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MODIFICATIONS OF CITRATE AND ISOCITRATE METABOLISM IN LIVER MITOCHONDRIA OF ETHANOL-FED RATS

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Abstract—The transports of citrate and isocitrate and the activity of enzymes involved in the metabolism of these substrates were studied in mitochondria isolated from rat alcoholic fatty liver. The reduction of intramitochondrial pyridine-nucleotides following on the addition of citrate with malate and phosphate to mitochondria was assayed with a double-beam spectrophotometer. A marked 79% increase was observed in mitochondria from rats fed ethanol compared to the control group. This increase was not observed with isocitrate. Alteration of transports might contribute to this observed change. The efflux of isocitrate exchanged for extramitochondrial citrate was markedly reduced (−50% in ethanol fed rats), while [^{14}C]citrate penetration into malate loaded mitochondria was but slightly inhibited and [^{14}C]malate transport was unchanged. Such alterations are similar to that produced by long-chain acyl-CoAs which are elevated in such alcoholic fatty liver. However, acceleration of pyridine-nucleotides reduction following on the addition of citrate is still observed in Triton-disrupted mitochondria. The following intramitochondrial changes may contribute to this acceleration: an increased activity of aconitate hydratase (+32%) and of NAD-linked isocitrate deshydrogenase (+11%), higher levels of mitochondrial pyridine-nucleotides (+21% to 35%). The increased utilization of citrate by mitochondria, along with the loss of activity of the cytosolic ATP-citrate lyase previously described in ethanol-fed rats may depress fatty acid synthesis, in response to the accumulation of triglycerides in the liver cell.

The interference of mitochondria with the metabolism of ethanol is very important since they contain 80% of liver aldehyde dehydrogenase [1] and since they reoxidize cytosolic $NADH_2$ [2, 3] by means of various shuttles. This and the conspicuous changes of mitochondrial morphology which follows chronic alcoholic intake [4, 5] have prompted several biochemical studies concerning mitochondrial alterations induced by such chronic intake of ethanol. The main results were an impairment of a certain number of enzyme activities [6–8] and of mitochondrial protein synthesis [8], an increase in α-glycerophosphate dehydrogenase in some strains of rats [9] and a decreased translocation of ADP supposedly due to increased long-chain acyl-CoAs [10]. In spite of previous observations [11], no changes in permeability to NADH or in the main metabolic shuttles were observed [12]. The present work describes a marked difference observed in the reduction of intramitochondrial pyridine-nucleotides with extramitochondrial citrate in the ethanol ingesting rat and other biochemical changes: diminished tricarboxylic acid exchange, and modified aconitate hydratase [citrate (isocitrate) hydrolyase (EC 4.2.1.3)] and pyridine-nucleotides levels, which may be related to the increased effect of citrate in the alcohol-fed animal.

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MATERIAL AND METHODS

Diets

Group I. Male Sprague–Dawley Rats (CD Rats supplied by Charles River Co.) were given an entirely liquid hyperlipidic diet similar to that used by Lieber *et al.* [13] except for the use of dextrin-maltose, that is, glucose instead of sucrose as the only carbohydrate. Ethanol was progressively substituted isocalorically to part of the carbohydrate, by weekly stages (25, 37·5, 43 and lastly 50 g/l.); these stages were extended if no growth was observed during the week. The final caloric composition of the diet was: ethanol 36%, proteins 15%, lipids 43%, carbohydrates 6% (Group IA). In pair-fed controls (Group IC) the 42% of carbohydrates calories was maintained during the whole experiment.

A few other animals received this diet *ad lib.* (Group ID). The average duration of the experiment was 11 weeks after the first full-dose ethanol intake. The animals were stunned in the morning without previous fasting, quickly opened, and the liver taken out for histologic samples and homogenization with 0·27 M cold sucrose.

Group II. The rats were administered a liquid diet (Group IIA) containing ethanol, in the same way as for Group I. This time, the caloric composition was ethanol 36%, proteins 15%, lipids 8%, carbohydrates 41%, that is, fewer lipids than for the first group. Pair-fed controls made up Group IIC.

Group III. Animals (Group IIIA) received the same diet as Group IA except for the last 8 days, during

which time they were fed the control diet. Controls made up Group IIIC.

Group IV. Biochemical analyses were carried out on normal rats of the same strain and weight given water and Extra labo biscuits *ad lib.*

Hepatic morphology

Liver tissues from each animal was studied following formalin fixation by light microscopy. Some liver fragments from Groups IA and IC were also studied with an Hitachi HU 11 CS electron microscope after Millonig osmic fixation and Epon embedding. (Details of the morphometric study of these livers using slight modifications of Weibel's method [14] will be the object of a later paper.)

