Quantitative Analysis of Metabolism of Hepatic Triglyceride in Ethanol-Treated Rats

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An acute intraperitoneal injection of ethanol (0.7 or 2.1 g/kg body wt.) causes the reversible, dose-dependent accumulation of hepatic triglyceride in rats. By using a pulse of [^{14}C]palmitate injected into a tail vein, it was found that ethanol (2.1 g/kg) had no effect on the flux of unesterified fatty acid of serum (4.3 $\mu\text{mol}/\text{min}$ per 100g body wt.). However, either dose increased the fraction of the total flux going to liver from 0.16 to 0.27 as rapidly as could be measured (30s), and it remained elevated until all ethanol had been cleared from the blood. The fraction of the total radioactivity in lipids of liver that was in triglyceride increased linearly for 1 h from 30 to 50% and there was a simultaneous decrease in phospholipid from 60 to 40%. The rate of synthesis of hepatic triglyceride derived directly from unesterified fatty acid of serum was calculated by using the flux rate of unesterified fatty acid in serum, the fractional hepatic uptake of this flux, and the percentage of liver fatty acid esterified to triglyceride. This contribution is related to the total synthetic rate of hepatic triglyceride (rate of accumulation+rate of release) to determine quantitatively how much of the developing fatty liver is attributable to increased uptake of unesterified fatty acid of serum. At the higher dose of ethanol, about half of the accumulating triglyceride is derived from this source, whereas with the lower dose of ethanol it can account for all of the build-up.

An acute dose of ethanol has been shown to cause a reversible accumulation of hepatic triglyceride in man and in experimental animals. Several theories have been proposed to explain this phenomenon, but the mechanism remains unresolved (Hawkins & Kalant, 1972). The most obvious general mechanisms and precipitating events are shown in Scheme 1.

Most items listed in Scheme 1 have been studied experimentally to various extents, although item 4 has received relatively little attention in the context of ethanol-induced fatty liver. One of the major shortcomings in trying to relate the available data to accumulation of hepatic triglyceride is that virtually nothing is known about the quantitative relationship between rate of accumulation of hepatic triglyceride and biochemical changes produced by ethanol. For example, it is inadequate simply to know that ethanol may increase the rate of incorporation of acetate into fatty acid of hepatic triglyceride *in vitro*. If this mechanism is to be accepted as a quantitative explanation for extra triglyceride in liver, we must know the control rate of synthesis of hepatic fatty acid *in vivo*, how it is altered by administration of ethanol and what fraction of the newly synthesized fatty acid is used for formation of triglyceride. In addition, these results must be related to the rate of accumulation of extra triglyceride. A similar analysis may be applied to each item listed in Scheme 1, and an examination

of the literature reveals a lack of information necessary to evaluate the merits of these proposals. The purpose of the work described below is to provide such an analysis of an hypothesis we consider to be worthy of study.

Mechanisms 1, 3 and 4 cited below (Scheme 1) relate in whole or in part to the supply of fatty acid available for synthesis of hepatic triglyceride. Two possible sources for extra fatty acid are dietary fat and adipose tissue. Brodie *et al.* (1961) concluded that increased mobilization of peripheral fat is responsible for ethanol-induced fatty liver, because the hepatic triglyceride had a fatty acid composition similar to adipose tissue. However, Lieber *et al.* (1966) reported that although there is a similarity in composition of triglyceride fatty acid in adipose tissue and liver following a single acute dose of ethanol, a different relationship occurs with prolonged ingestion of ethanol. Mendenhall (1972) also concluded that dietary lipid is the preferential source of fatty acid in hepatic triglyceride during chronic administration of ethanol. This led to the suggestion that enhanced peripheral mobilization of fat, after an acute dose of ethanol given to fed humans, has no relevance to hepatic steatosis, since in this situation the fatty acid is derived from the diet (Lieber, 1973). Since ethanol causes a fatty liver in either the fed state (preferentially from dietary fatty acid), or the starved state (presumably

Scheme 1. *Mechanisms and events involved in reversible accumulation of hepatic triglyceride*

1. Increased synthesis of hepatic triglyceride
 - (a) Decreased oxidation of fatty acid
 - (b) Increased synthesis of fatty acid
 - (c) Increased concentration and/or turnover of α -glycerol phosphate
 - (d) Diversion of common intermediates from synthesis of phospholipid
2. Decreased release of hepatic triglyceride
 - (a) Decreased synthesis of lipoprotein apoprotein(s)
 - (b) Decreased assembly of very-low-density lipoprotein
 - (c) Decreased release of very-low-density lipoprotein
3. Increased hepatic uptake of unesterified fatty acid from serum
 - (a) Increased concentration of unesterified fatty acid in serum
 - (b) Increased flux of unesterified fatty acid through serum
 - (c) Increased hepatic clearance of fatty acid from serum
4. Hepatic uptake of triglyceride from serum
 - (a) Uptake of intact lipoprotein
 - (b) Uptake of partially delipidated lipoprotein
 - (c) Uptake of triglyceride of lipoprotein

from fatty acid originating in adipose tissue), it seems unlikely to us that the alteration(s) involved in production of fatty liver is dependent on either mobilization of depot fat or utilization of dietary fat. A more attractive possibility is that ethanol induces a physiological change causing liver to obtain more fatty acid from the most readily available source, depending on the metabolic state. We therefore consider that the critical question is not where the fatty acid originates, but rather how and to what extent ethanol influences delivery of unesterified fatty acid of serum to liver. The purpose of the present study is to examine this question.

Experimental

Animal procedures

Male Sprague-Dawley rats (Carworth Farm, Portage, MI, U.S.A.) were maintained on Purina Chow for 10–14 days before use. They were housed in wire-bottom cages with free access to food and water. The room temperature was 22°C and the lights were on from 7:00 to 19:00 daily. The rats were starved 48 h before the start of the experiment and weighed 190–200 g after starvation. Ethanol was injected intraperitoneally as a 20% (v/v) solution, and control animals received an equivalent volume of water. In confirmation of previous work (Hiestand *et al.*, 1952) we found no evidence of intestinal irritation at any of the doses used. The solutions of water and ethanol were warmed to 37°C and injected with a 22-gauge 7 mm needle inserted in the mid-line equidistant from the xyphoid process and the pelvic region. At various times after intraperitoneal injection the rat was placed in a restraining cage and the protruding

