Effect of ingestion of saline, glucose, and ethanol on mobilization and hepatic incorporation of epididymal pad palmitate-1-^{14}C in rats

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ABSTRACT The effect of ingestion of saline, glucose, and ethanol (isocaloric with the glucose) on the mobilization of radiopalmitate from epididymal fat prelabeled in vivo and the incorporation of the mobilized label into liver lipids was investigated in rats. The mobilization of radiopalmitate from epididymal fat and the incorporation of the mobilized label into liver triglyceride were most markedly elevated by ingestion of ethanol. Increased mobilization and diversion of epididymal adipose tissue fatty acids to liver lipids of ethanol-treated rats were shown also by the close resemblance of the fatty acids of liver triglyceride to the fatty acids of epididymal fat.

The amount of radiopalmitate mobilized by the saline-treated rats, comprising approximately a third of that mobilized by the ethanol-treated animals, was larger than the amount mobilized by the rats treated with glucose; most of it was oxidized rather than incorporated into the liver fats. In glucose-treated rats a larger fraction of radiopalmitate mobilized from one prelabeled epididymal pad was diverted to and incorporated into the lipids of the contralateral pad of the same animal. The specific activity of hepatic triglyceride of ethanol- and saline-treated rats was similar and significantly higher than that of animals treated with glucose.

These data indicate that the ethanol-induced fatty liver can be attributed to an increased mobilization and incorporation of adipose tissue fatty acids into liver lipid and to an altered hepatic metabolism of fatty acids and triglyceride.

KEY WORDS saline · glucose · ethanol · effect · mobilization · palmitate · between epididymal fat pads · to liver · fatty acid compositions · fatty liver · rat

It has been repeatedly observed that the administration of a single intoxicating dose of ethanol produces an excessive accumulation of triglycerides (TG) in the liver (1–5). The possible mechanisms through which ethanol can affect the level of hepatic triglyceride fatty acids (TGFA) are related either to a peripheral action on adipose tissue (increased FA mobilization) or to a direct effect on the metabolism of lipids in the liver (increased FA synthesis, decreased FA oxidation, increased FA esterification to TG, decreased release of TG in the form of lipoproteins). There is evidence that ethanol affects all these mechanisms, but the quantitative importance of each mechanism in the pathogenesis of fatty liver has not yet been established.

It has been shown that the hepatic metabolism of FA is altered (increased FA synthesis and decreased FA oxidation) by ethanol in vivo and in vitro (3–5). However, the demonstration that similar effects were obtained with substances (glucose, sorbitol, xylitol, nicotinamide) which do not produce fatty liver (3) suggested that a direct effect of ethanol on the hepatic metabolism of FA cannot be a major factor. In addition, abolishment of FA mobilization from adipose tissue by hypophysectomy (3, 6), cordotomy (3), adrenalectomy (6, 7), or adrenergic blocking agents (7, 8) prevented development of fatty liver by ethanol, even in the presence of the usual effects of ethanol on the hepatic FA metabolism. Furthermore, the composition of TGFA in the alcohol-induced fatty liver closely resembled the FA composition of adipose tissue TG (5, 7) and was characterized by a high content of unsaturated acids, particularly linoleic acid (which is not synthesized by the mammalian liver). These observa-

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Abbreviations: TG, triglyceride(s); FA, fatty acid(s); FFA, free fatty acids.

tions suggested that an increased diversion of adipose tissue FA to the liver could be a major factor in the pathogenesis of ethanol-induced fatty liver. However, attempts to assess the effect of ethanol on FA mobilization from adipose tissue from examination of the level of FFA in the circulation have produced discrepant results (8–13). Detection of changes in the level of plasma FFA can be affected by many factors, such as dose and plasma level of alcohol, time of sampling, and duration of the period of sampling. In addition, if FA are removed from plasma as fast as they are released from adipose tissue, an increased mobilization will not be reflected in an increased level or circulating FFA.

The technique for selective labeling of epididymal adipose tissue TG in vivo (14) provides a convenient method for investigating the effect of ethanol on FA mobilization from a sample of peripheral adipose tissue. Experiments were therefore designed to test the effect of ethanol on FA mobilization from prelabeled epididymal pad TG and the extent to which the mobilized label is incorporated into liver TG. A similar experimental procedure was recently employed by Poggi and DiLuzio (15).