Preparation of the mitochondrial fraction. Mitochondria were prepared as described by Hogeboom [15] with two washings. Mitochondrial protein was measured using the biuret method [16] modified by the addition of cholate to dissolve mitochondria.

Polarographic determination of the respiratory control ratio. The respiratory control ratio (ACR) defined as the state III respiration rate/state IV respiration rate was determined using Chance's method [17] with a Gilson oxygraph and 15 mM glutamate at 25°.

Spectrophotometric recording of the intramitochondrial pyridine-nucleotides reduction

Using the method described by Chance [18], the reduction of mitochondrial NAD and NADP was assayed with a double-beam spectrophotometer (Perkin Elmer 356) at 374 and 340 nm. Mitochondria (2·4 mg protein) were incubated for 2 min at 21° in 3 ml 0·27 M saccharose 10 mM Tris pH 7·4 containing 0·8 μg CCP (Carbonyl cyanide *m*-chlorophenyl hydrazone) in order to oxidize the endogenous substrates; 20 μg of rotenone were then added and after the stabilisation of the optical density, sodium phosphate (final concn 1·2 mM) and, successively, sodium malate and citrate or DL-isocitrate were added. In most experiments, malate concentration was constant (0·6 mM) even when citrate or isocitrate concentrations varied from 0·4 to 12·8 mM. In other pilot experiments, the ratio of tricarboxylic acid to malate concentration was kept at 10. In a few experiments, tricarboxylic acids were used without previous addition of phosphate or malate. The negligible oxidation of added NADH was checked in a few experiments using uncoupled mitochondria without rotenone.

Reduction of pyridine-nucleotides following on the addition of citrate or isocitrate was also recorded using mitochondria which had been disrupted by the addition of 0·1% Triton X 100 to the mitochondrial suspension preincubated as above with CCP followed by rotenone. No malate or phosphate was added but $MgCl_2$ was used, the concentration ratio of tricarboxylic acid to Mg^{2+} being kept at 1/2.

All results were expressed as nmoles NAD(P)H formed/min per mg protein, that is the quantity of NADH and NADPH which cannot be distinguished by this method.

Measurement of enzymatic activities, and concentrations of mitochondrial pyridine-nucleotides and magnesium

Aconitate hydratase [citrate (isocitrate) hydrolyase (EC 4,2,1,3)]. All enzymatic activites were determined using mitochondria which had been ruptured by 0·1% Triton X 100. Disappearance of *cis*-aconitate was monitored using the method of Fansler and Lowenstein [19]. Mitochondrial protein (0·3 mg) was suspended in a medium (2·4 ml) pH 7·4 containing 80 mM Tris-HCl, 60 mM NaCl. The reaction was started by the addition of 2 mM *cis*-aconitate and was followed at 240 nm with a Zeiss PM Q II spectrophotometer. Addition of NADP isocitrate dehydrogenase and NADP without Mg^{2+} did not increase the disappearance rate of *cis*-aconitate.

Isocitrate dehydrogenases. Activities were determined according to the method of Plaut and Aogaichi [20] with modifications.

NAD-linked isocitrate dehydrogenase [Threo-D-isocitrate:NAD oxido reductase (decarboxylating) (EC 1.1.1.41)] activity was determined using the following incubation mixture: 100 mM Tris–HCl, pH 7·2, 5 mM $MgCl_2$, 1·5 mM ADP, 1·5 mM NAD containing 0·24 mg mitochondrial protein per ml mixture. The reaction was started at 24° with 5·6 mM DL-sodium isocitrate and was recorded at 340 nm with a Zeiss PM Q II spectrophotometer.

NADP-linked isocitrate dehydrogenase [Threo-D-isocitrate:NADP oxido reductase (decarboxylating) (EC 1.1.1.42)] activity was determined using 100 mM Tris–HCl pH 7·2, 5 mM Mg Cl_2, 60 μM NADP and 2·8 mM DL-sodium isocitrate containing 0·06 mg mitochondrial protein per ml mixture.

Mitochondrial pyridine-nucleotides. The NAD and NADP were extracted and assayed by the Williamson and Corkey method [21]. The NAD and NADP were extracted from mitochondria using perchloric acid. The supernatant was neutralized to pH 7·4 with 3 M K_2CO_3 containing 0·5 M triethanolamine base. NAD reduction was followed at 340 nm with a Perkin Elmer 356 spectrophotometer at 25°. The reaction mixture consisted of 0·5 ml perchloric extract, 2 ml buffer pH 8·5 (0·1 M Tris, 0·4 M hydrazine hydrate), 0·07 M ethanol. The reaction was started with 1·5 μg alcohol dehydrogenase. NADP reduction was followed at 340 nm with a reaction mixture consisting of 3 ml perchloric extract, 1·3 mM glucose-6-phosphate, 6·7 mM $MgSO_4$, and 0·8 μg glucose-6-phosphate dehydrogenase.

