prepared by modification of the method of Berry and Friend (7), as described previously (6). Livers were perfused with  $\text{Ca}^{++}$ -free Hanks' medium gassed with 95% oxygen, 5%  $\text{CO}_2$ , containing 0.05% collagenase (type I, Sigma Chemical Co.). Cells were suspended in  $\text{Ca}^{++}$ -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/dl (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  $\text{O}_2$  and utilize oxidizable substrates normally (6). Addition of  $\text{Ca}^{++}$  before or during the incubations did not affect [ $^{14}\text{C}$ ]oleate utilization. [ $^{14}\text{C}$ ] $\text{O}_2$

production and incorporation of [ $^{14}\text{C}$ ]oleate into lipids and water soluble products were determined as previously described (2).

**Assay of microsomal enzymes.** Microsomes were prepared from whole rat liver and were assayed for activities of long chain acyl CoA synthetase, acyl CoA: glycerol-3-phosphate acyltransferase, phosphatidate phosphohydrolase, and diglyceride acyltransferase as described (2).

Studies of the effects of hormonal manipulation were carried out in animals castrated by standard operative techniques at age 30 d. Some of these animals were provided with subcutaneous implants of silastic tubing containing either testosterone or estradiol-17 $\beta$ . Estradiol implants (containing  $7.7 \pm 0.1$  mg) were prepared as described by Legan et al. (8) and testosterone implants (containing  $29.9 \pm 0.1$  mg) as described by Cheung and Davidson (9). Hepatocytes, or liver 105,000 g supernate for assay of FABP (see below), were prepared from these castrated animals at age 60 d, i.e., after 30 d of either no hormone replacement or continuous estradiol or testosterone administration. There were no significant differences among the groups in ratios of cell number to cell protein (unpublished observations). Hormonal effect was documented by the greater body weights in testosterone-treated animals of both sexes (see below), by maturation of seminal vesicles in testosterone-treated males or uteri in estrogen-treated females, and by measurement of serum estradiol and testosterone concentrations in serum obtained at the time of the experiment (see below). In a separate series of experiments, hepatocytes from some animals were studied at intervals of 5–15 d after hormone implantation; in another, immature castrated rats were implanted with longer silastic capsules that contained five times the standard amount of hormones indicated above (i.e., "high dose").

**Radioimmunoassay of steroid hormones.** Plasma testosterone and estradiol concentrations were determined by radioimmunoassay after purification by Celite chromatography (Johns-Manville, Filtration & Indus. Minerals Div., Denver, Col.) (10, 11). The testosterone radioimmunoassay employs an antiserum to testosterone-3-carboxymethylloxime-bovine serum albumin, and the estradiol radioimmunoassay uses an antiserum to estradiol-6-carboxymethylloxime-bovine serum albumin. All hormone concentrations were corrected for procedural losses by an internal standard included in each sample. The average interassay coefficients of variation are <12 and 15%, respectively, for the testosterone and estradiol radioimmunoassays.

**Binding characteristics of partially purified FABP.** FABP was partially purified as the 12,000-mol wt fraction of liver cytosol by Sephadex G-50 chromatography as described previously (12), and was subjected to Sephadex G-25 chromatography with [ $^{14}\text{C}$ ]oleate. Oleate binding was calculated from the  $^{14}\text{C}$  that appeared with protein in the void volume. This method was used to compare binding characteristics of equal amounts of partially purified FABP among various experimental groups.

**Binding assay of FABP in whole liver cytosol.** Total concentration of available FABP-associated fatty acid receptors in 105,000 g supernate was estimated by the binding of [ $^{14}\text{C}$ ]oleate to the 12,000-mol wt fraction of cytosol, as determined by Sephadex G-50 chromatography. In this assay, 120% of the given amount of [ $^{14}\text{C}$ ]oleate was added in 5  $\mu\text{l}$  propylene glycol to 4.8 mg cytosolic protein in 0.24 ml 0.154 M KCl-0.01 M phosphate buffer, pH 7.4. Of this mixture, 0.2 ml (4 mg protein) was subjected to gel filtration on Sephadex G-50, 1.0  $\times$  40 cm, 20°C, flow rate 0.65 ml/min. Fractions (1.32 ml) were analyzed for OD at 280 nm and for  $^{14}\text{C}$ . Identity of eluate  $^{14}\text{C}$  under these conditions as fatty acid has been

established (12). Characteristic elution profiles are shown in Fig. 1, for three different amounts of added ligand. Two major  $^{14}\text{C}$  peaks were consistently observed. The first, corresponding to the excluded ("void") volume included residual albumin as well as other larger macromolecules and lipoproteins in the tissue supernate; this peak was just detectable at low ligand concentrations, but increased greatly as larger amounts were added. The second peak, corresponding to the elution volume of 12,000-mol wt proteins, represents FABP; this peak predominated at low ligand concentrations, but unlike the earlier peak did not increase proportionately as large amounts of ligand were added. Under all conditions, the two peaks were well separated.

