The Time-Course of the Effects of Ethanol on the Redox and Phosphorylation States of Rat Liver

By R. L. VEECH, R. GUYNN and D. VELOSO

Laboratory of Clinical Psychopharmacology, Division of Special Mental Health Research, IRP, and
 National Institute of Alcohol Abuse and Alcoholism, National Institute of Mental Health,
 Saint Elizabeths Hospital, Washington D. C., 20032, U.S.A.

(Received 29 September 1971)

1. The time-course of the effects of ethanol administration on the metabolite concentrations, redox states and phosphorylation state was studied in the freeze-clamped liver of starved rats. The response was found to vary with the time after ethanol administration. 2. Administration of ethanol caused an immediate decrease in the $[NAD^+]/[NADH]$ ratio of both cytoplasm and mitochondria, which persisted over the 30 min studied. 3. The free cytoplasmic $[NADP^+]/[NADPH]$ ratio in liver decreases immediately after ethanol administration but returns nearly to control values after 15 min. 4. The cytoplasmic $[ATP]/[ADP][HPO_4^{2-}]$ ratio is elevated 15 min after ethanol administration in the starved rat. 5. The rapid and large changes in most metabolite concentrations measured appeared to result from the maintenance of near-equilibrium in a wide inter-linked network. 6. Differences between fed and starved rats 15 min after ethanol administration were slight.

Ethanol is known to increase the $[lactate]/[pyruvate]$ ratio in liver (Forsander *et al.*, 1965b). This effect is generally believed to result from the oxidation of alcohol by NAD^+ acting through alcohol dehydrogenase (EC 1.1.1.1), which in liver shares a common NAD^+ pool with lactate dehydrogenase (EC 1.1.1.27). Previous studies on the effects of ethanol have shown changes in the concentration of one or another metabolite by a number of techniques *in vivo* and *in vitro* (Reboucas & Isselbacher, 1961; Lieber, 1967; Krebs, 1968; Williamson *et al.*, 1969; Lindros, 1970). However, neither the sequence of

Krebs & Veech (1969) have shown that changes in the cytoplasmic NAD couple must be reflected in changes of the mitochondrial NAD couple, the cytoplasmic NADP couple, or the cytoplasmic phosphorylation state, or by changes in certain metabolite concentrations. At equilibrium the cytoplasmic NADP couple is related to the cytoplasmic NAD couple in the following manner, through isocitrate dehydrogenase (EC 1.1.1.42), glutamate-oxaloacetate transaminase (EC 2.6.1.1) and malate dehydrogenase (EC 1.1.1.37), all enzymes of high activity:

$$\frac{[NADP_c^+]}{[NADPH_c]} = \frac{[NAD_c^+]}{[NADH_c]} \times \frac{[Glutamate][Malate][CO_2]}{[Isocitrate][Aspartate]} \times \frac{K_{GOT} K_{MDH}}{K_{IDH}} \quad (1)$$

events after the decrease in the cytoplasmic $[NAD^+]/[NADH]$ ratio nor the widespread effects of this change on the linked mitochondrial $[NAD^+]/[NADH]$ ratio, cytoplasmic $[NADP^+]/[NADPH]$ ratio and the cytoplasmic $[ATP]/[ADP][HPO_4^{2-}]$ system have been defined.

Likewise at equilibrium the cytoplasmic phosphorylation state is a function of the redox state of the cytoplasmic NAD couple because of the high activity of the 3-phosphoglycerate kinase (EC 2.7.2.3) and the glyceraldehyde 3-phosphate dehydrogenase reaction (EC 1.2.1.12) (Veech *et al.*, 1970):

$$\frac{[ATP]}{[ADP][HPO_4^{2-}]} = \frac{[NAD^+]}{[NADH]} \times \frac{[Glyceraldehyde\ 3-phosphate]}{[3-Phosphoglycerate]} \times K_{GAPDH} \cdot K_{3-PGK} \quad (2)$$

Finally the mitochondrial NAD couple will reflect changes in the cytoplasmic NAD couple through a number of systems, one of which would be mitochondrial glutamate dehydrogenase (EC 1.4.1.2), cytoplasmic glutamate-oxaloacetate transaminase and cytoplasmic malate dehydrogenase acting in the presence of α -oxoglutarate diffusible through the mitochondrial membrane:

$$\frac{[\text{NAD}_m^+]}{[\text{NADH}_m]} = \frac{[\text{NAD}_c^+]}{[\text{NADH}_c]} \times \frac{[\text{Malate}][\text{NH}_4^+]}{[\text{Aspartate}]} \times \frac{K_{\text{GOT}} \cdot K_{\text{MDH}}}{K_{\text{GDH}}} \quad (3)$$

This paper attempts to show that many of the effects of ethanol on metabolism result from the decrease in the $[\text{NAD}^+]/[\text{NADH}]$ ratio of the cytoplasm. The effects of this change in $[\text{NAD}^+]/[\text{NADH}]$ ratio are not limited solely to metabolites that are substrates of the cytoplasmic NAD^+ -linked enzymes such as lactate, malate and α -glycerophosphate, but are transmitted by a network of near-equilibria through the other $[\text{NAD}^+]/[\text{NADH}]$, $[\text{NADP}^+]/[\text{NADPH}]$ and $[\text{ATP}]/[\text{ADP}][\text{HPO}_4^{2-}]$ systems of the cell. Changes in the concentration of several liver metabolites, which might otherwise be thought to be unco-ordinated, can be explained by linking them to changes in nucleotide ratios. Particular emphasis has been placed on the time-course of the changes in the redox and phosphorylation states because of their rapidity.

