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ATP METABOLISM IN AN ETHANOL INDUCED FATTY LIVER

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Abstract—A marked decrease in the hepatic concentration of ATP lowers the phosphate potential of the liver of rats fed ethanol for prolonged periods of time. Either a decreased synthesis or an increased demand could account for this change in ATP. The experiments presented here were designed to investigate both possibilities. Changes in the utilization of ATP were assessed by measuring the activity of the $[Na^+ + K^+]$-activated ATPase system, and changes in the synthesis of ATP by the activity of the adenine nucleotide translocase system. Since the level of long-chain CoA derivatives of fatty acids regulate the translocation of ADP into mitochondria, their total cellular content and mitochondrial level were also determined. The experiments were conducted on male Sprague–Dawley rats (275–300 g) maintained on a liquid diet having 36 per cent of the caloric intake as ethanol. After 2 weeks, the experimental animals had a fatty liver. This change was not associated with any significant alteration in the ATP content of the liver or in the activity of the adenine nucleotide translocase system. After 4 weeks, the excess of neutral lipid in the liver still persisted, but was associated with a marked increase in the level of long-chain CoA derivatives of fatty acids. The level of ATP in the liver was only 50 per cent of normal, and the rate of translocation of ADP into the mitochondria was decreased. The activity of the adenine nucleotide translocase system could be restored to normal values if the long-chain CoA derivatives of fatty acids were removed from the surface of the mitochondrial membrane by treatment of the preparation with defatted albumin. At this time there was only a slight (15%) enhancement of the $[Na^+ + K^+]$-activated ATPase system. With the removal of ethanol from the diet the ATP level returned to normal rather quickly. Changes in the level of hepatic ATP correlated well with the activity of the adenine nucleotide translocase system but did not parallel changes in the activity of the Na^+ pump system. These findings indicate that the dominant feature leading to the decrease in the $(ATP/ADP \times P_i)$ ratio is decreased synthesis of ATP.

The state of phosphorylation of the hepatic cytoplasm, as measured by the ratio $(ATP/ADP \times P_i)$, is altered in different ways by the consumption of ethanol, depending on its mode of administration [1–3]. In contrast to an increase noted following a single dose of ethanol [1], prolonged consumption depresses the ratio below normal levels [2, 3]. The simultaneous observation of a minor elevation in the phosphate level, and an unchanged ADP value under these circumstances has indicated that a sizeable drop in hepatic ATP is responsible for the decrease in the $(ATP/ADP \times P_i)$ ratio [2, 3]. Two different mechanisms have been proposed to explain this decrease since either an increased utilization or a decreased synthesis could account for the observations. Evidence supporting the first mechanism has been presented by Israel's group, who claimed that the activity of the Na^+ pump is altered by the chronic ingestion of ethanol, causing an increased demand for ATP which exceeds the cells' capacity for synthesis [3]. Other investigators, however, have noted that mitochondria in an ethanol-induced fatty liver exhibit marked alterations in their ultrastructure, composition, and function, suggesting that the synthesis of ATP is depressed in these animals [3–7].

The experiments presented here were deisgned to investigate both possibilities. First, the length of time that ethanol must be present in the diet to cause a change in ATP metabolism was ascertained. Having established this, the changes in the utilization of ATP were assessed by measuring the activity of the $[Na^+ + K^+]$-activated ATPase system, and changes in the synthesis of ATP by the activity of the adenine nucleotide translocase system as this step regulates the process of oxidative phosphorylation [8, 9]. The total cellular content and mitochondrial level of long chain CoA derivatives of fatty acids was also determined since the level of these derivatives regulate the translocation of ATP into the mitochondria [10]. Since the ATP content of the liver returns to normal rather quickly upon removal of ethanol from the diet, the opportunity presented itself to examine these two pathways in response to a sudden withdrawal of ethanol from the diet. This was carried out by measuring changes in these systems as a function of time after the removal of ethanol from the diet.

METHODS AND MATERIALS

Animals. Male Sprague–Dawley rats (220–250 g) from Canadian Breeders, Quebec, Canada, were divided into two groups, the controls and experimental animals. Each control animal was maintained on a liquid diet [3] and pair-fed with an experimental animal. After 10 days, ethanol was introduced into the diet at a level of 20 cal % at the expense of an equivalent number of calories of carbohydrate. After a further 10 days the ethanol concentration was increased to 36 cal % at the expense of further carbohydrate. To save on routine analysis, in some of the

experiments, the regime of pair-feeding was changed so that each control animal was given the average daily intake of two experimental animals [3]. Animals were killed after 14 or 28 days on this regime, except for one group of experimental animals which was returned to the normal liquid diet and maintained thus for a further 1, 3 or 5 days, before being killed. All animals had free access to water and those on the liquid diets were given fresh diet in Richter tubes each day. The caloric intake was found to be adequate during the experimental period and no deaths occurred.