MATERIALS AND METHODS

Male Sprague-Dawley rats weighing 200–250 g and kept on a regular laboratory diet were lightly anesthetized with ether after an overnight fast. The epididymal fat pads of each rat were exposed and incubated in separate vessels by the technique of Stein and Stein (14). The incubation medium consisted of 2 ml of 5% bovine serum albumin (Fraction V, Armour Pharmaceutical Co., Kankakee, Ill.) in Krebs-Ringer phosphate (KRP) buffer (Ca^{++} omitted), pH 7.45, to which 1.0 μeq/ml of palmitic acid-1-^{14}C with a specific activity of 1.0 μc/μeq (New England Nuclear Corp., Boston, Mass.) was added. The bovine serum albumin was treated by the method of Goodman (16) to remove FFA, dissolved to a 5% solution in the KRP buffer, and dialyzed twice at 4°C against the buffer. The final FFA content of the albumin was 0.26 μeq/μmole. A 0.0125 N solution of sodium palmitate-1-^{14}C was prepared, heated until optically clear, and added to the warm (37°C) albumin solution. The epididymal pads of the lightly anesthetized rats were incubated for 20 min., each pad was then washed repeatedly with saline to remove adsorbed radioactivity, and unless otherwise stated, the two pads were replaced in the scrotal cavities and the skin sutured. Each rat received by a stomach tube equal volumes of either 0.9% solution of NaCl, 50% ethanol (9.3 g/kg of body weight) or an isocaloric amount of glucose, dissolved in saline. After an additional 16–18 hr of fasting the rats were sacrificed and their epididymal fat pads and liver removed. We perfused the liver in situ with 10 ml of saline through the portal vein in order to remove retained blood radioactivity. The tissues (liver and fat pads) were blotted dry, weighed, and homogenized in a Potter-Elvehjem all-glass homogenizer, and the lipids were extracted in chloroform–methanol 2:1 (17). The saline washings of each pad and the incubation media of each vessel were combined and the lipids extracted. The chloroform phase of the extracts was evaporated to dryness at 40°C in vacuo, the residue was redissolved in chloroform, and aliquots were taken for further analyses.

Triglycerides and FFA were separated by thin-layer chromatography in ether–*n*-hexane–glacial acetic acid 25:73:2. The compounds were located by means of iodine vapor and identified with the aid of simultaneously run standards. After the iodine had sublimed, the spots were scraped off into counting vials to each of which 12 ml of a toluene solution containing 0.01% *p*-bis[2-(-5-phenyloxazolyl)]-benzene and 0.3% 2,5-diphenyloxazole was added. Radioactivity was determined in a liquid scintillation spectrometer with a counting efficiency of 65% for ^{14}C. The samples were corrected for quenching by the channels ratio method (18).

Calculations

From the difference in the radioactivity of the incubation medium before and after the incubation of the epididymal pad, the fraction of the label incorporated into each pad was calculated. The fraction of the radioactivity that was mobilized from the pads during the test period was calculated from the difference in the activity incorporated originally into the pads and the activity at the time of sacrifice. From the radioactivity in the liver the fraction of the mobilized label that was incorporated in the liver was calculated. Percentage esterification of palmitic acid-1-^{14}C was estimated from the activity in the TG and FFA fractions.

The FA composition of TG was determined by gas–liquid chromatography. Methyl esters were prepared according to the method of Nelson and Freeman (19) and separated on a gas chromatograph (Research Specialties Co., Richmond, Calif., Model 600) equipped with an argon detector (10 mc ^{90}Sr foil). U-shaped stainless steel columns (1.8 m × 0.65 cm I.D.) packed with Gas-Chrom P, 80–100 mesh, coated with 17% diethylene glycol succinate polyester (w/w) were operated at 185°C. Quantitative results with the National Heart Institute Fatty Acid Standards agreed with the stated composition with a relative error of less than 8% for major and less than 15% for minor components.

Triglycerides were determined by the method of Van Handel and Zilversmit (20).

