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Effect of Prolonged Ethanol Ingestion on Hepatic Lipogenesis and Related Enzyme Activities

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1. Hepatic lipogenesis *in vivo* and the activities of enzymes associated with fatty acid synthesis in the liver were studied in rats fed for 21 days on liquid diets containing ethanol. 2. The ethanol-fed rats developed a moderate hepatic triacylglycerol accumulation during this period. When carbohydrate was replaced by ethanol in the diet, the rate of fatty acid synthesis was slower in the ethanol-fed rats on low-, medium- and high-fat diets than in the appropriate controls. However, when the fat/carbohydrate ratio was kept the same in the ethanol-fed and control rats, ethanol had no influence on the rate of fatty acid synthesis. 3. Glucose 6-phosphate dehydrogenase activity was lower in the ethanol-fed group. 'Malic' enzyme activity did not change during the ethanol treatment when the fat/carbohydrate ratio was kept unchanged. 4. The ATP citrate lyase activity was lower in the ethanol-fed rats on all diets, whereas acetyl-CoA synthetase activity was independent of the composition of the control diet, but was lower in the ethanol-fed rats, in which the concentration of the active form of pyruvate dehydrogenase was also lower. 5. It is concluded that hepatic fatty acid synthesis does not play any major role in ethanol-induced triacylglycerol accumulation. Careful design of the diets is necessary to reveal the specific effects of ethanol on the enzymes associated with lipogenesis.

Lipids accumulating in the ethanol-induced fatty liver can originate from three main sources: dietary lipids, adipose-tissue lipids and lipids synthesized in the liver itself. Those fatty acids that accumulate in the liver after prolonged ethanol intake are to a large degree dietary in origin (Lieber *et al.*, 1966; Lieber & Spritz, 1966). After low-fat diets hepatic triacylglycerols have a fatty acid composition with a predominance of endogenously synthesized fatty acids, in both human volunteers and rats fed on ethanol with adequate diets (Lieber & Spritz, 1966; Lieber *et al.*, 1966).

Stimulation of hepatic fatty acid synthesis has been suggested as one mechanism for the development of a fatty liver, at least when ethanol is given with low-fat diets (e.g., Lieber, 1973). The role of enhanced lipogenesis is supported by studies with rat liver slices (Lieber & Schmid, 1961) and with slices obtained from the livers of human alcoholics and non-alcoholics (Holmström, 1969). However, lipogenesis is sensitive to the disruption of normal intracellular structures, and thus rates of fatty acid synthesis obtained *in vitro* with broken-cell preparations or surviving slices are generally only one-fifth to one-tenth of those prevailing in the intact liver (Matthes *et al.*, 1960; Hanson & Ballard, 1967; Sabine *et al.*, 1968).

In the present investigation, incorporation of ^3H into the hepatic lipids from ^3H_2O was used to measure the rate of lipogenesis *in vivo*. This method is probably less influenced by the substrate than the methods involving ^{14}C-labelled substrates (Windmueller & Spaeth, 1966; Jungas, 1968; Salmon *et al.*, 1974). The activities of enzymes associated with acetyl-CoA metabolism and NADPH production were also measured, as were changes in some metabolite concentrations, in order to see what correlation these might have with the measured rates of fatty acid synthesis.

Materials and Methods

Chemicals

Intralipid, a stable soya-bean oil emulsion, was obtained from Vitrum AB, Stockholm, Sweden: 1 litre of this emulsion contains 200g of fractionated soya-bean oil, 12g of egg phosphatidylcholine and 25g of glycerol. Most of the fatty acids in the triacylglycerols are polyunsaturated. Aminosol, a solution of enzymically hydrolysed casein (10%, w/v, amino acids) was also acquired from Vitrum AB. Sucrose (AnalaR grade) was obtained from BDH Chemicals Ltd., Poole, Dorset, U.K., and ethanol

(94%, w/v) from the State Alcohol Monopoly (ALKO), Helsinki, Finland.

