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EFFECT OF AN INTOXICATING DOSE OF ETHANOL ON LIPID METABOLISM IN AN ISOLATED PERFUSED RAT LIVER

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Abstract—A dose of ethanol (200 mg/100 ml) produced marked alterations in the metabolism of two-carbon substrates in the liver. Utilizing radioactive tracers and the technique of perfusing an isolated rat's liver, it has been demonstrated that ethanol directly enhanced the synthesis of fatty acids from two-carbon sources (2-^{14}C-acetate, 3-^{14}C-pyruvate) and their esterification to triglycerides. This effect was not associated with any defect in the synthesis or with the release of triglyceride by the liver. These newly synthesized triglycerides were preferentially used to form the lipid portion of lipoproteins which were then released into the perfusate. An increase in the size of the two-carbon pools of the liver occurred during the oxidation of ethanol, but appropriate control experiments indicated that this change in pool size did not completely account for the changes observed in the distribution of the two-carbon sources in the various lipid pools of the liver.

AN ACCUMULATION of triglycerides in the liver has been repeatedly observed in both experimental animals and in man following the ingestion of ethanol.[1,2] The mechanisms underlying this abnormal deposition of lipid are still not completely resolved.[3,4] Under normal circumstances, triglycerides in the liver arising from synthesis or dietary sources are excreted as low density lipotroteins. An interference in the formation or excretion of these lipoproteins, such as has been shown to occur in choline deficiency,[5] or in carbon tetrachloride, ethionine or phosphorus poisonings,[6–8] results in the accumulation of neutral lipids in the liver. This interference mechanism has been proposed by Schapiro *et al.*[9] as the primary cause of an ethanol-induced fatty liver, but other investigators have been unable to confirm these findings.[10–13] Instead they have shown that, in the intact rat, an acute dose of ethanol has no effect or even enhances rather than inhibits the formation and release of low density lipoproteins from the liver.

It therefore seemed important to clarify this apparent disagreement, and a series of experiments have been carried out with the principal object of checking this point using a technique which removed as many of the objections to the previous investigations as could be managed. These experiments were conducted on an isolated rat's liver, which functions normally while maintained in an appropriate perfusion system. This technique eliminated the complication of any effect of ethanol on peripheral tissue which might indirectly affect hepatic lipid metabolism. In the experiments reported here, the effect of ethanol (200 mg/100 ml) was assessed by adding a trace amount of [^{14}C] labeled two-carbon substrates to the perfusate and by noting the subsequent pattern of incorporation of this radioactivity into the lipid fraction of the

perfusate. The activities of these metabolites were determined at different time-intervals during a 4-hr perfusion period, and from these data it was possible to determine if ethanol affected the synthesis of free fatty acids (FFA) and triglycerides as well as the formation and release of lipoproteins by the liver. Interpretation of results is frequently complicated by the fact that, during the oxidation of ethanol, acetate is formed at a greater rate than it can be utilized and results in an increase in the two-carbon pool of the liver.[14] This study also investigated whether or not a change in the size of the two-carbon pool might account for the observed changes in hepatic lipid metabolism following the ingestion of ethanol.

EXPERIMENTAL PROCEDURE

Methods and Materials

Liver perfusion. The apparatus and surgical techniques have been described in detail in the literature[15,16] and will not be discussed further here.

Male albino Wistar rats (250–350 g) were used and were allowed free access to water and Purina laboratory chow until the time of sacrifice. In all experiments, the perfusion medium (perfusate) consisted of freshly drawn defibrinated rat's blood which was diluted with 0·9% NaCl to give final hematocrit values which ranged from 22 to 28 per cent. Zero time (the time at which the first sample was taken) was always 30 min after the liver was introduced into the system. Throughout the experimental period, the temperature and pH of the perfusate were monitored. At specified time intervals, aliquots of the perfusate were taken for chemical analysis and were immediately replaced by equal amounts of fresh perfusate so that constant volume and hematocrit level were maintained in the system. Expired CO_2 were trapped in a measured volume of Hyamine-10X (Packard Instrument Co., Downers Grove, Ill., U.S.A.), which was replaced every 30 min. An aliquot of each sample of Hyamine-10X was used to determine the average [^{14}C] radioactivity which appeared in the expired CO_2 during that particular 30-min interval. At the end of the 4-hr experimental period, the liver was flushed with 0·9% NaCl, weighed and a portion taken for chemical analysis.