tail warmed for 10 min with a heat lamp to permit intravenous injection into a tail vein. The warming of the tail allowed a higher percentage of successful intravenous injections, but produced no change in rectal temperature and had no effect on any of the parameters measured. For experiments in which the interval between intraperitoneal and intravenous injections was short, the warming of the tail was omitted. The intravenous injections of 1–3 μ Ci of [U-¹⁴C]palmitic acid (New England Nuclear Corp., Boston, MA, U.S.A.)/rat and 0.04–0.1 μ Ci of ¹²⁵I-labelled human albumin (Squibb Co., New York, NY, U.S.A.)/rat were made in 0.5 ml of serum prepared from a starved rat just before use. No anaesthetics were ever used. At specified times after injection of isotope the rats were decapitated and the blood collected from the neck directly into a centrifuge tube for 5 s and allowed to clot (45–60 min) and centrifuged (20 min at 500 g) at room temperature. Livers were rinsed twice in cold 0.15 M-NaCl, blotted dry, divided as described below, weighed, and frozen in liquid N₂. The elapsed time from decapitation to freezing was 45–60 s. This time is not critical for reasons discussed below in the Results section. The small lobe (weighing about 1 g) located on the animals right side, dorsal to the large bifurcated lobe, was used. It was weighed and frozen separately from the remainder of the liver and was chosen for its convenient size. All lobes in any one liver were found to give identical results for accumulation of hepatic triglyceride and incorporation of [U-¹⁴C]palmitate. Rat carcasses (whole body minus liver and blood lost during decapitation) were rapidly cut into pieces smaller than 2 cm³ and frozen in liquid N₂. The entire operation from decapitation to freezing took about 2 min.

Lipid analysis

Serum lipids were extracted from a 0.5 ml sample by the method of Dole & Meinertz (1960). Samples (0.1–1.0 ml) of the heptane phase were dried at 50°C under N₂ and used for liquid-scintillation counting in experiments in which the irreversible disposal rate was determined. The ¹²⁵I-labelled albumin remained in the aqueous phase. There was no ¹²⁵I present in the experiments on incorporation of unesterified fatty acid of serum into lipids of liver, and therefore 0.1 ml of serum was counted directly for ¹⁴C radioactivity. When lipids of serum were to be separated by t.l.c., the dried heptane sample was taken up in 0.5 ml of chloroform and a 50 µl sample was plated.

The frozen 1 g liver lobe was homogenized in 20 ml of redistilled chloroform/methanol (2:1, v/v) and lipids extracted by the method of Folch *et al.* (1957). A 1.0 ml portion of the lower phase containing total lipids was dried under N₂ at 50°C in a 5 ml scintillation vial before counting for radioactivity. Lipids were extracted from frozen carcass by homogenization at room temperature in 1500 ml of chloroform/methanol (2:1, v/v) for 3 min at high speed in a large Waring blender, filtered through nylon gauze, and the sediment rehomogenized twice with 900 ml of chloroform/methanol (2:1, v/v) for 2 min each time and filtered through gauze. All filtrates were passed through a medium-porosity sintered-glass filter and a 10.0 ml portion of the single-phase filtrate was dried at 50°C under N₂ and used for liquid-scintillation counting.

Lipids were separated by t.l.c. on 20 cm × 20 cm glass plates of silica gel G (0.25 mm; E. Merck, A.-G., Darmstadt, Germany) following activation at 110°C for 30 min immediately before use. The solvent for t.l.c. was hexane/diethyl ether/acetic acid (70:30:1, by vol.) prepared fresh daily, and the chamber was allowed to equilibrate 30–60 min before use. A 5.0 ml portion of the chloroform phase of liver extracts was dried under N₂ at 50°C. The lipids were redissolved in 0.5 ml of chloroform, a 50 µl sample spotted in a 2 cm lane and the plates developed for 120 min at room temperature. Because of the relatively large sample size this development time was necessary for complete separation. The plates were air dried, sprayed with 10% (v/v) sulphuric acid in 50% methanol and heated at 160°C ± 5°C for 15 min at which time the spots had charred sufficiently for identification. These charring conditions were not, however, adequate for quantitation.

The lipids of the liver were quantified by chromatographing 25 µl portions of the initial chloroform phase on t.l.c. plates for 60 min and charring the plates for 30 min at 160°C ± 5°C after spraying as described above. For serum we used 100 µl samples of the heptane phase after the initial Dole & Meinertz (1960) extraction. The density of the charred spots was deter-

mined with a Photovolt model 52C photodensitometer and the area under the peak determined with a Photovolt model 42B integrating recorder. The areas were correlated with a standard curve obtained by using various amounts of lipid standards. To ensure proper charring on each plate a standard of each lipid was run in the centre lane. Any plate having standards that were not within ±5% of the normal values for density were discarded. The accuracy of the determination of fatty acid by charring was assessed by comparing these values with those obtained by direct colorimetric analysis of serum (Laurell & Tibbling, 1967). There was no significant difference ($t = 0.23$, $n = 20$).

Liquid-scintillation counting

Both the samples of serum and the silica scraped from the t.l.c. plates were made up to 1.0 ml with water, 3.0 ml of Aquasol (New England Nuclear Corp.) were added, and the mixture shaken to form a gel. The dried extracts from serum (heptane) and from liver (chloroform) were dissolved in 4.0 ml of Bray's solution (Bray, 1960). All ¹⁴C radioactivity counting was done on a Nuclear Chicago mark I liquid-scintillation counter by using an external standard to obtain the counting efficiency of each sample. The radioactivity is expressed in terms of d.p.m. Counting efficiencies were 50–80% for serum and liver extracts and 45–55% for the spots scraped from the t.l.c. plates. ¹²⁵I was counted on a Beckman gamma counter.

Physiological data

Rectal temperature was determined with a thermistor. Blood pressure was monitored with a catheter in the femoral artery connected to a mercury manometer. An electronic sensor to register respiration and a small microphone to detect heart rate were positioned on the chest by an elastic band and were both connected to a polygraph recorder.

Rate of release of hepatic triglyceride

This was determined by blocking the removal of triglyceride from serum with Triton WR 1339. In the starved rat the liver is the predominant, if not only, source of triglyceride in serum. The dosage of Triton was 100 mg/100 g body wt., administered intravenously into a tail vein as a solution (37°C) containing 200 mg/ml of 0.15 M-NaCl.

Each dose of ethanol had its own control series, since the volume of the intraperitoneal injection varies with dosage. Four groups of rats containing 36 animals per group were injected as follows: (1) low dose of ethanol (0.7 g/kg); (1a) an equivalent volume of water; (2) high dose of ethanol (2.1 g/kg); (2a) an

equivalent volume of water. After 45 min, 18 animals from each group were decapitated and serum prepared as described above. The other 18 animals in each group received Triton WR 1339, and 90 min later these animals were also decapitated and serum prepared. Serum triglyceride concentration was determined by the method described by Soloni (1971). The rate of release of triglyceride from liver into serum is calculated from the rate of increase over the 90 min interval by multiplying the increase in mg/min per ml of serum by 4.23 ml of serum/100 g body wt. (Table 3) to put it in the proper units. Annable & Cooper (1974) have demonstrated that there is a linear increase in serum triglyceride for 90 min after administration of Triton WR 1339. This estimation is predicated on the assumptions that: Triton WR 1339 completely blocks the removal of triglyceride from serum; that the rate of release from liver is linear over the 90 min interval; and that Triton WR 1339 has no effect on the rate of release from liver. Recknagel (1967) has reported that Triton inhibits removal of triglyceride from serum by more than 90%. Several laboratories have reported that the rate of increase of triglyceride in serum is linear for 90 min after administration of Triton (Otway & Robinson, 1967*b*; Recknagel, 1967; Annable & Cooper, 1974). The validity of the third assumption is discussed below.

