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COGNATE EFFECTS OF ETHANOL, HYDRAZINE AND TISSUE REGENERATION ON HEPATIC MITOCHONDRIAL ACTIVITIES

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Abstract—Some of the early hepatic responses to a single dose of ethanol or a subconvulsant dose of hydrazine are similar to those extant in regenerating liver. For example, in all three cases gluconeogenesis is inhibited, lipid accumulates, and varying degrees of structural change occur in mitochondria. The present communication establishes that, in addition, the concurrent mitochondrial generation of ATP is stimulated and that in each case such functions as activated respiration (state 3), phosphorylation rate, respiratory control and ADP:O ratios with both a flavin-linked and a pyridine nucleotide-linked mitochondrial substrate markedly exceed the corresponding activities in both fed and fasted control animals. Thus was observed the unusual consequence of a group of experimental treatments which *increased* rather than *decreased* the efficiency of intracellular energy retention as ATP. Such auxiliary mitochondrial activities as spontaneous and activated ATPases and high amplitude volume changes were minimally or not at all affected. It is proposed that elevated ATP generation was related to permeability changes of the organelles and that augmented ATP synthesis may be a concomitant of increased mitochondrial turnover. Such an explanation could account for the quite different mitochondrial response to chronic ethanol consumption and to typical hepatotoxic substances.

DESPITE the controversy over whether the toxic effects of ethanol on the liver are direct or indirect (see, for example, the reviews by Lieber[1] and by Porta *et al.*[2]), the fact remains that this compound predisposes toward hepatic fatty infiltration, fatty degeneration and, subsequently, often to cirrhotic changes. Likewise, a single subconvulsant dose of hydrazine increases hepatic lipid content several fold within 24 hr.[3,4] In any case, the acute effects of either ethanol or hydrazine return to normal in the absence of further treatment. Ingestion of ethanol is, of course, widespread in contemporary societies and exposure to hydrazine (a component of rocket propellants and fuel cells) and to hydrazides (herbicides, fungicides and several classes of commonly prescribed chemotherapeutic agents) is far from uncommon today. Some of these drugs (e.g. isoniazid, iproniazid) have been shown to produce an even more pronounced fatty accumulation in the liver than does hydrazine itself[5] and isoniazid[6] and other hydrazides yield hydrazine as one of their catabolites.[7] We showed previously that 4 weeks' subsistence on an ethanol containing liquid purified diet high in lipotropes produced a depression of hepatic protein biosynthesis and of mitochondrial electron transport linked phosphorylation.[8] In the present study we wished to investigate a relatively acute, rather than chronic, influence of ethanol on hepatic mitochondrial activities and to compare these with the acute changes produced by hydrazine.

Furthermore, the influence of partial hepatectomy was also studied since this treatment also increased liver lipid[9,10] and the increases in RNA and DNA contents and protein synthesis following a single dose of hydrazine[11] had been shown to be comparable to those occurring during hepatic regeneration.[12,13] Finally, it was of interest to determine if changes in hepatic mitochondrial functions induced acutely by ethanol were similar to those characteristic of hydrazine treatment and of tissue regeneration, and hence different from alterations produced by chronic ethanol ingestion.

METHODS

Treatment of animals. Male albino rats of the Holtzman strain (200–250 g) were maintained on Purina laboratory chow and water *ad lib.*

Hydrazine treatment. Rats were injected intraperitoneally (i.p.) with 4 mg of neutralized hydrazine per 100 g body weight 24 hr before sacrifice. Controls were similarly treated with an equal volume of physiological saline. The drug-treated animals ignore food during this period[3] and hence both groups were fasted but had free access to water.

Alcohol treatment. Except where indicated, rats had a 10-day pretreatment with 5% ethanol as their sole drinking fluid. Forty-five min before sacrifice they were injected i.p. with 3 ml of 10% ethanol in saline per 100 g body weight. Controls were injected with saline only and did not undergo pretreatment.

Partial hepatectomy. Approximately 67 per cent partial hepatectomy was accomplished by ablation of the median and left lateral lobes[14] 17 hr before sacrifice. Sham-operated controls underwent similar laparotomy, including exposure and handling of the liver, but without extirpation of hepatic tissue. Both groups fasted for the 17 hr post-operative period.

Preparation of mitochondria. All animals were sacrificed by decapitation and the mitochondrial fraction of the liver was prepared[15] immediately.

Respiratory rates (m-μatom O_2 min^{-1} mg mitochondrial $protein^{-1}$) were determined polarographically as described previously.[8] Conventions used in naming *respiratory states*, and *respiratory control* and *ADP:O ratios* were those of Chance and Williams.[16] *Phosphorylation rates* (m-μmole ATP synthesized min^{-1} mg mitochondrial $protein^{-1}$) were estimated by the method of Ozawa *et al.*[17]

ATPase (*ATP phosphohydrolase* EC 3.6.1.4). Liver mitochondria (2 mg mitochondrial protein) were incubated at 30° for 5 min in 2 ml of a pH 7·4 mixture of 150 mM sucrose, 15 mM tris, 5 mM ATP and, when present, 4 mM $MgCl_2$ or 0·1 mM 2,4-dinitrophenol (DNP). A blank was prepared at the same time with H_2O in place of mitochondria. P_i was determined according to Lowry.[18] Under these conditions hydrolysis of ATP was proportional with time and amount of mitochondria, and oligomycin (3 μg/mg mitochondrial protein) completely inhibited both Mg^{2+}- and DNP-stimulated ATPases.