^3H_2O and cyclic [G-^{3}H]AMP were purchased from NEN Chemicals G.m.b.H., Dreieichenhain, Germany, and [U-^{14}C]acetate and [1-^{14}C]pyruvate from The Radiochemical Centre, Amersham, Bucks., U.K. The enzymes were from Sigma Chemical Co., St. Louis, MO, U.S.A., or from Boehringer G.m.b.H., Mannheim, Germany. Standard reagents were purchased from E. Merck A.G., Darmstadt, Germany, coenzymes from Boehringer, avidin 10 units/mg; 1 unit will bind 1 μg of (+)-biotin and dithiothreitol from Sigma and glucose oxidase reagent (Glox) from Kabi AB, Stockholm, Sweden.

Animals and diets

Three sets of experiments were carried out. In each set, male Long–Evans rats from the Department's own stock were fed on a standard rodent chow diet (Hankkija Oy, Helsinki, Finland) until their weights reached 110–150g. The rats were then weight-paired into two (or three) groups of five rats and housed individually in cages with wire-mesh bottoms in a room automatically illuminated between 07:00 and 19:00h. During the experimental period of 21 days the ethanol-fed group was maintained on a liquid diet with 36% of their total energy requirement provided in the form of ethanol. The formula for this diet (Table 1) was based on those described by Lieber *et al.* (1965) and Lieber & DeCarli (1970). Intralipid, Aminosol, sucrose and ethanol were used in the preparation of the diets, with salts, vitamins, choline, methionine and cystine being added in the amounts used by Lieber *et al.* (1965).

In the first set of experiments (high-fat diets), 18% of the total energy was provided as protein, 40% as fat and the rest as carbohydrate or carbohydrate plus ethanol. Voluntary intake of this diet was sufficient to maintain a daily dosage of ethanol at 12–16g/kg body wt. For the pair-fed control animals, ethanol was replaced by an equivalent (isocaloric) amount of sucrose, the rest of the diet remaining unchanged. In the second set of experiments (low-fat diets), the fat content of both the ethanol and the control diet was decreased to 5% of the total energy requirement. In the third set of experiments (medium-fat diets), two different control diets were used. In the ethanol diet and the type I control diet 10% of the total energy supply was in the form of fat, the ethanol being replaced by sucrose in the controls, as described above. In the type II control diet the fat/carbohydrate ratio was adjusted to correspond to that in the ethanol diet. It has also been shown that protein malnutrition or the amount of dietary protein may affect the hepatic lipid metabolism (Bright Gaertner & Carroll, 1967), and this may be significant in ethanol-induced liver damage (Thorpe & Shorey, 1966). In order to observe possible ethanol-

Table 1. *Composition of the diets*

All-liquid diets provide 4.2J/g. Liquid-diet-ingredient sources were as follows: protein, casein hydrolysate (Aminosol; Vitrum AB); fat, fractionated soya-bean oil (Intralipid; Vitrum AB); carbohydrate, sucrose (AnalaR grade; BDH Chemicals); ethanol, grade A (ALKO). 'Solid chow' was pellet chow for rodents (Hankkija Oy, Helsinki, Finland; manufacturer's data). All values are given as percentages of total energy.

	High-fat diet	High-fat diet with ethanol	Low-fat diet	Low-fat diet with ethanol	Medium-fat diet type I	Medium-fat diet with ethanol	Medium-fat diet type II	Solid chow
Protein	18.2	18.2	18.2	18.2	18.2	18.2	18.2	36.4
Carbohydrate	41.8	5.8	76.8	40.8	71.8	35.8	63.9	54.7
Fat	40.0	40.0	5.0	5.0	10.0	10.0	17.8	8.9
Ethanol	—	36.0	—	36.0	—	36.0	—	—

specific effects, however, the dietary protein was kept constant in the present study. The animals always received freshly prepared diets between 16:00 and 17:00h, and gained weight steadily, weighing 170–190g by the end of the experiment, with no difference in body weights between the ethanol-fed and control groups. For comparison, one further group received the standard chow diet throughout the experimental period.