The relationship between these peaks is seen in greater detail in Fig. 2, in which the total nanomoles  $^{14}\text{C}$ oleate associated with each peak is plotted as a function of total nanomoles added in the assay. As noted above, binding to the FABP peak predominated at low ligand concentration, but as ligand concentration was increased, appearance of  $^{14}\text{C}$  in the void volume became dominant, and assumed a near-linear relationship to the amount of ligand added, with an approximate slope of 0.5. Simultaneously, total incremental binding to FABP decreased; the resulting curve can be resolved into two distinct components (13): (a) a saturable process with a maximal capacity of 8 nmol; and (b) a linear, nonsaturable process with an approximate slope of 0.03.

To assess the sensitivity of this assay to differences in FABP concentration, cytosol was artificially enriched in FABP by adding graded amounts of the partially purified 12,000-mol wt FABP fraction. Binding of  $^{14}\text{C}$ oleate by the resulting mixture was then measured. As shown in Fig. 3, the assay was reproducibly and linearly responsive to changes in FABP concentration over a biologically significant range. Although the assay actually depends on availability of

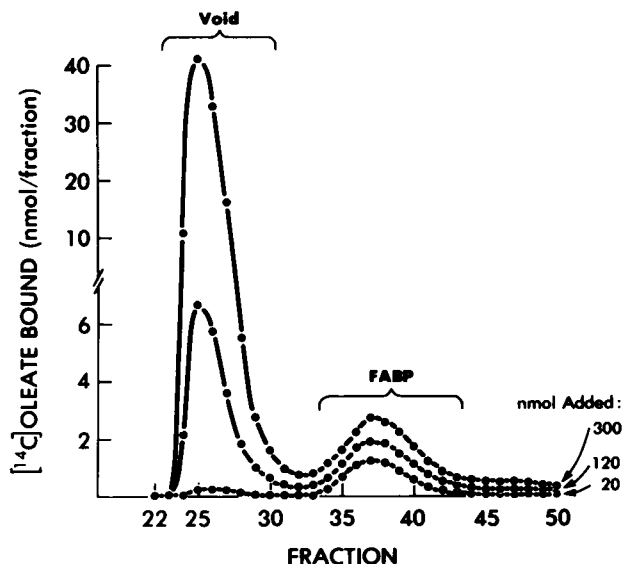


FIGURE 1 Sephadex G-50 chromatography of  $^{14}\text{C}$ oleate with rat liver 105,000 g supernate.  $^{14}\text{C}$ oleate was added to 4 mg supernatant protein and subjected to gel filtration chromatography as described in Methods. Over a wide range of added oleate, two well-separated peaks were consistently observed, corresponding to the void volume and the 12,000-mol wt FABP region, respectively.

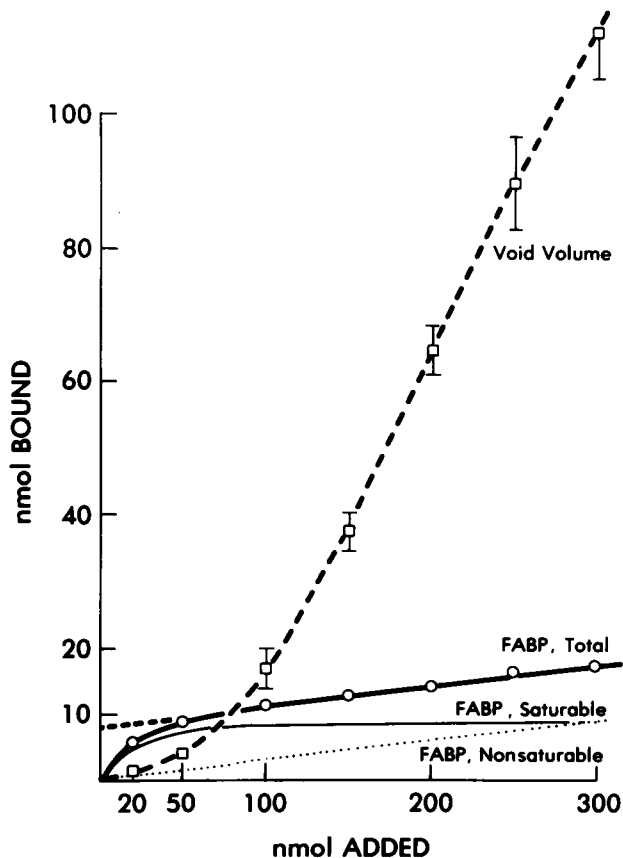


FIGURE 2 Binding of  $^{14}\text{C}$ oleate to Sephadex G-50 fractions of rat liver 105,000 g supernate. Total  $^{14}\text{C}$ oleate eluting with each of the two peaks shown in Fig. 1 is plotted as a function of total added ligand. The total FABP-associated  $^{14}\text{C}$  is resolved into saturable and nonsaturable components. Mean  $\pm$  SE (shown where SE exceeds symbol dimension);  $n = 4$  for each indicated point.

FABP-associated fatty acid receptors, the fact that the binding characteristics of FABP from immature and adult female and male rats are indistinguishable ([2] and unpublished observations) indicates that the availability of receptors per milligram FABP and their affinity do not differ among the various experimental groups and, therefore, that this method is valid for estimation of relative FABP concentrations. Throughout the text, the terms "concentration" or "estimated concentration" of FABP are used in this relative sense; accordingly the results are expressed as  $^{14}\text{C}$ oleate bound rather than as a quantity of protein. This assay was employed in studies of the effects of age, sex, and sex steroids on hepatic FABP concentration (see below).