TABLE 1 IN VIVO INCORPORATION OF PALMITATE-1-^{14}C BY THE TWO EPIDIDYMAL PADS OF THE SAME ANIMAL: COMPARISON OF TWO PROCEDURES FOR ASSAYING THE AMOUNT OF LABEL INCORPORATED

Rat No.	Pad 1			Pad 2			Difference between Pad 1 and Pad 2	
	Direct* Method	Indirect† Method	Difference	Direct Method	Indirect Method	Difference	Direct Method	Indirect Method
	dpm/pad		%	*dpm/pad*		%	%	
1	165,800	159,420	3.8	147,180	142,560	3.1	11.2	10.6
2	150,540	156,120	3.6	119,260	123,060	3.1	20.8	21.2
3	166,040	171,560	3.2	197,320	205,120	3.8	19.5	16.4
4	172,160	164,170	4.3	120,900	116,750	3.4	29.8	29.1
5	170,660	163,850	4.0	198,870	189,650	4.6	14.2	13.6
6	182,200	187,660	2.9	149,600	152,660	2.0	17.9	18.7
Mean ± SD			3.6 ±0.5			3.3 ±0.9	18.3 ±6.5	18.3 ±6.5

* Radioactivity determined in the total lipid extract of the pads.
† Calculated from the difference in the radioactivity of the incubation medium before and after the incubation of the pad.

RESULTS

Validity of the Experimental Procedure

In preliminary experiments it was found that the radioactivity incorporated by the left and right epididymal pads of the same animals may be markedly different (range 10.6–31.4%) (Table 1). A wide range of differences (5.4–32.0%) was observed also by Stein and Stein (14). Since it is impossible to know beforehand the amount of radioactivity that will be incorporated into each pad, calculations based on differences in the radioactivity between the control pad removed at the end of the incubation and the second pad removed at the time of sacrifice can be misleading. Indeed, when this procedure of calculation is employed, the activity in the second pad may be higher than the activity in the control pad, even after long periods of fasting. In these cases the fraction of the label mobilized from the pad replaced in the scrotum was smaller than the difference in the radioactivity between the two pads at the end of the incubation. Conversely, when the pad with lower activity is taken as a control, spuriously high values of ^{14}C mobilization will be obtained.

To circumvent these difficulties, we calculated the amount of label incorporated into each pad from the difference in the radioactivity of the incubation medium at the beginning and at the end of the incubation period. As can be seen in Table 1, the radioactivity incorporated into the fat pads determined by counting the lipid extract of the pads was very similar to that calculated from the differences in the activity of the incubation medium. The fraction of the label incorporated into the pads was 10.2 ± 2.6% per milliliter of incubation medium and 95.3 ± 4.2% of it was in the form of TG. The percentage difference between the radioactivities determined by the direct and the indirect procedures (3.6 ± 0.52 and 3.3 ± 0.86%) was considerably lower than the percentage difference in activity between the two pads of the same animal (18.3 ± 6.5%). There is no longer any need to remove one of the pads (control) at the end of the incubation, and mobilization of the label can then be calculated from the activity incorporated at the end of the incubation and the activity found in the fat pads at the time of sacrifice.

Effect of Saline, Glucose, and Ethanol on Mobilization and Reincorporation of Epididymal Pad Palmitate

To estimate the effect of saline, glucose, or ethanol administration on the outflow and inflow of palmitate-1-^{14}C from and to epididymal fat, we incubated only one fat pad in the labeling medium (see above) and replaced it in the scrotum. From the changes in the initial radioactivity of the incubated pad, the fraction of the label mobilized during the experiment was calculated. The fraction of the mobilized label which recirculated to and was incorporated into epididymal adipose tissue was determined from the activity in the intact (unincubated) pad removed at the end of the test period.

It can be seen in Table 2 that the fraction of radioactivity mobilized from the labeled pad depended strongly on the substance administered, and that ethanol-treated rats mobilized the most. Only a small fraction of the label incorporated in the incubated pad recirculated to the contralateral (unincubated) pad of the same animal. This fraction cannot be accounted for by the activity of blood retained in the pad because the activity in 1 ml of plasma at the time of sacrifice was much lower than that determined in the whole pad. When the label incorporated in the unincubated pad is expressed as a percentage of the radiopalmitate mobilized from the labeled pad, significant differences are obvious in the three groups of rats. The glucose-treated group, in agreement with the previously demonstrated tendency of fasted–

refed animals (21) to deposit FFA in adipose tissue, incorporated a much larger fraction of the mobilized label (9.8%). The saline-treated rats, which were deprived of caloric intake for approximately 32–34 hr, incorporated a much smaller fraction of the mobilized label (2.3%). This is consistent with the tendency of fasted animals to divert and supply depot FA for the oxidative metabolism of heart and other tissues (22). The ethanol-treated rats incorporated only 1.2% of the mobilized label, even though they had received the same caloric intake as the glucose-treated animals.

