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ETHANOL OXIDATION IN SYSTEMS CONTAINING SOLUBLE AND MITOCHONDRIAL FRACTIONS OF RAT LIVER

REGULATION BY ACETALDEHYDE

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Abstract—Systems containing soluble fraction of rat liver, with or without mitochondrial fraction, oxidised [1-^{14}C] ethanol to acetaldehyde, $^{14}CO_2$ and non-volatile ^{14}C-products of which acetate was the principal, and possibly the only, component. Ethanol oxidation was stimulated by pyruvate which served as an electron sink thereby allowing rapid regeneration of NAD. When no mitochondria were present acetaldehyde accumulated, rapidly at first but eventually reaching a plateau. The rate of ethanol oxidation in these systems was much lower than the measured maximum activity of alcohol dehydrogenase (ADH) and it was concluded that ADH was inhibited by the accumulated acetaldehyde. Mitochondria, because of their relatively high aldehyde dehydrogenase (ALDH) activity, prevented the accumulation of acetaldehyde, or quickly removed acetaldehyde already accumulated. This action was accompanied by a sharp increase in the rate of ethanol oxidation, presumably due to the deinhibition of ADH. Cyanamide, an inhibitor of mitochondrial ALDH, blocked the stimulatory effect of mitochondria on ethanol oxidation. It was concluded that, in the reconstituted systems, acetaldehyde played a dominant role in controlling the rate of ethanol oxidation. The possible importance of acetaldehyde in governing ethanol oxidation *in vivo* is discussed.

There are two contrasting views of how the metabolism of ethanol is regulated in mammalian liver. One is that there is ample alcohol dehydrogenase (ADH) present but that its activity is curtailed by the liver's limited capacity to reoxidise cytosolic NADH generated during ethanol oxidation [1–5]. The other is that the liver has a more than adequate capacity to reoxidise cytosolic NADH and that it is the ADH level which governs the rate of ethanol metabolism [6–8].

Arguments against the first hypothesis are based largely on the kinetic properties of ADH [6, 9] and are supported by the observation [10] that the malate–aspartate shuttle, through which the oxidation of cytosolic NADH may be partly mediated [11], probably has a much greater capacity than was at first thought [12]. On the other hand, the notion that the ADH level is the governing factor, while favoured by some theoretical and experimental evidence, is put in doubt by the fact that, except in one or two special instances [7, 13], the correlation between the rate of ethanol elimination *in vivo* and the activity of hepatic ADH is poor when both are expressed on a "per g liver" basis [8, 14, 15].

It appears, therefore, that the regulation of ethanol oxidation could involve other factors that have either been overlooked or have not yet been accorded their due weight. In an earlier article [16] we suggested that acetaldehyde might play a more prominent role than had previously been recognised. The suggestion arose from the observation that acetaldehyde strongly inhibits the oxidation of ethanol by soluble fraction of rat liver under near-physiological conditions, just as it inhibits the activity of purified ADH under non-physiological conditions [17, 18]. The influence of acetaldehyde on ethanol oxidation has now been explored further by using reconstituted ethanol-metabolising systems containing soluble and mitochondrial fractions of rat liver. It is shown that, even when the reaction catalysed by ADH lies far from equilibrium, the overall rate of ethanol oxidation appears to be governed by the removal of acetaldehyde rather than by the level of ADH.

MATERIALS AND METHODS

Chemicals. Biochemical grade substrates, cofactors, enzymes and inhibitors were purchased from Boehringer Mannheim Australia Pty Ltd (North Ryde, N.S.W., Australia) and Sigma Chemical Co. (St. Louis, MO). Acetaldehyde was from BDH Chemicals Australia Pty Ltd (North Sydney, N.S.W., Australia) and was redistilled before use. [1-^{14}C]Ethanol (56 mCi/mmole, radiochemical purity > 98%) was obtained from Amersham Australia Pty Ltd (Surry Hills, N.S.W., Australia) and was diluted with unlabelled ethanol for use in the experiments described later. Other reagents were analytical grade.

Tissue preparations. Livers from adult female Wistar rats were homogenised in a Potter–Elvehjem Teflon/glass homogeniser and the homogenate was fractionated by differential centrifugation. Soluble

fraction was obtained as described previously [16] except that microsomes were removed by centrifugation at 120,000 *g* for 1 hr instead of 50,000 *g* for 1 hr. The protein content of the final dialysed fraction was 35–45 mg/ml.

Mitochondria were isolated after homogenising the liver with 3 vol. of 0.3 M sucrose containing 2 mM HEPES buffer, pH 7.2 and 0.05% (w/v) defatted bovine serum albumin. The procedure was essentially that described by Bustamante *et al.* [19] and the washed mitochondrial pellet was suspended in fresh isolation medium at a concentration of 30–40 mg of protein/ml.