**Statistical methods.** Significance of differences among experimental groups was determined by the unpaired  $t$  test; linear regression and correlation coefficients were calculated by standard methods (14).

## RESULTS

**Effect of age on fatty acid utilization in isolated rat hepatocyte suspensions.** Hepatocyte suspensions from rats at various ages (body weights, Table I) from sexual

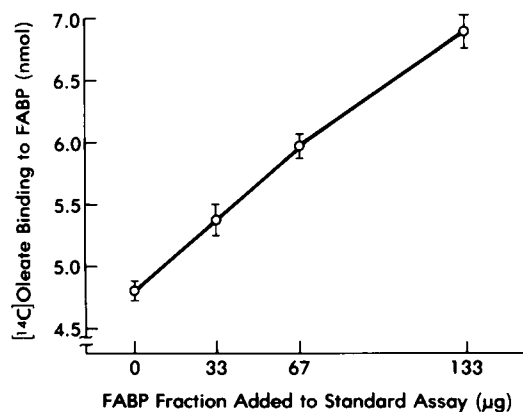


FIGURE 3 "Recovery" of FABP added to rat liver 105,000 g supernate as determined by gel filtration binding assay. Graded amounts of partially purified FABP fraction were added to 4 mg whole cytosol protein as indicated, and the resulting mixtures were subjected to the Sephadex G-50 gel filtration binding assay for total FABP described in Methods. Mean  $\pm$  SE;  $n = 4$  for each indicated point.

immaturity to adulthood were assayed for incorporation of [ $^{14}$ C]oleate into lipids and oxidation products as described in Methods. Results are shown in Table II for all measured products and in Fig. 4 for triglycerides. Among all groups of intact rats, few differences of significance in fatty acid oxidation were observed; triglyceride was the major product formed, so that total esterification and total utilization of [ $^{14}$ C]oleate generally paralleled its incorporation into triglycerides.

In cells from immature 30-d animals, there were no significant sex differences in any aspect of fatty acid utilization. In females, there was a moderate, but statistically significant ( $P < 0.001$ ) maturational increase in triglyceride biosynthesis and in total fatty acid esterification and utilization (Table II and Fig. 4).

TABLE I  
Effects of Castration and Hormone Administration  
on Body Weights of Rats at Age 60 d

	Female	Male
Intact controls		
Age 30 d	86 $\pm$ 8 (3)	96 $\pm$ 7 (4)
Age 60 d	207 $\pm$ 3 (8)	296 $\pm$ 6 (8)
Castrated at age 30 d		
No hormone	260 $\pm$ 7 (6)	304 $\pm$ 6 (8)
Estradiol-treated	203 $\pm$ 5 (5)	246 $\pm$ 8 (5)
Testosterone-treated	253 $\pm$ 7 (4)	290 $\pm$ 8 (5)

All rats were fed ad lib., and were 60 d old when weighed on the morning of the day of the experiment. Rats that had been castrated at age 30 d received, during the intervening 30 d, either no hormone replacement or standard-dose estradiol or testosterone, administered continuously via subcutaneous silastic implants as described in Methods. Mean  $\pm$  SE ( $n$ ).

However, the striking sex differences in mature 60-d-old animals were principally accounted for by the marked decrease associated with maturation in the male (Fig. 4). These sex differences also characterized hepatocytes from mature females and males of similar body weights (2). Other than with respect to incorporation into triglyceride, total esterification, and total utilization, cells from 60-d animals did not differ significantly from those 30-d animals of the same sex, except for a lower rate of [ $^{14}$ C]O<sub>2</sub> formation in cells from 60-d males.

*Effects of castration and sex hormone administration on hepatocyte fatty acid utilization.* To test the possibility that the sex steroids themselves accounted for these sex and age differences, rats were castrated at age 30 d, and hepatocytes were prepared from these animals at age 60 d, after 30 d of no hormone, or continuous administration of estradiol or testosterone (Methods). Body weights are shown in Table I. Serum estradiol and testosterone concentrations, measured in samples obtained immediately before preparation of the cell suspensions, are shown in Table III. Mean serum estradiol levels were  $<20$  pg/ml in all groups except intact adult females and estradiol-treated castrates. Despite the fact that standard-dose testosterone implants produced expected adult male patterns in terms of sexual development, body weight, and/or hepatocyte lipid metabolism (Methods, Table I, and below), the resulting serum testosterone levels were not greater than those in immature (30-d-old) males. This discrepancy between biological effect and serum hormone concentrations suggests that hormone concentrations may have fallen during the 30-d treatment period because of a decreasing rate of delivery of hormone. Testosterone concentrations in high-dose testosterone-treated castrates were similar to those in intact adult males.

Incorporation of [ $^{14}$ C]oleate into lipids and oxidation products by hepatocytes from these animals are shown in Table II, and are more readily compared for triglycerides in Fig. 5; in the latter, values for intact 30- and 60-d-old animals are shown for comparison. Certain important findings warrant emphasis.

First, castration at 30 d greatly diminished the age-related changes in fatty acid utilization in both sexes and blunted the expected differences between genotypic females and males at 60 d. Thus, cells from immature males and from castrated 60-d males were similar in all respects, whereas cells from castrated 60-d females differed from 30-d female cells only in that incorporation into phospholipids was greater in the former. Furthermore, although triglyceride biosynthesis, total esterification, and total utilization remained somewhat greater in cells from castrated 60-d females than males ( $P < 0.05$ ), these sex differences were far less impressive and significant than those between intact 60-d controls ( $P < 0.001$ ).