The NADH and NADPH were extracted from mitochondria using KOH according to the Klingenberg method [22]. The mixture was neutralized to pH 7·8 with buffer triethanolamine HCl phosphate. The NADH oxidation was observed in the following incubation mixture: 0·5 ml alkaline extract, 80 mM triethanolamine HCl pH 7·4, 3·3 mM sodium pyruvate and α-cetoglutarate and 25 μg lactate dehydrogenase. When the reaction was completed, 25 μg of glutamate dehydrogenase were added to catalyse the NADPH oxidation.

Results were expressed as nmoles NAD(P)H/mg of protein.

Mitochondrial magnesium. Mitochondrial magnesium was measured in a chlorhydric acid extract by the Weiner and Lardy method [23].

The magnesium content of the mitochondria was determined by extracting 1 mg of mitochondrial protein with 5 ml 0·1 N HCl. The 50,000 $g \times 30$ min supernatant of this extract was analysed for Mg^{2+} with an atomic absorption spectrophotometer (Perkin

Elmer 300 S). Results were expressed as nmoles Mg^{2+} per mg of protein.

Measurement of anion transport

Isocitrate efflux from mitochondria. The isocitrate efflux from mitochondria into a citrate-containing medium was determined by the method of Brand *et al.* [24]. Mitochondria (1·8 mg) were incubated in a medium (2·5 ml) pH 7·4 containing 120 mM KCl 20 mM Tris–HCl, 1 mM $MgCl_2$, 10 μg rotenone, 25 μM NADP, 25 μg isocitrate dehydrogenase. The reaction was started by the addition of sodium citrate (0·1–3·9 mM) and was followed at 340–374 nm with a Perkin Elmer 356 spectrophotometer. After 3 min 0·1% Triton X 100 was added and the new rate was determined. Results are expressed as nmoles NADPH formed per min and per mg of protein.

Intramitochondrial transport of labelled di- and tricarboxylic acids. [^{14}C]Malate transport was studied using the technique of Palmieri *et al.* [25]. Rat liver mitochondria (3·5 mg) were incubated at 4° in a medium (0·8 ml) pH 6·5 containing 100 mM KCl, 15 mM Tris–Mes, 0·5 mM EDTA, 2 μg/ml rotenone, 2 μg/ml antimycine, 2·5 μg/ml oligomycine. The uptake was started by the addition of 0·1 mM [^{14}C]malate and terminated by the rapid addition of 20 mM phenyl-succinate.

The [^{14}C]citrate transport into malate loaded mitochondria was studied using the technique of Palmieri [25, 26]. Rat liver mitochondria (5 mg) preloaded with malate, were incubated in a medium (0·8 ml) pH 7·2 at 9°. The exchange was started by adding 0·5 mM [^{14}C]citrate and terminated by the rapid addition of 10 mM 1,2,3 benzene-tricarboxylate, followed with rapid centrifugation.

The radioactivity was measured in a Packard Tricarb liquid scintillation spectrophotometer using Instagel as the liquid scintillator. The efficiency of counting was determined using an external standard. *Paired* t*-test and Student's* t*-test* were used for statistical analysis.

Chemicals

NAD, NADP, NADH, NADPH, ADP as well as the enzymes utilized in the various assays were supplied by Boehringer Mannheim Co. and the radioactive substrates by the Radiochemical Centre (Amersham).

CCP was supplied by Calbiochem, Rotenone by Aldrich and the other inhibitors by Sigma.

RESULTS

Caloric intake and growth curves. The increase in alcohol concentration in the diet was necessarily slow and progressive, to allow some growth although it was somewhat smaller than that of the pair fed controls, and much smaller than that of the animals fed *ad lib.* No death occurred—contrarily to what occurs with deficient diets [7] or when there is a very rapid or sudden increase in alcohol concentrations.

Ethanol consumption in relation with body weight decreased from 1·3 g/100 g per day to 1·1 g /100 g per day during the chronic ethanol intake.

Production of a fatty liver and morphologic changes. Morphologic changes were a slight increase in mitochondrial size, a proliferation of smooth endoplasmic reticulum and the occurrence of many fat droplets (the fat droplet volume density being 9·6% of hepatocytic unit volume in group IA compared to 2% in group IC, $P < 0·001$).

No fatty liver was observed in Groups IIA and IIC consuming low lipid diets, but fat droplets were observed in livers of group ID.