Abbreviations and symbols used in this paper have the following meanings. $[\text{NAD}_c^+]/[\text{NADH}_c]$, Ratio of the concentration of oxidized to reduced free cytoplasmic nicotinamide-adenine dinucleotides. $[\text{NAD}_m^+]/[\text{NADH}_m]$, Ratio of the concentration of oxidized to reduced free mitochondrial nicotinamide-adenine dinucleotides. $[\text{NADP}_c^+]/[\text{NADPH}_c]$, Ratio of the concentration of oxidized to reduced cytoplasmic nicotinamide-adenine dinucleotide phosphate. K_{GOT} , Equilibrium constant of the glutamate-oxaloacetate transaminase reaction. K_{MDH} , Equilibrium constant of the malate dehydrogenase reaction. $K_{\alpha\text{GPDH}}$, Equilibrium constant of α -glycerophosphate dehydrogenase. K_{LDH} , Equilibrium constant of the lactate dehydrogenase reaction. K_{IDH} , Equilibrium constant of the isocitrate dehydrogenase reaction. K_{GAPDH} , Equilibrium constant of the glyceraldehyde 3-phosphate dehydrogenase reaction. $K_{3\text{-PGK}}$, Equilibrium constant of the 3-phosphoglycerate kinase reaction. K_{TPI} , Equilibrium constant of the triose phosphate isomerase reaction.

Experimental

Rats

Male rats of the Sprague-Dawley strain (Zivic-Miller, Pittsburgh, Pa., U.S.A.) weighing 200–220 g

were used. 'Fed' rats were given Wayne Lablox containing 4% fat, 24% crude protein and 50.8% carbohydrate *ad libitum*. 'Starved' rats were kept without food for 48 h before death.

Reagents

Standard analytical-grade laboratory reagents were obtained from Mallinckrodt Chemical Co., St.

Louis, Mo., U.S.A. Florisil (100–200 mesh) was obtained from Fisher Scientific Co., Fairlawn, N.J., U.S.A. Enzymes and substrates were obtained from the Boehringer (Mannheim) Corp., New York, N.Y., U.S.A.

Treatment of liver

The rats were killed by cervical dislocation. The liver was rapidly removed and frozen (Wollenberger *et al.*, 1960). The time from cervical dislocation to freezing the liver was less than 8 s. The frozen liver was treated as described by Veech *et al.* (1969a). Before treatment with Florisil a sample of the supernatant was saved for the ATP, ADP, AMP and P_i determination.

Administration of ethanol

The rats were injected intraperitoneally with 2 ml of a 1 M-ethanol solution in 0.9% NaCl at various times before death. The control rats were injected with 0.9% NaCl solution. Since oral administration gave variable ethanol concentrations in the liver, the intraperitoneal route was chosen.

Determination of metabolites

Pyruvate was determined by the method of Bücher *et al.* (1963), dihydroxyacetone phosphate by the method of Bücher & Hohorst (1963), α -oxoglutarate by the method of Bergmeyer & Bernt (1963) and acetoacetate by the method of Williamson *et al.* (1962). These four metabolites were determined in phosphate buffer at pH 7.0. Lactate, malate and α -glycerophosphate were determined by the methods of Hohorst (1963b,c,d) by using tris-HCl buffer, pH 9.5. Phosphoenolpyruvate and 3-phosphoglycerate were determined by the method of Czok & Eckert (1963). Citrate was determined by the method of Gruber & Moellering (1966) in tris-HCl buffer, pH 7.4. The concentration of isocitrate was calculated from measured citrate by using the value of 15 for the citrate/isocitrate ratio (Krebs & Veech, 1969). The

value of 15 agrees well with the citrate/isocitrate equilibrium ratio of 13.5 found by Krebs (1953) using aconitase (EC 4.2.1.3). Previous studies had shown that there was no significant change in the [citrate]/[isocitrate] ratio after ethanol administration in the starved animal (Veech, 1968). Glucose 6-phosphate was determined by the method of Hohorst (1963a). Glutamate was determined by the method of Bernt & Bergmeyer (1963) by using tris-HCl buffer, pH 9.0, and β -hydroxybutyrate was determined by the method of Williamson *et al.* (1962) with pyro-

phosphate buffer, pH 8.5. ATP was determined by the method of Lamprecht & Trautshold (1963), ADP and AMP by the method of Adam (1963). P_i was determined by the method of Martin & Doty (1949).

Ethanol was determined by the method of Krebs & Perkins (1970). Samples were chosen to contain no more than 0.07 μ mol of ethanol and readings were taken for up to 5 min.

Recovery experiments were done on all compounds reported. Substrate amounts equal to the

Table 1. Concentrations of metabolites in freeze-clamped livers of starved rats after ethanol injection

Values are given in μ mol/g wet wt. $\pm$ S.E.M. Animals were killed at the times indicated after the injection.