Incubations. Reaction mixtures (total volume 3 ml) containing soluble fraction (up to 16 mg protein) and/or mitochondrial fraction (up to 8 mg protein) were incubated at 30° in sealed Warburg reaction vessels. The vessels were shaken at 110 cycles/min in a reciprocating shaking water bath for up to 90 min. The incubation medium, pH 7.2, comprised 210 mM sucrose, 14 mM KCl, 8 mM potassium phosphate buffer, 3.8 mM HEPES buffer, 1.5 mM $MgCl_2$, 0.6 mM NAD and, except where indicated otherwise, 12 mM sodium pyruvate. To avoid the possibility of mitochondrial respiration becoming limiting the mixture also contained 2.5 mM ADP, 10 mM D-glucose and 3 units of hexokinase, and was gassed with 100% medical-grade oxygen prior to incubation. Reactions were started by adding 30 mM [1-^{14}C]ethanol (9 μCi/mmole) and the centre well of each vessel contained 0.1 ml of 2 M NaOH to trap $^{14}CO_2$.

Incubations were routinely terminated by the addition of 0.2 ml of 3 M perchloric acid and the vessels were immediately placed on ice. After approximately 10 min the contents of the centre well were removed from each vessel and placed in scintillation vials while portions of the acidified reaction mixtures were transferred to capped Eppendorf tubes (1.5 ml capacity) and centrifuged to remove precipitated protein. The acid supernatants were then transferred to clean Eppendorf tubes, neutralised with 3 M KOH and centrifuged to remove the $KClO_4$ precipitates. The clear, neutral supernatants were retained on ice for use in metabolite determinations.

In some experiments in which NADH was to be determined directly incubations were terminated by treatment with 1 M KOH in ethanolic solution according to a standard procedure [20].

Metabolite determinations. Acetaldehyde, pyruvate and lactate were determined routinely by use of enzymatic methods adapted for a CentrifiChem 300 photometric analyser. Acetaldehyde was measured by following the reduction of NAD in the presence of yeast aldehyde dehydrogenase (ALDH) in a medium containing 50 mm sodium pyrophosphate, 50 mM potassium phosphate, 50 mM KCl and 6 mM 2-mercaptoethanol, pH 8 (cf. Ref. 21). Standard procedures were employed for the determination of pyruvate [22] and lactate [23, 24].

In some experiments glucose, glucose 6-phosphate and ATP were measured using minor modifications of a procedure employing hexokinase, glucose-6-phosphate dehydrogenase and NADP [25].

Acetate determinations were made by the method of Guynn and Veech [26] but with yeast acetyl-CoA synthetase instead of the beef heart enzyme, scaled down for the CentrifiChem system. NADH in samples from systems terminated with alkali was measured by a UV assay [20].

Non-volatile ^{14}C-labelled substances formed from [1-^{14}C]ethanol were measured by a method similar to that described by Berry *et al.* [27]. Samples of the neutralised extracts were transferred to scintillation vials and evaporated to dryness under a warm air stream. The dried material in each vial was redissolved in 0.5 ml of distilled H_2O and then 10 ml of a liquid scintillation cocktail [28] was added. Appropriate controls were treated in the same way and the counting was performed on a Packard Tri-Carb C2425 Liquid Scintillation Spectrometer.

Samples from the centre wells of the reaction vessels, in which $^{14}CO_2$ had been trapped, were also evaporated to dryness and then redissolved in distilled H_2O before adding the scintillation cocktail. This procedure was undertaken so that any ^{14}C-labelled ethanol or acetaldehyde that might have distilled into the centre wells during incubation was not falsely accounted for as $^{14}CO_2$.

Enzyme activities. ADH, lactate dehydrogenase (LDH) and ALDH activities of the soluble fraction were assayed spectrophotometrically at 30° under conditions very close to those used in routine incubations. Measurements were made either on the CentrifiChem analyser or on a Varian DMS 90 UV–VIS recording spectrophotometer. ADH was assayed in the same reaction mixture as that described for incubations except that pyruvate was omitted thereby permitting NADH formation to be monitored. For the assay of LDH ethanol was omitted and 0.15 mM NADH and 12 mM pyruvate were included. ALDH was determined with 0.05 or 0.5 mM acetaldehyde in systems from which both ethanol and pyruvate were omitted but to which 0.2 mM 4-methylpyrazole was added. It was demonstrated that methylpyrazole at this concentration completely inhibited the reduction of acetaldehyde by NADH in the presence of soluble fraction, so preventing the underestimation of ALDH due to reoxidation of NADH.

The oxidation of acetaldehyde and ethanol by mitochondrial fraction at 30° was measured using a Rank oxygen electrode. The reaction mixture, which was not gassed with oxygen, was that used for routine incubations but lacking pyruvate and NAD. Following the establishment of a basal rate of oxygen uptake ethanol (30 mM) or acetaldehyde (0.05 or 0.5 mM) was added and the increase, if any, in oxygen uptake was recorded. The effect of ethanol was also determined in the presence of added NAD (0.6 mM).

Protein estimations. The protein content of the liver fractions was determined by the method of Lowry *et al.* [29] with bovine serum albumin as the reference standard.