Experimental design. The rats were not starved before being killed and in each experiment were sacrificed at the same time of the day. The abdomen was quickly opened. Within 8 sec, a small portion of the liver was removed and dropped into liquid N_2, (or a portion of the liver was frozen *in situ*). The remaining lobes were used for the isolation of hepatic mitochondria, which were prepared by differential centrifugation according to the method of Johnson and Lardy [11].

Biochemical analysis. The piece of frozen liver was stored in liquid N_2 until the assays were performed. At the time, a portion of the frozen liver was weighed and homogenized in 0.6 M $HClO_4$ (1:5 w/v). The protein precipitate was spun off and the supernatant neutralized with 2.2 M $K_2H\ PO_4$ and utilized immediately for the determination of the hepatic levels of ATP, by the method outlined by Greengard [12]. Hepatic and mitochondrial protein levels were determined by the method of Lowry *et al.* [13]. Another portion of the frozen liver was weighed and homogenized in redistilled chloroform–methanol (2:1 v/v). The lipids were extracted and washed as outlined by Folch [14] and subsequently used for triglyceride analysis by semi-micro procedure outlined by Chernick [15].

Long-chain acyl CoA derivatives of fatty acids. The level of these derivatives of fatty acids was determined in the insoluble perchloric acid precipitate, prepared as outlined by Williamson and Corkey [16]. The amount of CoA formed after hydrolysis was estimated by the method of Kondrup and Grunnet [17].

Adenine nucleotide translocase. The activity of this system was measured by determining the translocation of [^{14}C]ADP into the mitochondria according to the method described by Lerner [18]. The basic reaction mixture of 40 mM Tris-HCl, pH 4.4; 100 mM KCl; 1.0 mM $MgCl_2$, and 0.5–4.0 mg of mitochondrial protein in a vol. of 1.0 ml was incubated for 4 min at 25°. The reaction was initiated by the addition of 0.125 μM to 0.200 μM [^{14}C]ADP (sp. act. ranged from 100–200 mCi/mmole). After 2 min the reaction was terminated with atractyloside. The reaction mixture was then centrifuged for 10 min at 500 *g* and the supernatant removed by suction. The pellet was washed and then allowed to solubilize overnight in N.C.S. reagent (Amersham Searle Co.). Aquasol (New England Nuclear) (15.0 ml) was added and the samples counted in a Packard Tri-Carb scintillation spectrometer model 2425. The efficiency of counting was determined by using an external standard and all the results were expressed in disintegrations per min. The reaction was carried out on four different aliquots of the mitochondria preparations. The simple diffusion of [^{14}C]ADP into the mitochondria was measured in each assay by adding atractyloside (5.0 mM) at zero time. Only if the results were linear were the data used to compute the translocation of [^{14}C]ADP into the mitochondria. Since the radioactivity added to the system was not always the same, the data were normalized by assigning a value of 100 to the activity obtained in the pair-fed liquid control group of the day and by expressing the data obtained for the experimental groups as a percentage of the control group's activity.

[$Na^+ + K^+$] activated ATPase. The [$Na^+ + K^+$] ATPase and Mg^{2+} ATPase activities were determined on liver homogenates as outlined by Ismail Beigi and Edelman [19]. The inorganic phosphate liberated was determined by the method of Penniall [20] exeept that the phosphomolybdic acid was measured in the unreduced form after extraction with 2 methyl propan-1-ol–benzene (1:1 v/v) [21].

Removal of long-chain acyl CoA derivatives. Mitochondria preparations were incubated at 0° for 5 min in an isolation media containing 1% defatted albumin. The suspension was then centrifuged at 15,000 *g* for 5 min, the supernatant discarded and the mitochondrial preparation re-suspended in 0.25 M sucrose.

RESULTS

After two weeks on a diet containing ethanol at a level of 36 cal % the animals exhibited fatty livers. The average triglyceride content in the ethanol-fed animals was 25.60 ± 2.4 mg/g wet wt of liver (n = 13) compared to 10.01 ± 1.0 (n = 8) in the controls. No significant change was observed in the hepatic level of ATP or in the activity of the adenine nucleotide translocase system at this time (Table 1).