Experimental design. The effect of ethanol (200 mg/100 ml of perfusate) on the incorporation of two-carbon substrates into the neutral lipid fractions of the perfusate and liver was investigated. Radioactive substrates utilized were [2-^{14}C]ethanol (sp. act., 2 mc/m-mole), [2-^{14}C]sodium acetate (sp. act., 2 mc/m-mole), and [3-^{14}C]-sodium pyruvate (sp. act., 1·5 mc/m-mole) (Amersham/Searle, Don Mills, Ont.). These substrates were added to the perfusate in trace amounts (50–100 μc substrate per experiment) at the beginning of the experiment in Krebs–Ringer phosphate buffer (pH 7·4) either alone or with ethanol (200 mg/100 ml of perfusate). Aliquots of the perfusate were taken at 0, 30, 60, 90, 120, 180 and 240 min, and each aliquot was analyzed for its concentration of glucose, pyruvate, lactate, triglyceride and free fatty acids (FFA), as well as for [^{14}C] radioactivity in the neutral lipid fractions of the serum. At the end of the experimental period, the liver was flushed with 0·9% saline, blotted, weighed and the lipids were extracted. The lipid extracts were utilized to ascertain the triglyceride level and the incorporation of [^{14}C] radioactivity into the neutral lipid fractions of the liver.

Chemical analysis. The lipids were extracted from the perfusate and the liver in redistilled chloroform–ethanol (2:1, v/v) and washed by the method of Folch *et al.*[17]

These extracts were used to ascertain the triglyceride levels by the method of Van Handel and Zilversmith.[18] The level of FFA in the perfusate was determined by the method of Novak.[19] The level of cholesterol in serum and liver, and the level of FFA in the liver were determined chemically after these metabolites were separated and quantitatively eluted from the neutral lipid chromatograms. The level of free cholesterol was determined by the method of Schoenheimer and Sperry.[20]

The incorporation of the [^{14}C] radioactivity into the neutral lipid fractions of the serum and the liver was determined by means of thin-layer chromatography. For the purposes of this work, glyceride, diglyceride, FFA, triglycerides, cholesterol and cholesterol esters are all defined as "neutral lipids". This is a convenient working definition, since all these fractions are separated on the same thin-layer chromatographic plate in the method of Skipski *et al.*[21] which was used in the present work. Standard neutral lipid samples (1-monopalmitin, cholesterol, 1,2-dipalmitin, 1,3-dipalmitin, palmitic acid, tripalmitin and cholesterol oleate) obtained from Applied Science Laboratories (State College, Pa., U.S.A.) were spotted on each chromatogram with unknowns. The neutral lipids having been identified were scraped from the Silica gel plate into counting vials which contained Gray's solution and a 4% thixotropic gel powder (Cab-o-sil; Packard Instrument Co., Downers Grove, Ill., U.S.A.). The Silica gel between the lipid bands was also scraped and counted so that the efficiency of the separation and extraction procedures could be ascertained and utilized in the calculations.

The radioactivity was measured at 4° in a Packard Tri-Carb liquid scintillation spectrophotometer (model 4222), using Bray's solution[22] as the liquid scintillator. The efficiency of counting was determined using an external standard, and all counting data were expressed in terms of total dis./min.

In this study, two sets of control experiments were used to distinguish between the direct effect of ethanol itself and an indirect effect, which might be produced by an increase in the size of the two-carbon pool of the liver (as mentioned in the introduction). The first set of controls (control group A) was intended to establish both the pattern and the absolute amount of radioactivity which could be expected in the neutral lipid fractions of the perfusate under normal conditions. In the second set of controls (control group B), the effect of the size of the two-carbon pool was investigated. Acetate containing trace amounts of [2-^{14}C]acetate was infused at a rate (30–36 mg/100 ml of perfusate/10 g liver/hr) similar to its rate of formation in the liver during the oxidation of ethanol, and the pattern and absolute amount of radioactivity were determined as with the first control group. A comparison of these two sets of controls made it possible to estimate what will be called the indirect effect of ethanol in these experiments.

Presentation of data. By using the isolated liver of a rat together with radioactive tracers, it was possible to trace ethanol or other two-carbon sources through specific pathways of lipid metabolism. However, a certain amount of caution had to be used in interpreting the results. This is because, in each pool, both formation and depletion go on concurrently in this active system, so that the rate of appearance of radioactivity depends on both the rate of formation and the position in the metabolic chain. Since changes in substrate levels in the perfusate are produced by the liver, a fundamental problem arises in any attempt to normalize the data, since two variables, perfusate volume and liver size, both have an influence on the effective radioactivity in the

metabolic pools: the liver size by affecting the total amount of substrate metabolized at a given rate of metabolic activity, and perfusate volume by changing the dilution of the pool and therefore changing the substrate level for a given amount of substrate formation. This problem was overcome by carefully selecting the experimental animals so that the weight of the liver in the different experiments varied less than 10 per cent, and by maintaining the perfusate volume at 70 ± 5 ml throughout the experimental period. In order to arrive at a reasonably correct picture of metabolic activity, it was necessary to determine the degree of radioactivity in the various lipid fractions as a function of time, and to keep the picture as clear and straightforward as possible, these changes have been expressed in terms of the total amount of radioactivity in the neutral lipid fraction. This is not the only possible way of expressing the data, but it has the advantage of emphasizing differences in the early stages of the experiment. It is further recommended by the known fact that approximately 70 per cent of the radioactivity in the serum lipids occurs in this fraction. Therefore, the total radioactivity appearing in all the neutral lipids of the perfusate was expressed in dis./min per milliliter of perfusate and was assigned the value of 100 per cent. The radioactivity (dis./min) appearing in each individual neutral lipid fraction was then expressed as a function of the total radioactivity appearing in all the neutral lipids at that time. By plotting these data, changes in the distribution of radioactivity in each fraction with time were obtained as well as the relative activity of each pool.