TABLE II

Effects of Age, Castration, and Sex Hormone Administration on [ $^{14}$ C]Oleate Utilization by Rat Hepatocyte Suspensions

Experimental group	Oxidation products			Esters					Total utilization
Age, sex, hormonal status	CO <sub>2</sub>	Water-soluble	Total	Phospho-lipids	Diglyc-erides	Triglyc-erides	Choles-terol esters	Total esters	
nmol [ <sup>14</sup> C]oleate incorporated/mg cell protein/10 min									
Intact									
30 d									
Female (3)	0.28±0.05	2.39±0.37	2.67±0.35	1.27±0.10	1.65±0.40	11.83±0.60	0.07±0.02	14.82±0.82	17.50±0.61
Male (4)	0.32±0.04	2.26±0.05	2.58±0.07	1.41±0.13	1.04±0.13	11.50±0.87	0.10±0.04	14.04±0.64	16.62±0.63
60 d									
Female (8)	0.26±0.02	2.80±0.13	3.06±0.13	2.48±0.47	2.35±0.34	15.18±0.33	0.10±0.02	20.34±0.63	23.16±0.69
Male (8)	0.18±0.02*	2.32±0.20	2.49±0.21†	1.17±0.06*	1.29±0.13*	6.70±0.51§	0.14±0.02	9.30±0.54§	11.79±0.67§
Castrated at age 30 d									
60 d, no hormone									
Female (6)	0.29±0.05	2.72±0.12	3.01±0.15	1.61±0.07	1.50±0.17	12.48±0.59	0.10±0.01	15.69±0.76	18.69±0.71
Male (8)	0.25±0.02	2.27±0.18	2.52±0.16	1.38±0.09	1.00±0.16	9.88±0.74†	0.13±0.02	12.39±0.97†	14.92±1.09†
60 d, estradiol									
Female (5)	0.34±0.04	2.64±0.22	2.97±0.26	1.66±0.13	2.37±0.28	11.87±0.67	0.10±0.02	16.00±1.03	18.97±1.22
Male (5)	0.29±0.03	2.73±0.09	3.02±0.10	1.78±0.09	1.72±0.12	15.46±1.22†	0.14±0.03	19.10±1.39	22.12±1.36
60 d, testosterone									
Female (4)	0.24±0.03	3.03±0.31	3.27±0.31	1.40±0.08	0.94±0.19	8.07±0.63	0.13±0.03	10.53±0.73	13.80±0.98
Male (5)	0.19±0.01	2.42±0.15	2.61±0.16	1.25±0.06	0.90±0.20	6.78±0.33	0.15±0.02	9.08±0.42	11.69±0.40

Hepatocyte suspensions were incubated with albumin-bound 0.44 mM [ $^{14}$ C]oleate for 10 min, and incorporation into oxidation products and lipids was measured as described in Methods. Animals castrated at age 30 d were studied at age 60 d after no hormone, estradiol, or testosterone treatment during the intervening 30 d. Mean±SE. Number of animals in each group indicated in parentheses. Significance of differences vs. corresponding values for females in same group:

\*  $P < 0.02$ ;

†  $P < 0.05$ ;

§  $P < 0.001$ .

Second, and with few exceptions, administration of exogenous hormones to castrated 30-d rats essentially reproduced the pattern of oleate utilization in cells from intact 60-d animals, regardless of the genotypic sex of the treated animal. Thus, cells from testosterone-treated female and male castrates and from intact 60-d males were similar in all respects. Likewise,

cells from estradiol-treated male castrates were similar to those from normal 60-d females.

Of interest was the finding that cells from standard-dose estradiol-treated female castrates incorporated significantly less fatty acid into triglyceride than did cells from similarly treated males and intact 60-d females. This sex difference in response to exogenous estradiol appeared to be dose-dependent, in that with high-dose estradiol, [ $^{14}$ C]oleate incorporation into triglycerides in cells from estrogen-treated females was further increased and no longer differed from similarly treated males and control adult females (Fig. 5).

Body weight, per se, did not appear to influence the results. For example, despite similar body weights, cells from castrated females, estradiol-treated females, and estradiol-treated males showed very different rates of [ $^{14}$ C]oleate utilization (Tables I and II).

In a separate series of experiments, the time-course of the exogenous testosterone effect generally paralleled or was slightly more rapid than the response to sexual maturation in the intact male (unpublished observations).

*Studies of the mechanism of age and sex differences in hepatocyte [ $^{14}$ C]oleate utilization.* These experiments addressed: (a) the concentration-dependence of oleate utilization in intact hepatocytes; (b) the activities of microsomal enzymes; and (c) the binding characteristics and concentration of hepatic FABP.