In the three groups of animals the TG specific activity of the incubated pads removed at the end of the test period was the same as the specific activity of the pads removed at the end of the incubation (332.8 ± 36.5 dpm/mg). This indicates that radioactive and unlabeled epididymal pad TG was mobilized at similar rates during the experimental period.

Effect of Saline, Glucose, and Ethanol on Hepatic Incorporation of Epididymal Pad Palmitate

It can be seen in Table 3 that after administration of ethanol a significantly larger fraction of epididymal fat radiopalmitate was mobilized and incorporated into liver lipid, predominantly in the form of TG. The differences in the liver radioactivity of the three groups of animals cannot be accounted for by differences in the metabolism of palmitate-1-^{14}C transported to the liver during the

TABLE 2 EFFECT OF SALINE, GLUCOSE, AND ETHANOL INGESTION ON MOBILIZATION AND REINCORPORATION OF EPIDIDYMAL PAD PALMITATE-1-^{14}C

		Pad 1*			Pad 2†		
Treatment	Rat No.	Original‡ ^{14}C Uptake	^{14}C after Treatment	^{14}C Mobilized	^{14}C after Treatment	^{14}C Re-circulated§	Mobilized ^{14}C Incorporated
		dpm/pad		%	*dpm/pad*	%	%
Saline	1	176,420	154,370	12.5	530	0.3	2.4
	2	184,260	159,940	13.2	550	0.3	2.3
	3	158,680	143,450	9.6	320	0.2	2.1
	4	182,800	156,840	14.2	730	0.4	2.8
	5	194,340	175,100	9.9	390	0.2	2.0
Mean ± SD				11.9 ±6.5			2.3 ±0.3
Glucose	1	148,260	140,550	5.2	740	0.5	9.6
	2	197,640	190,520	3.6	590	0.3	8.3
	3	201,120	191,470	4.8	800	0.4	8.3
	4	186,460	182,020	2.4	370	0.2	12.5
	5	198,420	192,670	2.9	600	0.3	10.3
Mean ± SD				3.8 ±1.2			9.8 ±1.7
Ethanol	1	164,840	119,010	27.8	450	0.3	1.0
	2	194.370	139,370	28.3	780	0.4	1.4
	3	156,840	119,980	23.5	630	0.4	1.7
	4	171,680	117,430	31.6	520	0.3	0.9
	5	196,840	149,790	23.9	400	0.2	0.8
Mean ± SD				27.0 ±3.4			1.2 ±0.4

* Pad incubated in labeling medium and replaced in the scrotal cavity.
† Unincubated pad removed at the time of sacrifice.
‡ Calculated from the difference in the radioactivity of the labeling medium before and after the incubation of the pad.
§ Per cent of palmitate-1-^{14}C originally incorporated in pad 1 which recirculated to pad 2.

TABLE 3 EFFECT OF SALINE, GLUCOSE, AND ETHANOL INGESTION ON HEPATIC INCORPORATION OF EPIDIDYMAL PAD PALMITATE-1-^{14}C

	^{14}C Mobilized	Mobilized ^{14}C Incorporated in Liver	Esterification	TG	Specific Activity	Liver Weight
	%	%	%	*mg/g*	*dpm/mg*	*g*
Saline (10)*	10.4 ± 5.6†	3.3 ± 1.6	68.2 ± 9.2	4.8 ± 1.5	16.8 ± 7.4	9.2 ± 1.9
Glucose (8)	4.2 ± 1.7	2.4 ± 1.7	71.6 ± 8.4	4.5 ± 1.8	4.3 ± 2.1	9.3 ± 1.2
Ethanol (12)	28.4 ± 4.2	8.8 ± 2.1	94.6 ± 9.7	23.4 ± 2.9	19.1 ± 9.2	9.4 ± 2.4

* Number in parenthesis denotes number of animals in each group.
† Mean ± SD.