High amplitude volume changes were followed as absorbance changes at 520 mμ with a Gilford recording spectrophotometer thermally controlled at 30°. Mitochondria (0·5 mg protein) were suspended in 125 mM KCl containing 20 mM tris, pH 7·4. Swelling was initiated by addition of either $CaCl_2$ or sodium oleate to a final concentration of 1 mM or 5 μM, respectively. When swelling was complete, contraction was induced by addition of (final concentrations) 5 mM ATP, 3 mM $MgCl_2$ and 2 mg/ml

dialyzed crystalline bovine serum albumin. All three components were necessary for contraction. Oligomycin (6 μg) added either at the start or after swelling was concluded prevented completely recontraction of Ca^{2+}- or oleate-swollen mitochondria.

Free fatty acids were extracted[19] immediately, in an ice bath, from freshly prepared mitochondria; this precaution was necessary since mitochondrial free fatty acid levels increase on aging.[20] These were estimated by the method of Novak[21] with palmitic acid as standard.

Protein was determined by a biuret method[22] in the presence of 1% deoxycholate with crystalline bovine serum albumin as standard.

Treatment of data. A two-tailed Student's *t*-test was used to ascertain the level of of significance.[23] A value of $P < 0.05$ was accepted as the level of significance. Means and standard errors of the means (S.E.M.) for each comparison between experimental and control groups are indicated in the Tables.

RESULTS

Rats refuse food during the 24-hr period following hydrazine treatment[3] and therefore the influence on mitochondrial function of the 24-hr fast alone was assayed. As shown in Fig. 1, fasting decreased the respiratory control ratio (RCR) with both succinate ($P < 0.001$) and glutamate ($P < 0.01$) when compared to fed rats. The 8 per

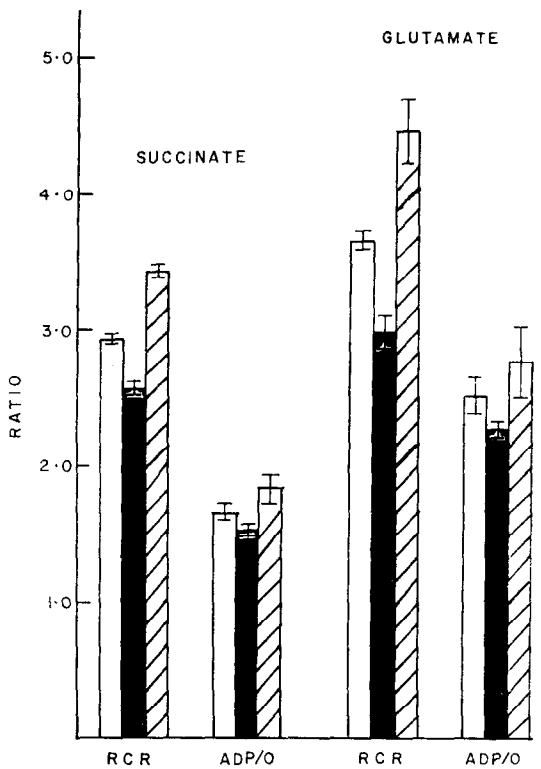

FIG. 1. Influence of 24-hr fast and of hydrazine plus 24-hr fast on RCR and ADP/O of hepatic mitochondria oxidizing succinate and glutamate. Open bars, *ad lib.* fed ($n = 42$); solid bars, 24 hr-fasted ($n = 18$); hatched bars, hydrazine treated followed by 24-hr fast ($n = 10$).

cent reduction due to fasting in ADP:O ratios with both substrates was not significant at the 0·05 level. Hydrazine treatment (with 24-hr fast) not only reversed the effects of fasting on respiratory control ($P < 0·001$ with both substrates) but actually increased this functional parameter above that for fed controls ($P < 0·001$ with succinate, $P < 0·05$ with glutamate). Increases in the RCR by hydrazine were due to augmented respiration in presence of P_i-acceptor (state 3); state 4 respiration was essentially unaffected. ADP:O ratios were also stimulated by hydrazine treatment ($P < 0·01$) when compared to the corresponding 24-hr fasted controls. Consequently, in all subsequent *in vivo* experiments, controls were fasted for the same period as was dictated in the experimental groups by the particular procedure employed.