Results

The major objective of these studies is to quantify parameters involved in turnover of hepatic triglyceride and to determine the effect of ethanol on these parameters. The area we focused on is the contribution of unesterified fatty acid of serum to synthesis of hepatic triglyceride. To measure this contribution and how it is affected by ethanol, several components must be determined quantitatively. These are: total flux of unesterified fatty acid in serum; the fraction of this flux taken up by liver; and percentage of unesterified fatty acids entering liver that are esterified specifically to triglycerides. All of these factors were measured, and the extent to which they contribute to synthesis of hepatic triglyceride in normal and ethanol-treated animals was calculated.

Since ethanol-induced hepatic steatosis is reversible and varies with content of ethanol in blood, it is essential to make all measurements in the interval during which the build-up of triglyceride in liver is occurring at a constant rate.

Effect of dose of ethanol on concentration of ethanol in serum and rate of accumulation of triglyceride in liver

The intraperitoneal injection of ethanol produces a rapid rise in its concentration in serum and both maximum concentration in serum and time of clear-

ance are dose-related (Fig. 1). The rapid reproducible response to intraperitoneal injection of ethanol makes this route of administration well suited for the work described below. Most measurements of ethanol in serum were made on samples from a tail vein. However, for some experiments to be described below, pooled blood obtained by decapitation was used and different results were obtained. Variations in concentration of ethanol in serum stemming from differing sampling sites have been previously noted by Kalant (1971).

The accumulation of hepatic triglyceride is also dependent on the dose of ethanol (Fig. 2). From a control concentration of slightly more than 4 mg/g of liver there is a reasonably linear increase in the value for 2 h either to 6.0 mg/g with a dose of 0.7 g/kg, or to 9.4 mg/g with a dose of 2.1 g/kg, giving accumulation rates of 0.015 ± 0.002 and 0.041 ± 0.003 (mean $\pm$ s.d.) mg of hepatic triglyceride/min per g of liver respectively. These rates were determined by linear-regression analysis. Other major lipids of liver were also measured at various times after the 2.1 g/kg dose, and all remained constant over the first 4 h after administration of ethanol (Table 1). Cholesterol and diglycerides are not completely separated in the t.l.c.

Fig. 1. Concentration of ethanol in serum

Different doses of 20% (v/v) ethanol were injected intraperitoneally into rats starved 48 h. At indicated times blood was collected and the serum analysed for ethanol by g.l.c. Blood from a tail vein was used for 0.2 g/kg ($\blacktriangle$, three animals/time-point); 0.7 g/kg ($\square$, five to ten animals/time-point); and 2.1 g/kg ($\bullet$, 10–20 animals/time-point). Blood collected by decapitation from animals given the high dose (2.1 g/kg) was also analysed ($\circ$, five to ten animals/time-point). Each point represents the mean $\pm$ s.e.m.

system we used as a routine, and therefore the quantitative values reported here make no distinction between these two lipids. Similarly, all polar lipids such as phospholipids, phosphatidic acid, monoglyceride, and acyl-CoA and acyl-carnitine derivatives remain at the origin, and the values in Table 1 for polar lipids therefore represent the sum of these compounds; re-chromatography in a different solvent (chloroform/methanol/water, 65:25:4, by vol.), however, indicates this material consists almost entirely of phospholipids. The concentration of unesterified fatty acid in liver was also measured and this very small, rapidly turning-over pool (0.036 mg/g of liver) remained unchanged. This cannot be accounted for by the presence of blood in the isolated liver because we measured this parameter (Abrams & Cooper, 1976) as well as the concentration of unesterified fatty acid in serum. By 4 h the concentration of hepatic triglyceride began to decline. We focused on the first 2 h, which shows a linear rate of increase to simplify quantification and interpretation.

Effect of dose of ethanol on physiological parameters

Some physiological parameters were measured to evaluate the effect on the animals of the different doses of ethanol used. Since merely handling an animal causes some excitatory responses, the data are expressed as changes beyond those obtained with control animals handled in a similar manner. The magnitude of these changes are dose-dependent (Fig. 3). As dose is increased there is a proportionally greater decrease in rectal temperature, blood pressure and respiration, and a rise in heart rate. It is important to emphasize that the highest dose used (2.1 g/kg) produces physiological alterations, whereas the

lower dose (0.7 g/kg) does not. This agrees with the findings of Pohorecky & Jaffe (1975) that an intraperitoneal dose of 2.5 g/kg produces a marked hypothermia, whereas no effect is seen at a dose of 1.0 g/kg or lower.

Fig. 2. Accumulation of hepatic triglyceride

Groups of rats starved 48 h were injected intraperitoneally with water (Δ), 20% ethanol at doses of 0.7 g/kg ($\circ$) or 2.1 g/kg ($\bullet$). At indicated times the animals were decapitated, livers removed and analysed for triglyceride as described in the text. Each point represents the mean $\pm$ S.E.M., five to ten rats being used.

Table 1. *Lipids of liver after administration of ethanol*

Rats were given either water or ethanol (2.1 g/kg) intraperitoneally; they were decapitated at the indicated times, and the livers removed and analysed for content of lipid as described in the Experimental section. Results are expressed as means $\pm$ S.E.M., with n = number of animals. As mentioned in the text, 'phospholipids' are defined here as any lipid which does not migrate in our solvent system for neutral lipid. However, rechromatography in a different solvent system showed this material to be over 95% phospholipid. Similarly, cholesterol migrates with diglycerides, but diglycerides were shown (as described in the Results section) to be less than 5% of the material quantified here.