RESULTS

The results of these experiments are shown in Figs. 1–6 and in Tables 1–3.

The perfusate levels of FFA, cholesterol and triglycerides are presented in Table 1.

TABLE 1. TRIGLYCERIDE, FFA AND CHOLESTEROL LEVELS OF THE PERFUSATE

	Triglyceride (mg/100 ml)*		FFA (μmoles/100 ml)*		Free cholesterol (mg/100 ml)	
Treatment time (min)	Control† (9)§	Ethanol‡ (6)	Control† (8)	Ethanol‡ (5)	Control (8)	Ethanol (5)
0	53 ± 4·0	54·6 ± 3·6	49·0 ± 4·2	43·3 ± 4·3	57 ± 2·0	52 ± 4·0
60	54 ± 5·6	52·0 ± 4·4	50·2 ± 3·1	55·0 ± 6·7		
120	46 ± 4·9	51·0 ± 4·4	48·1 ± 5·8	53·9 ± 8·8	62 ± 5·2	53 ± 3·5
180	52 ± 2·3	51·0 ± 4·1	52·3 ± 2·0	51·0 ± 12·7		
240	48 ± 4·9	49·0 ± 3·8	53·2 ± 1·2	59·0 ± 6·5	55 ± 3·0	50 ± 4·9

* Mean values ± S.E.M.
† Average of data collected from control groups A and B.
‡ Perfusate, 200 mg/100 ml, added at zero time.
§ Figures in parentheses indicate number of animals per group.

The level of these metabolites did not change significantly during the 4-hr perfusion, nor were there any statistical differences noted between the different groups. The average triglyceride, FFA and cholesterol levels, as found in the liver at the end of the experimental period, are summarized in Table 2. The average triglyceride level at the end of the 4-hr experimental period was 6·20 ± 0·22 mg/g of wet wt of the liver in the perfused controls. This was not significantly different from the levels observed in

TABLE 2. TRIGLYCERIDE, FFA AND CHOLESTEROL LEVELS IN THE LIVER*

Treatment	No. of animals	Triglyceride level (mg/g wet wt of liver)	FFA levels (μmoles/g wet wt of liver)	Free cholesterol (mg/g wet wt of liver)
Controls (fed *ad lib.*)	11	9·20 ± 0·44		
Controls (A and B)	10	6·50 ± 0·20†	3·8 ± 0·3	3·6 ± 0·3
[2-^{14}C]ethanol‡	9	7·72 ± 0·49	4·0 ± 0·4	3·4 ± 0·3

* Mean values ±S.E.M.
† Statistically significant with respect to control groups at P = 0·001.
‡ Ethanol, 200 mg/100 ml, added to perfusate at zero time.

the experimental animals, although there existed a significant difference between all perfused and non-perfused animals. This presumably represents the effect of operational trauma. The addition of ethanol to the system did not change significantly the level of cholesterol or FFA in the liver.

The pattern of incorporation of [^{14}C] radioactivity in the FFA of the perfusate during a 4-hr perfusion is presented in Fig. 1(a) for the different groups of experi-

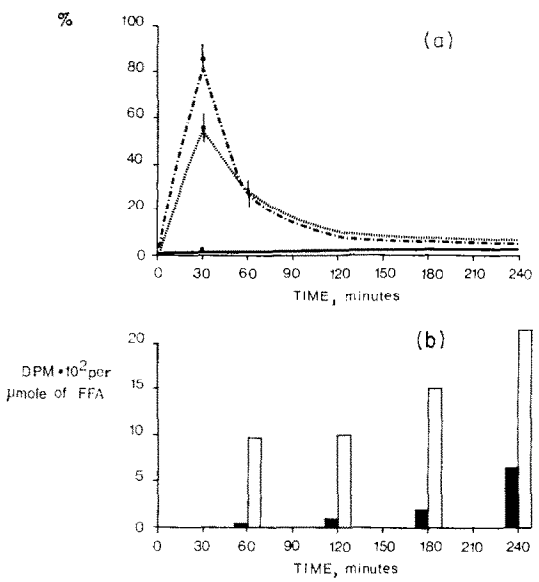

FIG. 1. (a) Incorporation of radioactivity into FFA fraction of the perfusate is expressed as a per cent of the total radioactivity appearing in the neutral lipids of the perfusate. At zero time, the following substrates were added to the perfusate in different experiments. The range of percentage is shown. Trace amount of [2-^{14}C]ethanol, – – –; average of six experiments. Trace amount of [2-^{14}C]ethanol in addition to a load of ethanol (200 mg/100 ml of perfusate/10 g of liver),———; average of three experiments. Trace amount of [2-^{14}C]acetate infused with a load of acetate at a rate of 30–36 mg/100 ml of perfusate/10 g of liver/hr, –.–.–; average of two experiments. (b) Specific activities of the FFA of the perfusate at various time intervals are presented as a histogram. The open bars represent data from an experiment in which a trace amount of [2-^{14}C]acetate was infused with a load of acetate at a rate of 30–36 mg/100 ml of perfusate/10 g of liver/hr. The closed bars represent data from an experiment in which a trace amount of [2-^{14}C]ethanol in addition to a load of ethanol (200 mg/100 ml of perfusate/10 g of liver) was added at zero time.

