Biochemical Aspects Associated with an Ethanol-Induced Fatty Liver

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Isocaloric replacement of either the fat or carbohydrate content of the diet by ethanol (36% of the total caloric intake) produced fatty infiltration of the liver in rats. The increase in hepatic triglyceride content was associated with a decrease in both ATP and glycogen contents. Increased activity of mitochondrial Mg^{2+} -stimulated adenosine triphosphatase paralleled the increase in the free P_i content of the liver homogenate. During the regression of the fatty liver, glycogen contents returned to normal within 24 h of the removal of ethanol from the diet. Not until the third day after the withdrawal of ethanol had the Mg^{2+} -stimulated adenosine triphosphatase activity and free P_i content of the homogenate returned to normal. A slow regression of the triglyceride content from the liver occurred and by the fifth day both ATP and triglyceride concentrations had returned to the values observed in the rats given the liquid control diet.

Fatty infiltration of the liver occurs in rats after the prolonged ingestion of ethanol in spite of a nutritionally adequate liquid diet (Lieber & Rubin, 1969). Many investigators have shown that alterations in lipid metabolism can produce marked hepatic triglyceride accumulation. However, alterations in lipid metabolism do not, as yet, adequately explain the ethanol-induced fatty liver (Isselbacher & Greenberger, 1964a,b; Lieber & Rubin, 1969). A group of experiments were designed to investigate if other metabolic disturbances in the liver were produced by chronic ethanol ingestion and if these metabolic alterations could be correlated with the marked accumulation of hepatic triglyceride.

EXPERIMENTAL

Materials and methods

Animals. Male albino Sprague-Dawley rats (100-120 g; Carworth Inc., New City, N.Y., U.S.A.) were divided randomly into four groups. Groups 1 and 2 were used as controls and groups 3 and 4 as experimental animals. During the experimental period, group 1 was maintained on Purina Laboratory Chow and group 2 on a liquid diet (Table 1), as described by Lieber, Jones, Mendelson & DeCarli (1963) and Lieber & Spritz (1966), except that the source of fat was corn oil. This was used as its fatty acid composition (Kritchevsky & Rabinowitz, 1966) is similar to that of Purina Chow (Scheig & Isselbacher, 1965). Animals in groups 3 and 4 were maintained on the normal liquid diet for 10 days, then ethanol was added to partially replace isocalorically either the fat (group 4) or the carbohydrate (group 3) at 20% of the total caloric intake. After 10 days, the ethanol concentration was increased to 36% of the caloric intake. After 20 days at this ethanol

concentration animals of groups 3 and 4 were killed, with an adequate number of control animals. Some animals of group 3 were now designated as group 5 and were given the normal liquid diet for 1, 3, 5 or 6 days before being killed. All animals had free access to water and those on the liquid diets were given fresh diet daily in Richter tubes. The caloric intake was found to be adequate during the experimental period, and no deaths occurred. The results presented are the mean values from experiments carried out at two different times.

Experimental procedure. The rats were not starved before being killed but in each experiment they were killed at the same time of day. At the time of death, the rat was stunned by a blow on the head; the abdomen was quickly opened, and a portion of the right median lobe of the liver was removed and dropped immediately into liquid N_2 . This frozen piece was used for ATP, glycogen and lipid analyses. Another portion of the liver was taken for examination by light microscopy and electron microscopy. The remaining liver lobes were perfused with ice-cold 0.25 M-sucrose, removed, weighed and then homogenized in ice-cold 0.25 M-sucrose (1:9, w/v). Mitochondria were isolated from the homogenate by the method of Schneider & Hogeboom (1950).

Biochemical analyses

Mg^{2+} -stimulated ATPase*. Mitochondrial Mg^{2+} -stimulated ATPase activity (Pullman, Penefsky, Datta & Racker, 1960) was measured (5 min incubations) in duplicate (1-2 mg of mitochondrial protein). The P_i released was determined by the method of Mozersky, Pettinatti & Kolman (1966).

P_i . Liver homogenate (2-3 mg of protein) and mitochondria (2-4 mg of protein) were taken for determination

*Abbreviation: ATPase, adenosine triphosphatase.