	Time after ethanol (min) ... No. of observations ...	Concn. (μ mol/g wet wt.)				
		0 22	5 9	10 7	15 5	30 9
L-Lactate		0.27 ± 0.02	1.65 ± 0.30	0.84 ± 0.08	0.63 ± 0.06	0.77 ± 0.08
Pyruvate		0.019 ± 0.001	0.022 ± 0.002	0.019 ± 0.002	0.019 ± 0.002	0.017 ± 0.001
L-Malate		0.36 ± 0.03	1.13 ± 0.13	1.05 ± 0.09	0.89 ± 0.06	0.97 ± 0.03
Phosphoenolpyruvate		0.072 ± 0.007	0.031 ± 0.004	0.021 ± 0.004	0.012 ± 0.001	0.010 ± 0.001
3-Phosphoglycerate		0.14 ± 0.02	0.061 ± 0.050	0.042 ± 0.006	0.030 ± 0.003	0.022 ± 0.003
Dihydroxyacetone phosphate		0.020 ± 0.001	0.022 ± 0.001	0.022 ± 0.003	0.019 ± 0.001	0.018 ± 0.001
α -Glycerophosphate		0.23 ± 0.02	1.36 ± 0.11	1.45 ± 0.15	0.98 ± 0.05	1.14 ± 0.06
Glucose 6-phosphate		0.071 ± 0.007	0.079 ± 0.009	0.051 ± 0.004	0.044 ± 0.002	0.040 ± 0.001
Citrate		0.43 ± 0.02	0.62 ± 0.05	0.68 ± 0.08	0.58 ± 0.02	0.46 ± 0.05
Isocitrate		0.028 ± 0.001	0.041 ± 0.003	0.045 ± 0.006	0.039 ± 0.002	0.031 ± 0.003
α -Oxoglutarate		0.120 ± 0.007	0.069 ± 0.006	0.112 ± 0.010	0.140 ± 0.009	0.105 ± 0.010
L-Glutamate		2.53 ± 0.12	2.26 ± 0.14	3.17 ± 0.09	3.15 ± 0.11	3.58 ± 0.29
Acetoacetate		0.65 ± 0.04	0.23 ± 0.05	0.29 ± 0.02	0.26 ± 0.01	0.24 ± 0.02
β -Hydroxybutyrate		1.82 ± 0.13	1.62 ± 0.22	1.77 ± 0.22	1.47 ± 0.09	1.85 ± 0.17
ATP		2.32 ± 0.08	2.12 ± 0.14	2.18 ± 0.07	2.44 ± 0.06	2.28 ± 0.05
ADP		1.48 ± 0.03	1.48 ± 0.07	1.38 ± 0.07	1.10 ± 0.03	1.14 ± 0.04
AMP		0.47 ± 0.04	0.84 ± 0.10	0.98 ± 0.13		0.72 ± 0.10
P_i		3.69 ± 0.12	4.09 ± 0.28	3.34 ± 0.19	2.49 ± 0.10	2.88 ± 0.10

endogenous amount were frozen in a centrifuge tube before the addition of frozen liver powder. The assay was then carried out as usual and the endogenous amount subtracted. Recoveries of all metabolites were between 90 and 105%. The spectrophotometric measurements were carried out on a Zeiss PMQ II spectrophotometer modified to contain 10 cuvettes.

Results

Concentrations of ethanol

The amount of ethanol injected was calculated to produce a 10mM concentration in total body water, the concentration found to produce maximum rates of alcohol oxidation by alcohol dehydrogenase *in vitro* (Krebs, 1968). Actual concentrations of ethanol measured in the livers at 5min ranged from 8 to 10mM, with values falling approx. 50% 30min after injection. These values do not represent the exact concentration in liver, owing to slight adherence of ethanol to the liver surface. Assuming that approx. 5mm-ethanol, distributed through the total body water of a 220g rat, was being metabolized in 30min by a rat liver weighing 7g, the rate of ethanol oxidation can be calculated to be about 4 μ mol/min per g wet wt. of liver.

Time-course of the effect of ethanol on the concentrations of metabolites in the livers of starved rats

At 5min after an ethanol injection, there was a sixfold increase in [lactate] and [α -glycerophosphate] and a threefold increase in [malate], followed by a gradual decline of these metabolite concentrations towards normal (Table 1). [Citrate] and [AMP] followed a similar pattern, increasing 1.5-fold and twofold respectively at 10min. [Glucose 6-phosphate] showed a gradual fall throughout the time-period studied, decreasing 1.7-fold 30min after ethanol administration. The largest proportionate changes induced by ethanol were decreases in [3-phosphoglycerate] and [phosphoenolpyruvate], both of which decreased sevenfold over the 30min studied. To have equal scales for increases or decreases of metabolite concentrations, decreases are expressed as the reciprocal of the factor of change. Total ketone-body concentration showed a slight and variable decrease after ethanol administration. This decrease was due mainly to a fall in [acetoacetate], which persisted for 30min. [Glutamate] fell slightly at 5min, followed by a slow rise to a value 1.4 times the control at 30min. [α -Oxoglutarate], the common intermediate between citrate and glutamate, showed an initial fall at 5min with return to control values thereafter. In

Table 2. Cytoplasmic and mitochondrial redox and phosphorylation ratios in freeze-clamped liver at time-intervals after a single dose of ethanol

Ratios are calculated from the metabolite values given in Table 1 and given as means $\pm$ S.E.M. The *P* values given in parentheses refer to differences between the means of the saline and ethanol injections as judged by Student's *t* test. For methods of calculation see the text. Animals were killed at times indicated after the injection.