Expression of results. Where possible results are expressed as mean values ± S.E.M. for three or four replicate experiments. However, with some experiments it was not possible to replicate conditions exactly from one experiment to the next and so the results of typical individual experiments are given in these cases. However, data from individual experi-

Table 1. Enzyme activities of soluble and mitochondrial fractions of rat liver

Enzyme	Substrate	Enzyme activity (nmoles/min per mg protein) Soluble fraction	Mitochondrial fraction
ALDH	0.05 mM acetaldehyde	0.54 ± 0.03	11.7 ± 1.3
	0.5 mM acetaldehyde	1.06 ± 0.06	7.7 ± 1.2
ADH	30 mM ethanol	15.8 ± 0.8	ND
LDH	12 mM pyruvate	4148 ± 380	NT

Cell fractions were incubated at 30° in buffered medium containing 10 mM glucose and 2.5 mM ADP. The activities of alcohol dehydrogenase (ADH), aldehyde dehydrogenase (ALDH) and lactate dehydrogenase (LDH) in the soluble fraction were measured spectrophotometrically by following changes in absorbance at 340 nm as described under Materials and Methods. ALDH and ADH activities of mitochondrial fraction were estimated by monitoring changes in the rate of oxygen consumption as described under Materials and Methods. Results are expressed as the mean values ± S.E.M. for four separate preparations in each case.
ND = not detected; NT = not tested.

ments are presented only if they accurately represent observations made in each of three or more separate experiments performed under similar conditions.

The amount of ethanol oxidised could not be determined directly because of its high initial concentration and the relatively small amount metabolised. Therefore, ethanol oxidation is calculated as the sum of the measured products, acetaldehyde, non-volatile ^{14}C-products and $^{14}CO_2$ [27].

Calculations of the equilibrium factor [9] were made as described previously [16] except that the NAD/NADH ratio was calculated from the lactate/pyruvate ratio rather than being determined directly. The equilibrium constants at 30° for the reactions catalysed by LDH and ADH were those reported by Williamson *et al.* [30] and Backlin [31] respectively.

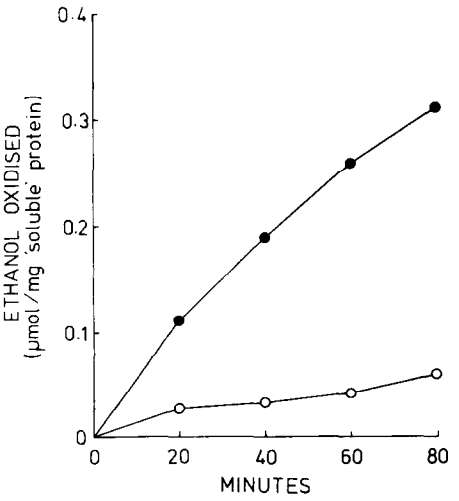

Fig. 1. Effect of pyruvate on ethanol oxidation by soluble fraction. Soluble fraction (7.4 mg protein) was incubated at 30° in 3 ml of buffered medium, pH 7.2, containing 30 mM [1-^{14}C]ethanol, 0.6 mM NAD, 2.5 mM ADP, 10 mM glucose and 3 units of hexokinase with or without added pyruvate. The amount of ethanol oxidised was taken to be equal to the sum of the non-volatile ^{14}C-products, $^{14}CO_2$ and acetaldehyde present at the end of the incubation. ○, no pyruvate; ●, 12 mM pyruvate.

RESULTS

The activities of ADH, ALDH and LDH in the soluble and mitochondrial fractions are given in Table 1. Acetaldehyde, at either 0.05 or 0.5 mM, stimulated the uptake of oxygen by mitochondria, the greater response being elicited by the lower concentration of acetaldehyde. In contrast, ethanol had no detectable effect on respiration even in the presence of added NAD. The results suggest that the mitochondria contained ALDH but were devoid of significant ADH activity. This position was reversed in the soluble fraction in which the activity of ADH exceeded by a very wide margin that of ALDH. Also, the ALDH activity of soluble fraction was seen to be much lower than that of the mitochondria, especially at the lower concentration of acetaldehyde. Very high LDH activity was found in the soluble fraction, reduction of pyruvate taking place over 200 times more rapidly than the oxidation of ethanol by ADH.

Metabolism of [1-^{14}C]ethanol in systems containing only soluble fraction was very slow when no pyruvate was added (Fig. 1). Non-volatile ^{14}C-labelled compounds and $^{14}CO_2$ were the major metabolic products and very little acetaldehyde accumulated (approximately 6 nmoles/mg protein). However, it was found that the concentration of NADH in the incubation mixture rose from zero to more than 0.05 mM during the course of the incubation (data not shown). This rise in NADH was abolished by pyruvate which also caused a marked increase in the rate of ethanol metabolism as shown in Fig. 1. Accompanying this increase was a change in the metabolic pattern, illustrated in Fig. 2. During the early part of the incubation acetaldehyde accumulated rapidly but the concentration of acetaldehyde in the system tended towards a plateau as the incubation progressed. $^{14}CO_2$ was formed at a very slow rate throughout the incubation period and constituted less than 5% of the total products of ethanol metabolism. Non-volatile ^{14}C-products were formed at a relatively constant rate and eventually became the major product. The precise nature of the non-volatile ^{14}C-products was not examined in detail but it was found that their formation was paralleled by