ments. When a trace amount of [2-^{14}C]ethanol (50–100 μc) (control group A) was added to the system at zero time, 55 per cent of the total radioactivity appearing in all the neutral lipids of the perfusate was found in the FFA fraction 30 min later. During the next 3 hr, this fraction slowly decreased until only 5 per cent could be found in this fraction. In the experiments in which sodium [2-^{14}C]acetate (control group B) was infused at a rate of 30–36 mg/100 ml of perfusate/10 g of liver/hr, 85 per cent of the total radioactivity appearing in all neutral lipids of the perfusate appeared in the FFA fraction at 30 min (Fig. 1(a)). This was a significantly greater proportion than was noted in control group A ([2-^{14}C]ethanol in trace amounts). However, after 1 hr, no significant differences were observed between the two groups. When a load of ethanol (200 mg/100 ml of perfusate/10 g of liver) in addition to a trace amount of [2-^{14}C]ethanol was added to the system at zero time, only 1–2 per cent of the radioactivity could be found in FFA at the 30-min period and this fraction did not change significantly during the remainder of the experiment. In summary, a significant difference occurs in the pattern of incorporation of radioactivity in the FFA even between the control groups A and B in the first hour, but an even greater difference exists between the control groups and the experimental group. This difference in distribution was still significant 2 hr after the start of the experiment. In terms of the original assumption, this represents a small but significant indirect effect due to an increase in the two-carbon pool size superimposed on a direct effect due to ethanol itself.

In Fig. 1(b), the specific activities of the FFA fractions of the perfusate at various time intervals during the experiment are presented as a histogram. The different bars represent data obtained from a typical experiment from each group and indicate that in all instances the specific activity of the FFA of the perfusate was changing with time. When the data are expressed in terms of specific activity, the same changes are observed.

The pattern of incorporation of [^{14}C] radioactivity in the triglyceride fractions of low density lipoproteins of the perfusate is given in Fig. 2(a). In the control groups A and B, less than 10 per cent of the radioactivity appearing in the neutral lipids of the perfusate would be accounted for in the triglyceride fraction at 30 min; with time, this increased to 60 per cent. When a load of ethanol was added to the system, within 30 min, 74 per cent of the radioactivity could be accounted for in the triglyceride fraction. No significant alteration in the distribution pattern was observed in any of the groups after 60 min. The difference between triglyceride pattern and free fatty acid pattern is presumed to relate to the difference in position in the metabolic chain. The fact that in the ethanol group the triglyceride fraction incorporated greater amounts of radioactivity than the free fatty acid fraction has the appearance of an anomaly, if the role of consumption is neglected. Presumably, however, a larger percentage of the newly synthesized free fatty acid is used to form triglyceride in the ethanol experiment, resulting in a relatively greater tryglyceride activity.

In Fig. 2(b), the specific activities of the triglyceride fraction of the perfusate at various time intervals are presented as a histogram. The different bars represent data obtained from a typical experiment from each group, and indicate that in all instances the specific activity of the triglycerides of the perfusate was changing with time. The same pattern was observed when the data were expressed in terms of specific activity.

Figure 3 illustrates the pattern of [^{14}C] incorporation into the cholesterol fraction