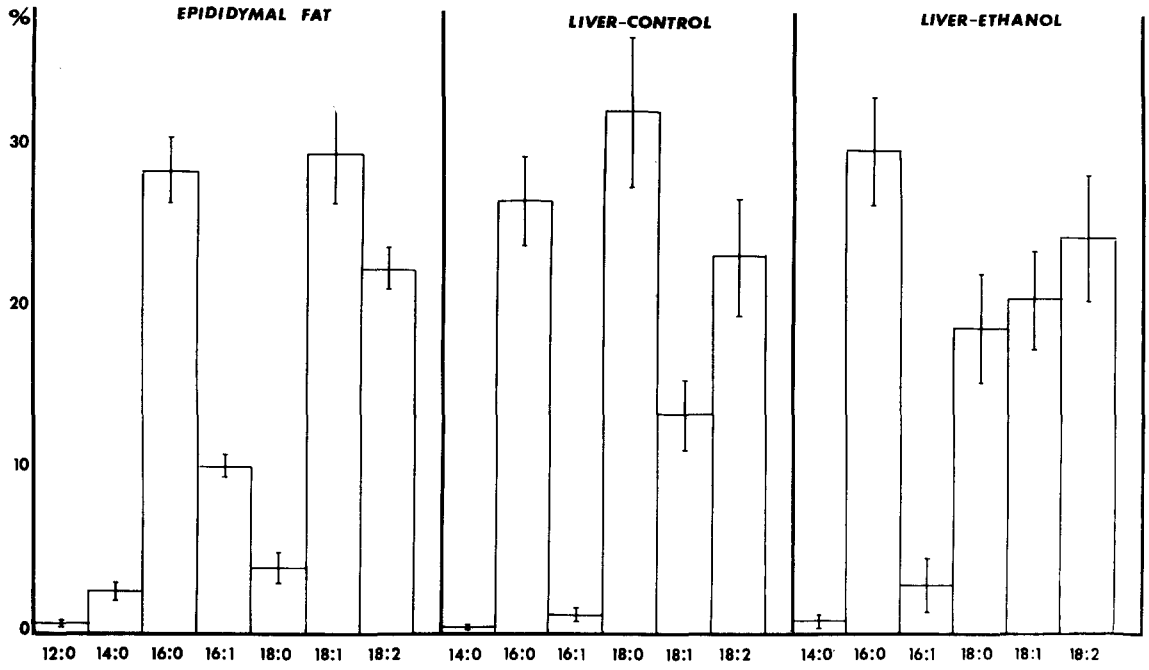

FIG. 1. Effect of saline, glucose, and ethanol ingestion on the FA composition of epididymal pad and liver TG. "Epididymal fat": TGFA composition (mean ± SD) of epididymal fat of rats treated with saline (15), glucose (13), or ethanol (17) (compositions were so similar in the three groups that values have been pooled). "Liver–control": FA composition (mean ± SD) of liver TG of rats fed saline (15) and glucose (13)." Liver–ethanol": TGFA composition for the ethanol-treated rats (17).

incubation of the epididymal pads in the labeling medium because the liver radioactivity at the end of the incubation is negligible (14). The specific activity of liver TG of glucose-treated rats was much lower than that of animals treated with saline or ethanol.

Effect of Saline, Glucose, and Ethanol on the FA Composition of Epididymal Pad and of Hepatic TG

The compositions of epididymal pad and liver TG are shown in Fig. 1. The composition of epididymal fat TG remained fairly constant regardless of the treatment of the rats and was characterized by a high proportion of unsaturated FA (18:1 and 18:2). The composition of liver TG was identical in saline- and glucose-treated rats and was characterized by a predominance of saturated acids (16:0 and 18:0). Administration of ethanol modified markedly the composition of liver TGFA. The proportion of unsaturated acids increased to such an extent that the FA composition of liver TG resembled closely that of epididymal adipose tissue. Similar results were previously reported by Brodie, Butler, Horning, Maickel, and Maling (7), by Scheig and Isselbacher (5), and recently by Lieber, Spritz, and DeCarli (23).

DISCUSSION

The experiments reported here were specifically designed to demonstrate a possible effect of ethanol on mobilization of palmitate-1-^{14}C from epididymal adipose tissue prelabeled in vivo. The ethanol-treated rats mobilized a significantly larger fraction of epididymal palmitate-1-^{14}C than did the animals treated with saline or glucose. Since the major FA of epididymal fat (palmitic, oleic, linoleic) were shown to be mobilized at similar rates (24), and since our preliminary experiments showed that treatment with saline, glucose, and ethanol did not significantly alter the specific activity of epididymal pad TG, any preferential mobilization of the label from newly formed TG can be reasonably excluded. From our results it appears that the response of the control animals (saline- and glucose-treated) was dependent on their nutritional state. Saline-treated (fasted) rats mobilized a larger fraction of epididymal fat radioactivity than did the animals treated with glucose. The contrasting effect of ethanol, however, was independent of the caloric intake of the animals since ethanol and glucose were given in equicaloric amounts.