Fatty acid synthesis in vivo

At the end of the 21-day period the rate of hepatic fatty acid synthesis *in vivo* was determined by measuring ^3H incorporation into long-chain fatty acids as described by Lowenstein (1975). No starvation preceded the experiments, which were always begun between 08:30 and 09:00h. The rats were injected intracardially with 1mCi of ^3H_2O as a 0.9% NaCl solution. The rats on the low-fat diet received 10mCi/animal to facilitate the subsequent analysis of the various lipid classes. Then 1h later the abdominal cavity was opened under ether anaesthesia, and samples from the liver and epididymal fat-pads were obtained by the freeze-clamp technique (Wollenberger *et al.*, 1960). Immediately afterwards, a piece of fresh liver was removed and chilled in ice. Blood samples were drawn from the inferior vena cava.

The liver samples obtained by the freeze-clamp technique were stored in a liquid-nitrogen refrigerator. A piece of the frozen liver was weighed and dropped into 19vol. of chloroform/methanol (2:1, v/v) and homogenized immediately with an Ultra-Turrax homogenizer. Fatty acids and non-saponifiable lipids were extracted and counted for radioactivity in a toluene-based scintillator (Lowenstein, 1975). The specific radioactivity of the body water of each animal was determined by counting a suitably diluted sample of plasma, and calculated as described by Windmueller & Spaeth (1966). The amount of ^3H_2O incorporated into the fatty acids was converted into nmol of the acetyl group incorporated by multiplying by 1.15. The results were expressed as nmol of acetyl group incorporated/s per g wet wt.

As pointed out by Brunengraber *et al.* (1974), the $^3H/^{14}C$ incorporation ratio for ethanol differs from that for the carbohydrates. In isolated perfused livers the contribution of ethanol or acetate can rise to approx. 25% of the total. After correction of the values for ethanol incorporation by that proportion, about 10% higher values had been obtained for lipogenesis. This difference, however, does not influence the interpretation of the present data. The same experimental design and timing has also been used to measure hepatic lipogenesis *in vivo* (Lowenstein, 1971), and from the tissue distribution of the lipogenic activity and fatty acid turnover in the liver (Romsos & Leveille, 1974; Johnson *et al.*, 1975) it can be estimated that the results obtained by this method for hepatic lipogenesis are not significantly distorted by extrahepatic processes.

Pyruvate dehydrogenase

Active pyruvate dehydrogenase (EC 1.2.4.1) was measured by determining the rate of decarboxylation of [1-^{14}C]pyruvate essentially by the method described by Hiltunen & Hassinen (1976). The method was modified by adding avidin to the reaction mixture to inhibit pyruvate carboxylase. The final assay medium contained 70mM-potassium phosphate buffer, pH8.0, 0.17mM-EDTA, 1mM-dithiothreitol, 1.5mM-NAD^+, 1.4mM-$MgCl_2$, 4.3mM-[1-^{14}C]pyruvate (0.3μCi/mol), 0.42mM-CoA, 0.03mg of avidin, 50μg of lactate dehydrogenase, 10μg of phosphotransacetylase and a tissue homogenate equivalent to 1.5–3.5mg of protein. The total volume of the reaction mixture was 1.15ml. Correction for the non-enzymic production of CO_2 was accomplished by subtracting the $^{14}CO_2$ produced when boiled homogenate was used as the sample. For the measurement of the active pyruvate dehydrogenase the freeze-clamped sample was homogenized in 10mM-potassium phosphate containing 1mM-EDTA, pH7.4, and used immediately in the assay. When the homogenate was incubated for 40min in the presence of 0.4 unit (for unit definition, see Siess & Wieland, 1972) of pyruvate dehydrogenase phosphatase/ml (isolated as described by Denton *et al.*, 1972), 10mM-$MgCl_2$ and 1mM-$CaCl_2$ to fully activate the pyruvate dehydrogenase, 19.8% of total pyruvate dehydrogenase was in the active form in the liver of chow-fed rats.