Determination of respiration control ratio. The respiration control ratio in the ethanol group (3·83 ± 0·26) was significantly lower compared to group IC (4·42 ± 0·31) but the difference was significant when group IA was compared to group IV (4·50 ± 0·15), $P < 0·05$.

Reduction of intramitochondrial pyridine-nucleotides following the addition of exogenous substrate. The reduction rates of mitochondrial pyridine-nucleotides by exogenous citrate or isocitrate are reported in Table 1. A marked 79% increase ($P < 0·001$) is observed in mitochondria from rats fed ethanol and a high lipid diet following the addition of citrate when compared to the control Group IC.

The increase was confirmed with different concentrations of extra-mitochondrial citrate; for low concentrations this difference was not significant but graphic determinations of initial rates become inaccurate in such conditions (Fig. 1). When a constant citrate/malate concentration ratio, instead of a fix concentration of malate was used, the results were similar.

In group III, which comprised two pairs of animals, the difference between ethanol-fed rats and their controls was still present 8 days after ethanol withdrawal (mean values: 3·10 vs 1·89 nmoles NAD(P)H/min per mg protein).

The 43% increase observed in Group IIA compared to group IIC was not significant in this small 6-animal group (Table 1). Differences between groups IIA and IA and between groups IIC and IC, that is between groups receiving different amounts of lipids were not significant. On the other hand, in animals having higher caloric intake, the reduction rate of mitochondrial pyridine-nucleotides by exogenous

Table 1. Reduction of pyridine-nucleotides (nmoles NAD(P)H/min per mg protein) following the addition of 6·4 mM citrate or DL-isocitrate (0·6 mM malate) to rat liver mitochondria

Group	Citrate–malate P_i (nmoles NAD(P)H/min per mg protein)	Isocitrate–malate P_i
IA	3·68 ± 0·43*(34)	6·55 ± 1·17 (16)
IC	2·06 ± 0·34 (32)	5·62 ± 1·30 (14)
ID	2·63 ± 0·12†(7)	3·96 ± 0·23 (7)
IIA	3·26 ± 0·37 (6)	6·50 ± 0·88 (6)
IIC	2·27 ± 0·19 (6)	5·00 ± 0·46 (6)
IV	1·89 ± 0·34 (19)	7·41 ± 2·12 (8)

Figures in parentheses indicate number of animals in Group. Values given are mean ± S.E.M.

* Statistically significant with respect to Group IC, $P < 0·001$.

† Statistically significant with respect to Group IV, $P < 0·005$.

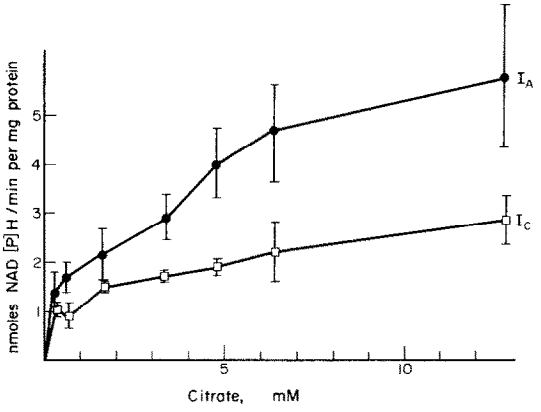

Fig. 1. Reduction of intramitochondrial pyridine-nucleotides following the addition of exogenous sodium citrate (0·6 mM malate). Values given are mean ± S.E.M. of 4 pairs.

citrate was markedly higher (Group ID compared to Group IV, $P < 0{\cdot}005$).

This more rapid reduction of pyridine-nucleotides did not occur when isocitrate was the substrate. (Table 1 and Fig. 2). Neither did it occur when citrate penetration was partially prevented by the non-addition of small amounts of malate and P_i.

Group IA (19): 0·95 ± 0·70 nmoles NAD(P)H/min per mg of protein and

Group IC (18): 0·90 ± 0·51 nmoles NAD(P)H/min per mg of protein.

Reduction of pyridine-nucleotides following the addition of exogenous substrate to a suspension of ruptured mitochondria. This difference between alcohol-fed rats and controls in Group I was still observed when mitochondria were ruptured by Triton X 100 before the addition of citrate (Fig. 3); no difference was observed with isocitrate (Fig. 4).