Time after ethanol (min)	n	Content (mg/g of liver)			
		Phospholipid	Cholesterol	Triglyceride	Cholesterol ester
0	60	12.55 $\pm$ 0.58	2.46 $\pm$ 0.27	4.18 $\pm$ 0.12	2.03 $\pm$ 0.09
5	9	12.30 $\pm$ 0.18	2.61 $\pm$ 0.07	4.44 $\pm$ 0.09	2.18 $\pm$ 0.07
10	8	12.66 $\pm$ 0.60	2.96 $\pm$ 0.09	4.99 $\pm$ 0.10	2.01 $\pm$ 0.07
15	9	12.16 $\pm$ 0.29	3.13 $\pm$ 0.23	5.16 $\pm$ 0.18	2.13 $\pm$ 0.09
30	5	12.85 $\pm$ 0.90	2.79 $\pm$ 0.40	5.85 $\pm$ 0.51	2.11 $\pm$ 0.14
60	9	12.46 $\pm$ 0.61	2.64 $\pm$ 0.20	6.28 $\pm$ 0.22	1.99 $\pm$ 0.07
90	5	12.35 $\pm$ 0.54	2.58 $\pm$ 0.14	8.03 $\pm$ 0.26	2.07 $\pm$ 0.08
120	9	12.44 $\pm$ 0.24	2.49 $\pm$ 0.09	9.40 $\pm$ 0.25	2.01 $\pm$ 0.05
240	6	12.49 $\pm$ 0.57	2.45 $\pm$ 0.10	8.31 $\pm$ 0.24	1.99 $\pm$ 0.66

Fig. 3. Response of physiological parameters to different doses of ethanol

Each point is the mean $\pm$ S.E.M. of results obtained with three rats. ●, Temperature; △, blood pressure; ○, respiration; and ▲, heart rate.

Table 2. Disappearance of unesterified fatty acid from serum in control and ethanol-treated rats

Rats injected intraperitoneally with either water or ethanol (2.1 g/kg) were injected 60 min later with 125 I-labelled albumin plus [U- 14 C]palmitate into a tail vein. At the specified times the animals were decapitated and the percentage of injected dose of labelled fatty acid remaining in serum was determined (see under 'Lipid analysis' in the Experimental section). The area under the corresponding disappearance curve was calculated as described by Corney & Heath (1970). Data are expressed as means $\pm$ S.E.M., with n = number of rats.

Time after injection (s)	Injected dose remaining (%) (mean $\pm$ S.E.M.)			
	Control	n	Ethanol	n
8	64.83 $\pm$ 1.87	5	63.99 $\pm$ 2.52	5
15	56.08 $\pm$ 1.31	8	55.63 $\pm$ 1.21	8
26	33.76 $\pm$ 0.96	5	34.65 $\pm$ 1.47	5
40	23.83 $\pm$ 0.37	4	24.63 $\pm$ 0.90	4
120	9.20 $\pm$ 0.20	9	9.10 $\pm$ 0.20	9
180	4.41 $\pm$ 0.13	4	4.56 $\pm$ 0.24	4
Area under activity/time curve (% $\times$ min)	64.13 $\pm$ 0.69		65.13 $\pm$ 1.09	

Flux of unesterified fatty acid in serum

The irreversible disposal rate (R) of unesterified fatty acid in serum was determined as described by Cunningham (1973). It is calculated from the equation $R = kQ$, where Q is the pool of unesterified fatty acid (the product of concentration of unesterified fatty acid in serum and volume of serum) and k is the irreversible disposal rate coefficient. 'Irreversible' applies to movement of tracer only for the duration of the experiment. Recycling is a known phenomenon, but it is insignificant in the very brief experimental periods used in the present study. The irreversible disposal rate coefficient [k , (min^{-1})] for unesterified fatty

Table 3. Irreversible disposal rate of unesterified fatty acid from serum in control and ethanol-treated rats

Irreversible disposal rate coefficients are derived from data in Table 2, and the relationship dose (100%) divided by the area under activity/time curve. The volume of serum was calculated from dilution of injected 125 I-labelled albumin in serum and concentration of unesterified fatty acid in serum determined in samples of serum from each individual rat (see the Experimental section). Irreversible disposal rate = (concn. of unesterified fatty acids in serum) $\times$ (vol. of serum) $\times$ (irreversible disposal rate coefficient). All data are expressed as means $\pm$ S.E.M., n = number of animals, and t = time after intraperitoneal injection of water or ethanol (2.1 g/kg).

	Controls	+Ethanol
Concn. of unesterified fatty acid in serum ($\mu\text{equiv./ml}$, $n = 36$)	0.64 $\pm$ 0.06	0.65 $\pm$ 0.07
Volume of serum (ml/100 g body wt., $n = 36$)	4.23 $\pm$ 0.18	4.24 $\pm$ 0.14
Irreversible disposal rate coefficient (min^{-1})		
$t = 10$ min	1.59 $\pm$ 0.06	1.61 $\pm$ 0.06
$t = 60$ min	1.56 $\pm$ 0.02	1.54 $\pm$ 0.03
Irreversible disposal rate ($\mu\text{equiv./min}$ per 100 g body wt.)		
$t = 10$ min	4.30 $\pm$ 0.47	4.30 $\pm$ 0.52
$t = 60$ min	4.22 $\pm$ 0.44	4.11 $\pm$ 0.48

acid in serum is equal to D/A where D is the injected pulse of radioactivity (100%) and A is the area under the radioactivity disappearance curve determined from the data in Table 2. The reasons for using the indicated time-points are discussed by Cunningham (1973). An extra time-point (15s) is also reported, since this is a period of extremely rapid disappearance of isotope and it is considered to be helpful in further defining the disappearance curve of isotope. The analysis of these curves to obtain the area, and the validity of this approach have been discussed in detail previously (Corney & Heath, 1970; Heath & Barton, 1973). The data used to calculate the irreversible disposal rate (flux) of unesterified fatty acid in serum 10 and 60 min after intraperitoneal injection of either water or ethanol (2.1 g/kg) are shown in Table 2. The values presented for concentration of unesterified fatty acid in serum and volume of serum are the means of the six time-points, since only minor variations occurred over the 3 min course of the experiment. No significant difference in flux rate was found between control and experimental animals at either time after intraperitoneal injection. Our values for irreversible disposal rate coefficient (1.57, see Table 3) and plasma volume (4.24, see Table 3) are almost identical to the values of 1.53 and 4.29 obtained by Cunningham (1973) for 24h starved rats.

Fig. 4. Effect of ethanol on incorporation of $[U-^{14}C]$ palmitate into total lipids of liver

Rats were injected intraperitoneally with either water or ethanol (2.1 g/kg). After 60 min $[U-^{14}C]$ palmitate was injected into a tail vein. At indicated times animals were decapitated, livers removed, and the percentage of injected labelled unesterified fatty acid incorporated into total lipids of liver determined. Each point is the mean $\pm$ S.E.M., obtained with five to ten rats. All values are corrected for residual blood in liver (Abrams & Cooper, 1976). ●, Ethanol-treated; ○, control.