Time after ethanol (min) ... No. of observations ...	Concentration ratio				
	0	5	10	15	30
[NAD ⁺]/[NADH] from lactate dehydrogenase (cytoplasm)	719 ± 64	132 ± 16	223 ± 44	285 ± 33	211 ± 19
		(<0.001)	(<0.001)	(<0.01)	(<0.001)
[NADP ⁺]/[NADPH] from malic enzyme (cytoplasm)	0.0018 ± 0.0001	0.00067 ± 0.00008	0.00081 ± 0.00004	0.00074 ± 0.00006	0.00056 ± 0.00004
		(<0.001)	(<0.02)	(<0.002)	(<0.001)
[NADP ⁺]/[NADPH] from isocitrate dehydrogenase (cytoplasm)	0.0043 ± 0.0002	0.0018 ± 0.0002	0.0026 ± 0.0003	0.0036 ± 0.0002	0.0035 ± 0.0002
		(<0.001)	(<0.001)		(<0.05)
[ATP]/[ADP][HPO ₄ ²⁻] (M ⁻¹) (cytoplasm)	735 ± 50	654 ± 100	840 ± 97	1511 ± 119	1189 ± 92
				(<0.001)	(<0.001)
[NAD ⁺]/[NADH] from β -hydroxybutyrate dehydrogenase (mitochondria)	7.67 ± 0.37	2.67 ± 0.35	3.57 ± 0.35	3.56 ± 0.11	2.71 ± 0.09
		(<0.001)	(<0.001)	(<0.001)	(<0.001)
[ATP]/[AMP] [ADP ²]	0.49 ± 0.04	0.91 ± 0.18	1.14 ± 0.14		1.22 ± 0.15
		(<0.002)	(<0.001)		(<0.001)

contrast with α -oxoglutarate, the other measured oxidized partners of the powerful redox couples, pyruvate and dihydroxyacetone phosphate, showed no significant change. [ATP] was unaffected by ethanol. In contrast, [ADP] decreased slightly 10min after ethanol injection, falling 1.3-fold at 15min. Although there was a net increase of $1\mu\text{mol/g}$ in [α -glycerophosphate] 5min after ethanol injection, the [P_i] did not decrease. By 10min, however, [P_i] had decreased below the control value, falling by 1.5-fold at 15min and remaining low at 30min.

Time-course of the effects of ethanol on the redox state of rat liver cytoplasm and mitochondria in the starved animal

Ethanol decreased the [NAD^+]/[NADH] ratio in the cytoplasm 5.5-fold 5min after administration (Table 2). There was a gradual rise in this ratio thereafter until at 30min the ratio decreased 2.4-fold below the control value. The [NAD^+]/[NADH] ratio of the mitochondria decreased 2.9-fold 5min after ethanol was given. This ratio remained low throughout the next 25min. The [NADP^+]/[NADPH] ratio of the

Table 3. *Effects of ethanol on metabolite concentrations of freeze-clamped liver from fed rats*

Concentrations are expressed as $\mu\text{mol/g}$ fresh wt. and are given as means $\pm$ S.E.M. of the number of observations indicated. Animals were killed 15min after injection.

	No. of observations ...	Saline injection 6	Ethanol injection 6
L-Lactate		0.59 ± 0.06	1.28 ± 0.20
Pyruvate		0.11 ± 0.01	0.031 ± 0.008
L-Malate		0.40 ± 0.04	0.78 ± 0.06
Phosphoenolpyruvate		0.14 ± 0.01	0.023 ± 0.007
3-Phosphoglycerate		0.31 ± 0.03	0.050 ± 0.016
Dihydroxyacetone phosphate		0.024 ± 0.004	0.019 ± 0.001
α -Glycerophosphate		0.084 ± 0.005	0.43 ± 0.08
Glucose 6-phosphate		0.12 ± 0.01	0.12 ± 0.01
Citrate		0.48 ± 0.05	0.45 ± 0.06
Isocitrate		0.032 ± 0.004	0.030 ± 0.004
α -Oxoglutarate		0.27 ± 0.02	0.25 ± 0.04
L-Glutamate		2.76 ± 0.16	2.72 ± 0.24
Acetoacetate		0.094 ± 0.011	0.055 ± 0.005
β -Hydroxybutyrate		0.13 ± 0.01	0.14 ± 0.02
ATP		2.83 ± 0.07	2.67 ± 0.04
ADP		1.06 ± 0.04	0.99 ± 0.06
AMP		0.21 ± 0.02	0.27 ± 0.02
P_i		3.59 ± 0.13	3.31 ± 0.13

Table 4. *Effects of ethanol on cytoplasmic and mitochondrial redox and phosphorylation ratios in freeze-clamped liver of fed rats*

Ratios are calculated from the metabolite values given in Table 3 and are given as means $\pm$ S.E.M. The *P* values refer to differences between the means of the normal state and ethanol injection as judged by Student's *t* test. For method of calculation see the text.