In vitro treatment with hydrazine of isolated mitochondria did not reverse the depression in respiratory control caused by a 24-hr fast (Fig. 2). In fact, at all levels of hydrazine tested, the RCR and ADP:O ratios were much lower in mitochondria from both fed and fasted rats than were the corresponding ratios in untreated organelles. Hence the implication is that contrary to the *in vivo* experience, hydrazine may be a weak uncoupler *in vitro*. The only exception was the ADP:O ratio for fed rats which

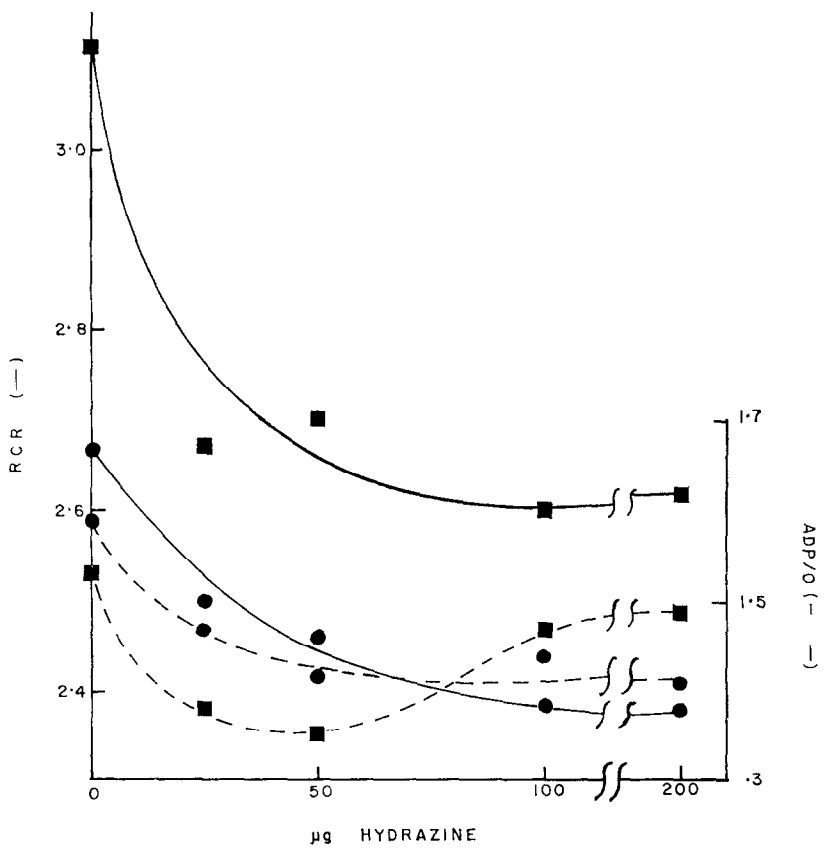

FIG. 2. Effect of hydrazine *in vitro* on respiratory control and oxidative phosphorylation in mitochondria isolated from fed and fasted rats. Hydrazine was added in the amount indicated immediately before assay (2·3–2·5 mg mitochondrial protein); substrate, succinate. (———) RCR; (– – – –) ADP:O; ■, *ad lib.* fed; ●, fasted 24 hr.

at relatively high levels of hydrazine was similar to the ADP:O for untreated mitochondria. In a subsequent experiment hydrazine was included in the mitochondrial isolation medium at a level of 1 mg/g liver. (One-half of each liver was homogenized in the usual isolation medium and the other half in the hydrazine-containing medium.) This value is comparable to the *in vivo* dose of 40 mg/kg body weight, assuming that the entire i.p. dose was absorbed by the hepatocytes. This *in vitro* treatment also failed to elevate RCR and ADP:O ratios. Therefore, neither isolation of mitochondria in the presence of hydrazine nor exposure to the compound immediately before assay reversed the depression in phosphorylation caused by the 24-hr fast and it is concluded that the influence of hydrazine in restoring respiratory control and oxidative phosphorylation *in vivo* must be indirect.

Ethanol is another agent capable of severely modifying many aspects of metabolism in the liver and when administered throughout a 4-week period as 40 per cent of the calories of a nutritionally adequate, purified liquid diet, it depressed acceptor control of hepatic mitochondrial respiration with both succinate and glutamate.[8] In order to

TABLE 1. INFLUENCE OF ETHANOL ON ACCEPTOR CONTROL OF RESPIRATION IN RAT LIVER MITOCHONDRIA

Treatment	Respiratory control ratio	
	Succinate	Glutamate
Control (18)	2·57 ± 0·05	2·99 ± 0·12
Ethanol		
No pretreatment (8)	3·02 ±0·11*	3·26 ± 0·14
Pretreatment‡ (8)	3·28 ± 0·07†	4·64 ± 0·18†

Number of animals in parentheses. Means ± S.E.M.
* $P < 0·005$.
† $P < 0·001$.
‡ 5 per cent Ethanol drinking fluid for 10 days.