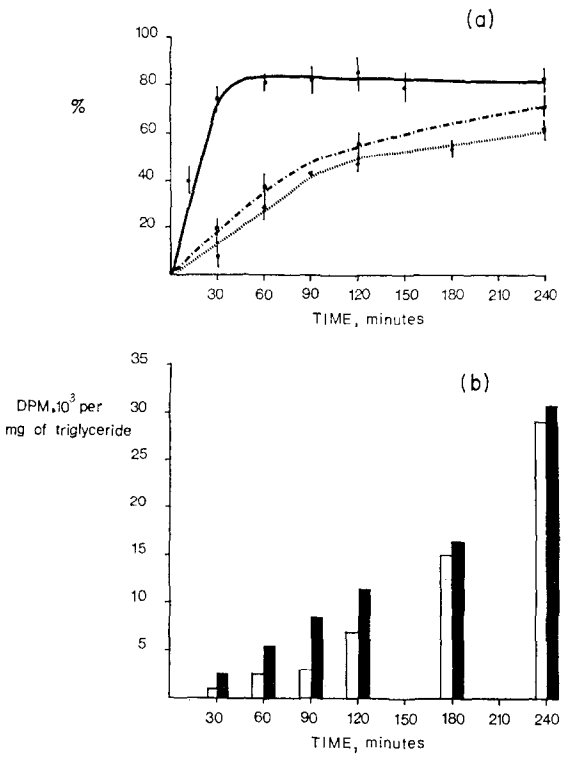

FIG. 2. (a) Incorporation of radioactivity into the triglyceride fraction of the perfusate is expressed as a per cent of the total radioactivity appearing in the neutral lipids of the perfusate. At zero time, the following substrates were added to the perfusate in the different experiments. The range of percentage is shown. Trace amount of [2-^{14}C]ethanol, – – –; average of six experiments. Trace amount of [2-^{14}C]ethanol in addition to a load of ethanol (200 mg/100 ml of perfusate/10 g of liver), ———; average of three experiments. Trace amount of [2-^{14}C]acetate infused with a load of acetate at a rate of 30–36 mg/100 ml of perfusate/10 g of liver/hr, –·–·–; average of two experiments. (b) Specific activities of triglycerides in the perfusate at various time intervals are presented as a histogram. The open bars represent data from an experiment in which a trace amount of [2-^{14}C]acetate was infused with a load of acetate at a rate of 30–36 mg/100 ml of perfusate/10 g of liver/hr. The closed bars represent data from an experiment in which a trace amount of [2-^{14}C]ethanol in addition to a load of ethanol (200 mg/100 ml of perfusate/10 g of liver) was added at zero time.

of the perfusate. After 1 hr, the incorporation of radioactivity was lower in the ethanol experiments compared to that noted in either of the control groups (either A or B). This difference was statistically significant at the 95 per cent level. Since cholesterol represents a separate pathway for the utilization of two-carbon fragments, any difference in the pattern of incorporation of radioactivity is of interest. The first observation to be made is that the per cent of radioactivity in cholesterol is about one-third that found in triglyceride. In the early stage, the effect of ethanol is not so profound as in the case of FFA, and ethanol tends to convert the over-all pattern from one resembling the triglyceride normals to one having the appearance of the free fatty acid normals. After the first 30 min, the fraction of radioactivity appearing in the ethanol group is only one-half to one-third of that appearing in the normal. The specific activity of the cholesterol fraction of the perfusate is given in the lower portion of Fig. 3. The different bars represent data obtained from a typical experiment from each group and indicate

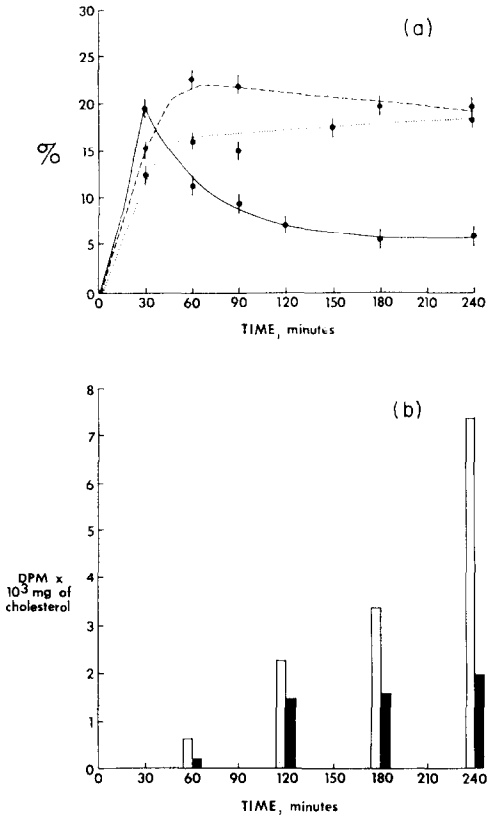

FIG. 3. (a) Incorporation of radioactivity into the cholesterol fraction of the perfusate is expressed as a per cent of the total radioactivity appearing in the neutral lipids of the perfusate. At zero time, the following substrates were added to the perfusate in different experiments. The range of percentage is shown. Trace amount of [2-^{14}C]ethanol, – – –; average of six experiments. Trace amount of [2-^{14}C]-ethanol in addition to a load of ethanol (200 mg/100 ml of perfusate/10 g of liver), ———; average of three experiments. Trace amount of [2-^{14}C]acetate infused with a load of acetate at a rate of 30–36 mg/100 ml of perfusate/10 g of liver/hr –.–.–; average of two experiments. (b) Specific activities of free cholesterol in the perfusate at various time intervals are presented as a histogram. The open bars represent data from an experiment in which a trace amount of [2-^{14}C]acetate was infused with a load of acetate at a rate of 30–36 mg/100 ml of perfusate/10 g of liver/hr. The closed bars represent data from an experiment in which a trace amount of [2-^{14}C]ethanol in addition to a load of ethanol (200 mg/100 ml of perfusate/10 g of liver) was added at zero time.

that the specific activity of the perfusate cholesterol was changing with time. It should be noted that when the data are expressed in terms of specific activity the same differences are observed.