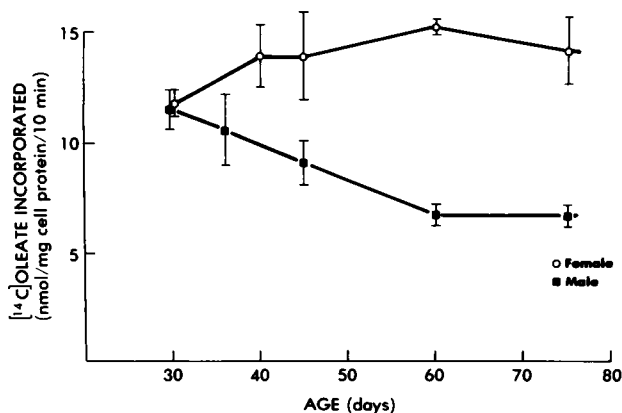


FIGURE 4 Effect of age on incorporation of [ $^{14}$ C]oleate into triglyceride by rat hepatocyte suspensions. Hepatocyte suspensions from female and male rats of the indicated ages were incubated with albumin-bound 0.44 mM [ $^{14}$ C]oleate for 10 min; incorporation into triglycerides was measured as described in Methods. Mean±SE;  $n = 3-5$  for all groups except 60-d females and males (eight each).

TABLE III

Effect of Castration and Hormone Administration on Serum Estradiol and Testosterone Concentrations

	Estradiol	Testosterone
	pg/ml	ng/ml
Intact		
30 d		
Female	15.7±1.5(3)	0.45±0.5(3)
Male	15.3±4.3(3)	1.30±0.24(3)
60 d		
Female	31.8±5.5(5)	0.34±0.03(4)
Male	15.0±3.8(3)	8.85±1.29(4)
Castrated at age 30 d		
60 d, no hormone		
Female	20.0±3.8(5)	0.28±0.01(6)
Male	14.8±2.2(6)	0.22±0.02(6)
60 d, estradiol (standard dose)		
Female	58.0±5.2(5)	0.27±0.03(5)
Male	51.4±4.9(5)	0.30±0.01(5)
60 d, testosterone (standard dose)		
Female	11.4±3.2(5)	1.48±0.10(5)
Male	16.2±2.2(5)	2.18±0.19(5)
60 d, estradiol (high dose)		
Female	227±112(3)	0.44±0.06(4)
Male	108±8.6(4)	0.35±0.01(4)
60 d, testosterone (high dose)		
Female	17.3±1.3(4)	6.63±0.92(4)
Male	16(13, 19)	8.42±2.13(3)

Sex steroid concentrations were measured by radioimmunoassay in serum obtained at the time that liver cell suspensions were prepared as described in Methods. Mean±SE. Sample size or individual values in parentheses.

In Fig. 6 is shown the effect of [ $^{14}$ C]oleate concentration and oleate:albumin molar ratio on oleate incorporation into triglycerides and oxidation products by hepatocytes from immature 30-d-old female and male rats. Curves for 60-d animals (2) are presented for comparison. In contrast to cells from sexually mature animals, those from 30-d females and males showed no significant differences in [ $^{14}$ C]oleate incorporation. At lower substrate concentrations, rates of incorporation into triglycerides were intermediate between those for adult females and males; at high substrate concentration, rates for all groups were similar. Thus, as was the case for sex differences in adult cells, differences in [ $^{14}$ C]oleate incorporation into triglycerides between cells from immature and adult rats are not evident at very high substrate concentrations. Oxidation was generally greater in immature than mature hepatocytes (lower dotted lines, Fig. 6), but no sex differences were observed.

Activities of microsomal acyl CoA synthetase, glycerol-3-phosphate acyltransferase, phosphatidate phosphohydrolase, and diglyceride acyltransferase

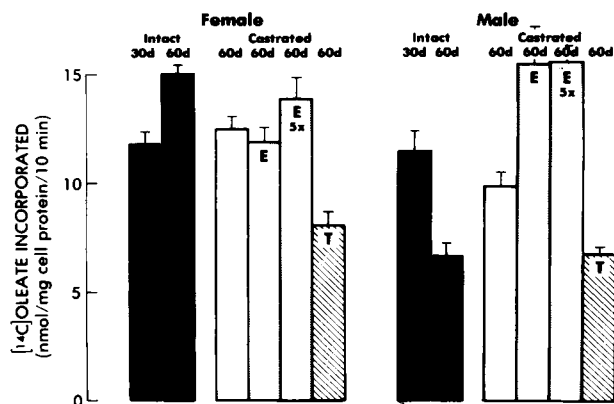


FIGURE 5 Effects of castration and hormone administration on [ $^{14}$ C]oleate incorporation into triglyceride by rat hepatocytes. Hepatocyte suspensions were prepared from 60-d-old rats that had been castrated at age 30 d and treated subsequently with no hormone, standard-dose estradiol (E), or testosterone (T), or high-dose estradiol (5 $\times$ ). Cells were incubated in triplicate for 10 min with albumin-bound 0.44 mM [ $^{14}$ C]oleate and incorporation into triglycerides was measured as described in Methods. Values for unoperated control 30- and 60-d-old rats are shown for comparison. Mean±SE for all castrated groups,  $n = 4-8$ .

were similar in livers of 30-d-old females and males and, in the case of acyl CoA synthetase and glycerol-3-phosphate acyltransferase, this similarity existed over a wide range of substrate concentrations (2–20  $\mu$ M fatty acid and acyl CoA, respectively). Because there