Table 1. *Composition of chow and liquid diets*

Liquid diet ingredient sources were as follows: casein hydrolysate (enzymic) (Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.); fat, Lipomul (10g of corn oil/15ml; Upjohn Co. Ltd., Kalamazoo, Mich., U.S.A.); carbohydrate, domestic sugar; vitamin diet fortification mixture (General Biochemicals Corp., Chagrin Falls, Ohio, U.S.A.); Tween 80 (Fisher Scientific Co. Ltd., Montreal, Que., Canada). The caloric value was 1 kcal/ml. Values are given as percentages.

	Group 1	Group 2	Group 3	Group 4
Protein	23.4	19.4	19.4	20.0
Carbohydrate	50.6	40.8	4.8	29.0
Fat	3.8	39.8	39.8	15.0
Ethanol	0.0	0.0	36.0	36.0
Residue	22.2	0.0	0.0	0.0

Table 2. *Effects of ethanol on protein and DNA contents of the liver*

Mean values are given $\pm$ S.E.M.

	No. of animals	Protein (mg/g wet wt. of liver)	Mitochondrial protein (mg/g wet wt. of liver)	DNA (mg/g wet wt. of liver)
Group 1	14	238 $\pm$ 6	40.8 $\pm$ 1.6	1.99 $\pm$ 0.06
Group 2	7	219 $\pm$ 7	38.1 $\pm$ 1.6	1.94 $\pm$ 0.07
Group 3	11	239 $\pm$ 6	38.7 $\pm$ 1.6	1.91 $\pm$ 0.06
Group 4	6	281 $\pm$ 9*†	42.2 $\pm$ 1.3	1.52 $\pm$ 0.05*†
Group 5				
Days‡				
1	5	229 $\pm$ 12	35.4 $\pm$ 1.0*†	1.65 $\pm$ 0.12*†
3	4	226 $\pm$ 9	30.9 $\pm$ 2.4*†	1.33 $\pm$ 0.07*†
5	5	217 $\pm$ 11	30.9 $\pm$ 2.0*†	1.58 $\pm$ 0.21*†
7	4	239 $\pm$ 11	40.8 $\pm$ 4.7	1.23 $\pm$ 0.04*†

* Statistically significant with respect to group 1 at $P = 0.01$.

† Statistically significant with respect to group 2 at $P = 0.01$.

‡ Time of removal of ethanol from the diet.

of free P_i (Penniall, 1966). However, the phosphomolybdic acid was measured in the unreduced form (Mozersky *et al.* 1966) after extraction with 2-methylpropan-1-ol-benzene (1:1, v/v).

Glycogen. A portion of the frozen liver was weighed and then dropped into 30% (w/v) KOH at 100°C. The glycogen was precipitated and then hydrolysed to glucose (Somogyi, 1934). The glucose concentration was determined by using the Glucostat kit (Worthington Biochemical Corp., Freehold, N.J., U.S.A.).

Lipids. Another portion of the frozen liver was weighed and homogenized in redistilled chloroform-methanol (2:1, v/v). The lipids were extracted, Folch-washed (Folch, Lees & Sloane-Stanley, 1957) and used for triglyceride analysis (Van Handel & Zilversmit, 1957).

ATP. Another portion of the frozen liver was weighed and homogenized in ice-cold 0.6M-HClO₄ (1:10, w/v). The protein precipitate was spun off and the supernatant was neutralized with 2.2M-K₂HPO₄. ATP was determined by using the Boehringer ATP Biochima test combination [Boehringer (Mannheim) Corp., New York, N.Y., U.S.A.] adapted for use with the Turner fluorimeter. Analysis was carried out within 1 h of extraction and care

was taken to keep the samples ice-cold. Proportionality was obtained for three different portions of each sample and for a standard ATP solution (1–10nmol). The standard was run daily.

Protein and DNA. Total hepatic and mitochondrial protein contents were determined by the method of Lowry, Rosebrough, Farr & Randall (1951) and DNA by the method of Dische (1930).

All spectrophotometric measurements were made on a Coleman model 6C Junior Spectrophotometer unless stated otherwise.

Statistical analysis. The data were analysed statistically by using Student's *t* test (Goldstein, 1964).