Figures 4, 5 and 6 illustrate the pattern of incorporation of radioactivity in the FFA, triglyceride and cholesterol fraction of the perfusate when a trace amount of [3-^{14}C]-pyruvate was added instead of a trace amount of [2-^{14}C]ethanol. As might be expected, very little difference can be observed when compared with the group of experiments in which the radioactive tracer added was ethanol. There seems to be a significant increase in the percentage of radioactivity observed in the 30-min peak in the cholesterol fraction (Fig. 6), but this is probably most easily explained by assuming that the

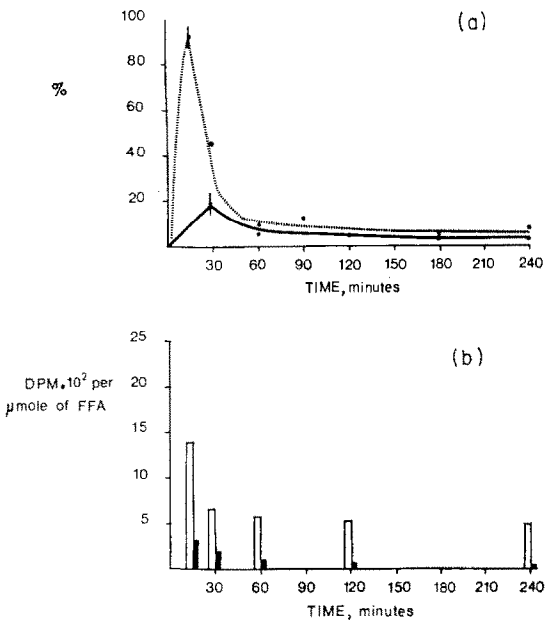

FIG. 4. (a) Incorporation of radioactivity into FFA fraction of the perfusate is expressed as a per cent of the total radioactivity appearing in the neutral lipids of the perfusate. At zero time, the following substrates were added to the perfusate in different experiments. The range of percentages is shown. Trace amount of [3-^{14}C]pyruvate, – – –; average of three experiments. Trace amount of [3-^{14}C]pyruvate in addition to a load of ethanol (200 mg/100 ml of perfusate/10 g of liver), ———; average of three experiments. (b) Specific activities of the FFA of the perfusate at various time intervals are presented as a histogram. The open bars represent data from an experiment in which a trace amount of [3-^{14}C]pyruvate was added at zero time. The closed bars represent data from an experiment in which a trace amount of [3-^{14}C]pyruvate in addition to a load of ethanol (200 mg/100 ml of perfusate) was added at zero time.

radioactivity pattern required a few more minutes to establish itself when the radioactive tracer used was pyruvate.

In all the experiments, a variety of metabolic indices were used to measure the viability of the liver. The glucose, lactate and pyruvate levels of the perfusate were determined at the specified time intervals during the 4-hr experiment. The concentration of these metabolites did not differ significantly from values already published[10] and therefore are not included. Initially, the average pH value of the perfusate was 7·42. However, during the 4-hr experiment, the pH of the perfusate fell slowly to 7·38 in the control experiments (A and B). When ethanol was added, the change in the pH was greater and the average final pH was 7·30.

While levels in the liver itself were determined only at the end of the experiment, the distribution of radioactivity among the various neutral lipid fractions reflected the changes observed in the perfusate. There was a significant difference in the incorporation of radioactivity into the triglyceride, FFA and cholesterol between the two groups. In the experiments in which ethanol (200 mg/100 ml of perfusate/10 g of liver) was added, 70 per cent of the total radioactivity appeared in the triglyceride, 1·2 per cent in the FFA and only 13 per cent in the cholesterol fraction, while in the initial

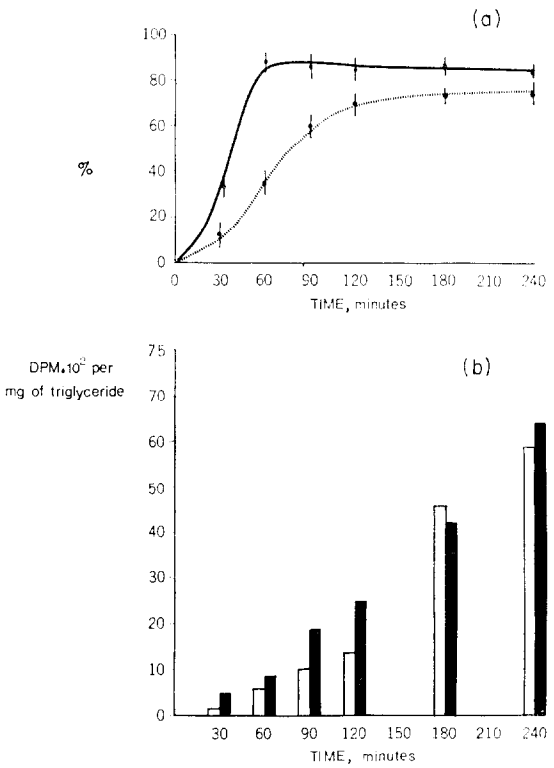

FIG. 5. (a) Incorporation of radioactivity into the triglyceride fraction of the perfusate is expressed as a per cent of the total radioactivity appearing in the neutral lipids of the perfusate. At zero time, the following substrates were added to the perfusate in the different experiments. The range of percentages is shown. Trace amount of [3-^{14}C]pyruvate, ---; average of three experiments. Trace amount of [3-^{14}C]pyruvate in addition to a load of ethanol (200 mg/100 ml of perfusate), ——; average of three experiments. (b) Specific activities of triglycerides of the perfusate at various time intervals are presented as a histogram. The open bars represent data from an experiment in which a trace amount of [3-^{14}C]pyruvate was added at zero time. The closed bars represent data from an experiment in which a trace amount of [3-^{14}C]pyruvate in addition to a load of ethanol (200 mg/100 ml of perfusate was added at zero time.