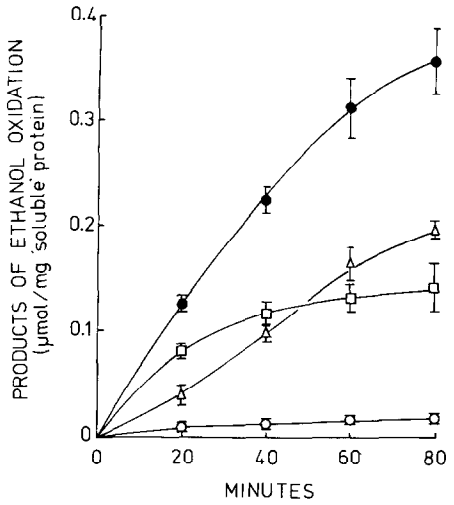

Fig. 2. Products of ethanol oxidation by soluble fraction. Soluble fraction (7–8 mg protein) was incubated at 30° as described for Fig. 1 in the presence of 12 mM pyruvate. The curves show the amount of $^{14}CO_2$ formed (○), the amount of non-volatile ^{14}C-products formed (△), the amount of acetaldehyde accumulated (□) and the sum of these, taken to represent total ethanol oxidation (●). Each point represents the mean value ± S.E.M. for four separate experiments performed with different preparations.

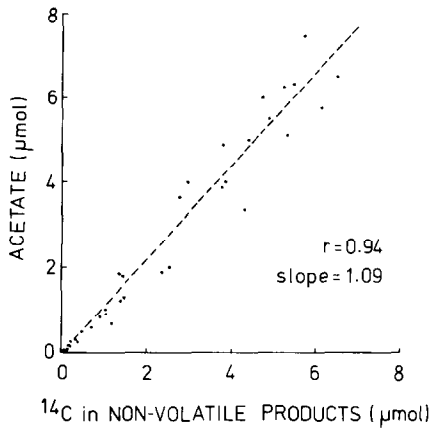

Fig. 3. Correlation between the amount of acetate formed and the amount of non-volatile ^{14}C-products formed. Eight of the points shown were from experiments in which only soluble fraction was present, and the remaining 24 points were from experiments in which both soluble and mitochondrial fractions were present. The correlation coefficient, r, was equal to 0.94.

the production of an almost stoichiometrically equal amount of acetate. The acetate formed was equal to 99 ± 7% of the ^{14}C incorporated into non-volatile products (mean ± S.E.M.; N = 8) suggesting that acetate was the main, if not exclusive, non-volatile ^{14}C-product. The close correlation between acetate and non-volatile ^{14}C-products in these and other experiments is shown in Fig. 3. The data in Table 2 show that pyruvate was used, and lactate produced, at a rate sufficient to account fully for the oxidation of NADH generated during the conversion of ethanol to acetaldehyde and acetate. It was therefore concluded that the oxidative removal of NADH was responsible for the acceleration of ethanol metabolism in the presence of pyruvate. None the less, it is important to note that, even under these conditions, the rate of ethanol metabolism was less than 30% of that expected if ADH were assumed to operate at maximum activity (cf. Table 1). The low rate was not attributable to a loss of ADH during incubation as soluble fraction that was preincubated for 60 min at 30° before exposure to ethanol had an ADH activity almost identical to that of the soluble fraction which was not preincubated. Nor could the low rate be attributed to a reversal of the reaction catalysed by ADH. Even by 80 min the lactate/pyruvate ratio had risen to only 0.21 ± 0.04 (mean ± S.E.M.; N = 3) and the ethanol/acetaldehyde ratio had fallen to only 85 ± 13 (mean ± S.E.M.; N = 3). If it is assumed that the LDH reaction was at effective equilibrium it can be calculated that the ADH reaction lay very far from equilibrium (equilibrium factor > 0.99) and the rate in the reverse direction was therefore negligible. It was concluded that the low rate of ethanol oxidation was probably due to the direct inhibition of ADH by a component of the system, most likely acetaldehyde.

Experiments in which ethanol metabolism was investigated in systems containing different amounts of the same preparation of soluble fraction gave

Table 2. Generation and reoxidation of NADH during ethanol oxidation by soluble fraction of rat liver

Metabolite	Change (nmoles/mg protein)	NADH equivalents (nmoles/mg protein)	
Acetaldehyde	+132 ± 13	+132	
Non-volatile ^{14}C-products (acetate)	+164 ± 16	+328	Total + 490
$^{14}CO_2$	+15 ± 1	+30*	
Pyruvate	−499 ± 55	−499	Average − 517
Lactate	+533 ± 52	−533	

Soluble fraction (7–8 mg protein) was incubated for 60 min at 30° in 3 ml of buffered medium containing 30 mM [1-^{14}C]ethanol, 0.6 mM NAD, 12 mM pyruvate, 2.5 mM ADP, 10 mM glucose and 3 units of hexokinase. Changes in the metabolite levels are presented as the mean values ± S.E.M. for three separate experiments. The calculated amount of NADH produced or used as a consequence of the changes in metabolite levels is shown.