No. of animals ...	Saline injection 6	<i>P</i>	Ethanol injection 6
[NAD ⁺]/[NADH] from lactate dehydrogenase (cytoplasm)	1620 ± 97	<0.001	212 ± 46
[NADP ⁺]/[NADPH] from malic enzyme (cytoplasm)	0.0097 ± 0.0017	<0.001	0.0015 ± 0.0005
[NADP ⁺]/[NADPH] from isocitrate dehydrogenase (cytoplasm)	0.0087 ± 0.0009		0.0084 ± 0.0008
[ATP]/[ADP][HPO ₄ ²⁻] (M ⁻¹) (cytoplasm)	1260 ± 94		1410 ± 125
[ATP][AMP] [ADP] ²	0.53 ± 0.02	<0.01	0.75 ± 0.05
[NAD ⁺]/[NADH] from β -hydroxybutyrate dehydrogenase (mitochondria)	14.8 ± 1.06	<0.01	8.51 ± 1.37

cytoplasm calculated from the isocitrate dehydrogenase reaction initially decreased 2.4-fold below the control value. This decrease was less marked than the fall in either the cytoplasmic or mitochondrial [NAD⁺]/[NADH] ratios. After an initial fall, cytoplasmic [NADP⁺]/[NADPH] returned almost to the control value at 15 and 30 min. The cytoplasmic [NADP⁺]/[NADPH] ratio calculated from the malic enzyme reaction did not agree with the [NADP⁺]/[NADPH] value calculated from the isocitrate dehydrogenase reaction after ethanol injection. The agreement between the [NADP⁺]/[NADPH] ratio calculated from either the isocitrate dehydrogenase reaction or the malic enzyme reaction is usually within a factor of about 2 (Veech *et al.*, 1969a). However, after ethanol administration the factor becomes twice as large.

Time-course of the effect of ethanol on the cytoplasmic [ATP]/[ADP][HPO₄²⁻] and [ATP][AMP]/[ADP]² ratios in the liver of starved rats

After a lag period of 10 min, there was a 2.1-fold increase in the cytoplasmic [ATP]/[ADP][HPO₄²⁻] ratio (Table 2). At 30 min this ratio was still elevated. The increase in [ATP]/[ADP][HPO₄²⁻] was not due to an increase in [ATP], but rather to an almost equal proportionate decrease in [ADP] and [HPO₄²⁻]. The [ATP][AMP]/[ADP]² ratio rose by a factor of about 2 at 5 min and remained constant.

Effects of ethanol in the fed rat

The effects of ethanol on the fed rat were determined 15 min after a single injection (Tables 3 and 4).

Whereas the effects were generally similar to those seen in the starved rat, five differences in the response to an ethanol dose were noted: (1) [pyruvate] fell threefold in the fed rat but was unchanged in the starved animal; (2) [glucose 6-phosphate] was unchanged in the fed rat but decreased 1.6-fold in the starved rat; (3), (4) and (5) [glutamate], [AMP] and [citrate] increased in the starved animal but did not change in the fed animal.

Discussion

Maintenance of near-equilibrium by the cytoplasmic NAD⁺-couple

The decreased cytoplasmic [NAD⁺]/[NADH] ratio resulting from the coupled oxidation of ethanol has been observed previously in the perfused liver (Forsander *et al.*, 1965b) and *in vivo* (Rawat, 1968). Increases in [lactate], [malate] and [α -glycerophosphate] are related to the decrease in the cytoplasmic [NAD⁺]/[NADH] ratio.

The dehydrogenases that catalyse the reactions of these metabolites have been shown to be in near-equilibrium with the cytoplasmic NAD couple in different dietary states (Hohorst *et al.*, 1959). Our experiments indicate that near-equilibrium is maintained during conditions of rapid flux and large changes in metabolite concentrations. This conclusion is supported by the value of the [pyruvate][α -glycerophosphate]/[lactate][dihydroxyacetone phosphate] ratio, which may be calculated from the results in Tables 1 and 3. At equilibrium this ratio should be 0.85 (see Williamson *et al.*, 1967; Rüssman, 1967). The value of this expression has now been found to

be in the range 0.55–1.55 *in vivo*. The agreement between the value calculated for equilibrium and the value determined *in vivo* suggests that α -glycerophosphate dehydrogenase is able to maintain a condition of near-equilibrium in this situation of high $[\alpha\text{-glycerophosphate}]$ and rapid flux. This contradicts suggestions that α -glycerophosphate dehydrogenase might be significantly inhibited by high concentrations of α -glycerophosphate (Blanchaer, 1965; Black, 1966; Krebs, 1968), or might fail to maintain equilibrium during conditions of rapid flux (Bücher & Rüssman, 1964).

In the rat liver perfused with glucose (Forsander *et al.*, 1965b; Salaspuro & Mäenpää, 1966) and in the fed rat, the change in $[\text{lactate}]/[\text{pyruvate}]$ ratio after ethanol results largely from a decrease in $[\text{pyruvate}]$. In contrast, the increase in $[\text{lactate}]/[\text{pyruvate}]$ ratio found in the starved animal *in vivo* results from a rise in $[\text{lactate}]$.