experiments (acetate infused) 26 per cent appeared in cholesterol, 4·8 per cent in FFA and 55 per cent in the triglyceride fraction. When the same data are expressed in terms of specific activity, the same significant differences are observed (Table 3).

TABLE 3. EFFECT OF ETHANOL ON THE DISTRIBUTION OF TWO-CARBON SOURCES IN THE NEUTRAL LIPIDS OF THE LIVER*

Treatment†	Triglyceride (dis./min/mg)	FFA (dis./min/μmole)	Cholesterol (dis./min/mg)
Control (A and B) (6)	13,007 ± 575	2290 ± 195	12,618 ± 1077
Ethanol (200 mg/100 ml) (6)	18,560 ± 348‡	619 ± 216‡	6513 ± 706‡

* Mean values ±S.E.M.
† Figures in parentheses indicate number of animals per group.
‡ Statistically significant with respect to control at P = 0·91.

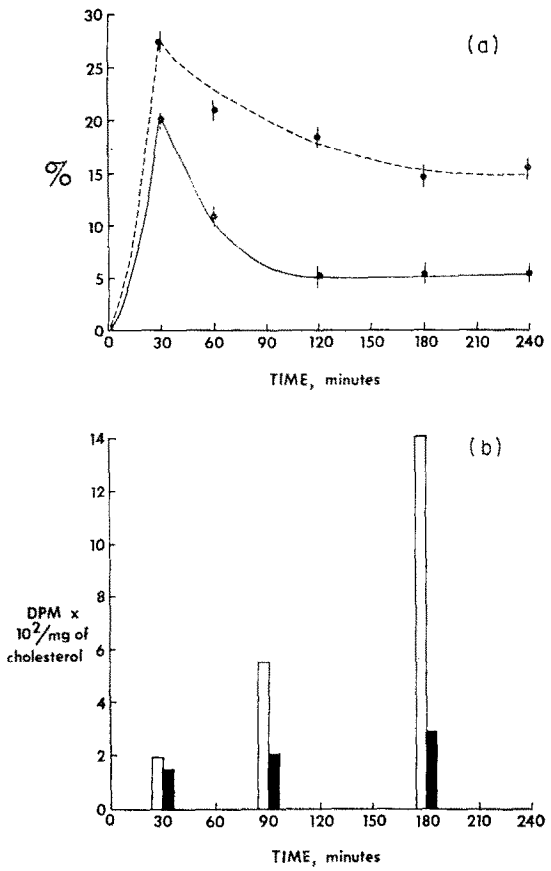

FIG. 6. (a) Incorporation of radioactivity into the cholesterol fraction of the perfusate is expressed as a per cent of the total radioactivity appearing in the neutral lipids of the perfusate. At zero time, the following substrates were added to the perfusate in different experiments. The range of percentages is shown. Trace amount of [3-^{14}C]pyruvate, – – –; average of three experiments. Trace amount of [3-^{14}C]pyruvate in addition to a load of ethanol (200 mg/100 ml of perfusate/10 g of liver) ———; average of three experiments. (b) Specific activities of free cholesterol in the perfusate at various time intervals are presented as a histogram. The open bars represent data from an experiment in which a trace amount of [3-^{14}C]pyruvate was added at zero time. The closed bars represent data from an experiment in which a trace amount of [3-^{14}C]pyruvate in addition to a load of ethanol (200 mg/100 ml of perfusate) was added at zero time.

DISCUSSION

There was no indication in the present investigation that ethanol inhibited the formation or release of low density lipoproteins from the liver, as has been claimed elsewhere.[9] No significant difference in triglyceride or FFA levels was observed between any of the three groups investigated. In fact, the enhanced incorporation of radioactivity in the triglyceride fraction might well be interpreted to mean an increase in the rate of formation of this fraction. However, since this change might also be explained as the result of a change in the proportion of the various precursors forming triglycerides, such an interpretation cannot be successfully defended. The findings are in substantial agreement with those of Baraona and Lieber[13] and appear to

contradict the conflicting evidence of Schapiro *et al.*[9] and other contributors.[10–12] The experimental technique of Baraona and Lieber[13] differed markedly from the technique used here and is subject to a variety of interpretations. Since they used the intact animal and introduced a substantial fat load at the outset, it is possible to suggest that the observed changes were induced, in part, by the flooding of the lipid pools of the liver. The experimental techniques used in the experiments described here most closely resemble those of Schapiro *et al.*,[9] although the findings seem to be at considerable variance. One possible reason can be suggested for the difference. Schapiro *et al.*[9] used ethanol doses that were significantly greater than the level used here. An ethanol concentration of 400 mg/100 ml, a level which produces anesthesia in rats, was utilized by Schapiro's group,[9] while in this investigation an ethanol concentration of 200 mg/100 ml, a level which produces intoxication in rats, was used. The fact that Schapiro *et al.*[9] used radioactive palmitate would not be expected to produce a significant difference in view of the findings of this work.