* The amount of NADH associated with $^{14}CO_2$ formation is uncertain.

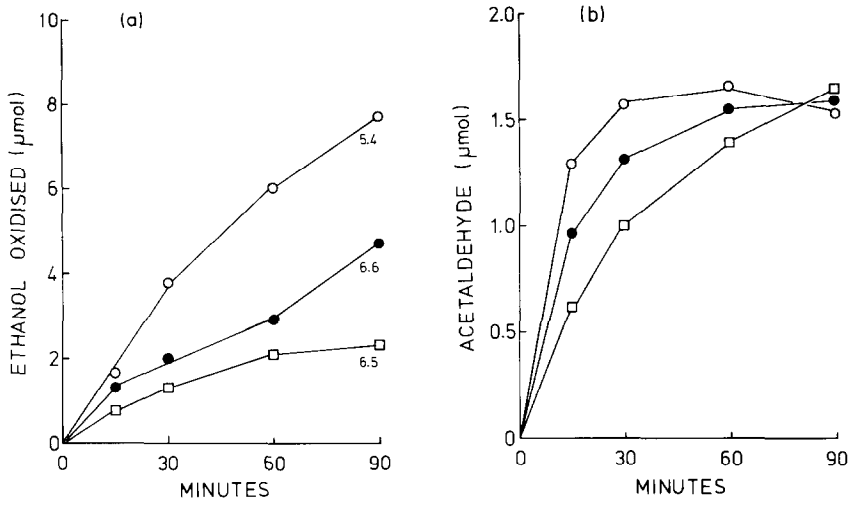

Fig. 4. Effect of varying amount of soluble fraction on: (a) ethanol oxidation and (b) acetaldehyde accumulation. Soluble fraction was incubated under the conditions described for Fig. 2. □, 3.6 mg protein; ●, 7.2 mg protein; ○, 14.4 mg protein. The values beside each of the curves in (a) give the average rate of ethanol oxidation over the full incubation period in nmoles/min per mg protein.

results which supported this conclusion. These are presented in Fig. 4. The values against each of the three curves in Fig. 4a give the rates of ethanol oxidation in nmoles/min per mg of protein and show that the rate was almost directly proportional to the amount of soluble fraction added. However, the curves in Fig. 4b show that, although the initial rate of accumulation of acetaldehyde mirrored the overall rate of ethanol oxidation, the level to which acetaldehyde finally accumulated was the same in all systems. Given that the acetaldehyde concentration stopped rising when its production was balanced by its removal, and accepting that the relative levels of ADH and ALDH were the same whatever the amount of soluble fraction present, it may be inferred that the concentration of acetaldehyde itself was the critical factor in bringing about the balance between production and removal. Moreover, the fact that the rate of ethanol metabolism declined throughout the incubation, most noticeably when the least amount of soluble fraction was present, suggests that the balance was achieved mainly through a fall in the activity of ADH.

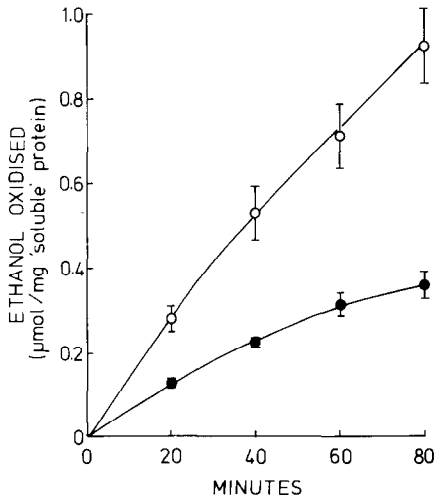

Fig. 5. Effect of mitochondria on the oxidation of ethanol. Systems containing soluble fraction alone (7–8 mg protein) or together with mitochondrial fraction (7–10 mg protein) were incubated as described for Fig. 2. ●, without mitochondrial fraction; ○, with mitochondrial fraction. Each point represents the mean value ± S.E.M. for four separate experiments performed with different tissue preparations.