After 4 weeks on the liquid diet the ethanol-fed animals still exhibited fatty livers (Table 2). At this time, however, the ATP content of the liver was only 50 per cent of the control value. This change was associated with a decreased activity in the adenine nucleotide translocase system (35 per cent of the control value– Table 3) and an increase in the content of long-chain CoA derivatives of fatty acids in both the total liver and in the mitochondrial preparations

Table 1. The level of ATP and the activity of the adenine nucleotide translocase system in the liver of rats chronically fed ethanol*

Treatment	ATP (μmoles/g wet wt of liver)†	Adenine nucleotide translocase activity units‡
Control	2.53 ± 0.14 (5)	100 (5)
Ethanol	2.36 ± 0.10 (12)	86.2 ± 7.7 (8)

* Rats maintained on the liquid diet containing ethanol at a level of 36 cal % for 2 weeks.

† Values given are mean ± S.E.M.

‡ Units are defined in the text.

Figures in parentheses indicate number of animals in the group.

Table 2. The hepatic content of long-chain acyl CoA derivatives of fatty acids and triglycerides in an ethanol-induced fatty liver*

Treatment	Triglycerides† mg/g wet wt	Long chain acyl CoA derivatives of fatty acids† Total liver nmoles/g wet wt	Long chain acyl CoA derivatives of fatty acids† Mitochondria nmoles/5.0 mg protein
Control	9.2 ± 0.3 (12)	38.5 ± 2.6 (12)	2.11 ± 0.13 (12)
Ethanol	35.6 ± 2.3‡ (8)	55.5 ± 2.7‡ (8)	2.89 ± 0.15§ (8)
Days off ethanol			
1	26.4 ± 0.3‡ (4)	39.5 ± 8.9 (4)	2.03 ± 0.13 (4)

* Rats maintained on the liquid diet containing ethanol at a level of 36 cal % for 4 weeks.

† Values given are mean ±S.E.M., and the numbers in parentheses denote the number of animals in the group.

‡ Statistically significant with respect to Control $P < 0.001$.

§ Statistically significant with respect to Control $P < 0.01$.

(Table 2). The consumption of ethanol appeared to enhance the activity of the $[Na^+ + K^+]$ activated ATPase system. However this increase was not highly significant since the levels observed in the ethanol-fed rat were only 15 per cent greater than those observed in the pair-fed controls.

Upon withdrawal of ethanol from the diet, both the level of ATP in the liver, and the activity of the mitochondrial adenine nucleotide translocase system increased slowly and by day 5 had returned to control levels (Table 3). A further increase was observed in the activity of the $[Na^+ + K^+]$ activated ATPase system on day 1, but by day 3 the activity of this system had decreased to a level that was slightly lower than that observed in the controls (Table 4). One day after the removal of ethanol from the diet, the level of long-chain CoA derivatives of fatty acids in the liver and in the mitochondrial fraction had returned to levels observed in the controls. Treatment of the mitochondrial preparations with defatted albumin lowered the concentration of long-chain CoA derivatives of fatty acids to 185 ± 18.6 ($n = 5$) and 211 ± 24.2 ($n = 5$) pmoles/mg of mitochondrial protein in preparations from control and ethanol-fed rats respectively. The effect of this treatment on the translocation of ADP into the membrane of the mitochondria is presented in Table 5. The rate of translocation of ADP into the mitochondria was increased by the removal of the derivatives of fatty acids. In fact this treatment abolished the differences between the ethanol-treated animals and their pair-fed controls. This effect was observed in all mitochondiral preparations isolated from the ethanol-fed animals, and also in preparations from animals which had ethanol removed from the diet (Table 5). Varying concentrations of palmityl CoA were added to the albumin treated mitochondrial preparation from the ethanol-fed and control animals. In both preparations, a 50 per cent inhibition of the translocation of $[^{14}C]$ADP into the mitochondria was noted with concentrations of palmityl CoA ranging from 2.0 to 3.0 nmoles/mg

Table 3. ATP content and adenine translocase activity in an ethanol-induced fatty liver*

Treatment	ATP (μmoles/g wet wt)†	Adenine nucleotide translocase activity units‡
Controls	2.62 ± 0.13 (17)	100 (22)
Ethanol	1.37 ± 0.08§ (10)	35.4 ± 5.5§ (14)
Days off ethanol		
1	1.61 ± 0.10§ (5)	44.0 ± 7.4§ (8)
3	1.74 ± 0.25§ (5)	66.3 ± 13.3§ (10)
5	2.55 ± 0.18 (6)	93.3 ± 19.9 (12)

* Rats maintained on the liquid diet containing ethanol at a level of 36 cal % for 4 weeks.