study more immediate effects of ethanol on these organelles, rats were fasted for 24 hr either with no pretreatment or after 10 days with 5 per cent ethanol as their sole drinking fluid. Animals were then injected i.p. with either ethanol or saline (see Methods) and sacrificed 45 min later. Table 1 shows that in contrast to observations on rats treated chronically with ethanol,[8] the acute dose of ethanol augmented respiratory control and that the pretreatment further increased the RCR with both succinate and glutamate. In order to ascertain the temporal extent of this protective effect of ethanol in fasting animals, a group of rats pretreated as before with ethanol was divided into five groups of eight each and fasted for 0, 24, 39, 48 and 72 hr, respectively. At the end of each period of fast the group was administered ethanol i.p. and hepatic mitochondria were prepared from each rat as previously. Equal numbers of untreated rats, injected with saline only, served as controls. Figure 3 shows that respiratory control with either substrate was higher in ethanol-treated rats than in controls throughout the 3-day fast ($P < 0·001$ with glutamate at each point; $0·01 > P < 0.001$ with succinate throughout). Although a 24-hr fast depressed respiratory control in mitochondria from untreated animals ($P < 0·0025$ with succinate;

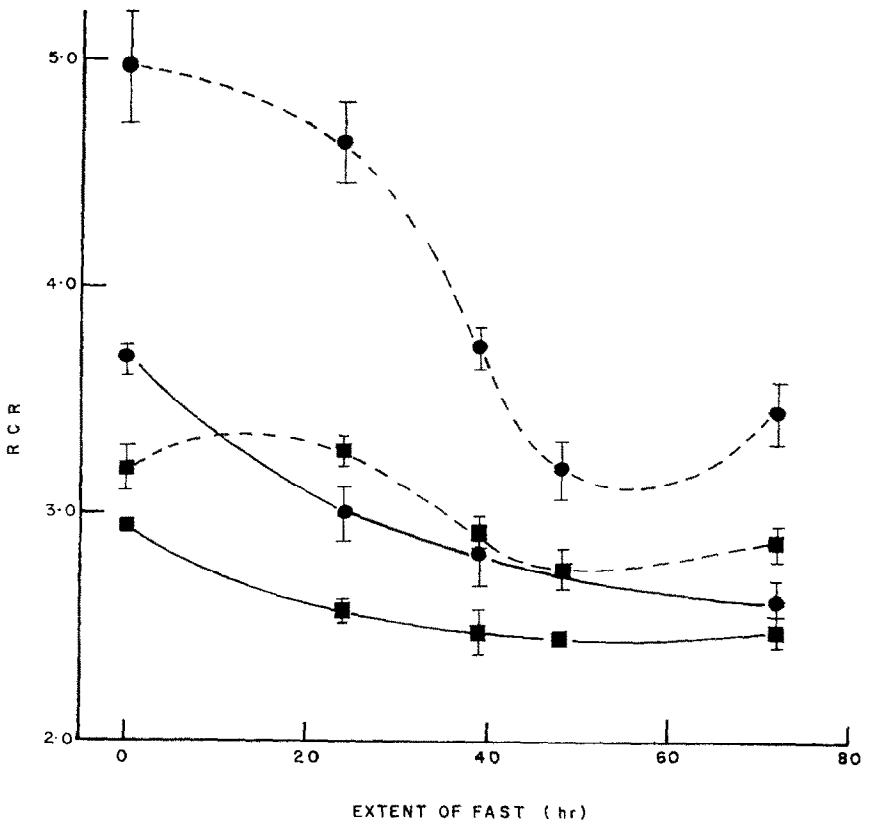

FIG. 3. Maintenance by ethanol treatment of respiratory control in liver mitochondria during 24-hr fast. Rats were injected i.p. with either ethanol or saline (see Methods) 45 min before sacrifice. Abscissa indicates period each group was fasted preceding the injection. Each point represents the mean of at least eight rats ± S.E.M. (- - - -) Ethanol treated; (———) controls; ●, glutamate oxidation; ■, succinate oxidation.

$P < 0{\cdot}025$ with glutamate), ethanol prevented this loss in the treated group (0 and 24 hr values not significantly different). However, prolonging the fast exhausted this stimulatory effect by ethanol, particularly in the case of glutamate respiration. There were no significant differences in mitochondrial protein contents (mg/g liver) between control and alcoholic groups, nor during the 72-hr fast within a group. At each point the higher respiratory control ratios in the ethanol treated groups, compared to controls, resulted from elevated state 3 respiratory rates accompanied by no change in state 4 rates. In this regard the ethanol-treated and hydrazine-treated rats were similar and hence the augmentation of mitochondrial function could not be explained on the basis of calories administered as ethanol during the fasting periods.