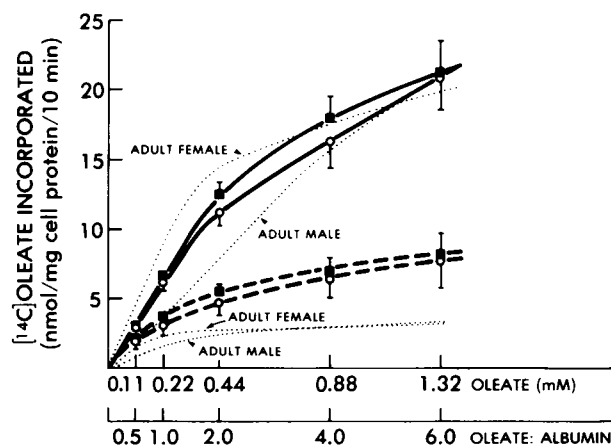


FIGURE 6 Incorporation of [ $^{14}$ C]oleate into triglycerides and oxidation products by hepatocytes from immature rats. Cells from 30-d-old female and male rats were incubated for 10 min with the indicated concentration of albumin-bound [ $^{14}$ C]oleate, and incorporation into triglycerides and oxidation products was measured as described in Methods. (Previously determined curves for cells from 60-d rats [2] are shown for comparison; [■], male; [○], female; upper dotted lines, triglycerides; lower dotted lines, oxidation products). Mean±SE;  $n = 5$  for all points except male triglycerides, 0.11 mM oleate ( $n = 4$ ).

were no significant sex differences in microsomal yield per gram liver, the sexes were similar in total activity of these enzymes as well. Although there were no important sex differences among 30- or 60-d rats, specific activities of acyl CoA synthetase, phosphatidate phosphohydrolase, and diglyceride acyl-transferase were 40–80% greater in the immature (30-d) animals, whereas the specific activity of glycerol-3-phosphate acyltransferase was ~50% greater in the mature animals. It is impossible to draw from these comparisons any conclusions regarding the overall activities of the triglyceride pathway. Conceivably, however, differences in enzyme activity could contribute to the apparently “anomalous” behavior of immature hepatocytes with respect to [ $^{14}$ C] oleate utilization and FABP concentration (see below).

Binding characteristics of FABP, partially purified from 30-d female and male rat liver, were examined by Sephadex G-25 chromatography and, as indicated in Methods were not significantly different, and were essentially superimposable on those published previously for hepatic FABP from mature rats (2). This suggested that FABP binding characteristics were similar in all groups and, therefore, that any differences observed in binding activity of the FABP fraction in whole cytosol could be taken to represent differences in FABP concentration rather than in binding affinity. On this basis, a binding assay was developed for the estimation of relative FABP concentrations in whole cytosol, and is described in detail in Methods.

Results for animals 30, 45, and 60 d of age are shown in Fig. 7. It can be seen that cytosolic FABP concentrations were similar in 30-d females and males. Previously observed differences between the sexes at maturity (2) were confirmed and were found by this

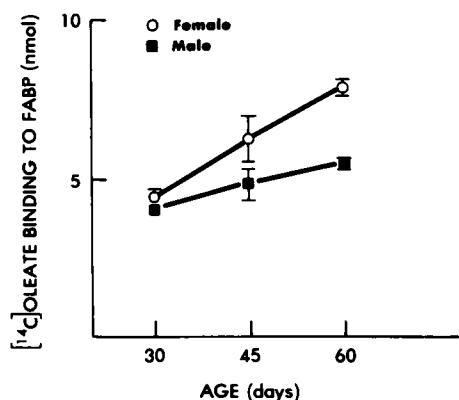


FIGURE 7 Effects of maturation on binding of [ $^{14}$ C]oleate to FABP fraction of rat liver cytosol. Binding of [ $^{14}$ C]oleate to the FABP fraction of 4 mg cytosolic protein was determined for 20 nmol added ligand by Sephadex G-50 gel filtration as described in Methods. Mean  $\pm$  SE;  $n = 4$  for each point. Sex differences are significant only at 60 d ( $P < 0.001$ ).

improved assay to be highly significant ( $P < 0.001$ ). Furthermore, there was a statistically significant maturational increase in cytosolic FABP concentration in both sexes; this increase was substantial in females (78%,  $P < 0.001$ ), but relatively modest in males (36%,  $P < 0.001$ ). Significant sex differences were present only in the 60-d rats. Yields of cytosolic protein were similar in adult females and males ( $88.9 \pm 1.7$  vs.  $90.8 \pm 1.5$   $\mu$ g/g tissue, respectively); yields were somewhat lower in 30-d animals ( $76.7 \pm 1.5$  vs.  $81.9 \pm 2.5$   $\mu$ g/g), but as with adults the sexes were similar.

In a final series of experiments, the influence of sex steroids on hepatic FABP concentration was examined in rats castrated at 30 d and treated as described above with no hormone, estradiol, or testosterone, over the ensuing 30 d. Duplicate assays were performed at 60 d for each animal; 20 nmol ligand were added, because virtually all binding to FABP under these conditions is attributable to the saturable process (Fig. 2). The results are shown in Fig. 8; values for intact 30- and 60-d-old animals are presented for comparison.