Fraction of flux of unesterified fatty acid in serum going to the liver

The uptake of unesterified fatty acid from serum by liver was measured by the rate of appearance of isotope in lipids of liver. The effect of ethanol on this rate is shown in Fig. 4. The hyperbolic curves reflect the kinetics of the irreversible movement of an isotopic pulse from one compartment (serum) to another (liver). The data indicate there is an enhanced rate of incorporation of isotope into lipids of liver of ethanol-treated rats and Fig. 5 quantifies this effect. In this instance the data are presented as fractional uptake of unesterified fatty acid from serum by liver at various times after injection of labelled fatty acid. This fraction is obtained by dividing the percentage of injected radioactivity in liver lipids (corrected for residual blood) by the percentage of injected radioactivity that has left the serum at any chosen time. The fractions are constant and measure the percentage of total flux of unesterified fatty acid through serum that is attributable to liver. Although ethanol has no effect on total flux of unesterified fatty acid through serum (Table 3), the fraction of total flux going to liver is increased from 0.16 ± 0.02 for controls, to 0.27 ± 0.03 (mean $\pm$ S.E.M.) for animals receiving ethanol. These values have been corrected for serum

Fig. 5. Effect of ethanol on fractional hepatic uptake of $[U-^{14}C]$ palmitate

Data from Fig. 4 expressed as (percentage of injected radioactivity in total lipid of liver)/[radioactivity remaining in serum (100%)]. ●, Ethanol-treated; ○, control.

Table 4. Effect of ethanol on fractional hepatic uptake of $[U-^{14}C]$ palmitate in fed and starved animals

Data for starved rats taken from Fig. 4. Fed rats were without access to food for 3 h before injection of $[U-^{14}C]$ palmitic acid into a tail vein. The ethanol-treated rats received an intraperitoneal injection of 2.1 g/kg 1 h before injection of isotope.

Nutritional state	Ethanol	Fractional hepatic uptake (mean $\pm$ S.E.M.)	n
Starved	—	0.16 ± 0.02	56
	+	0.27 ± 0.03	62
Fed	—	0.17 ± 0.01	7
	+	0.27 ± 0.01	6

present in liver after its removal from the animal (Abrams & Cooper, 1976).

Experiments similar to those shown in Fig. 5 were also done by using fed rats and identical results were obtained (Table 4). This indicates: (1) that the fractional hepatic uptake of $[U-^{14}C]$ palmitate into total lipids of liver is unaffected by the nutritional state of the animals; and (2) that this uptake is enhanced to a similar extent by ethanol in both fed and starved rats.

Before these data can be interpreted as showing that there is an ethanol-induced alteration in the fraction of total flux of unesterified fatty acid through serum taken up by liver, it is necessary to rule out possible sources of error. The most apparent source of error is that the differences may reflect variation

Table 5. *Recovery of radioactivity injected as [U-¹⁴C]palmitate*

Rats were injected intraperitoneally with either water or ethanol (2.1 g/kg), and 60 min later [U-¹⁴C]palmitate was injected into a tail vein. Either 2 or 3 min later the animals were decapitated, and recovery of injected isotope determined as described in the text. Data are expressed as means $\pm$ S.E.M. and n = number of animals.

	Time after injection (min) ...		2		3	
	Ethanol ...		+		+	
	n ...		6	6	4	4
Percentage of injected dose						
Lipids of liver			15.3 $\pm$ 1.0	25.3 $\pm$ 0.9	15.8 $\pm$ 0.9	25.9 $\pm$ 2.3
Lipids of carcass			75.4 $\pm$ 4.3	62.4 $\pm$ 5.2	50.5 $\pm$ 2.1	50.2 $\pm$ 2.5
Carcass acid-soluble					25.9 $\pm$ 1.8	16.6 $\pm$ 0.7
Serum			9.2 $\pm$ 0.2	9.1 $\pm$ 0.2	4.4 $\pm$ 0.1	4.6 $\pm$ 0.2
Total recovery (%)			99.9 $\pm$ 4.4	96.8 $\pm$ 5.3	96.6 $\pm$ 2.9	97.3 $\pm$ 3.5

Fig. 6. *Fractional hepatic uptake of [U-¹⁴C]palmitate at different times after ethanol administration*

Data are expressed as in Fig. 5, except that the pulse time of [U-¹⁴C]palmitate was constant (2 min) and time after intraperitoneal injection of water or ethanol (2.1 g/kg) was varied. Each point represents the mean $\pm$ S.E.M. obtained with 5–15 rats. ●, Ethanol-treated; ○, control.

in recovery of isotope rather than a change in fractional uptake of unesterified fatty acid by liver. A change in recovery of isotope could result from variation in exit of isotope from liver in some other form (i.e. very-low-density lipoprotein or ketone bodies) or the conversion of hepatic fatty acid to non-lipid material (i.e., HCO_3^- or ketone bodies) that would not be present in the extracts of hepatic lipid. These possibilities can be evaluated by accounting for 100% of the injected isotope. At either 2 or 3 min after injection, all of the isotope was recovered (Table 5). At 2 min all radioactivity that had left the blood was

recovered as lipid and the 9% remaining in serum was present as unesterified fatty acid. This demonstrates that, at this pulse-interval, none of the injected [U-¹⁴C]palmitate was oxidized or incorporated into lipoprotein of serum. However, by 3 min it was necessary to make a perchloric acid extract of the delipidated carcass to completely recover the injected isotope, but even at this time there was no loss of radioactivity from hepatic lipids. All of the above measurements on the fractional uptake by liver were made 1 h after administration of ethanol.

The effect of ethanol on accumulation of hepatic triglyceride begins immediately after administration of ethanol (Fig. 2). If the enhanced fractional hepatic uptake of unesterified fatty acid from serum (Figs. 4 and 5) has any quantitative relevance to the hepatic steatosis, it is important to know the temporal relationship between accumulation of hepatic triglyceride and increased hepatic fractional uptake of unesterified fatty acid from serum. The results of experiments testing this point are shown in Fig. 6. At the earliest time tested under our experimental conditions (35 s after intraperitoneal injection of ethanol the pulse of palmitate was administered intravenously and the animals killed 2 min later), the effect on the fractional hepatic incorporation was maximal, and remained constant as long as ethanol was present in serum. Three different doses of ethanol (0.2, 0.7 and 2.1 g/kg) gave identical results as long as ethanol was present in serum, even though maximum concentrations of ethanol in serum and times of clearance were very different (Fig. 1). At 8 h after the 2.1 g/kg dose every rat having ethanol present in its serum (ranging from 20–60 mg% in blood samples obtained by decapitation) showed about the same excess incorporation of unesterified fatty acid from serum into lipids of liver. However, at 9 h every animal showed complete clearance of ethanol from blood and the hepatic fractional uptake had also returned to the control value. In

Fig. 7. Pattern of incorporation of $[U-^{14}C]$ palmitate into different classes of lipid within livers of control and ethanol-treated rats

Groups of rats were injected intraperitoneally with either water or ethanol (2.1 g/kg), then 60 min later injected with $[U-^{14}C]$ -palmitate in a tail vein. At indicated times, rats were decapitated and livers removed and analysed. Each point represents the mean $\pm$ S.E.M., four to ten rats being used. ●, Polar lipids; ○, triglyceride; ▲, diglyceride; △, cholesterol ester. (a) Control; (b) ethanol-treated.

summary, as long as there is ethanol in serum the fractional uptake of unesterified fatty acid of serum by liver is increased.