Consequently, more detailed studies of mitochondrial function were undertaken to explore similarities of action of these two chemically unrelated compounds and to compare them with regenerative events in 67 per cent partially hepatectomized rats. For comparative purposes all of these results are summarized in Table 2. Hydrazine treatment (rats were fasted for 24 hr following injection of either hydrazine or saline)

TABLE 2. ACUTE INFLUENCE OF CHEMICAL AND SURGICAL HEPATIC TRAUMAS ON MITOCHONDRIAL ACTIVITIES

	Hydrazine		Ethanol		Partial hepatectomy	
	Control	Experimental	Control	Experimental	Control	Experimental
Succinate oxidation						
State 3 rate	116·3 ± 3·0	145·1 ± 3·0*	109·3 ± 2·7	134·3 ± 3·9*	128·5 ± 1·6	150·7 ± 3·0*
State 4 rate	43·8 ± 1·2	41·3 ± 1·0	42·9 ± 1·0	43·2 ± 1·5	50·3 ± 0·9	45·1 ± 1·0†
RCR	2·67 ± 0·05	3·53 ± 0·06*	2·65 ± 0·05	3·13 ± 0·05*	2·57 ± 0·05	3·35 ± 0·07*
ADP:O	1·48 ± 0·03	1·73 ± 0·05*	1·60 ± 0·06	1·61 ± 0·04	1·41 ± 0·04	1·57 ± 0·04‡
PR	171·4 ± 4·0	250·7 ± 6·8*	185·6 ± 2·5	215·5 ± 7·9†	181·8 ± 5·7	235·3 ± 5·8*
Glutamate oxidation						
State 3 rate	62·2 ± 2·1	70·4 ± 2·8‡	63·3 ± 2·0	80·4 ± 3·1*	68·1 ± 2·3	87·9 ± 2·6*
State 4 rate	20·1 ± 0·6	16·9 ± 0·4*	19·8 ± 0·7	19·0 ± 0·8	22·0 ± 0·8	22·9 ± 0·6
RCR	3·10 ± 0·07	4·14 ± 0·13*	3·23 ± 0·07	4·28 ± 0·14*	3·13 ± 0·1	3·87 ± 0·13*
ADP:O	2·18 ± 0·06	2·54 ± 0·12‡	1·97 ± 0·04	2·41 ± 0·03*	1·98 ± 0·04	2·17 ± 0·05§
PR	134·3 ± 5·0	174·8 ± 7·0*	124·6 ± 4·6	187·4 ± 11·1*	135·3 ± 5·6	191·3 ± 8·0*
ATPase						
Spontaneous	3·41 ± 0·26	3·29 ± 0·39	3·79 ± 0·19	3·56 ± 0·27	3·49 ± 0·41	3·96 ± 0·38
DNP-stimulated	17·85 ± 0·92	16·80 ± 1·1	15·49 ± 0·82	14·94 ± 0·62	12·46 ± 1·3	7·57 ± 1·5§
Mg-activated	9·15 ± 0·49	6·78 ± 0·62*	10·93 ± 0·42	9·72 ± 0·55	12·27 ± 0·92	10·86 ± 0·52
Volume changes						
Oleate swelling	−0·155 ± 0·046	−0·113 ± 0·014	−0·098 ± 0·010	−0·156 ± 0·016†	−0·079 ± 0·011	−0·114 ± 0·020
Contraction	+0·096 ± 0·006	+0·088 ± 0·008	+0·107 ± 0·006	+0·123 ± 0·011	+0·096 ± 0·009	+0·065 ± 0·007§
Ca^{2+}-swelling	−0·289 ± 0·016	−0·214 ± 0·018*	−0·262 ± 0·019	−0·292 ± 0·017	−0·246 ± 0·015	−0·255 ± 0·083
Contraction	+0·108 ± 0·010	+0·111 ± 0·014	+0·106 ± 0·008	+0·123 ± 0·009	+0·086 ± 0·010	+0·083 ± 0·034
Free fatty acids	—	—	69·9 ± 3·6	75·8 ± 4·0	—	—
Mitochondrial protein	27·8 ± 0·6	24·8 ± 0·6†	25·9 ± 0·5	24·4 ± 0·5	24·0 ± 0·6	20·6 ± 0·5†

Food was withheld from controls during same period that respective experimental group fasted. See Methods for additional details. *Respiratory rates* = m-μatoms O_2 min^{-1} mg $protein^{-1}$, *phosphorylation rate (PR)* = m-μmole ATP synthesized min^{-1} mg $protein^{-1}$, *ATPases* = μmole ATP hydrolyzed hr^{-1} mg $protein^{-1}$, *volume changes* = ΔA_{520} mg $protein^{-1}$, *free fatty acids* = m-μequiv. mg $protein^{-1}$, *mitochondrial protein* = mg protein g $liver^{-1}$. Nonannotated comparisons were not significantly different at the 0·05 level. Degrees of freedom: Hydrazine = 22, Ethanol = 26, Partial Hepatectomy = 16. Means ± S.E.M.

* $P < 0·001$. ‡ $P < 0·01$.

† $P < 0·005$. § $P < 0·02$.

increased the RCR with both substrates by one-third. These changes were due predominantly to elevated stated 3 respiratory rates although in this experiment state 4 respiration was also depressed when the substrate was glutamate. ADP:O ratios were increased by 17 per cent and the phosphorylation rates for succinate and for glutamate were stimulated by 46 and 30 per cent, respectively. Although the Mg^{2+}-activated ATPase was partially masked in mitochondria from treated rats, the spontaneous, as well as DNP-stimulated activities were unaffected. Moreover, except for minor protection against Ca^{2+}-induced swelling, treated and control organelles responded similarly in assays of energized contraction and oleate-swelling. Contraction of mitochondrial membranes is one of the physiological roles assigned to the membrane bound ATPases.