Measurement of other enzyme activities in vitro

A piece of fresh liver was homogenized in ice-cold buffer containing 0.25M-sucrose, 1mM-EDTA and 5mM-Tris/HCl, pH7.4, in a Potter–Elvehjem homogenizer fitted with a Teflon pestle. The homogenate was then centrifuged at 25000*g* and 2°C for 20min, and the supernatant used for enzyme assays without further manipulation.

The activity of 'malic' enzyme (EC 1.1.1.40) was determined spectrophotometrically by the method of Hsu & Lardy (1969). The reaction was initiated by the addition of L-malate and the increase in A_{340} was observed for 5min at 25°C. The rate of NADPH production before the addition of the substrate was subtracted. Glucose 6-phosphate dehydrogenase (EC 1.1.1.49) was assayed by a method that corrects for the 6-phosphogluconate dehydrogenase present in liver homogenates (Rudack *et al.*, 1971). ATP citrate lyase (EC 4.1.3.8) was assayed spectrophotometrically as described by Takeda *et al.* (1969). The decrease in A_{340} at 37°C was

measured. Acetyl-CoA synthetase (EC 6.2.1.1) activity was determined by measuring the incorporation of radioactivity from [1-^{14}C]acetic acid (sodium salt, sp. radioactivity 2.9Ci/mol) into non-volatile compounds, as described by Tove *et al.* (1964).

Initial rates were measured in all the foregoing enzyme assays. The enzyme activities were expressed as nmol of substrate utilized or product formed/s (nkat) per g of protein. Protein was determined by the biuret method. The biuret method was corrected for turbidity by the cyanide method of Szarkowska & Klingenberg (1963).

Measurement of metabolite concentrations

The hepatic triacylglycerols were determined by the method of Carlson (1963), and the hepatic glycogen by that of van Handel (1965). Plasma unesterified fatty acids were determined by the method of Novák (1965), and blood glucose was measured, after deproteinization with $Zn(OH)_2$ (Hjelm & de Verdier, 1963), by the method of Huggett & Nixon (1957), with a glucose oxidase reagent supplied by Kabi AB.

Cyclic AMP was extracted and assayed by the method of Gilman (1970), by using the binding protein and protein kinase inhibitor of bovine heart muscle and cyclic [^{3}H]AMP (sp. radioactivity 38.15Ci/mmol) as the indicator ligand. The binding protein and protein kinase inhibitor were extracted as described by Gilman (1970). Bound cyclic [^{3}H]AMP was assayed in a Wallac liquid-scintillation spectrometer, and the radioactivity data were processed and converted into amounts of cyclic AMP on a Honeywell 1642 computer by a program modified from that of Burger *et al.* (1972).

For the assays of CoA and its derivatives, liver samples were pulverized in a mortar under liquid N_2. Initial extraction was with ice-cold aq. 8% (v/v) $HClO_4$. Extraction was then repeated with 6% $HClO_4$, and the filtrate was neutralized to pH6.5 with 3.74M-K_2CO_3 containing 0.5M-triethanolamine hydrochloride. The $HClO_4$-insoluble tissue fraction was washed with 2% $HClO_4$ and the precipitate stored at −70°C.

CoA was assayed as described by Garland (1964), with α-oxoglutarate dehydrogenase extracted from pig heart as described by Sanadi (1969). Acetyl-CoA was determined in the same assay by the subsequent addition of phosphotransacetylase (Tubbs & Garland, 1969). Both assays were completed within 2h of the termination of the experiment. Long-chain fatty acyl-CoA was determined in the $HClO_4$-insoluble fraction. The hydrolysis was carried out at pH11 in the presence of 10mM-dithiothreitol (Williamson & Corkey, 1969), and the CoA released was assayed by the α-oxoglutarate dehydrogenase reaction.

Determination of radioactivity

All radioactivity measurements were carried out in a Wallac liquid-scintillation spectrometer. Values in d.p.s. were obtained by quenching correction by using an automatic external-standardization procedure.