Fraction of unesterified fatty acid of serum taken up by liver that is converted into triglyceride

To quantify the contribution of unesterified fatty acid of serum to hepatic triglyceride it is essential to know the fraction of the fatty acid taken up by liver that is converted into triglyceride. It should be emphasized that the purpose of these experiments is to determine the maximum conversion of fatty acid into hepatic triglyceride and not the kinetics of the movement of fatty acid through various hepatic lipid pools.

The pattern of incorporation into different classes of lipids of liver as a function of the length of the pulse of $[U-^{14}C]$ palmitate is shown in Fig. 7. These measurements were made 1 h after injection of ethanol. The incorporation into phospholipid and triglyceride reaches a plateau at 60 and 30% respectively by about 2 min in control animals. Incorporation also reaches a plateau after 2 min in the experimental animals, but these values are markedly different from those of the controls. In both cases these plateau values are maintained until about 10 min after palmitate injection, at which time very-low-density lipoprotein starts to be released from liver (J. Wilson &

C. Cooper, unpublished work). In this case the value for phospholipid decreased from 60 to 40% whereas that for triglyceride showed a comparable increase from the 30% (obtained with control animals) to 50%.

The decline in radioactivity in diglyceride (similar in both control and experimental animals) concomitant with a rise in radioactivity in triglyceride is probably a result of a precursor-product relationship between these lipids. Diglyceride migrates almost identically with cholesterol in the t.l.c. system we used and additional procedures were necessary to determine the relative proportions of isotope in the two types of lipids. The cholesterol diglyceride spot was eluted from the t.l.c. plate with chloroform/methanol (2:1), and the solvent evaporated under N_2 at 30°C. The residue was subjected to mild alkaline hydrolysis as described by Kates (1972) and the resulting material was rechromatographed. Two spots corresponding to cholesterol and fatty acid methyl ester were found. All of the radioactivity was accounted for and was located exclusively in the fatty acid methyl ester spot. This means the radioactivity in the fraction obtained following chromatography in the first solvent was present entirely in diglyceride. A similar problem was encountered in defining the reason for the decline in the fraction of radioactivity in phospholipid from ethanol-treated animals during the first 2 min of the $[U-^{14}C]$ palmitate pulse (Fig. 7).

Fig. 8. Changes in relative fractions of radioactivity of liver in phospholipids and triglycerides with time after ethanol administration

Groups of rats were injected intraperitoneally with either water or ethanol (2.1 g/kg), then at indicated times injected with [U-¹⁴C]palmitate in a tail vein. At 2 min after the isotopic pulse, rats were decapitated, livers removed and analysed. Each point represents the mean $\pm$ S.E.M., five to ten rats being used. (a) Phospholipid; (b) triglyceride. ●, Controls; ○, ethanol-treated.

A number of polar compounds potentially capable of becoming labelled all fail to migrate in the solvent system used. The spot of polar lipid was eluted and concentrated as described above and the material rechromatographed by using chloroform/methanol/water (65:25:4, by vol.) as the solvent. At 8 s after injection of palmitate, 36% of the isotope in the fraction containing polar lipid is in phosphatidic acid, and by 2 min this value has declined to 4%. The decline in content of isotope in the fraction containing polar lipid in this case also is presumably the result of a precursor-product relationship between phosphatidic acid and triglyceride.

The manner in which the relative incorporation of [U-¹⁴C]palmitate (2 min after an intravenous injection) into triglyceride and phospholipid is affected by time after administration of ethanol is seen in Fig. 8. With either 0.7 or 2.1 g of ethanol/kg the fraction of liver ¹⁴C in triglyceride increases linearly over the first hour from about 30% to about 50%. It should be emphasized that both the concentration (Table 1) and specific activity (not shown) of phospholipid is constant for each of these measurements. The changes in percentage incorporation shown in Fig. 8 reflect the ethanol-induced increases in (1) uptake of un-

esterified fatty acid by liver, and (2) rate of formation of hepatic triglyceride.

Discussion

Our data show that an acute dose of ethanol results in a marked increase in the amount of unesterified fatty acid taken up by liver, even though the concentration and flux of fatty acid in serum is unaltered. The purpose of the present experiments is to relate quantitatively the accumulation of hepatic triglyceride and the increased utilization of unesterified fatty acid of serum for formation of triglyceride in liver. In control animals the concentration of triglyceride in liver is constant (Fig. 2) and represents a steady state between biosynthetic rate and rate of release as very-low-density lipoprotein. The latter rate was estimated by injecting Triton WR 1339 intravenously to block lipoprotein lipase and by using the subsequent rate of accumulation of triglyceride in serum as a measure of release of hepatic triglyceride. This then also serves as an estimate of the hepatic biosynthetic rate. The value obtained is 0.15 mg/min per 100 g body wt. Several assumptions are inherent in this measurement and they are discussed below. Our value agrees with previously reported estimates of 0.15 mg of triglyceride/min per 100 g body wt. (Schotz *et al.*, 1964), 0.145 (Recknagel, 1967) and 0.14 (Annable & Cooper, 1974). The latter two values were also obtained with the use of Triton WR 1339.

Since the rate of accumulation of hepatic triglyceride is linear for 2 h at either dose of ethanol (Fig. 2), the rate of build-up can be estimated from the slopes of these lines and by applying a correction factor of 2.8 to convert grams of liver to 100 g of body weight. The value of 2.81 ± 0.13 was determined by us with over 400 animals starved 48 h and weighing about 200 g each. It may be calculated that an extra 0.04 or 0.11 mg of hepatic triglyceride/min per 100 g body wt. is being synthesized at the low and high doses respectively, if there is no change in the rate of exit (see Fig. 9). When similar measurements were made 90 min after injection of ethanol, it was found that the rate of release increases from a control value of 0.15 ± 0.01 to 0.18 ± 0.01 (mean $\pm$ S.E.M.) (Fig. 9). Therefore under these experimental conditions the total rate of synthesis of hepatic triglyceride is the sum of the rate of accumulation of excess of triglyceride and the rate of release of very-low-density lipoprotein. It is assumed that triglyceride in serum is not taken up by liver and converted into hepatic triglyceride. Since the release rate varies, this calculation is made immediately after ethanol injection (before the exit rate has changed) and 90 min later, which is well after this rate has reached a higher steady state.