The relationship between enzyme activities, rates of ethanol oxidation and the degree of acetaldehyde accumulation was explored further by introducing mitochondria into the systems. Mitochondria were earlier shown to possess much more ALDH activity than did soluble fraction, but negligible ADH activity. In Fig. 5 it is shown that addition of an amount of mitochondrial fraction protein roughly equal to the amount of soluble fraction protein led to a very marked increase in the rate of ethanol oxidation. Over the 80-min period the average rate rose from 4.4 ± 0.4 to 11.5 ± 1.1 nmoles/min per mg soluble fraction protein (means ± S.E.M.; N = 4). Experiments with mitochondria alone revealed that less than one fifth of the increase could be attributed to a simple additive effect, indicating that the increase must have required a rise in the ADH activity provided by the soluble fraction. However, despite this increase in ADH activity, no acetaldehyde accumulated in these systems. Non-volatile ^{14}C-products, of which acetate again appeared to be the main, if not exclusive, component, made up 98% of the total products and $^{14}CO_2$ constituted the remaining 2%. Lactate was produced at a near constant rate of 15 nmoles/min per mg soluble fraction protein throughout the 80-min incubation, whereas pyruvate, which can be oxidised by mitochondria, was used at twice that rate during the early part of the incubation but at a rate much closer to that of

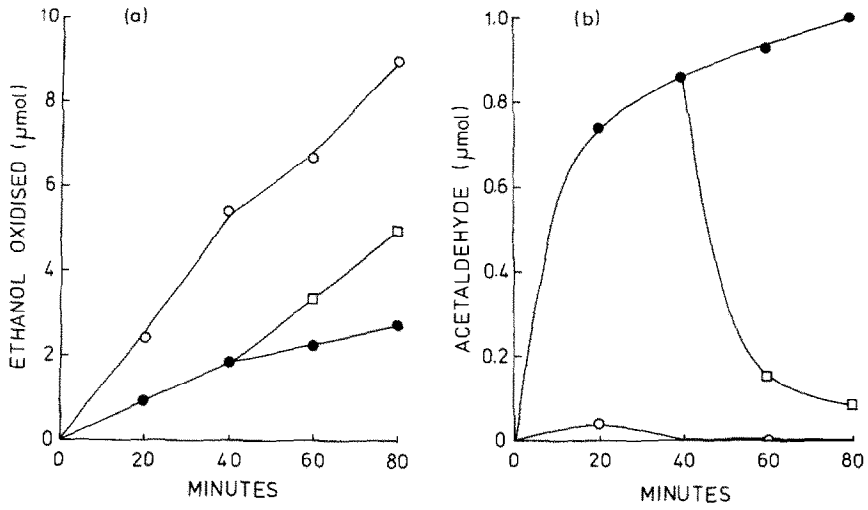

Fig. 6. Effect of mitochondria on the oxidation of ethanol. Soluble fraction (7.9 mg protein) was incubated as described for Fig. 2. Mitochondrial fraction (7.9 mg protein) was absent (●) or was added at either 0 min (○) or 40 min (□). The curves in (a) show the amount of ethanol oxidised; those in (b) show the amount of acetaldehyde accumulated.

lactate formation during the later stages. Reduction of pyruvate to lactate could account for the reoxidation of only 55–65% of the NADH generated by ethanol oxidation and, as no NADH accumulated, it was concluded that the rest was oxidised by the mitochondria. This conclusion seems reasonable in view of the intramitochondrial location of most of the ALDH activity which was assumed to be responsible for up to half of the total NADH production. Data not presented here showed that there was a continuous disappearance of glucose and a smaller accumulation of glucose 6-phosphate during the incubation, consistent with there having been a continuous turnover of ATP. ATP itself was not detectable and it therefore appears unlikely that mitochondrial respiration limited the oxidation of ethanol.

The role of mitochondria in ethanol metabolism was investigated further by adding mitochondria midway through the incubation period when the concentration of acetaldehyde was already approaching its plateau level. The results in Fig. 6 show that upon addition of mitochondria there was an immediate increase in the rate of ethanol oxidation (Fig. 6a) accompanied by a dramatic fall in the level of accumulated acetaldehyde (Fig. 6b). Less than one-third of the additional non-volatile ^{14}C-products and $^{14}CO_2$ formed during the 40 min fol-

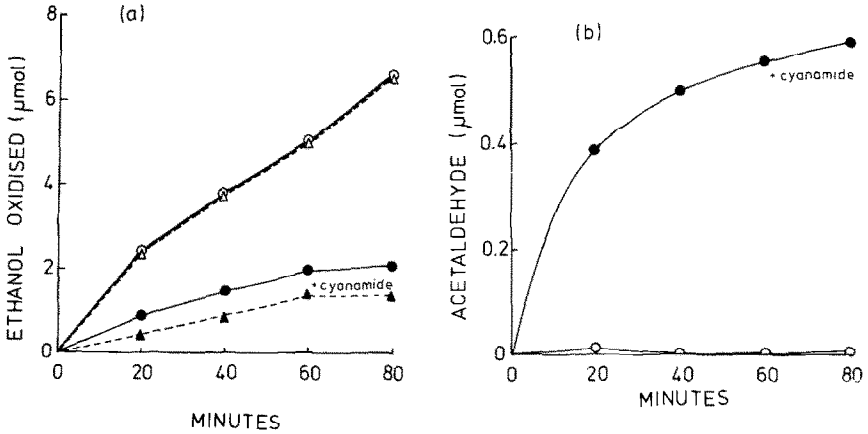

Fig. 7. Effect of cyanamide on the oxidation of ethanol. Systems containing soluble fraction (7.9 mg protein) and mitochondrial fraction (8.4 mg protein) were preincubated at 30° for 8 min with or without 0.2 mM cyanamide before addition of 30 mM [1-^{14}C]ethanol. The incubation then proceeded as described for Fig. 2. The curves in (a) show the amount of ethanol oxidised (○, ●) and the amount of non-volatile ^{14}C-products formed (△, ▲). The curves in (b) show the amount of acetaldehyde accumulated (○, ●). In each case the open symbol represents the absence of cyanamide and the closed symbol represents the presence of cyanamide.