Transport studies. Transport studies were carried out in Groups IA, IC, and IV. The efflux of isocitrate from mitochondria incubated in citrate, determined by the method of Brand *et al.*, was markedly reduced (Fig. 5). Pretreatment with Triton suppressed this

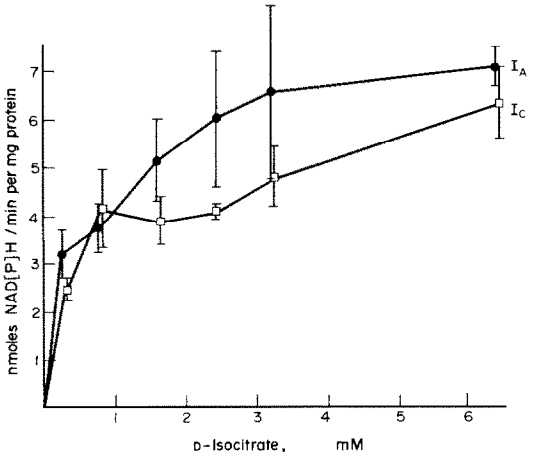

Fig. 2. Reduction of intramitochondrial pyridine-nucleotides following the addition of exogenous sodium D-isocitrate (0·6 mM malate). Values given are mean ± S.E.M. of 4 pairs.

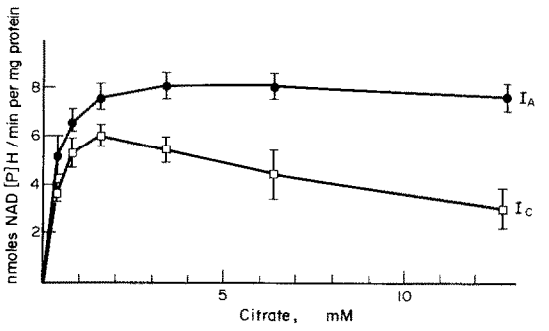

Fig. 3. Reduction of intramitochondrial pyridine-nucleotides following the addition of sodium citrate to mitochondria ruptured by Triton X 100 (Citrate/Mg^{2+} = 1/2). Values given are mean ± S.E.M. of 3 pairs.

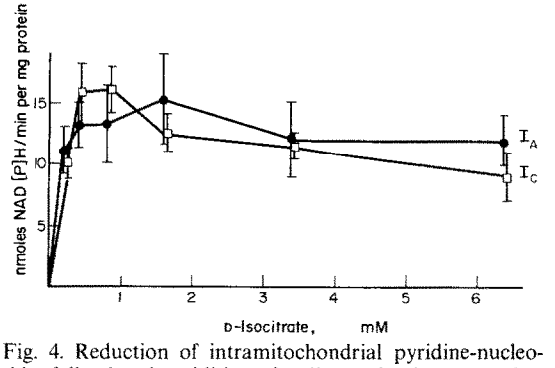

Fig. 4. Reduction of intramitochondrial pyridine-nucleotides following the addition of sodium D-isocitrate to mitochondria ruptured by Triton X 100. Values given are mean ± S.E.M. of 4 pairs.

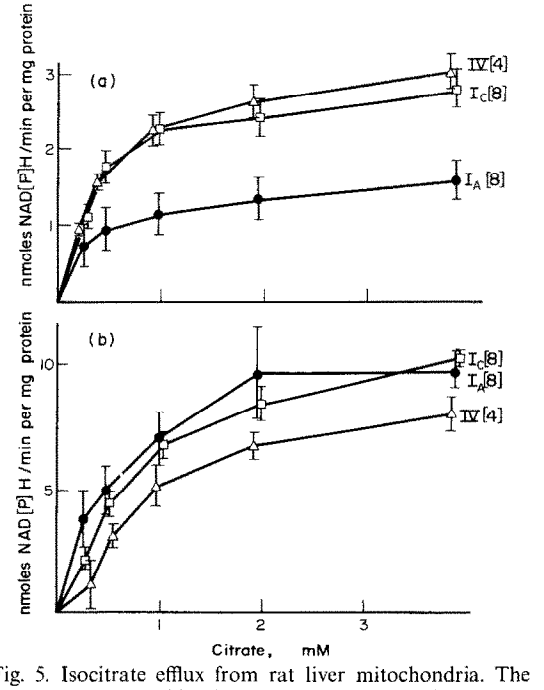

Fig. 5. Isocitrate efflux from rat liver mitochondria. The reaction was started by the addition of sodium citrate (0·1–3·9 mM) and was followed at 340–374 nm with a Perkin Elmer 356 spectrophotometer. (a) Unbroken mitochondria. (b) Mitochondria were ruptured by Triton X 100. Figures in parentheses indicate number of animals in the group. Values given are mean ± S.E.M.