Our results indicate that the starved rat metabolizes ethanol *in vivo* at about $4\mu\text{mol/min per g wet wt. of liver}$, which would produce $8\mu\text{mol of NADH/min per g}$, with acetate assumed to be the product. This rate agrees well with other studies *in vivo* in the rat (Hawkins *et al.*, 1966). Since the liver perfused with ethanol does not significantly change its oxygen consumption (H. A. Krebs & R. Hems, personal communication), it may be estimated that in the starved animal the liver is using about $2.2\mu\text{mol of oxygen/min per g of tissue}$ (Hems *et al.*, 1966), assuming the consumption of oxygen during starvation is similar to that occurring during the metabolism of endogenous liver lipid and amino acids. This rate of oxygen consumption would utilize 4.4 of the $8\mu\text{mol of NADH/min per g}$ produced by ethanol metabolism. The remaining $3\text{--}4\mu\text{mol of NADH produced/min per g}$ must leave the liver after transfer of hydrogen to form lactate, discounting the small fraction utilized for synthetic processes. The production of $3\text{--}4\mu\text{mol/min per g}$ of lactate would require the delivery to the liver of an equivalent amount of pyruvate precursors (presumably amino acids), since the $[\text{pyruvate}]$ does not change. The failure of $[\text{pyruvate}]$ to decrease *in vivo* is in contrast to the situation seen in perfusion, where $[\text{pyruvate}]$ does decrease after ethanol administration (Krebs, 1968), presumably because of the lack of these extra-hepatic precursors.

Effects of the decrease in the cytoplasmic NAD couple upon the mitochondrial NAD couple

The redox state of the mitochondrial NAD couple is also decreased after ethanol administration. The $[\beta\text{-hydroxybutyrate}]/[\text{acetoacetate}]$ ratio was increased in the liver of a starved rat perfused with ethanol (Forsander *et al.*, 1965a) and in the liver of the fed rat (Rawat, 1968). Williamson *et al.* (1967) showed that the $[\beta\text{-hydroxybutyrate}]/[\text{acetoacetate}]$

and the $[\alpha\text{-oxoglutarate}]/[\text{NH}_4^+]/[\text{glutamate}]$ ratios both reflect the redox state of the $[\text{NAD}^+]/[\text{NADH}]$ ratio in the mitochondria. The precise mechanism for the transfer of reducing equivalents from cytoplasm to mitochondria is not known. It appears likely, however, that the redox state of the NAD couple in these two compartments is governed by the concentration of certain metabolites to which the inner mitochondrial membrane is permeable, and by the equilibrium constants of the reactions involved as described in eqn. (3) (Krebs & Veech, 1969).

Response of the cytoplasmic NADP couple to ethanol

There are no previous reports on the effect of ethanol on the $[\text{NADP}^+]/[\text{NADPH}]$ ratio of the cytoplasm. Unlike the persistent decrease in the NAD couples of cytoplasm and mitochondria, the $[\text{NADP}^+]$ of cytoplasm shows a small and transient decrease, returning nearly to control values at 15 min, coinciding with the rise of the cytoplasmic phosphorylation state.

It could be argued that the return of the $[\text{NADP}^+]/[\text{NADPH}]$ ratio to normal is the result of the metabolism of ethanol by the NADPH-requiring microsomal ethanol-oxidizing system (Lieber & DeCarli, 1970). Although such an interpretation is not impossible it seems unlikely. In the starved animals reported here, ethanol was being metabolized at about $4\mu\text{mol/min per g wet wt.}$ The capacity of glucose 6-phosphate dehydrogenase to generate NADPH is $3\mu\text{mol/min per g}$ at 38°C (Veech *et al.*, 1969a). Another $3\mu\text{mol/min per g}$ could be produced by 6-phosphogluconate dehydrogenase and about $1.5\mu\text{mol/min per g}$ by the malic enzyme for a total capacity of $7.5\mu\text{mol/min per g}$ at 38°C . It seems unlikely, even if ethanol was totally metabolized *in vivo* by this proposed microsomal pathway, that it could overwhelm the capacity of the cell to regenerate NADPH. What does seem likely is that the return of the $[\text{NADP}^+]/[\text{NADPH}]$ ratio to normal is related to the rise of the cytoplasmic phosphorylation state. A clear consequence of the change in $[\text{NADP}^+]/[\text{NADPH}]$ ratio is the transient rise in citrate concentration seen in the starved animals. The activity of the isocitrate dehydrogenase-aconitase system is sufficiently high to be the determinant of the steady-state citrate concentration under most conditions. This is so because the isocitrate concentration is the product of $[\alpha\text{-oxoglutarate}]$, $[\text{CO}_2]$ and the $[\text{NADPH}]/[\text{NADP}^+]$ ratio (Veech *et al.*, 1969a).