lowing the addition of mitochondria was directly attributable to the oxidation of acetaldehyde accumulated during the previous 40 min, again emphasizing that with mitochondria present the activity of ADH rises thus generating an increase in total flux through the metabolic pathway.

The simplest explanation of these results is that, by oxidising acetaldehyde, mitochondria brought about the deinhibition of ADH activity. This interpretation was supported by other observations, one of which was that addition of a smaller amount of mitochondrial fraction than those used in the experiments shown in Fig. 5 was less effective both in preventing acetaldehyde accumulation and in stimulating ethanol oxidation (data not shown). However, more conclusive support came from experiments in which cyanamide, an inhibitor of mitochondrial ALDH, was used. The results of one such experiment, presented in Fig. 7, show that preincubation with cyanamide led to a marked decrease in ethanol oxidation due to a very steep fall in the formation of non-volatile ^{14}C-products (Fig. 7a). Although acetaldehyde accumulation rose very substantially (Fig. 7b) the rise was far lower than the fall in acetaldehyde oxidation. Hence, the pattern that emerged in systems containing mitochondria and cyanamide was virtually identical to that seen in systems lacking mitochondria, strongly suggesting that the stimulatory action of mitochondria on ethanol oxidation was entirely due to their ability to oxidise acetaldehyde. The decrease in the rate at which ethanol was oxidised to acetaldehyde in the presence of cyanamide was closely followed by a decrease in the rate at which pyruvate was reduced to lactate but other avenues of pyruvate metabolism were apparently unaffected, indicating that the effect of cyanamide on ALDH was specific. These data suggest that ALDH activity and, consequently, the removal of acetaldehyde influences the activity of ADH and hence the rate of flux through the ethanol-metabolising pathway.

DISCUSSION

Despite the apparent simplicity of the metabolic pathway leading from ethanol to acetate considerable controversy has surrounded the question of what factors are most important in regulating the rate of ethanol oxidation in mammalian liver. The three factors thought to be potentially important are the concentrations of free NAD and/or NADH in the cytosol [1–5], the real level of hepatic ADH [6–8] and the intrahepatic concentration of acetaldehyde [8, 9, 16]. However, it must be pointed out that although many workers have elected to favour one or other of these factors as being uniquely important there is no reason why all should not make a significant contribution to the regulation of ethanol metabolism. The purpose of the present study was to look at possible regulatory mechanisms by using an ethanol-metabolising system reconstituted from soluble and mitochondrial fractions of rat liver.

The fully reconstituted systems metabolised high concentrations of ethanol in a similar way to other *in vitro* preparations of liver [27, 32, 33], acetate apparently being the principal end product and only very small quantities of CO_2 being formed. Accumulation of acetaldehyde was variable and depended on the particular experimental circumstances. Pyruvate, which readily accepts electrons from NADH in the presence of LDH, stimulated ethanol metabolism especially when little or no mitochondrial fraction was present, indicating that ethanol oxidation in the reconstituted systems may be restricted by a lack of adequate NADH-oxidising activity. The same phenomenon has been reported frequently in studies with isolated hepatocytes [34–38] but, as Crow *et al.* [37] and Berry *et al.* [38] have pointed out, pyruvate merely restores ethanol oxidation in hepatocytes to the *in vivo* rate and, despite claims to the contrary [39], it seems unlikely that administration of pyruvate to intact rats has any impact on ethanol oxidation [8].

When NADH oxidation was eliminated as the rate-limiting process in the reconstituted systems other regulatory mechanisms appeared. In systems lacking mitochondria a pronounced accumulation of acetaldehyde occurred and the rate of ethanol metabolism was far lower than would have been the case had ADH been fully active. This was a clear indication that the ADH level *per se* was not the sole governing factor. However, when mitochondrial fraction was added in sufficient quantity to prevent acetaldehyde accumulation the rate of ethanol metabolism almost tripled despite the fact that the mitochondria contributed no detectable ADH activity. Cyanamide, a potent inhibitor of mitochondrial ALDH both *in vitro* and *in vivo* [40–45], abolished the stimulatory effect of mitochondria and returned ethanol metabolism to the rate and pattern observed in systems containing only the soluble fraction.

These findings strongly suggest that ALDH activity and acetaldehyde accumulation were important factors in determining the rate of ethanol oxidation in reconstituted systems. Furthermore, the observations bore a strong similarity to those made in studies with isolated hepatocytes in which inhibitors of ALDH, especially in the presence of pyruvate, promote the accumulation of acetaldehyde and cause the rate of ethanol metabolism to fall [35, 46, 47].