Table 2. Kinetics of (a) [^{14}C]malate uptake and (b) [^{14}C]citrate uptake by rat liver mitochondria

Group	[^{14}]malate uptake (nmoles substrate/min per mg protein)	[^{14}C]citrate uptake
IA	3·14 ± 0·41 (7)	2·07 ± 0·90*(8)
IC	3·29 ± 0·82 (7)	2·98 ± 1·47 (9)
IV	4·08 ± 1·87 (7)	3·89 ± 1·08 (8)

(a) Rat liver mitochondria (3·5 mg) were incubated at 4° in a medium pH 6·5. The uptake was started by the addition of 0·1 mM [^{14}C]malate and terminated by the rapid addition of 20 mM phenyl succinate.

(b) Rat liver mitochondria (5 mg) preloaded with malate, were incubated at 9° in a medium pH 7·2. The uptake was started by adding 0·5 mM [^{14}C]citrate and terminated by the rapid addition of 10 mM 1,2,3 benzene tricarboxylate.

v was measured in nmoles substrate/min per mg protein. Figures in parentheses indicate number of animals in group. Values given are mean ± S.E.M.

* Statistically significant with respect to Group IV, $P < 0·02$.

difference. Uptake of [^{14}C]malate was not significantly changed following on ethanol intake (Table 2, Fig. 6). [^{14}C]citrate penetration into malate loaded mitochondria seemed to be slightly lower in Group IA than in groups IC and IV. When initial velocity was computed from the initial 30-sec linear segment of the experimental curves, significant difference was noted only between Groups IA and IV (Table 2) ($P < 0·02$). Significant differences, however, were observed between Groups IA and IC when the citrate contents of mitochondria were compared at 5 and 10 min ($P < 0·05$), (Fig. 7).

Enzyme activities and coenzyme concentrations. Aconitate hydratase activities were significantly increased in ethanol group compared to controls (+32%, $P < 0·001$) and in Group ID compared to Group IV (32%, $P < 0·001$) (Table 3). Ethanol intake also induced an increase in NAD-linked isocitrate dehydrogenase (+11%) in Group IA compared to Group IC; NADP-linked isocitrate dehydrogenase was unchanged (Table 3).

Mitochondrial concentrations of pyridine-nucleotides were also increased in Group IA compared to

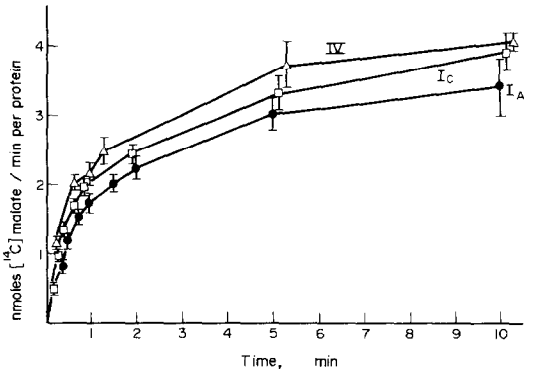

Fig. 6. Kinetics of [^{14}C]malate uptake by rat liver mitochondria. Values given are mean ± S.E.M. of 4 animals. See legend to Table 2 for experimental details.

Group IC: NAD + NADH = +35%, NADP + NADPH = +21%; Mg^{2+} was unchanged (Table 4).

DISCUSSION

Administration of a liquid diet resulted in ethanol consumption in relation to body weight comparable to that obtained in other work [10]. This was not

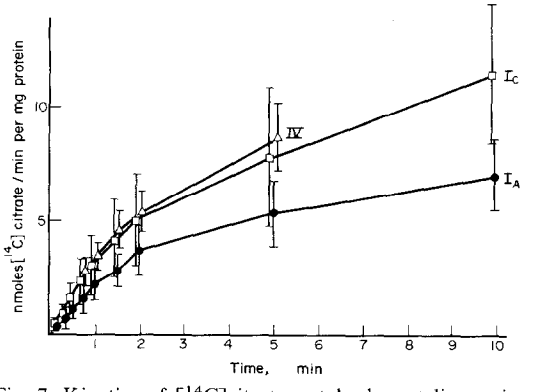

Fig. 7. Kinetics of [^{14}C]citrate uptake by rat liver mitochondria preloaded with malate. Values given are mean ± S.E.M. of 5 animals. See legend to Table 2 for other experimental details.