Disequilibrium after ethanol administration

It has previously been shown that the values of the cytoplasmic $[\text{NADP}^+]/[\text{NADPH}]$ ratio calculated

from the malic enzyme reaction, the 6-phosphogluconate dehydrogenase reaction and the isocitrate dehydrogenase reaction agreed quite well with one another in most cases (Veech *et al.*, 1969a). This finding was taken to demonstrate that these reactions were in near-equilibrium with the same cytoplasmic NADP couple. However, after ethanol administration the value of the $[NADP^+]/[NADPH]$ ratio calculated from the malic enzyme was five times lower than the value calculated from the isocitrate dehydrogenase reaction. The isocitrate dehydrogenase reaction is likely to represent the true $[NADP^+]/[NADPH]$ ratio of the cytoplasm more closely than the malic enzyme largely because the V_{max} of the isocitrate dehydrogenase reaction is 17 i.u. and that of the malic enzyme is 0.7 i.u. in the starved animal. Both isocitrate dehydrogenase and the malic enzyme operate in the direction of decarboxylation at tissue concentrations of substrate that are well above their respective K_m values (Ochoa, 1948; Hsu *et al.*, 1967). Therefore the most important determinant of the net flux through these enzymes is the V_{max} of each enzyme and a factor that represents the distance each reaction is from equilibrium. This factor can be thought of as the net chemical energy driving the reaction toward equilibrium, given by the well-known expression: $\Delta G = -RT \ln[\text{products}]/[\text{reactants}]$. At true equilibrium $\Delta G = 0$ and there is no net flux through the system. In a 'near-equilibrium' system the net flux is a function of ΔG or the distance from equilibrium, the V_{max} of the system, and the various K_m values of the enzyme. The mathematical formulation of velocity of a five-membered reaction has been given by Cleland (1963). However, the

particularly in the starved animal, where the $[NADP^+]/[NADPH]$ ratio calculated from the malic enzyme reaction differs by a factor of 2.4 (Veech *et al.*, 1969a) from the value calculated from the isocitrate dehydrogenase reaction, and after ethanol administration, where the values differ by a factor of 5. After ethanol administration, the malic enzyme appears unable to decarboxylate the increased malate resulting from the more decreased cytoplasmic $[NAD^+]/[NADH]$ ratio and thus stays significantly away from equilibrium during the 30 min studied.

Changes in the $[ATP]/[AMP]/[ADP]^2$ ratio, which is the K_{app} for the myokinase reaction, have been found previously after ethanol administration (Williamson *et al.*, 1969) and were confirmed in the experiments reported here. The value of K_{app} in the control group agrees well with the value of 0.44 found *in vitro* with low Mg^{2+} concentrations (Eggleston & Hems, 1952). After ethanol administration the apparent value of this reaction *in vivo* increased 2.5-fold. Myokinase is such an active enzyme that it is unlikely to be significantly inhibited under these conditions. It follows that the cause of the change in K_{app} for this reaction was due either to a radical change in the Mg^{2+} concentration (Blair, 1970; Rose, 1968) or to a segregation of AMP in a place inaccessible to myokinase, for instance, within the mitochondrial matrix.

Rise of the cytoplasmic phosphorylation state

The rise in the cytoplasmic phosphorylation state 15 min after ethanol administration was totally unexpected. From eqn. (2):

$$\frac{[NAD^+]}{[NADH]} = \frac{[ATP]}{[ADP][HPO_4^{2-}]} \times \frac{[3\text{-Phosphoglycerate}]}{[\text{Glyceraldehyde 3-phosphate}]} \times \frac{1}{K_{GAPDH} \cdot K_{3-PGK}}$$

precise K_m values needed for calculation *in vivo* are not known. Another factor bearing on the disequilibrium in the malic enzyme reaction after ethanol administration is the high activity of the other enzymes that affect malate concentration. Malate concentration can be rapidly changed by oxaloacetate transaminase and malate dehydrogenase with activities of 80 i.u. and over 400 i.u. respectively in liver. In contrast, the only net producer of citrate, apart from isocitrate dehydrogenase, is the condensing enzyme (EC 4.1.3.7) with about 6 i.u. This enzyme is far less active than the isocitrate dehydrogenase-aconitase system and is therefore unlikely to produce citrate at a rate sufficient to cause a marked disequilibrium in the isocitrate dehydrogenase reaction. Attempts to calculate the precise $[NADP^+]/[NADPH]$ ratio from the reactants of the malic enzyme reaction are therefore likely to be erroneous,

Since the primary effect of ethanol in liver was a large decrease in the cytoplasmic NAD couple, we expected smaller decreases in both $[ATP]/[ADP] \times [HPO_4^{2-}]$ and $[3\text{-phosphoglycerate}]/[\text{glyceraldehyde 3-phosphate}]$ ratios. All previous conditions studied showed that cytoplasmic $[NAD^+]/[NADH]$ and $[ATP]/[ADP][HPO_4^{2-}]$ ratios changed in the same direction (Veech *et al.*, 1970). Ethanol is unique in its ability to cause simultaneously a decrease of the cytoplasmic NAD couple and an increase in the cytoplasmic phosphorylation state. Whereas the precise mechanism by which it does this is not known, existing evidence shows that the elevation of the cytoplasmic phosphorylation state is due to the further metabolism of ethanol beyond the aldehyde step. Stubbs *et al.* (1972) showed that administration of crotyl alcohol or the administration of the dimer of ethanol, butane-1,3-diol (Veech & Mehlman,

1972), both of which are metabolized by alcohol dehydrogenase but not by aldehyde dehydrogenase or acetate thiokinase (EC 6.2.1.1), causes reduction of the cytoplasmic NAD couple, but no elevation of the cytoplasmic $[ATP]/[ADP][HPO_4^{2-}]$ ratio.