The relationship between raised acetaldehyde levels and lowered rates of ethanol oxidation is seen not only in reconstituted systems and hepatocytes but has also been noted in experiments with perfused liver [2] and intact animals [48, 49]. At first sight it appears paradoxical that acetaldehyde levels should rise in the face of decreased acetaldehyde production but the phenomenon can be explained relatively easily in either of two ways. Both explanations accept the obvious point that for acetaldehyde to accumulate the mechanisms responsible for its removal, principally ALDH, must be less active than those responsible for its production, principally ADH. Both also accept that, because the low K_m mitochondrial ALDH, which is thought to be of prime importance in hepatic ethanol metabolism [50, 51], must be saturated at about 10 μM acetaldehyde [52–55] and may be inhibited by concentrations in excess of 25 μM [56], it is highly unlikely that acetaldehyde removal can accommodate to a constant

rate of acetaldehyde formation. Even if this were theoretically possible it would require the attainment of intolerably high acetaldehyde concentrations in order to raise sufficiently the activity of the high K_m ALDH isoenzymes. Hence, to reach a steady state, there must be a downward adjustment in the rate of acetaldehyde production to balance the more fixed rate of acetaldehyde removal. Where the two explanations differ is in the way they each account for this downward adjustment of acetaldehyde production. One suggests that the accumulation of acetaldehyde leads to the reversal of the ADH reaction until the net flux in the forward direction is just enough to balance the loss of acetaldehyde through the ALDH reaction. This explanation has been used frequently [2, 46, 47, 49, 57] and implies that ADH behaves as an equilibrium enzyme [57]. The alternative explanation [16] is that acetaldehyde directly inhibits, in a concentration-dependent fashion, the oxidation of ethanol by ADH thereby regulating its own production. Hence, in simple terms, the first explanation has the net rate of acetaldehyde production being determined by the concentration of all the reactants of the ADH reaction since all affect the equilibrium position, whereas the second considers the acetaldehyde concentration alone to be important.

When the merits of the two explanations are examined in relation to the observations made in the present study, it becomes clear that, provided one major assumption is made, the second is the more satisfactory. The assumption is that because LDH activity is very high in the reconstituted systems the reaction it catalyses will be maintained at, or very close to, equilibrium. If this is so it can be calculated, using the appropriate equilibrium constant [30], that even at the highest lactate/pyruvate ratio achieved in the systems, the NAD/NADH ratio exceeded 10,000. When this value is used together with the observed ethanol and acetaldehyde concentrations and the equilibrium constant for the reaction catalysed by ADH [31], it is found that the reaction lay so far from equilibrium than any reversal would have diminished net flux in the forward direction by less than 1%. Therefore, the decrease in acetaldehyde formation that accompanies the increase in acetaldehyde concentration cannot be attributed to reversal of the ADH reaction and must instead be due to a direct inhibition of ADH, presumably by acetaldehyde itself.

The conclusion that acetaldehyde is the inhibitory agent is supported by previous studies in which it was demonstrated that the concentrations of acetaldehyde attained in the reconstituted systems strongly depressed the oxidation of ethanol by rat liver soluble fraction [16] or by purified ADH [17, 18]. Given that concentrations of a similar magnitude may also occur in systems containing isolated hepatocytes [35, 46, 47] and in the liver *in vivo* [8, 58, 59], the possiblity exists that regulation of ADH by acetaldehyde could extend to these systems. Indeed, the situation in hepatocytes incubated with pyruvate and ALDH inhibitors [46, 47] resembles so closely that in reconstituted systems that this might be the only satisfactory explanation of the lowered rates of ethanol metabolism that accompany rises in the acetaldehyde concentration. In intact animals too there is little evidence to suggest that ADH acts as an equilibrium enzyme. Guynn and Pieklik [60] observed that the cytoplasmic NAD/NADH ratio in the liver increased at higher ethanol doses, contrary to what might be expected if ADH were maintaining equilibrium unless acetaldehyde were also to rise dramatically. Similarly it has been found that the rate of ethanol metabolism in rats is positively correlated with the lactate/pyruvate ratio in the blood [7, 8], an observation which is difficult to reconcile with the idea of the ADH reaction lying at, or close to, equilibrium.

It is concluded, therefore, that in the reconstituted systems the rate at which ethanol is oxidised by ADH may be dictated to a large degree by the concentration of acetaldehyde, and that this constitutes the mechanism by which the rates of production and removal of acetaldehyde are balanced. The steady-state concentration of acetaldehyde could vary widely depending on the relative activities of ADH and ALDH, and the overall rate of ethanol oxidation must be primarily determined by the lower of the two activities. The degree to which this mode of regulation operates *in vivo* remains to be established, though the fact that relatively high concentrations of acetaldehyde may be found in the liver and hepatic venous blood of experimental animals suggests that its importance might so far have been underestimated.

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