Table 3. Activity of rat liver mitochondria enzymes involved in citrate metabolism

Group	NAD isocitrate dehydrogenase (nmoles NAD(P)H/min per mg protein)	NADP isocitrate dehydrogenase	Aconitate hydratase (nmoles *cis*-aconitate/min per mg protein)
IA	18·7 ± 0·6 (6)	75·2 ± 1·7 (17)	98·4 ± 2·8*(10)
IC	16·9 ± 0·7 (6)	73·9 ± 2·2 (19)	74·5 ± 3·3 (12)
ID	—	—	82·5 ± 5·3‡(7)
IIA	—	—	90·1 ± 5·3†(7)
IIC	—	—	75·4 ± 5·1 (7)
IV	15·5 ± 1·1 (3)	64·7 ± 4·9 (14)	62·7 ± 1·6 (13)

Isocitrate-dehydrogenase activity. NAD(P) reduction was followed at 340 nm with a Zeiss PM Q II spectrophotometer.
Aconitate-hydratase activity. Disappearance of *cis*-aconitate was monitored at 240 nm.
Figures in parentheses indicate number of animals in each group. Values given are mean ± S.E.M.
* Statistically significant with respect to Group IC, $P < 0·001$.
† Statistically significant with respect to Group IIC, $P < 0·005$.
‡ Statistically significant with respect to Group IV, $P < 0·001$.

Table 4. Concentrations of intramitochondrial magnesium and pyridine-nucleotides

Group	NADH + NAD (nmoles/mg protein)	NADPH + NADP (nmoles/mg protein)	Mg^{2+} (nmoles/mg protein)
IA	3·15 ± 0·23* (6)	2·65 ± 0·07† (6)	45·2 ± 4·5 (4)
IC	2·34 ± 0·11 (6)	2·19 ± 0·14 (6)	51·1 ± 4·1 (4)

Figures in parentheses indicate number of animals in each group. Values given are mean ± S.E.M.

* Statistically significant with respect to Group IC, $P < 0·01$.

† Statistically significant with respect to Group IC, $P < 0·05$.

higher than that observed previously in animals fed solid laboratory food and drinking 30% (v/v) alcohol (unpublished data). Liquid diets therefore offered no other advantage than that of fixity of caloric composition through suppression of a limited degree of free choice between food and diluted alcohol.

Chronic ethanol intake over 3–4 months without any protein or vitamin dificiency induced a moderately fatty liver. The observed 4·8-fold increase in fat droplet volume density at the 3rd month of alcoholic intake is quite comparable to the 3·7-fold increase in triglycerides per mg wet tissue observed by Gordon [10] and the 8-fold increase observed by Lieber *et al.* [13]. An increase in smooth endoplasmic reticulum was observed as previously described [27–29]. There were slight changes in mitochondrial morphology (no obvious megamitochondria were observed). Very rough tests for mitochondrial functions, such as the respiratory control ratio, also showed only slight differences between intoxicated and control animals.

A quantitatively striking difference was nevertheless observed when the effect to exogenous citrate upon the oxido-reduction of intramitochondrial pyridine-nucleotides was studied. The increase following ethanol intake was observed in almost all the pairs under study and the reduction rate was doubled in most Group IA rats. This was not due to the presence of ethanol in the mitochondria, since the blood alcohol level of such animals [8] allows one to compute that less than 2 nmoles of ethanol was present in the test cuvette and the observed difference between alcohol-fed rats and controls persisted in the few pairs which were studied 8 days after alcohol withdrawal.

The observed reduction is the result of several successive phenomena. Citrate enters the mitochondria in exchange for malate [25]. However, a second mechanism for citrate entry has been recently proposed [30]. By means of aconitate hydratase, citrate is transformed into *cis*-aconitate and isocitrate. As described by Chappell [24], part of the isocitrate generated from citrate is exchanged for extramitochondrial citrate and leaves the organelle. The exchange is supposedly effected by the same carrier as the citrate–malate exchange [31]. Isocitrate is also oxidized into oxo-glutarate by several different enzymes. The respective importance of NAD, NADP-linked dehydrogenases and *trans*-hydrogenases is very differently evaluated [32–35]. The kinetics of NAD-linked dehydrogenase, although extensively studied as a classical example of allosteric regulation, are still very diversely interpreted. While ADP activation and ATP and NADH inhibition [36] are well known, the interaction between Mg^{2+} or Mn^{2+}, isocitrate and other chelating molecules, like citrate, has been variously interpreted. Recently, the complex Mg^{2+}–isocitrate has been described as the substrate of the reaction that is inhibited by excess free Mg^{2+} [37, 38]. Citrate 'activation' that has been described in yeast and micro-organisms [39–41] and denied [42, 43] or confirmed [44] in some mammalian tissues might be due to the chelation by citrate of excess Mg^{2+}. No data is available about citrate 'activation' of the liver mitochondrial enzyme. However, citrate or isocitrate may, without entering the mitochondria, stimulate the fatty acid shuttle [45].

Such stimulation without penetration into the mitochondrion cannot account for the difference between alcohol-fed rats and controls which was observed after addition of citrate, malate, and P_i but not with isocitrate, malate, and P_i or citrate by itself.