Chemical sources. ATP (disodium salt) was obtained from Sigma Chemical Co., St Louis, Mo., U.S.A. All other reagents, certified A.C.S., were obtained from Fisher Scientific Co. (Montreal) Ltd., Montreal, Que., Canada, unless stated otherwise.

RESULTS

The hepatic protein contents (Table 2) were similar in groups 1, 3 and 5, but that of group 2 was

Table 3. *Effects of ethanol on triglyceride, ATP and glycogen contents of the liver*Mean values are given $\pm$ S.E.M.

	No. of animals	Triglyceride (mg/g wet wt. of liver)	ATP (μ mol/g wet wt. of liver)	Glycogen (mg/g wet wt. of liver)
Group 1	14	11.2 $\pm$ 0.9	3.16 $\pm$ 0.15	54.6 $\pm$ 3.1
Group 2	7	21.9 $\pm$ 1.7*	2.66 $\pm$ 0.05	51.5 $\pm$ 7.4
Group 3	11	60.5 $\pm$ 4.3*†	1.50 $\pm$ 0.09*†	16.8 $\pm$ 1.5*†
Group 4	6	41.7 $\pm$ 3.4*†	1.89 $\pm$ 0.10*†	30.6 $\pm$ 2.8*†
Group 5				
Days‡				
1	5	53.5 $\pm$ 3.4*†	1.61 $\pm$ 0.10*†	49.1 $\pm$ 8.8
3	4	37.6 $\pm$ 1.2*†	1.49 $\pm$ 0.10*†	72.2 $\pm$ 3.8*†
5	5	25.3 $\pm$ 1.7	2.59 $\pm$ 0.21	50.2 $\pm$ 6.6
7	4	28.9 $\pm$ 1.1	2.53 $\pm$ 0.08	65.7 $\pm$ 2.2

* Statistically significant with respect to group 1 at $P = 0.01$.† Statistically significant with respect to group 2 at $P = 0.01$.

‡ Time of removal of ethanol from the diet.

slightly but not significantly lower. In group 4 the protein content was significantly higher than that of the controls. Mitochondrial protein contents (Table 2) of the diet groups 3 and 4, and the control groups 1 and 2, were not significantly different. However, in group 5, during the regression of the fatty liver, the mitochondrial protein content was significantly lower than that of the controls for up to 5 days. By 7 days after the withdrawal of ethanol the protein contents of the mitochondria were within the control range.

The hepatic DNA contents (Table 2) of groups 1, 2 and 3 were not found to differ significantly. However, a significant decrease in DNA content of the liver was noted in group 4 given the low-fat diet and in group 5 given the high-fat diet from which ethanol had been removed.

The hepatic triglyceride content (Table 3) of group 2 was significantly greater than that of group 1. With chronic ingestion of ethanol the hepatic triglyceride accumulation in groups 3 and 4 was enhanced to a value two to three times that of group 2. In group 3 given the high-fat diet the triglyceride content was significantly greater ($P = 0.01$) than that in group 4 given the low-fat diet. The release of triglyceride from the liver was slow as the triglyceride contents did not return to normal until 5 days after ethanol had been withdrawn from the diet.

In group 2 given the liquid control diet the ATP content was significantly lower ($P = 0.01$) than that in group 1 given the chow diet (Table 3). However, both groups 3 and 4 had ATP contents that were significantly lower than that of group 2. The ATP content for group 4 was slightly greater than that

for group 3 ($P = 0.05$). At 1 and even 3 days after the removal of ethanol from the diet the ATP content was still depressed. By the fifth day the ATP content had returned to values similar to those observed in the animals given the liquid control diet.

The glycogen contents (Table 3) of the livers of groups 1 and 2 were the same. However, in groups 3 and 4 there was a marked depletion of glycogen, which was confirmed histologically (J. Lough, J. E. C. Walker & E. R. Gordon, unpublished work). The hepatic glycogen content of group 3 was significantly lower ($P = 0.01$) than that of group 4. A dramatic change was observed in the glycogen stores, in that 24h after the removal of ethanol from the diet the glycogen content had returned to normal. By the third day, the glycogen concentration in the liver was significantly greater than that in either control group. However, by the fifth and seventh days the levels had returned to normal.