It is apparent that the maturational increase in hepatic FABP concentration was not prevented by castration in either sex, and was not augmented by administration of estradiol to castrated females or testosterone to castrated males. In contrast, testosterone treatment of castrated females resulted in FABP concentrations essentially identical to those in intact adult males, whereas estradiol treatment of male castrates produced the highest FABP concentrations observed.

Thus, hepatic FABP concentration appears to be influenced by at least two determinants: (a) an age-related effect that is independent of gonadal steroids (a possible contribution of extragonadal sex steroids is not excluded); and (b) a sex steroid effect (testosterone

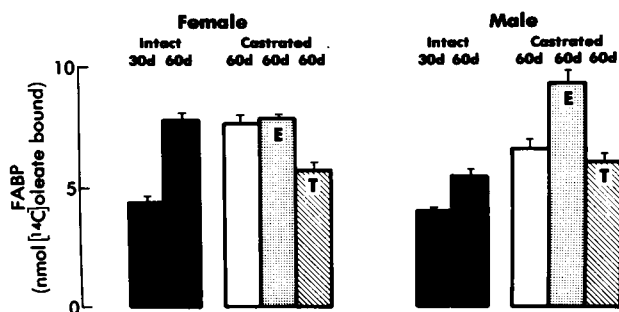


FIGURE 8 Effects of castration and hormone administration on hepatic FABP concentration. FABP concentration was determined in duplicate in 105,000 g supernate from livers of 60-d-old rats that had been castrated at age 30 d and treated subsequently with no hormone, estradiol (E), or testosterone (T), employing a binding assay as described in Methods. Values for unoperated control 30- and 60-d-old rats are shown for comparison, mean  $\pm$  SE. For castrated groups:  $n = 7$  (female);  $n = 9$  (male);  $n = 3$  (female + E);  $n = 4$  (male + E, female + T, male + T).

inhibitory, estradiol stimulatory) demonstrated only in animals of the genotypic sex in which that sex steroid is not normally predominant.

The hepatic FABP concentrations shown in Fig. 8 are graphically related to the values in the corresponding experimental groups for hepatocyte incorporation of [ $^{14}$ C]oleate into triglycerides (Fig. 5). This comparison is shown in Fig. 9. It is apparent that among all groups of 60-d-old animals, FABP concentration and triglyceride biosynthesis are closely correlated. A similar correlation was demonstrated between FABP concentration and total oleate utilization. In contrast, 30-d animals deviated significantly from these correlations. The nature of this deviation implies that fatty acid utilization was severalfold greater in cells from these animals than would be predicted from the estimated FABP concentration.

## DISCUSSION

In contrast with adult hepatocytes, no sex differences in [ $^{14}$ C]oleate utilization were demonstrated in cells from immature 30-d-old rats. With maturation, there

was a moderate increase in [ $^{14}$ C]oleate utilization and triglyceride biosynthesis in female hepatocytes, and a more striking decrease in fatty acid utilization in male cells. The decreased utilization in adult male cells, and the resulting differences from adult female cells, also characterized [ $^{14}$ C]oleate incorporation into other glycerolipids and, at very low substrate concentrations (2), [ $^{14}$ C]oleate oxidation. Thus, sex differences in adult cells reflected differences in net entry of fatty acid into the cell, not simply in its distribution among metabolic pathways.

The differences between adult (60-d) females and males were prevented or blunted by castration at age 30 d; cells from these castrated "adults" did not differ significantly from cells from immature animals of the same genotypic sex. However, if the animals received estradiol or testosterone during the 30 d after castration, the patterns of fatty acid utilization in their hepatocytes were generally similar to those of cells from intact adult females and males, respectively, regardless of the sex of the castrated animal to which the hormone had been administered.

In these studies, standard-dose estradiol appeared to have a lesser effect on hepatocytes from female castrates than from male castrates, whereas the sexes did not differ in response to high-dose estradiol. The basis for this sex difference in estradiol dose response is not known, but it could reflect differences in hepatic receptors (15) or the effects of nongonadal sex steroids.

It remains unclear whether the observed effects reflect direct sex steroid interaction with the liver cell, or are secondary to extrahepatic hormonal and metabolic responses. Either or both mechanisms may be involved. Evidence for a direct effect of estrogen on the liver cell includes the finding that estradiol induces the synthesis of VLDL apoprotein messenger RNA (16–18). Estrogens also appear to increase directly the hepatic synthesis of other proteins (19), to bind to liver surface membrane receptors (20), and to induce changes in liver surface membrane properties (20–22), and bile secretion (23). Estrogens also influence lipid metabolism in rat hepatocyte cultures (24).

Estrogens also may modulate liver function indirectly, however. For example, pharmacologic doses of estradiol increase rat portal venous insulin:glucagon ratios, associated with increased activity of hepatic lipogenic enzymes (25). Thus, estrogens may increase the availability of newly synthesized fatty acids as well as FFA uptake from plasma. On the other hand, it seems unlikely that sex differences in hepatocyte FFA utilization are mediated solely through insulin and/or glucagon because these differences persist in hepatocytes from fasted rats (2).