† Values given are mean ±S.E.M., and the numbers in parentheses denote the number of animals in the group.

‡ As defined in the text.

§ Statistically significant with respect to Control, $P < 0.01$.

Table 4. ATPase activity of liver homogenate from rats chronically red ethanol*

	Mg^{2+} ATPase μmole P_i/hr/mg of protein	$[Na^+ + K^+]$ATPase μmole P_i/hr/mg of protein
Controls	8.79 ± 0.61 (12)	1.37 ± 0.08 (12)
Ethanol	8.46 ± 0.81 (10)	1.56 ± 0.13 (10)
Days off ethanol		
1	9.12 ± 1.01 (10)	1.79 ± 0.28† (10)
3	7.6 ± 0.70 (6)	1.25 ± 0.19 (6)
5	8.43 ± 0.72 (2)	1.19 ± 0.04 (3)

* Values given are means ±S.E.M., and figures in parentheses indicate the number of animals per group.

† Statistically significant with respect to control, $P < 0.01$.

Table 5. [^{14}C]ADP translocation into mitochondria DPM/mg of protein*

Treatment of mitochondria	Control	Ethanol
	17.199 ± 854	4142 ± 606†
Defatted albumin	34.781 ± 5997	31.150 ± 4532

* All values represent mean ± S.E.M. of five experiments in which same source of [^{14}C]ADP was utilized.
† Statistically significant from control. $P < 0.01$.

of mitochondrial protein. An effect similar to that observed by other investigators [10, 18].

DISCUSSION

The data obtained in these experiments support the concept that a decrease in the rate of ATP synthesis produces the change in hepatic ATP content in rats consuming ethanol for prolonged periods of time. Since the drop in the ATP level occurs slowly over a period of 4 weeks, it can be argued that it is the result of an indirect effect brought about by some cumulative alteration in the metabolic interrelationship rather than by a direct response to ethanol itself. Thus, after maintaining the rats on the diet containing ethanol for 2 weeks, an increase in the lipid content of the liver was noted, but no significant change in the ATP content or in the activity of the adenine nucleotide translocase system. Since the rate of formation of ATP is limited by the translocation of ADP into the mitochondria [22] and because the level of long chain CoA derivatives of fatty acids regulate the activity of this carrier system, the observation of Bustos, Kalant and Khanna [23] that the level of these derivatives of fatty acids was not increased at this time agrees with our observation of a lack of any change in the translocation of ADP into the mitochondria. After 4 weeks on the diet, the lipid content of the liver was still higher than normal but was now associated with an increase in the level of long chain CoA derivatives of fatty acids. A significant drop in the ATP content was noted as well as a marked decrease in the activity of the adenine nucleotide translocase system. The increase in the content of long chain CoA derivatives of fatty acids in the mitochondria appeared to parallel the decreased rate of translocation of ADP into mitochondria. This data strongly suggests that there is an actual decrease in ATP synthesis at this time.

With the removal of ethanol from the diet, this simple relationship no longer seemed to apply. The mitochondrial level of long chain CoA derivatives of fatty acids rapidly decreased to levels noted in the controls, but the rate of translocation of ADP into the mitochondria remained depressed. The experiments in which the mitochondrial preparations were treated with defatted albumin seemed to clarify this point. This procedure lowered the level of long chain CoA derivatives of fatty acids in the mitochondrial preparations and any difference in the rate of translocation of ADP into the mitochondria between controls and experimental animals disappeared. Apparently the total level of long-chain CoA derivatives of fatty acids in these preparations does not necessarily reflect the level at the surface of the inner mitochondrial membrane. This data can then be taken as evidence that a long-term effect of chronic ethanol consumption is produced by the slow accumulation of these derivatives at the mitochondrial membrane. These long chain CoA derivatives of fatty acids reversibly inhibit the translocation of ADP into the mitochondria and thus regulate the synthesis of ATP [10]. At present biochemical basis for this accumulation is the ethanol-fed rats is not understood. These results do not preclude other factors which may be involved in altering the activity of this system.

While it seems that a reduction in the synthesis of ATP does occur, this does not rule out a simultaneous increase in the utilization of ATP. In our experiments, the activity of the Na^+ pump as measured by the [$Na^+ + K^+$]-activated ATPase system was enhanced by only 15 per cent. Since it is estimated that this system utilizes only 7–10 per cent of the total ATP formed in the liver [2, 24, 25], this increase would represent only a 1–2 per cent change in the overall demand for ATP. Other investigators [2] report an increase of 190 per cent in their system but even this represents only a 15 per cent increase in the demand for ATP. It would not seem likely that such changes would have any significant effect on the phosphate potential, so that the effect, if it occurs at all, would not appear to be an important factor in the overall response.