It has been previously shown that the effect of ethanol on adipose tissue is mediated through the pituitary–adrenal axis (7). In addition, Perman (25) had shown that ethanol increases the catecholamine secretion from the adrenal medulla. Since release of adipose tissue FA is preceded by enzymatic hydrolysis of TG, it seems likely that ethanol affects mobilization of FFA by increasing the release of substances known to activate the hormone-sensitive lipase of adipose tissue (26, 27). This assumption is substantiated by the finding that cordotomy (3),

adrenalectomy (6, 7), and adrenergic blocking agents (7, 8) abolished the effect of ethanol on liver TG.

The administration of ethanol also produced an increased diversion to the liver of FA mobilized from adipose tissue. A larger fraction of epididymal fat radioactivity was incorporated into livers of ethanol-treated rats, and the hepatic TGFA of these animals strikingly resembled epididymal fat in FA composition (Fig. 1). In addition, a greater fraction of the label incorporated in the liver of the ethanol-treated rats was esterified as TG (Table 3). An increased TG synthesis by livers of ethanol-treated rats was demonstrated also by Scheig and Isselbacher (5) and recently by Nikkilä and Ojala (28). The latter have attributed it to an increased esterification of excess α-glycerophosphate from the metabolism of ethanol in the presence of an increased influx of FFA from adipose tissue.

Of interest are the changes in the specific activity of hepatic TG (Table 3). Although the specific activity of epididymal pad TG remained within a very close range during the test period, the specific activity of hepatic TG of the glucose-treated rats was significantly lower than that of saline- and ethanol-treated animals. Similar changes in the specific activity of hepatic TG of ethanol- and glucose-treated rats were recently reported by Nikkilä and Ojala (28). If unlabeled FA was not preferentially mobilized from epididymal fat, the low specific activity of hepatic TG could have resulted from the smaller amount of epididymal pad radiopalmitate incorporated into liver TG and from dilution of the label by FA synthesized in the liver from carbohydrate. The latter assumption is substantiated by the presence of high proportions of saturated FA in liver TG of glucose-treated rats (Fig. 1), for saturated FA are synthesized preferentially by the mammalian liver (29). The possibility of a preferential incorporation and release of labeled TG in the form of plasma lipoprotein or an increased hepatic oxidation of palmitate-1-^{14}C was not investigated. However, it is unlikely that glucose could have affected the hepatic metabolism of radioactive palmitate so selectively.

The similar specific activities of hepatic TG of saline- and ethanol-treated rats (Table 3) are also interesting. Because there are marked differences in the amount of radioactive and unlabeled TG in livers of saline- and ethanol-treated rats (Table 3), the similar specific activities of hepatic TG indicate that administration of ethanol produced an impairment in the hepatic metabolism of TG in addition to an increased mobilization and incorporation of adipose tissue FA into liver TG. This assumption is consistent with the results of Poggi and DiLuzio (15) and with the previously shown inhibition of hepatic oxidation of FA (3) and decreased incorporation and release of hepatic TG in the form of lipoproteins (30).

The possibility of increased FA synthesis in livers of ethanol-treated rats, as suggested by Lieber and Schmid (4), is not supported by our results since a lowering of the specific activity of hepatic TG would have resulted because of dilution of the label by newly synthesized, stable FA. The high content of unsaturated TGFA (Fig. 1) in livers of rats treated with ethanol (5, 7, 22) argues further against the possibility of increased hepatic FA synthesis, since the mammalian liver preferentially synthesizes saturated FA (5, 29).

Part of our results are different from the results reported recently by Poggi and DiLuzio (15). These authors have found that the amount of label mobilized from epididymal fat, calculated from the difference in the radioactivity between the right pad removed at the end of the incubation and the left pad removed at the time of sacrifice, was the same in glucose- and ethanol-treated rats (6 g/kg). It is difficult to explain the discrepancy of our results, although differences in the age and the weight of the animals and in the doses of glucose and ethanol used may be sufficient to do so. In addition, the response of adipose tissue to large doses of ethanol could have been modified by possible differences in the nutritional state of the animals used, and in the diet before the experiment. Further investigation might, therefore, elucidate important differences in the pathogenesis of ethanol-induced fatty liver related to the dose of ethanol and to the age and nutritional state of the animals.

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