In the ethanol-induced fatty liver (group 3) mitochondrial Mg^{2+} -stimulated ATPase activity (Table 4) was significantly increased over that of groups 1 and 2. With removal of ethanol from the diet the Mg^{2+} -stimulated ATPase activity was observed to decrease to the control values within 3 days.

No significant change in the P_i contents (Table 4) occurred in the mitochondrial fractions of groups 1, 2, 3 and 5. However, the free P_i content of the liver homogenate of group 3 was greater than that of either control group. Paralleling the change in Mg^{2+} -stimulated ATPase activity, within 3 days of ethanol removal from the diet the free P_i contents returned to normal.

Table 4. *Effects of ethanol on the free P_i content and Mg^{2+} -stimulated ATPase activity of the liver*

Mean values are given $\pm$ S.E.M.

	No. of animals	Free P_i (mg/g wet wt. of liver)		Mg^{2+} -stimulated ATPase (mg of P_i released/h per g equiv. wet wt. of liver)
		Total	Mitochondrial	
Group 1	14	0.72 ± 0.04	152 ± 6	11.0 ± 0.7
Group 2	7	0.76 ± 0.06	162 ± 6	12.3 ± 0.8
Group 3	11	$1.41 \pm 0.10^{*†}$	147 ± 8	$16.4 \pm 1.1^{*†}$
Group 5				
Days†				
1	5	$1.02 \pm 0.07^{*†}$	128 ± 7	$17.0 \pm 2.5^{*†}$
3	4	0.89 ± 0.15	131 ± 10	10.8 ± 1.1
5	5	0.79 ± 0.08	133 ± 11	11.9 ± 0.4
7	4	0.80 ± 0.09	156 ± 9	12.1 ± 1.8

* Statistically significant with respect to group 1 at $P = 0.01$.

† Statistically significant with respect to group 2 at $P = 0.01$.

‡ Time of removal of ethanol from the diet.

DISCUSSION

A fatty liver was produced in rats maintained on a nutritionally adequate diet in which ethanol provided 36% of the total caloric intake for a period of 20 days. These results support previous findings by Lieber *et al.* (1963) and Lieber & Spritz (1966). In the present experiments, associated with hepatic triglyceride accumulation there was a marked change in the glycogen and ATP contents, which suggests a disturbance in carbohydrate metabolism. Alterations in the morphology of cellular components (J. Lough, J. E. C. Walker & E. R. Gordon, unpublished work) and in the activity of a mitochondrial membrane-bound enzyme indicate that hepatic metabolism is disturbed by the chronic ingestion of ethanol. The biochemical changes during the regression of the fatty liver also indicate that the effects of ethanol are extensive. These studies indicate that an ethanol-induced fatty liver is not due to a simple alteration in lipid metabolism.

There is no question that the composition of the diet influences liver metabolism (Anonymous, 1965). In fact significant differences were observed between the chow and liquid control groups (Table 3). However, in these experiments group 2, given the liquid control diet, is the valid control. Although the dietary fat content appears to influence the degree of steatosis, a fatty liver has been produced in rats when ethanol isocalorically replaced either the carbohydrate or the fat content of the diet (Table 3; Lieber & Rubin, 1969). Therefore an alteration in the proportions of dietary carbohydrate, fat and protein by ethanol cannot fully explain an ethanol-induced fatty liver as suggested by Hartroft & Porta (1968).

It is unlikely that a simple enhancement of the dietary choline requirements by ethanol (Klatskin, Krehl & Conn, 1954) would explain the fatty infiltration of the liver, since it has been shown that a 20-fold increase in dietary choline concentration did not fully protect against the ethanol-induced steatosis (Lieber & DeCarli, 1966). In addition, fatty livers induced by choline deficiencies are not associated with lowered ATP contents (Simon, Scheig & Klatskin, 1969).

During starvation the hepatic ATP content is lowered (Kowadlo & Laron, 1963), the glycogen stores are markedly depleted (Tzur, Tal & Shapiro, 1964; Rubinstein & Scholefield, 1969) and lipid metabolism is affected (Bortz & Lynen, 1963; Nørby, 1965; Scheig & Isselbacher, 1965). Consequently in the present experiments the animals were not starved before being killed.