Statistical treatment of results

Student's paired *t* test was used for significance calculations on the results in Tables 2–4.

Results

Hepatic lipogenesis

After high-fat and medium-fat diets the rate of fatty acid synthesis in the ethanol-fed rats was significantly lower than in the control animals (Table 2), but an intake of ethanol with 5% of total energy as fat led to a rate of incorporation equal to that in the control animals. Since the specific radioactivity of the body water was determined in each animal, variations in the hydration state did not affect the results.

To check whether the results could be caused by the large difference in carbohydrate/lipid ratio (see Volpe & Vagelos, 1973) between the ethanol and control diets, an additional control diet was used in the third experiment, in which carbohydrate and lipid were given in the same ratio as in the corresponding ethanol diet. The rats in both ethanol and type II control diet groups incorporated ^{3}H into fatty acids at an equal rate.

The radioactivity of the non-saponifiable fraction accounted for 5–15% of that incorporated into the liver fatty acids (Table 2).

Pyruvate dehydrogenase

The hepatic concentration of active pyruvate dehydrogenase was highly dependent on diet composition (Table 3). Prolonged ingestion of ethanol caused a decrease of 80% ($P<0.05$) in the active pyruvate dehydrogenase in rats on a medium-fat diet.

Cytoplasmic acetyl-CoA-synthesizing enzymes

The activity of ATP citrate lyase showed a marked adaptation to different nutritional conditions (Table 3). More than a tenfold increase was induced by decreasing dietary fat from 40 to 5% of total energy. In all cases prolonged ingestion of ethanol caused a significant ($P<0.01$) decrease in activity to about one-half the original value.

In contrast, the activity of cytoplasmic acetyl-CoA synthetase appeared to be independent of diet composition in the controls, but the addition of ethanol to the diet decreased this activity by about 20% ($P<0.05$ after both high-fat and low-fat diets).

Table 2. *Effect of prolonged ethanol ingestion on lipid synthesis in rat liver in vivo*

The animals on liquid diets (Table 1) were pair-fed for 21 days. For the measurement of the rate of lipogenesis, rats were injected with 1 mCi (in low-fat diet groups with 10 mCi) of ^3H_2O and killed 60 min later. Liver lipids were isolated and counted for radioactivity (Lowenstein, 1975). Values are given as the means ± S.E.M. for five animals, except in the low-fat diet group (as the means ± S.E.M. for three animals). Statistical significances were calculated by the paired *t* test. * $P<0.05$ and ** $P<0.01$ compared with values in rats on a control diet containing the same amount of fat. † $P<0.05$ compared with values in rats on the type II control diet.

	High-fat diet	High-fat diet with ethanol	Low-fat diet	Low-fat diet with ethanol	Medium-fat diet type I	Medium-fat diet with ethanol	Medium-fat diet type II	Solid chow
Hepatic fatty acid synthesis (nmol of C_2 units incorporated/s per g wet wt. of liver)	1.60±0.18	0.72±0.10**	4.75±0.92	3.98±0.78	4.92±0.92	3.12±0.55*	3.03±0.38	1.78±.027
Synthesis of non-saponifiable lipids (nmol of ^3H_2O incorporated/s per g wet wt. of liver)	0.14±0.01	0.05±0.01**	0.26±0.03	0.10±0.03	0.43±0.07	0.26±0.05*†	0.47±0.06	0.53±0.04

Table 3. *Effect of prolonged ethanol ingestion on enzymes associated with fatty acid synthesis in rat liver*

The samples for pyruvate dehydrogenase activity determinations were obtained by the freeze-clamp method, stored in liquid N_2, homogenized with an Ultra-Turrax homogenizer and assayed immediately for pyruvate dehydrogenase, as described in the Materials and Methods section. Other enzymes were measured in the 25000*g* supernatant. Values are the means ± S.E.M. of measurements on five animals (§ indicates a value which is the mean ± S.E.M. from three animals). * $P<0.05$ and ** $P<0.01$ compared with values in control rats. †† $P<0.01$ compared with data in rats receiving the type II control diet. n.d., Not determined.