When citrate is used by itself, the fact that there is no difference shows that there is no increased transport of citrate into Group IA mitochondria due to higher levels of endogenous malate.

The isocitrate exit which appears following the addition of citrate to intact mitochondria is less important in group IA compared to Group IC; pretreatment with Triton X 100 suppresses this difference, which may therefore be reasonably ascribed to an impaired exchange between intramitochondrial isocitrate and external citrate. When assayed by the method of Palmieri *et al.*, the entrance of citrate into malate-loaded mitochondria is however less affected by ethanol intake, although the isocitrate–citrate and malate–citrate exchanges are supposed to be effected by the same tricarboxylic acid carrier. Halperin *et al.* [46] have already observed that long-chain acyl-CoAs inhibit to a greater extent the entrance of citrate when exchanged for intramitochondrial citrate than when exchanged for malate. On the other hand, the present results (impairment of the citrate entrance into malate-loaded mitochondria being more marked than the entrance of malate) are in agreement with the various K_i which have been determined *in vitro* by the method of Palmieri *et al.* for the inhibition by long-chain acyl-CoAs of citrate and malate transports. The K_i is twice as small for citrate than for malate [47]. The acyl-CoAs are elevated in the fatty livers of alcohol-fed rats [10] and Gordon has ascribed to them the 68% inhibition of ADP translocase that she observed.

Such modifications in mitochondrial transport cannot, however, completely explain the observed difference in pyridine-nucleotides reduction following the addition of citrate to intact mitochondria since most of the difference persists when mitochondria have been disrupted by Triton X 100. Some intramitochondrial reactions responsible for the reduction are modified in ethanol fed animals. The increase in NAD-linked isocitrate dehydrogenase activity and in pyridine-nucleotides concentrations might promote a somewhat quicker reduction of NAD and NADP following the addition of citrate but this effect must be slight since it does not lead to a similar increase in reduction rate when the substrate is isocitrate.

The higher aconitate hydratase activity in Group IA could explain the increased reduction observed when only citrate is the substrate. The disappearance rate of *cis*-aconitate is increased in a suspension of ruptured mitochondria from ethanol-fed rats: it is not accelerated when NADP and isocitrate-dehydrogenase are added. The increased disappearance rate of *cis*-aconitate may therefore probably be ascribed to a greater aconitate hydratase activity. No significant difference in reduction of NADP between Groups IA and IC is observed, however, following addition of citrate to ruptured mitochondria supplemented with NADP, Mg^{2+} and isocitrate dehydrogenase.

Lower isocitrate exit, greater aconitate-hydratase and NAD dehydrogenase activity and higher pyridine-nucleotides levels might cumulate their effects to account for the difference between Groups IA and IC. This conclusion cannot be firmly drawn in the absence of a complete quantitative model of the phenomenon. Increased activation by citrate of isocitrate dehydrogenation should not be entirely excluded in trying to account for the lack of differences observed with isocitrate. More generally, the observed increased enzymatic activities may result from some activation or from an increased amount of enzymatic protein.

The greater part of this study has been performed by comparing alcohol-fed rats to paired fed controls in a group which received a high lipid diet. Less extensive investigation of other groups receiving various diets attempted to discriminate the effects of ethanol and fat. Alcohol alone increased the reduction rate of pyridine-nucleotides and the activity of aconitate hydratase as it appears from the comparison of Groups IA vs IC and IIA vs IIC. Lipids, however, when taken in large amounts seemed to promote a slight but significant increase both in pyridine-nucleotides reduction rate by citrate and of aconitate hydratase activity (Group ID vs IV). The highest reduction rate of pyridine-nucleotides and aconitate hydratase activity were observed in Group IA when alcohol and high lipid diet were combined.

Extrapolation from isolated mitochondria to a whole cell is always hypothetical. Long-chain acyl-CoAs, for instance, will be more concentrated in whole cells than in the test cuvette and in mitochondria submitted to several washings. Citrate concentration in normal cells is 0·5 mM according to Krebs [48], that is the lowest concentration here used *in vitro*. Its levels in livers from alcohol-fed rats is unknown. The increased utilization of citrate by mitochondria demonstrated here might, along with the loss of activity of the cytosolic ATP citrate lyase observed in ethanol-fed rats [49], depress fatty acid synthesis. Cytosolic citrate activates acetyl CoA carboxylase which initiate fatty acid synthesis and citrate is transformed by ATP citrate lyase into acetyl CoA which is used for elongation of the fatty acid chain. The mitochondrial changes in citrate metabolism which follow chronic intake could therefore be an adaptative response to the accumulation of triglycerides in the liver cells.

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