The other experiments reported in the literature[23–27] appear to have been carried out at later time periods when the metabolic pattern may well have changed, so that significant differences in the results might well occur. Since most of these experiments were also carried out in the intact animal, the fact that they agree with this report in finding an enhanced synthesis of FFA and triglyceride and no defect in the formation or release of lipoproteins from the liver after 2 hr is gratifying. The fact, reported here, that significant changes do occur in the first hour, however, seems to be a new finding. The finding that an indirect effect can be produced by the increase in two-carbon pool size due to the production of excess acetate is not unexpected, but these experiments show the magnitude of the effect and that it is smaller than the direct effect of ethanol. The further finding that ethanol affects the metabolism of free fatty acids, triglycerides and cholesterol in different ways is of interest and will be discussed in further detail.

The exact mechanisms at work are not clearly understood. However, the time sequence of changes in the radioactivity pattern described here allows some inferences to be drawn. However, it is often difficult to decide whether changes are produced by a direct pharmacological effect of ethanol, by a more indirect effect mediated through the metabolic products of ethanol oxidation, or by dietary changes associated with ethanol intake. In the experiments reported here, the liver is isolated and not affected by dietary intake or by the effects of ethanol on peripheral tissue, which also might indirectly affect the over-all hepatic lipid metabolism. In the subsidiary experiments, using control groups A and B, it was possible to isolate the indirect effect of ethanol oxidation produced by excess acetate production. While this is not the only possible indirect effect, it is the most obvious one and did not prove to be large. It is therefore felt that the changes observed in these experiments are essentially those produced by the direct effect of ethanol.

The changes in triglyceride and FFA levels of the perfusate in this system are the result of lipid metabolism in the liver alone. For these reasons it has been assumed that the incorporation of [^{14}C] radioactivity from trace amounts of [3-^{14}C]pyruvate, [2-^{14}C]acetate and [2-^{14}C]ethanol into the triglyceride and FFA fractions of the perfusate was clear evidence that lipid synthesis was occurring. Since, in any of the experiments, the actual levels of triglyceride, cholesterol and FFA in the perfusate did not change significantly during the experimental period, while the specific activity of each of these fractions increased, this behavior is taken as evidence for a continued

turnover of both these substrates. Therefore, the isolated liver was not only synthesizing lipoproteins and releasing them into the perfusate, but was also utilizing them.

The exact manner in which ethanol affects the rate of synthesis of the lipid fraction of serum lipoprotein is not known. It has been proposed that the shift in redox potential of the liver to a more reduced level, which has been shown to occur as a result of the oxidation of ethanol, is involved.[28–31] The studies of Lieber and Schmid,[24] which indicate that in liver slices ethanol favors the incorporation of labeled precursors into lipids, and that these effects could be mimicked by an alternate NADH generating system, namely sorbitol, would tend to support this theory. Whether or not this change could influence, in this time interval, the distribution of two-carbon sources in the metabolic pools of the liver has yet to be established.

Although incorporation of two-carbon fragments into lipids was markedly affected by an acute dose of ethanol (Figs. 1, 2, 5 and 6), it should be noted that no change in the total neutral lipid content of the liver was detected at the end of the 4-hr perfusion period (Table 2). However, in the intact animal, 3 hr after an intoxicating dose of ethanol, there is an increase in the neutral lipid content of the liver.[32] This lack of increase in neutral lipid reported here, therefore, appears to support the theory proposed by Kessler and Yalovsky-Mishkin[33] and Brodie *et al.*[34] that the early accumulation of lipid in the liver after ethanol administration is the result of an increased peripheral mobilization of FFA from adipose tissue.

The pattern of distribution of radioactivity in the liver at the end of a 4-hr experiment also indicated that ethanol in some manner changed the distribution of two-carbon fragments in the neutral lipid pools of the liver. A greater proportion of the radioactivity appeared in the cholesterol fraction of the liver in both sets of control experiments and less in triglycerides, compared to experiments in which ethanol was added. These observations are consistent with the hypothesis that ethanol itself has a direct effect on metabolic pathways of the liver.

It should be emphasized again that during the oxidation of ethanol by the liver there must be an increase in the size of the two-carbon pools of the liver. This change in pool size, however, does not completely account for the changes observed in the distribution of the carbon atoms in the various lipid compartments of the liver. Ethanol directly enhanced the synthesis of fatty acids from two-carbon sources and their esterification to triglycerides. Apparently, this newly synthesized triglyceride pool was preferentially used to form the lipid portion of lipoproteins which were released into the perfusate.

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