	Activity [nmol/s (nkat) per g of protein]							
	High-fat diet	High-fat diet with ethanol	Low-fat diet	Low-fat diet with ethanol	Medium-fat diet type I	Medium-fat diet with ethanol	Medium-fat diet type II	Solid chow
Active pyruvate dehydrogenase	5±2	3±1	34± 6§	40± 6	30±15	6± 2*	11± 8	24± 3
ATP citrate lyase	28±3	13±2**	298±35	113±12**	256±43	138±22**	219±63	42± 9
Acetyl-CoA synthetase	84±4	68±7*	95± 7	76± 7*	104±28	83± 7	104±10	137±12
'Malic' enzyme	124±9	74±8**	500±49	265±40**	440±27	351±88	374±34	42± 4
Glucose 6-phosphate dehydrogenase	n.d.	n.d.	329±48	176±20**	575±128	257±25**††	446±84	12± 1

Table 4. *Effect of prolonged ethanol ingestion on metabolite concentrations in rats*

Experimental conditions were as in Table 2. Results are the means±S.E.M. from five rats in each group (§ indicates a value which is the mean±S.E.M. from three rats). * $P<0.05$ and ** $P<0.01$ compared with values in rats on a control diet containing the same amount of fat. † $P<0.05$ and †† $P<0.01$ compared with values in rats on a type II control diet. n.d., Not determined.

	High-fat diet	High-fat diet with ethanol	Low-fat diet	Low-fat diet with ethanol	Medium-fat diet type I	Medium-fat diet with ethanol	Medium-fat diet type II	Solid chow
Hepatic triacylglycerols (μmol/g wet wt.)	19.3±1.1	76.2±6.9**	27.6±1.8	45.2±9.7*	20.5±5.7	46.3±14.1*†	24.9±3.8	6.5±0.7
Hepatic glycogen (mg/g wet wt.)	26.4±3.2	13.4±2.5	27.5±1.6§	14.4±2.1	58.4±6.1	23.4±3.4**††	51.2±5.4	39.1±6.3
Blood glucose (mmol/l)	n.d.	n.d.	7.1±0.2	7.1±0.5	7.4±0.3	6.9±0.5	7.2±0.6	7.3±0.6
Hepatic cyclic AMP (nmol/g wet wt.)	1.04±0.03	0.92±0.03	1.01±0.09§	1.17±0.17	1.16±0.08	0.95±0.04*	1.04±0.08	1.20±0.16
Hepatic CoA (nmol/g wet wt.)	52.8±3.2	59.4±6.5	49.3±3.5§	61.6±6.9	52.1±3.7	52.1±5.9	43.8±5.4	49.8±2.7
Hepatic acetyl-CoA (nmol/g wet wt.)	45.2±6.3	42.7±8.1	55.0±5.9§	51.5±5.9	43.1±3.6	37.3±3.5	41.3±1.9	37.6±2.3
Hepatic long-chain fatty acyl-CoA (nmol/g wet wt.)	n.d.	n.d.	n.d.	n.d.	32.0±6.2	29.4±3.6	34.7±4.3	36.4±3.5
Plasma unesterified fatty acids (mmol/l)	0.50±0.08	0.52±0.09	0.68±0.03	0.49±0.08	0.51±0.13	0.51±0.10	0.60±0.16	0.41±0.06

NADPH-generating enzymes

To test the possible regulatory effects of the NADPH-forming enzymes on lipogenesis, especially in the light of the suggested role of lipogenesis in the disposal of reducing equivalents (Lieber, 1973), the activities of 'malic' enzyme and glucose 6-phosphate dehydrogenase were measured. Both enzymes were highly dependent on diet composition (Table 3), and their activities were significantly lower in ethanol-treated rats.