It has been pointed out [2] that many of the changes in hepatic metabolism noted in animals fed ethanol are similar to those observed in animals given thyroid hormones. In animals treated with thyroxine, an increase in [$Na^+ + K^+$]-activated ATPase system is associated with a decrease in the level of ATP, and a reduction in the phosphate potential of the cell [26]. None the less, different mechanisms appear to be involved. In the thyroxine-treated animals for instance, the decreased hepatic levels of ATP are associated with an increase in the hepatic levels of ADP; in the ethanol-treated animal the decreased ATP levels are not associated with any significant change in ADP level [3].

If the decrease in the phosphate potential of the liver is produced by changes in the synthesis of ATP, then an explanation other than increased utilization of ATP must be found for the observations that the endogenous rate of oxygen consumption as well as the rate of ethanol oxidation is increased in the ethanol-fed animal [27, 28]. In a normal animal such a marked drop in ATP levels would cause an increase flux of electrons through the respiratory chain and as the rate of ethanol oxidation in the alcohol dehydrogenase system is limited by the re-oxidation of NADH [29–31], this could account for the increased rate of removal of ethanol. Indeed an increased turnover of ATP has been associated with an increased rate of ethanol removal in parenchyma preparations from normal rats starved 48 hr [32]. However, this concept if applied to rats consuming ethanol for prolonged periods of time requires the assumptions that pathways of ethanol metabolism other than by alcohol dehydrogenase are of little importance, and that the prolonged ingestion of ethanol has no effects on the translocation of reducing equivalents from the

cytosol to the mitochondria or on their subsequent oxidation in the process of oxidative phosphorylation. There is considerable evidence that ethanol can be oxidized in pathways other than alcohol dehydrogenase [33–36], although exact interpretation of the evidence is much disputed [37–39]. However, one cannot ignore the evidence which indicates that the increased rate of ethanol oxidation can be accounted for in part by its metabolism in the microsomal system [39]. Also one cannot ignore the evidence which indicates that prolonged ingestion of ethanol alters the functions and morphology of the mitochondria [3, 7]. Thus further experiments are required to show that the chronic consumption of ethanol produces changes within the parenchyma cell causing an increased turnover of ATP.

The data obtained in these experiments also raise a number of other interesting points. It is clear, for instance, that two different mechanisms control the response to alcohol depending on whether the dose is acute or chronic. Contrary to a common point of view, the change in phosphate potential is not reflected in the redox state, since the latter state is shifted to a lower level when ethanol is introduced into the diet and remains at this level as long as ethanol is being consumed, whereas the former ratio (i.e. the state of phosphorylation) is shown to drop slowly. It should be recalled that the cytoplasmic ($ATP/ADP \times P_i$) ratio has been related to the $NADH/NAD^+$ ratio through the glyceraldehyde-3-phosphate dehydrogenase and 3-phosphoglycerate reactions [1]. Under these circumstances one would therefore expect that a decrease in the state of phosphorylation would be associated with an increase in the $NADH/NAD^+$ ratio. While this is true for the chronic case, an increase in the state of phosphorylation is associated with an increase in the $NADH/NAD^+$ ratio when a single dose of ethanol is administered [1]. Moreover, when ethanol is removed from the diet in the former experiments the redox state returns to normal levels while the the state of phosphorylation remains depressed [3]. Clearly this simple picture is inadequate.

Changes observed in these experiments indicate that the prolonged ingestion of ethanol alters the metabolism of long chain CoA derivatives of fatty acids in the liver. A direct correlation appeared to exist between the increased level of these derivatives of fatty acids, changes in the activity of the adenine nucleotide translocase system, and the decreased ATP content of the liver in the ethanol-fed rats. An increased demand for ATP to maintain the ion balance could not account for the lowered phosphate potential of the liver. However, this should not be interpreted to mean that other energy requiring systems have not been affected by the consumption of ethanol. As the level of ATP is a function of its rate of synthesis and utilization in many metabolic pathways, these results may only reflect a small portion of a very complex problem. Our results suggest that changes in fatty acid acyl CoA metabolism develop with time in animals consuming ethanol as part of their diet and that this in turn alters the translocation of ADP into the mitochondrial membrane, and this limits the process of oxidative phosphorylation. Therefore the decreased phosphate potential of the cytoplasm can be explained in part by changes in the rate of synthesis of ATP.

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