Since the water content of the livers did not differ significantly in the various groups ($P = 0.01$), it is evident that changes in the protein and the DNA contents of the livers of groups 4 and 5 (Table 2) reflect metabolic changes in the liver; consequently in these experiments all data are expressed per g wet wt. of liver.

Prolonged ingestion of ethanol results in a marked accumulation of hepatic triglyceride, which was, in these experiments, associated with a marked depletion in glycogen (Table 3). These findings are in agreement with previous findings (Clarke & Evans, 1960; Mirone, 1965). Although the carbohydrate content of the diet of group 4 was greater than that of the diet given to group 3 (Table 1), chronic ingestion of ethanol still resulted in the depletion of hepatic glycogen (Table 3). The rapid increase in glycogen stores after the removal of

ethanol from the diet (Table 3) indicates an immediate effect of ethanol on hepatic metabolism. As triglyceride contents are much slower to return to control values than are those of glycogen, the rapid return of glycogen to normal therefore cannot be completely explained by a lowered demand for glycerol 1-phosphate. In conjunction with the rapid change in glycogen contents, the $[NADH]/[NAD^+]$ ratios have been observed to return to normal within 16h of removal of ethanol from the diet (H. Kalant, personal communication). Therefore it appears that ethanol or one of its immediate metabolites markedly influences carbohydrate metabolism, and these changes appear to precede the disturbances in lipid metabolism.

There is a wide variation in the hepatic ATP contents ($0.278\text{--}1.49\mu\text{mol/g}$ wet wt. of liver) reported in the literature for chow-fed rats (French, 1966; Hymans & Isselbacher, 1964; Marchetti, Ottani, Zanetti & Pudden, 1968). However, Schenker & O'Donnell (1965) have stressed that rapid freezing techniques and rapid analysis after extraction are prerequisites for accurate ATP determinations. As liquid N_2 was used and the samples were analysed within 1h after extraction, this could explain the higher ATP values we observed for chow-fed rats (Table 2).

Lowered ATP contents (Table 3) in an ethanol-induced fatty liver may be the result, not only of increased utilization, but also of decreased synthesis. Both fatty acid and triglyceride synthesis require ATP, and therefore an enhancement of these two processes by ethanol (Lieber & Schmid, 1961; Mendenhall, Bradford & Furman, 1969; Zakim, 1965) may result in a decrease of available ATP. In fact it was observed that, with chronic ethanol ingestion, the triglyceride accumulation and the ATP depletion were greater in group 3 given the high-fat diet than in group 4 given the low-fat diet.

If the major source of energy for hepatic metabolism is fatty acid oxidation (Fritz, 1961), then ethanol-depressed fatty acid oxidation (Lieber, 1967; Scheig & Isselbacher, 1965) may also contribute to lowered ATP contents. ATPase activity may also decrease ATP contents and increase P_i contents, since Racker (1965) indicates that ATPase activity of intact mitochondria represents a reversal of oxidative phosphorylation.

Alteration in the metabolism of ATP in the fatty liver was shown by the increased activity of Mg^{2+} -stimulated ATPase (Table 4), increased free P_i contents in the homogenate (Table 4) and decreased ATP contents (Table 3). Consequently the free P_i contents in the homogenate may be correlated with ATP turnover and usage. However, in the regression of the ethanol-induced fatty liver, mitochondrial Mg^{2+} -stimulated ATPase activity and free P_i contents of the homogenate return to normal

within 3 days, but the ATP content does not return to normal until the fifth day. A gradual decrease in hepatic triglyceride concentration was observed after the removal of ethanol from the diet (Table 3). The change in ATP content was not gradual, but appeared to increase abruptly between 3 and 5 days after ethanol was withdrawn from the diet (Table 3). The metabolic factors contributing to lowered ATP contents are complex, but appear to be associated with the alteration in triglyceride metabolism.

The results presented clearly indicate that the effects of ethanol or one of its immediate metabolites on hepatic metabolism are widespread. The rapid changes in glycogen content, in contrast with the changes in those of the other hepatic constituents after the withdrawal of ethanol from the diet, emphasizes the fact that the metabolic pathways in the liver are differently affected by ethanol. There seems to be a direct relationship between energy metabolism and the fatty liver; however, these experiments do not indicate the extent of the relationship.

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