Metabolite concentrations

As shown in Table 4, rats on ethanol diets developed a marked accumulation of triacylglycerols in the liver. The degree of fatty liver, however, varied depending on the composition of the diet. A decrease in dietary fat from 40 to 10% or 5% of total energy was accompanied by a significant diminution in hepatic triacylglycerol accumulation. With diets containing low or moderate amounts of fat, however, the results were more variable. In some animals ethanol increased hepatic triacylglycerol content three- to four-fold, whereas in other rats the fat accumulation was only slightly above the control values. A similar finding has been reported by Lieber & DeCarli (1970). The triacylglycerol content in the liver did not correlate with the rate of fatty acid synthesis.

A marked depletion of hepatic glycogen occurred in the rats fed on ethanol diets for 21 days (Table 4), but there were no significant differences in blood glucose concentrations between the groups. Prolonged ingestion of ethanol decreased hepatic cyclic AMP concentrations after diets containing high or moderate amounts of fat, but no changes were noted after a low-fat diet, or after a medium-fat diet when compared with the type II control diet (Table 4). No significant changes took place in unesterified fatty acid concentrations in the plasma. Neither the dietary regimen nor the administration of ethanol had any significant effect on the hepatic acetyl-CoA, long-chain fatty-acyl-CoA or CoA concentrations (Table 4).

Discussion

The effects of ethanol on lipid synthesis have been studied by incorporation experiments with various substrates of different metabolic fates and variable numbers of metabolic control points and with differing susceptibility to isotope dilution under conditions *in vivo*. Interpretation of the somewhat contradictory reports on the effects of ethanol is also confused in many cases by the use of labelled substrates requiring acetyl-CoA synthetase, an enzyme of probably minor importance in lipid synthesis in non-ruminant mammals, except possibly during acetate production caused by the metabolism of ethanol. Moreover, only the acute effects

of ethanol on fatty acid synthesis *in vivo* have been studied by modern methods (Guynn *et al.*, 1973). Dramatic adaptive changes in the tissue content of the fatty acid synthetase complex have been demonstrated, however, and fatty acid synthetase content may vary by a factor of 20 under different nutritional conditions (Volpe *et al.*, 1973).

The use of liquid diets is unavoidable when one wishes to increase the proportional caloric intake of ethanol by small laboratory rodents to an amount comparable with that consumed by human victims of alcoholic liver damage (Lieber, 1967) and to pair-feed the animals exactly. The present results indicate, however, that the commonly accepted practice in metabolic studies of substituting only carbohydrate for ethanol in the diet (Lieber, 1967; Lieber & DeCarli, 1970) leads to effects that may not have any specific connexions with those of ethanol. As the development of ethanol-induced fatty liver is influenced by the amount of dietary fat (Lieber & DeCarli, 1970), three diets with different fat content were used initially. However, when the effects of ethanol in combination with these diets were examined, it became obvious that this type of dietary substitution was not necessarily optimal to reveal the possible specific effects of ethanol on fatty acid synthesis. Nutritional factors have marked effects on the rate of hepatic fatty acid synthesis, as demonstrated by the increased rates of lipogenesis in animals on low-fat diets and decreased rates in animals on high-fat diets (Hill *et al.*, 1958). Some evidence has also been presented of the inhibition of hepatic lipogenesis by unesterified fatty acids perfusing the liver (Mayes & Topping, 1974). It is plausible that dietary carbohydrate may effect hepatic fatty acid synthesis by means of hormonal adaptation and a resulting decrease in the concentration of plasma unesterified fatty acids (Gordon *et al.*, 1957). The effects of these mechanisms were tested by introducing the type II control diet. The results showed that this dietary manipulation eliminated the effects of ethanol on the rate of fatty acid synthesis, but did not cause any corresponding changes in the activities of the auxiliary enzymes of lipogenesis. This, however, does not explain the mechanism of the decrease in the rate of lipogenesis in the animals on diets in which only carbohydrate was replaced by ethanol. That non-specific mechanisms may be involved is suggested by the hepatic glycogen concentrations observed in the various experimental groups (Table 4). Hepatic glycogen was invariably lower in the ethanol-treated animals than in the corresponding controls. Therefore the availability of carbohydrates, not necessarily based on their precursor nature in lipogenesis (Salmon *et al.*, 1974), may also be significant *in vivo*, provided that the dietary fat content does not change.