Using (a) the irreversible disposal rate of unesterified fatty acid of serum (Table 3), (b) the fraction of that total flux that is incorporated into lipids of liver

Fig. 9. Quantitative data on effect of ethanol on synthesis of hepatic triglyceride

The contribution by unesterified fatty acid of serum to synthesis of hepatic triglyceride was calculated as described in the Discussion section. The rate of formation of excess triglyceride is calculated from Fig. 2. The rate of exit of triglycerides was determined by using Triton WR 1339. The broken lines represent calculated contributions from an unidentified source. These rates are the difference between the measured rate of total synthesis of triglyceride (accumulation + exit) and rate of synthesis of hepatic triglyceride from unesterified fatty acids of serum. (a) Control; (b) ethanol-treated.

(Fig. 6), and (c) the fraction of this uptake that is esterified into hepatic triglyceride (Figs. 7 and 8), it is possible to quantify the contribution made to hepatic triglyceride formation by fatty acid taken up from serum:

Since all parameters involved in making these calculations are either unaltered or affected to the same extent by both 0.7 and 2.1 g/kg doses of ethanol, the rates are the same for both. By using $1 \mu\text{mol}$ of hepatic triglyceride/ $3 \mu\text{mol}$ of fatty acid as a correction

$$\text{Irreversible disposal rate of unesterified fatty acid of serum} \times \text{fractional hepatic uptake} \times \text{fraction of hepatic } ^{14}\text{C in triglyceride} \times \frac{1 \mu\text{mol of triglyceride}}{3 \mu\text{mol of fatty acid}} \times \frac{0.85 \text{ mg of triglyceride}}{\mu\text{mol of triglyceride}}$$

Controls:

$$(4.23) \times (0.16) \times (0.30) \times (1/3) \times (0.85) = 0.060 \text{ mg} \pm 0.01 \text{ (S.E.M.) hepatic triglyceride/min per 100g body wt.}$$

Immediately after ethanol:

$$(4.23) \times (0.27) \times (0.30) \times (1/3) \times (0.85) = 0.10 \text{ mg} \pm 0.01 \text{ (S.E.M.; } P < 0.01) \text{ hepatic triglyceride/min per 100g body wt.}$$

At 90 min after ethanol administration:

$$(4.23) \times (0.27) \times (0.50) \times (1/3) \times (0.85) = 0.16 \text{ mg} \pm 0.02 \text{ (S.E.M.; } P < 0.005) \text{ hepatic triglyceride/min per 100g body wt.}$$

factor we assume that all triglyceride being synthesized requires three fatty acids from the serum, and this has been shown to be the case in similar experiments (Farquhar *et al.*, 1965). The percentage of excess fat accumulating in liver that can be accounted for by excess uptake of unesterified fatty acid of serum and its conversion into hepatic triglyceride can be determined by the following equation and by using the data shown in Fig. 9.

$$\text{Accountability} = \frac{\left(\begin{array}{c} \text{hepatic triglyceride synthesis} \\ \text{via unesterified fatty acid} \\ \text{of serum after ethanol} \end{array} \right) - \left(\begin{array}{c} \text{hepatic triglyceride synthesis} \\ \text{via unesterified fatty acid} \\ \text{of serum in controls} \end{array} \right)}{\left(\begin{array}{c} \text{total synthesis} \\ \text{after ethanol} \end{array} \right) - \left(\begin{array}{c} \text{total synthesis} \\ \text{in controls} \end{array} \right)} \times 100$$

The rate of total synthesis of hepatic triglyceride is taken to be the sum of the rate of release into serum plus the rate of accumulation of excess triglyceride in liver.

With 2.1 g of ethanol/kg: (a) immediately after injection: $(0.10 - 0.06) / [(0.15 + 0.11) - 0.15] \times 100 = 36\%$; (b) 90 min after injection: $(0.16 - 0.06) / [(0.18 + 0.11) - 0.15] \times 100 = 71\%$.

With 0.7 g of ethanol/kg: (a) immediately after injection: $(0.10 - 0.06) / [(0.15 + 0.04) - 0.15] \times 100 = 100\%$; (b) 90 min after injection: $(0.16 - 0.06) / [(0.18 + 0.04) - 0.15] \times 100 = 143\%$. With the larger dose of ethanol, approximately half (36–71%) of the excess fat being synthesized can be accounted for by enhanced hepatic uptake of unesterified fatty acids of serum. There must be some additional source(s) contributing fatty acid. The magnitude of the unaccounted-for fatty acid contribution that results in the synthesis of 0.09 mg of hepatic triglyceride/min per 100 g body wt. in control animals is obtained by difference between the measured total synthetic rate (0.15) and the amount we measured as coming from unesterified fatty acid of serum (0.06). A very different picture is observed with the smaller dose (0.7 g/kg). Quite soon after this low dose was administered all the excess hepatic triglyceride can be accounted for quantitatively by enhanced uptake of unesterified fatty acid from serum by liver. However, by 90 min the increased uptake by liver provides more-than-enough fatty acid (143%). The fact that the contribution of 'endogenous' fatty acid is markedly increased at 2.1 g/kg indicates there may be complicating effects caused by the high dose of ethanol. The physiological alterations seen at 2.1 g/kg are not observed at doses below 1.0 g/kg (Fig. 3). In addition, the contribution by 'endogenous' fatty acid decreases with time after ethanol administration at both the low (0.09–0.06) and high (0.16–0.13) doses (Fig. 9). This may result from a secondary, time-dependent effect of ethanol on the unidentified source of 'endogenous' fatty acid contri-

buted to hepatic triglyceride synthesis. Although the notation 'endogenous' fatty acid is used here, it should be noted that the rate of synthesis *de novo* of fatty acid by rat liver *in vivo*, in starved rats with or without ethanol, is much too low for it to be an important factor in explaining the 'endogenous' contribution. By using the data of Guynn *et al.* (1973) and the information in Fig. 8 above, it may be estimated that the contribution of fatty acid synthesis *de novo* to hepatic

triglyceride formation in the starved rat would be 0.002 mg of triglyceride/min per 100 g body wt. for control animals and 0.006 for animals receiving an acute dose of ethanol. The most apparent source of the 'endogenous' fatty acid is serum triglyceride, and this possibility requires investigation.