Despite uncertainties as to whether they are direct or indirect, accumulating evidence indicates that, although sex steroids influence rates of triglyceride

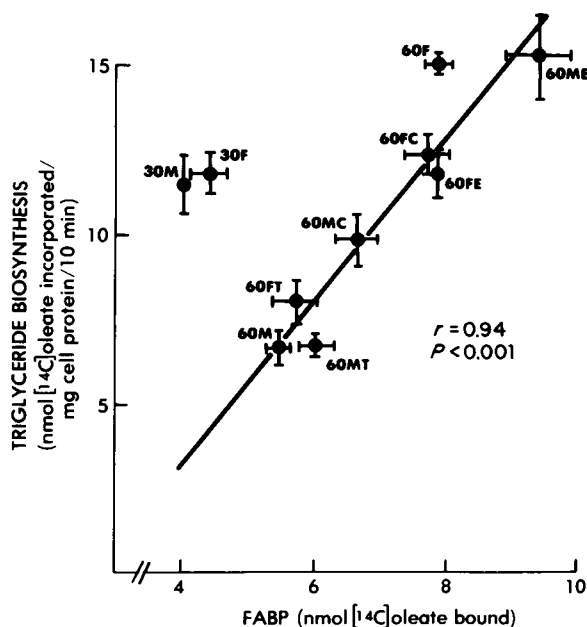


FIGURE 9 Triglyceride biosynthesis in hepatocyte suspensions: relationship to cytosolic FABP concentration. Group mean values for incorporation of [ $^{14}$ C]oleate into triglycerides by hepatocyte suspensions shown in Fig. 5 (calculated from the individual means of triplicate incubations for each of 59 animals) are plotted as a function of the corresponding group mean values for FABP concentration in hepatic cytosol (calculated from the individual means of duplicate assays for each of 39 animals). The linear regression is calculated for 60-d-old animals only. Symbols: 30, 60 = age; F = female; M = male; E = estradiol-treated; T = testosterone-treated; C = castrated.

removal from plasma (26–29), their effects on plasma lipid concentrations are mediated to a large extent via changes in hepatic lipoprotein production (26, 30–35). Furthermore, it is clear that an increased hepatic VLDL secretion rate can be sustained only if there is a corresponding increase in the availability of long-chain fatty acids. The absence of consistent sex hormone effects on plasma FFA concentration and turnover (31) suggests that the sex steroid effect on fatty acid availability must be mediated largely or exclusively via mechanisms intrinsic to the liver cell.

In these and in previously reported studies (2), cellular mechanisms by which an increase in the availability of fatty acid might be mediated have been investigated. In neither adult nor immature animals were there substantial sex differences in the activity of enzymes in the triglyceride pathway. However, the estimated concentration of FABP in liver is significantly greater in adult females than males, and correlates closely with total [ $^{14}\text{C}$ ]oleate utilization and incorporation into triglycerides by hepatocytes in a variety of experimental conditions. Only hepatocytes from immature animals deviate from this correlation, and do so in a manner indicating that net fatty acid entry into the cell and the triglyceride pathway is greater than would be predicted from the relationship between fatty acid utilization and FABP concentration in the older animals. The basis for this difference is not known; it could reflect in part the observed differences in microsomal enzyme activities. Whatever its mechanism, however, it appears independent of gonadal steroids, because fatty acid utilization by hepatocytes from 60-d castrates that had not received hormones was related to FABP concentration in a manner similar to that of intact and hormone-treated adult rats (Fig. 9).

In addition to providing further evidence in support of the concept that sex differences in hepatic fatty acid utilization are associated with corresponding differences in estimated hepatic FABP concentration, the present studies demonstrate that hepatic FABP responds to hormonal influences. Thus, in all groups in which endogenous or exogenous estradiol was the dominant hormone, estimated FABP concentration was greater than in those groups in which testosterone predominated. The mechanism of this effect remains to be determined. As noted above, however, estimated hepatic FABP concentrations were greater at age 60 d than at 30 d in all animals including castrates, indicating that FABP concentration also is influenced by one or more factors in addition to gonadal steroids. This could reflect extragonadal sex steroids, or other humoral or nutritional factors.

The mechanism by which FABP might influence hepatocyte fatty acid utilization and triglyceride biosynthesis has been considered previously in some

detail (6). It is clear that FABP increases the activity of a number of the enzymes of the triglyceride pathway (6, 36–39), but it remains to be determined whether FABP also acts as a fatty acid “carrier.”

It is also conceivable that the changes in FABP concentration are the result, rather than the cause, of changes in cellular fatty acid flux rates. A primary influence of fatty acid flux probably accounts for the response of intestinal mucosal FABP concentration in rats to dietary fat content (38), and warrants investigation in the present instance. Consistent with this alternative interpretation, we have recently obtained evidence for a saturable interaction between the fatty acid-albumin complex and the liver cell, implying the possible participation of a surface membrane receptor in the observed sex differences (40, 41).

In demonstrating the important influence of sex steroids on the net entry of fatty acids into the hepatocyte and the pathway of triglyceride biosynthesis, and on hepatic FABP concentrations, these studies raise the possibility that the recognized clinical effects of several of these agents on plasma lipoprotein concentrations may be mediated to a large extent at this level. These clinical effects include increased plasma triglyceride levels associated with pharmacological estrogen treatment, oral contraceptive administration, and pregnancy (42–44), and the decreased levels produced by androgens and progestational agents including norethindrone (45) and oxandrolone (46). Additional studies in this important area may be expected to further clarify the determinants of hepatic fatty acid uptake and utilization, and may have therapeutic implications as well.

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