Observations obtained as a result of ethanol treatment were quite comparable to those achieved with hydrazine. Rats drank 5 per cent ethanol for 10 days, were fasted for 24 hr, then given ethanol i.p. according to techniques described earlier. Controls were carried through the same procedure, but without ethanol. During glutamate oxidation, the ethanol treatment increased the RCR by one-third (as a result of an augmented state 3 rate), ADP:O rose by 22 per cent, and the phosphorylation rate by 50 per cent. With succinate the RCR increased by 18 per cent due to state 3 stimulation, the ADP:O was unaltered, but the phosphorylation rate rose by 16 per cent. The various ATPase activities were unaffected and although the swelling and contraction cycles appeared to be minimally influenced, a trend did exist toward slightly enhanced changes in each of these parameters by the ethanol treatment. Consequently, the possibility was considered that ethanol may have increased mitochondrial permeability to some degree (see Discussion). It is known that ethanol administration, under various conditions, may lead to hepatic lipid accumulation and that fasting mobilizes fatty acids from fat depots to the liver. Moreover, such fatty acids are natural swelling agents and uncouplers. Nevertheless, none of our results could be attributed to an alteration in mitochondrial fatty acid content. The various parameters related to oxidative phosphorylation (state 3 respiration, RCR, ADP:O and phosphorylation rate) were *increased* by the ethanol treatment and there was no significant difference in the mitochondrial free fatty acid contents (Table 2).

As seen also in Table 2, 17 hr after 67 per cent partial hepatectomy the RCR with both substrates was elevated also by about one-third over that of sham-operated controls similarly fasted for 17 hr. These changes were brought about by stimulated state 3 respiratory rates although state 4 oxidation of succinate was also lower than in laparotomized controls. ADP:O ratios were augmented by 10 per cent and phosphorylation rates by 30 and 40 per cent in the posthepatectomized rats. Although the DNP-stimulated ATPase was partially masked, the spontaneous and Mg^{2+}-activated activities were not altered significantly. Also, volume changes were essentially unaffected although energized contraction in oleate-swollen mitochondria was impaired ($P < 0{\cdot}02$) in regenerating livers. Within 4 weeks after the partial hepatectomy all of the mitochondrial activities had returned essentially to control values (not shown in Table).

The RCR is generally considered to be a more sensitive and rigorous test of the degree of coupling of oxidation to phosphorylation than is the ADP:O ratio[24, 25] and hence it would not be surprising for the former quantity to be affected more severely than the latter by certain metabolic alterations. For example, state 3 respira-

tion and respiratory control were found to be depressed in mitochondria from diabetic animals while the ADP:O remained unchanged.[26] Indeed, in every case in Table 2 respiratory control ratios were influenced much more than ADP:O ratios by the various treatments. Furthermore, the minor effects on ATPases and high amplitude volume changes produced by those treatments, and which were accompanied by major changes in those parameters influenced by the "phosphate potential" [ATP]/[ADP] [P_i], tend to implicate a rather selective effect related to adenine nucleotide translocations. In addition, it has been reported[27] that the adenylate kinase mass action ratio [ATP] [AMP]/[ADP]^2 increased 3-fold during ethanol oxidation in perfused rat liver and also that ethanol decreased ADP, increased AMP and maintained the ATP: ADP ratio at slightly elevated levels. Those results might have a bearing on our observations, provided mitochondrial entry of ADP were augmented by our ethanol treatment. Addition of exogenous ADP would then overcome the adenine nucleotide imbalance and permit a higher stimulation of state 3 respiration, RCR and phosphorylation rate without influencing ATPase activities or high amplitude volume changes. We cannot explain the increased ADP:O ratios on this basis and, since the changes are of lower magnitude than the comparable changes in RCR, there may be another mechanism which modifies the ADP:O.

Mitochondrial protein was unaltered by ethanol but was depressed by 11 and 14 per cent in hydrazine treated and in partially hepatectomized animals, respectively (Table 2). A depression of 18 per cent has been reported in posthepatectomized male Wistar strain rats.[28]

DISCUSSION

Although ethanol oxidation depresses the citrate cycle in liver mitochondria,[29] French[30] has observed a transient but significant increase in hepatic ATP which may have resulted either from electron transport from cytoplasmic NADH to the mitochondrial respiratory chain or from ethanol-induced depression of ATP utilization or degradation. The present results (elevated phosphorylation rate, RCR and ADP:O ratios) are consistent with increased formation of ATP, rather than decreased breakdown. Moreover, in chronic ethanol feeding the liver ATP content decreased[30] and we have observed a marked depression of oxidative phosphorylation after 4 weeks' feeding of an ethanol-containing liquid purified diet.[8]