The fraction of total flux of unesterified fatty acids in serum attributable to uptake by liver (Fig. 6) has been studied previously by others. The experiments reported in the literature have either been carried out by methods similar to those used by us or by implanting catheters in the hepatic artery and vein, and in the portal vein, and directly measuring the difference in concentrations flowing into and out of liver (Spitzer *et al.*, 1969). When direct measurement is coupled with a measure of liver blood flow, the fraction taken up can be determined. The values in the literature for starved laboratory animals (both types of measurements being used) range from 14–54%, with most values clustered around 25–30% compared with our control value of 16%. The catheterization technique suffers from the complication that it is almost invariably carried out on dogs under deep anaesthesia using surgical conditions. The values obtained in measurements similar to ours are not easily compared to the ones reported here because one or more of the following experimental conditions essential to an accurate measurement were not met: (a) all injected isotope must be accounted for; (b) none of the isotope delivered to liver has been released from liver; and (c) there is no recirculation of isotope from other tissues back to the liver. Also, many experiments were carried out with continuous infusion of isotope into female or anaesthetized animals. In one study (Baker & Schotz, 1967) starved, unanaesthetized, male rats were injected in a tail vein. The total hepatic uptake of $[1-^{14}\text{C}]$ palmitate was 14% after 10 min, which correlates well with our value (Figs. 5 and 6). Baker & Schotz (1961) reported that it takes at least 10 min for radioactive triglyceride in liver to appear in serum,

and we have confirmed this. In the experiments reported here no anaesthetics were used, all injected isotope was recovered (Table 4), none of the isotope delivered to the liver was released (all ^{14}C in serum was still in the form of unesterified fatty acid) and there was no significant recirculation of isotope from other tissues back to the liver (radioactivity in lipids of liver did not change significantly in the 3–10 min interval after the intravenous injection of $[\text{U-}^{14}\text{C}]$ palmitate). The fulfillment of all of these conditions should make our fractional-uptake values reliable.

The fraction of radioactivity in lipids of liver esterified to phospholipid and triglyceride has been reported as ranging from 16–40% and 60–78% respectively (Havel *et al.*, 1962) in starved rats. These results are very different from those we obtained (Figs. 7 and 8). Havel *et al.* (1962) extracted lipids from liver by an ethanol/acetone (1:1, v/v) procedure that included overnight incubation at 45°C. Their method was compared by us with the Folch extraction on the same livers. We were only able to extract $15 \pm 2\%$ of the injected dose in total lipids of liver of ethanol-treated rats by the ethanol/acetone extraction, as compared with $27 \pm 1\%$ recovered with the chloroform/methanol procedure. The percentage of isotope in phospholipid and triglyceride found after ethanol/acetone extraction was $23 \pm 1\%$ and $56 \pm 1\%$ respectively, whereas the method we used gave $37 \pm 2\%$ and $50 \pm 2\%$. Thus it seems that not all of the radioactivity present in phospholipids is recovered in the method used by these investigators, which accounts for the discrepancy in the results.

All calculations presented above are dependent on two major assumptions: (1) that no triglyceride of serum can be taken up by liver, thereby causing an overestimation in the rate of synthesis of triglyceride, and (2) that the detergent action of Triton does not interfere with metabolism of lipids in the whole body, thereby giving an erroneous rate of exit of triglyceride from liver. The first point has been extensively studied, but a definite answer is not presently available (Stein & Shapiro, 1960; Belfrage *et al.*, 1965; Belfrage, 1966; Mayes & Felts, 1967; Fielding, 1972; Krauss *et al.*, 1973). Triton does have some known effects. In particular it has been shown to inhibit esterification of cholesterol in plasma *in vitro* (Klauda & Zilversmit, 1974), enhance activity of hydroxymethylglutaryl CoA reductase (Goldfarb, 1975) and possibly synthesis and release of lipoprotein (Otway & Robinson, 1967*a,b*). However, the manner in which these alterations might possibly influence the parameters studied here is not clear. Several of the important determinations used for making calculations, such as flux, fractional hepatic uptake, and the pattern of incorporation of unesterified fatty acid of serum into lipids of liver have been measured by us in control animals in the presence of Triton and were all found to be unaffected. One final assumption we made is

that measurements involving only palmitic acid gives an accurate appraisal of similar events involving all fatty acids in unesterified serum. This assumption appears reasonable, because Hagenfeldt *et al.* (1972) reported that the metabolic behaviour of palmitate in serum is close to the average of that of all unesterified fatty acids in serum, thus making it a suitable tracer for the entire class.

Annable & Cooper (1974) previously reported a rate of release of 0.14 mg/min for hepatic triglyceride into serum as determined with Triton WR 1339 and with the same type of control animals. This is very similar to the value of 0.15 mg/min reported for control animals in the present study. However, Annable & Cooper (1974) also reported that 2.0 g of ethanol/kg caused a marked inhibition of the rate of release, whereas we have found the same dose of ethanol causes a slight increase from 0.15 to 0.18 mg of triglyceride/min per 100 g body wt. Whereas the data of Annable & Cooper (1974) regarding the effect of ethanol on the release rate are reproducible, they were subsequently found to result from the manner in which the intraperitoneal injection of ethanol was made, and are therefore considered by us to be inapplicable to the other data presented above. The reasons why the results of Annable & Cooper (1974) with ethanol are reproducible, but different, require investigation.

The carbon tetrachloride-induced fatty liver is caused by a block in release of hepatic lipoprotein (Recknagel, 1967), and the same mode of action has been proposed for ethanol (Schapiro *et al.*, 1964). This model cannot explain our results because the 2 min pulse interval used (during which time ethanol causes an enhanced incorporation of unesterified fatty acid of serum into hepatic triglyceride) is far less than the minimum time (10–15 min) it takes to convert unesterified fatty acid of serum into triglyceride in very-low-density lipoprotein in serum (Baker & Scholtz, 1961).

It has also been suggested that decreased oxidation of fatty acid of liver could result in increased formation of triglyceride. Since we can account for all injected fatty acid as lipid (primarily triglyceride and phospholipid) during the 2 min pulse in both control and experimental animals, the differences in incorporation of fatty acid into triglyceride are unrelated to oxidation of preformed fatty acid arriving via absorption from blood.

The proposed role of concentration of α -glycerophosphate and ratio of NADH/NAD in the development of fatty liver could possibly explain our results (Hawkins & Kalant, 1972). If the intracellular concentration or flux of α -glycerophosphate is rate-limiting for formation of triglyceride, then such changes could lead to an enhanced hepatic uptake of unesterified fatty acid from serum, as well as increased esterification into triglyceride. Evidence presented in

the following paper (Abrams & Cooper, 1976) shows that other factors are, however, of primary importance. Our conclusion is that, at least in the acute situation, alterations in the ratio of NADH/NAD and concentration of α -glycerophosphate are related only to the metabolism of ethanol and not to the hepatic steatosis.

Another mechanism of action of ethanol previously proposed is increased mobilization of fat from adipose tissue (Hawkins & Kalant, 1972). Increased output of fatty acid from the depots will result in either elevated concentration or enhanced flux of unesterified fatty acid in serum. The data in Table 2 show clearly that neither of these parameters changes throughout the course of our experiments, and therefore this mechanism cannot provide an explanation for our data.

We have described an effect produced by an acute dose of ethanol that occurs almost immediately and that has not been reported previously. Many possibilities have been eliminated as reasonable explanations for the observed data. An ethanol-induced physiological alteration that could account for our results is an increased hepatic blood flow. This would result in the opening of additional liver sinusoids, thereby providing an increased absorptive surface area and increased hepatic clearance of unesterified fatty acid from serum. The following paper (Abrams & Cooper, 1976) presents experimental results bearing on this hypothesis.

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