The present results show that fatty acid synthesis and the auxiliary enzymes of lipogenesis show differential patterns of long-term control in rats on diets containing ethanol. The activities of glucose 6-phosphate dehydrogenase and citrate lyase were 50% lower in the ethanol-fed group than in the type II control group in spite of the equal rates of fatty acid synthesis. The results may indicate that these adaptive enzymes are not rate-limiting under these conditions and merely belong to the same co-ordinatively controlled group of enzymes. The 'malic' enzyme seems to stand out among these auxiliary enzymes of lipogenesis in that it is not specifically influenced by the long-term administration of ethanol. Similarly the lower content of the active form of pyruvate dehydrogenase in the ethanol-treated group is not reflected in the rate of lipogenesis and is probably due to activation of pyruvate dehydrogenase kinase by NADH (Batenburg & Olsen, 1976) and the subnormal concentration of pyruvate (Hucho *et al.*, 1972) resulting from the ethanol-induced redox change (Forsander *et al.*, 1958). In this respect the liver seems to differ from adipose tissue, in which the activity of pyruvate dehydrogenase is closely related to the rate of fatty acid synthesis (Coore *et al.*, 1971).

The acetyl-CoA synthetase activity shows very little dependence on the constituents of the control diet. However, among the dietary groups used in the present study, the lowest activities of this enzyme were always found in the ethanol-treated animals. This is surprising, since increased concentrations of acetate should occur in the latter, which could have an activating effect on the enzyme or a stimulating effect on its synthesis.

The different pattern of regulation of the auxiliary enzymes of fatty acid synthesis compared with the observed rates of fatty acid synthesis may indicate that either the fatty acid synthetase complex or acetyl-CoA carboxylase is limiting for lipogenesis.

The hepatic concentration of long-chain acyl-CoA derivatives did not show any significant changes in the three types of medium-fat diet studied in spite of changes in fatty acid synthesis. This may indicate that the allosteric regulation of acetyl-CoA carboxylase or fatty acid synthetase by fatty acyl-CoA is probably not significant under these conditions and that the changes in lipogenesis are due to adaptive changes in the concentrations of the enzymes instead.

It is possible that the observed effects of the dietary constituents are partly mediated by the peptide hormones involved in the regulation of carbohydrate metabolism, and as a probe for their action the hepatic concentration of cyclic AMP was measured. Acetyl-CoA carboxylase has been shown to be subject to covalent interconversions (Carlson & Kim, 1973), but the possible involvement of cyclic AMP is

still in dispute (Raskin *et al.*, 1974). The present results suggest that cyclic AMP is not involved in the regulation of fatty acid synthesis in the liver.

The well-known accumulation of triacylglycerols in the liver during the long-term administration of ethanol has no correlation with hepatic lipid synthesis. In acute experiments *in vivo* and in isolated perfused livers, ethanol has been shown to have no effect on lipid synthesis (Guynn *et al.*, 1973; Brunengraber *et al.*, 1974). As pointed out above, the enzymes involved in lipid synthesis show a striking adaptive behaviour, so that the results of acute experiments are not applicable to the effects of chronic administration of ethanol *in vivo*. Comparison of the ethanol-fed and type II control animals in the present study suggests that long-term administration of ethanol produces no adaptive response in fatty acid synthesis under conditions involving developing hepatic steatosis. At the same time, as indicated by the unaltered plasma unesterified fatty acid concentration, major changes in peripheral lipolysis probably play no significant part in the development of chronic ethanol-induced fatty liver. By exclusion, the results of the present paper indicate rather that changes in the hepatic disposal of fatty acids may be the main determining factors for this disturbance.

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