Electron microscopic and enzymic evidence suggested that chronic ethanol ingestion may alter the outer mitochondrial membrane, increase fragility of the organelles and subsequently lead to their disintegration.[31, 32] Similarly, after *chronic* ethanol consumption, phosphorylation and respiratory control are markedly depressed[8] at a time when gross alteration of mitochondrial structure, decreased ATP, and disintegration of the organelles occur.[31, 32] The observations that control mitochondria in the present study had lower state 3 respiratory rates and respiratory control ratios than did the ethanol-treated organelles, and that these depressions were unaccompanied by changes in state 4 respiratory rates, imply that ethanol in some way stimulated mitochondrial translocation of the adenine nucleotides. Altered permeability due to structural changes might be less marked after moderate treatment with ethanol, compared to chronic treatment,[31, 32] and would afford a plausible explanation for these phenomena. Such behavior might also account for the mitochondrial responses

to hydrazine since the most striking morphological feature of hydrazine treatment is swelling of the organelles[33] and acute[34] as well as chronic[35–37] ethanol administration similarly produce mitochondrial swelling and deformity.

In a comparable fashion, mitochondria from regenerating livers had higher state 3 respiratory rates, RCR and ADP:O ratios, and phosphorylation rates than did organelles from laparotomized controls. The same behavior was true of hydrazine treatment. Adenine nucleotides require the specific carrier for exchange diffusion of ADP and ATP, molecule for molecule (the atractyloside-sensitive site), and hence they cannot pass the inner membrane by simple diffusion in normal mitochondria. Possibly newly synthesized mitochondria are more permeable to adenine nucleotides than are "normal" mitochondria and if so, these responses could result from the same benign structural changes accountable for the similar augmentation of activities seen in the ethanol-treated animals.

All of the mitochondrial activities which were elevated by ethanol, hydrazine or tissue regeneration are inner membrane functions. Since spontaneous or unmasked ATPase activities were not affected appreciably, it would appear that the postulated alteration in membrane structure did not involve gross disruption of the inner membrane. Interpretation of the volume changes is somewhat more difficult since there was no consistency in response to the various treatments. The surface area of the inner membrane, because of its extensive invaginations and foldings in the form of cristae, is considerably greater than that of the outer membrane and high amplitude swelling involves the unfolding and expansion of the inner membrane with consequent distension and rupture of the outer membrane.[38, 40] Hence the increased susceptibility of the ethanol-treated mitochondria to swelling by oleate ($P < 0{\cdot}005$) could be due to weakening of the outer membrane by ethanol[31, 32] making it more sensitive to rupture by the expanding inner membrane. The phenomenon might be inversely comparable to that observed in mitochondria enriched with cholesterol *in vitro*.[41] Such organelles are much more resistant to high amplitude and low amplitude volume changes, apparently because of increased strength of the outer membrane, than are control mitochondria.

The half-life of mitochondria has been estimated by radioactive labelling techniques to be 10·3 days[42] and the mitochondrial fraction isolated by differential centrifugation normally contains a heterogeneous mixture of the organelles in terms of age.[28] One may speculate, therefore, that agents which are capable of mitochondrial damage and compensatory hypertrophy and regeneration may cause to result a larger proportion of "incomplete" mitochondria with increased permeability properties. The phenomenon of induced compensatory hypertrophy of mitochondria has been observed in response to low protein feeding in the rat[43] and is accompanied by increased oxidative phosphorylation in not only the rat,[44] but also in hepatic mitochondria of children with kwashiorkor.[45] In view of the foregoing it is of interest that the mean number of mitochondria per cell is decreased in liver both from rats fed low protein diets[46] and from partially hepatectomized rats from the first through the seventh postoperative day.[47] Therefore, it would seem that any event which increases mitochondrial turnover may in general be accompanied by an elevated rate of ATP generation. Expectedly, gluconeogenesis is consequently inhibited by acute ethanol treatment,[2] by acute hydrazine treatment,[3, 48] and immediately following partial hepatectomy.[49] Because of the transient nature of the various changes discussed in this paper, they are not

considered comparable to the severe biochemical and morphological alterations produced by classical hepatotoxins such as carbon tetrachloride or phosphorus, for example. Additionally, the possibility of increased mitochondrial efficiency of energy production in response to the less severe types of injury in the present study might constitute an important mechanism for maintaining cellular homeostasis.

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REFERENCES

1. C. S. Lieber, *A. Rev. Med.* **18**, 35 (1967).
2. E. A. Porta, O. R. Koch and W. S. Hartroft, *Exp. molec. Path.* **12**, 104 (1970).
3. J. S. Amenta and E. H. Johnston, *Lab. Invest.* **11**, 956 (1962).
4. D. L. Trout, *Aerospace Med.* **35**, 357 (1964).
5. H. McKennis, A. S. Yard and E. V. Pahnelas, *Am. J. Tuberc.* **73**, 956 (1956).
6. K. Obojska, *Acta physiol. pol.* **11**, 469 (1960).
7. H. McKennis, A. S. Yard, E. J. Adair and J. H. Weatherby, *J. Pharmac. exp. Ther.* **131**, 152 (1961).
8. W. L. Banks, Jr., E. S. Kline and E. S. Higgins, *J. Nutr.* **100**, 581 (1970).
9. S. Ludewig, G. R. Minor and J. C. Hortenstine, *Proc. Soc. exp. Biol. Med.* **42**, 158 (1939).
10. G. Fex, *Biochim. biophys. Acta* **202**, 415 (1970).
11. W. L. Banks, Jr., *Biochem. Pharmac.* **19**, 275 (1970).
12. N. L. R. Bucher, *Int. Rev. Cytol.* **15**, 245 (1963).
13. H. M. Rabes and H. Brändle, *Cancer Res.* **29**, 817 (1969).
14. G. M. Higgins and R. M. Anderson, *Archs Path.* **12**, 186 (1931).
15. W. C. Schneider and G. H. Hogeboom, *J. biol. Chem.* **183**, 123 (1950).
16. B. Chance and G. R. Williams, *Adv. Enzymol.* **17**, 65 (1956).
17. K. Ozawa, O. Kitamura, T. Ohsawa, H. Araki, T. Murata and I. Honjo, *J. Biochem. (Tokyo)* **67**, 149 (1970).
18. O. H. Lowry, in *Methods in Enzymology* (Eds. S. P. Colowick and N. O. Kaplan), Vol. IV, p. 372, Academic Press, New York (1957).
19. V. P. Dole, *J. clin. Invest.* **35**, 150 (1956).
20. W. Chefurka, *Biochemistry, N.Y.* **5**, 3887 (1966).
21. M. Novak, *J. Lipid Res.* **6**, 431 (1965).
22. A. C. Gornall, C. J. Bardawill and M. M. David, *J. biol. Chem.* **177**, 751 (1949).
23. N. T. Bailey, *Statistical Methods in Biology*, pp. 23, 45–49, John Wiley, New York (1959).
24. A. L. Lehninger, *The Mitochondrion*, p. 135, W. A. Benjamin, New York (1964).
25. K. Ozawa, K. Seta, H. Takeda, K. Ando, H. Handa and C. Araki, *J. Biochem. (Tokyo)* **59**, 501 (1966).
26. A. A. Boveris, M. C. de Peralta Ramos, A. O. M. Stoppani and V. G. Foglia, *Proc. Soc. exp. Biol. Med.* **132**, 171 (1969).
27. J. R. Williamson, R. Scholz, E. T. Browning, R. G. Thurman and M. H. Fukami, *J. biol. Chem.* **244**, 5044 (1969).
28. A. R. L. Gear, *Biochem. J.* **95**, 118 (1965).
29. O. A. Forsander, N. Räihä, M. Salaspuro and P. Mäenpää, *Biochem. J.* **94**, 259 (1965).
30. S. W. French, *Proc. Soc. exp. Biol. Med.* **121**, 681 (1966).
31. S. W. French, *Gastroenterology* **54**, 1106 (1968).
32. S. W. French, A. Sheinbaun and R. J. Morin, *Proc. Soc. exp. Biol. Med.* **130**, 781 (1969).
33. C. E. Ganote and A. S. Rosenthal, *Lab. Invest.* **19**, 382 (1968).
34. W. S. Hartroft and E. A. Porta, *Gastroenterology* **46**, 304 (1964).
35. O. A. Iseri, C. S. Lieber and L. S. Gottlieb, *Am. J. Path.* **48**, 535 (1966).
36. K. H. Kiessling and U. Tobe, *Expl Cell Res.* **33**, 350 (1964).
37. E. A. Porta, W. S. Hartroft and F. A. de la Iglesia, *Lab. Invest.* **14**, 1437 (1965).
38. P. Wlodawer, D. F. Parsons, G. R. Williams and L. Wojtczak, *Biochim. biophys. Acta* **128**, 34 (1966).
39. E. A. Munn and P. V. Blair, *Z. Zellforsch. mikrosk. Anat.* **80**, 205 (1967).
40. G. A. Blondin and D. E. Green, *Proc. natn. Acad. Sci., U.S.A.* **58**, 612 (1967).
41. J. M. Graham and C. Green, *Europ. J. Biochem.* **12**, 58 (1970).
42. M. J. Fletcher and D. R. Sanadi, *Biochim. biophys. Acta* **51**, 356 (1961).
43. E. A. Porta, G. Sugioka and W. S. Hartroft, *Am. J. clin. Nutr.* **22**, 676 (1969).

44. N. HARADA, *J. Nutr.* **93**, 263 (1967).
45. J. METCOFF, *A. Rev. Med.* **18**, 377 (1967).
46. O. R. KOCH, E. A. PORTA and W. S. HARTROFT, *Lab. Invest.* **18**, 379 (1968).
47. C. ALLARD, G. DE LAMIRANDE and A. CANTERO, *Cancer Res.* **12**, 58 (1952).
48. P. D. RAY, R. L. HANSON and H. A. LARDY, *J. biol. Chem.* **245**, 690 (1970).
49. R. D. HARKNESS, *Br. med. Bull.* **13**, 87 (1957).