In previous studies on the effect of ethanol on gluconeogenesis it was suggested (Williamson *et al.*, 1969) that the change in 3-phosphoglycerate concentration is due to a 'crossover point' at glyceraldehyde 3-phosphate dehydrogenase. This is usually interpreted as indicating a change in the kinetic constants of glyceraldehyde 3-phosphate dehydrogenase, leading to changes in metabolite concentrations. However,

metabolites are the result of the changes in the redox state of the NAD couple and the phosphorylation state of the cytoplasm.

Control of metabolite concentration by near-equilibrium reactions

The important control functions of the redox and phosphorylation states of the cell are well illustrated by the changes in metabolite concentration induced by ethanol. The largest of these are a fivefold increase in $[\alpha\text{-glycerophosphate}]$ and a fivefold decrease in $[3\text{-phosphoglycerate}]$. Put in a formal way:

$$[\alpha\text{-Glycerophosphate}] = \frac{[\text{Dihydroxyacetone phosphate}]}{K_{\alpha\text{GPDH}}} \cdot \frac{[\text{NADH}_c]}{[\text{NAD}_c^+]^2} \quad (4)$$

where $K_{\alpha\text{GPDH}} = 1.3 \times 10^{-4}$ (Rüssman, 1967)

$$[\text{Glyceraldehyde 3-phosphate}] = \frac{[\text{Dihydroxyacetone phosphate}]}{K_{\text{TPI}}} \quad (5)$$

where $K_{\text{TPI}} = 9$ in liver tissue (Veech *et al.*, 1969b)

$$K_{\text{GAPDH}} \cdot K_{3\text{-PGK}} = \frac{[3\text{-Phosphoglycerate}]}{[\text{Glyceraldehyde 3-phosphate}]} \cdot \frac{[\text{ATP}_c]}{[\text{ADP}_c][\text{HPO}_4^{2-}]} \cdot \frac{[\text{NADH}_c]}{[\text{NAD}_c^+]^2} \quad (6)$$

where $K_{\text{GAPDH}} \cdot K_{3\text{-PGK}} = 59 \text{ M}^{-1}$ (Veech *et al.*, 1970)

Combining eqn. (5) and eqn. (6):

$$\frac{K_{\text{GAPDH}} \cdot K_{3\text{-PGK}}}{K_{\text{TPI}}} = \frac{[3\text{-Phosphoglycerate}]}{[\text{Dihydroxyacetone phosphate}]} \cdot \frac{[\text{ATP}_c]}{[\text{ADP}_c][\text{HPO}_4^{2-}]} \cdot \frac{[\text{NADH}_c]}{[\text{NAD}_c^+]^2}$$

Then eliminating the common $[\text{dihydroxyacetone phosphate}]$ in eqns. (4) and (6):

$$[\alpha\text{-Glycerophosphate}] = [3\text{-Phosphoglycerate}] \cdot \frac{K_{\text{TPI}}}{K_{\text{GAPDH}} \cdot K_{3\text{-PGK}} \cdot K_{\alpha\text{GPDH}}} \cdot \frac{[\text{ATP}_c]}{[\text{ADP}_c][\text{HPO}_4^{2-}]} \cdot \frac{[\text{NADH}_c]^2}{[\text{NAD}_c^+]^2} \quad (7)$$

our study shows that the changes in 3-phosphoglycerate concentration, in both fed and starved animals, are due simply to the maintenance of near-equilibrium. The value of $K_{\text{GAPDH}} \cdot K_{3\text{-PGK}}$ at 38°C , $I = 0.25$ and $\text{pH } 7.0$, is 59 M^{-1} (Veech *et al.*, 1970). The value of this expression can be calculated directly from the metabolite concentrations, assuming that dihydroxyacetone phosphate concentrations are nine times the concentration of glyceraldehyde 3-phosphate (Veech *et al.*, 1969b).

We obtained the following values for $K_{\text{GAPDH}} \cdot K_{3\text{-PGK}}$ *in vivo*: fed control, 73; fed plus ethanol, 124; starved control, 51; starved plus ethanol (5 min), 97; (10 min), 51; (15 min), 59; (30 min), 49.

These values, both in fed and starved rats, are in good agreement with the value of 59 expected at equilibrium. The above analysis shows that this 'crossover point' should not be construed as a functionally important change in the activity of glyceraldehyde 3-phosphate dehydrogenase but rather that the changes in the concentration of

Eqn. (7) states that $[\alpha\text{-glycerophosphate}]$ is determined by the square of the cytoplasmic NAD couple, the cytoplasmic phosphorylation state and the 3-phosphoglycerate concentration. The validity of this equation is easily seen by comparing the values of $[\alpha\text{-glycerophosphate}]$ calculated from eqn. (7) with the determined $[\alpha\text{-glycerophosphate}]$ in Table 5.

The two sets of values agree within a factor of two, which is quite good considering that there are changes of 25-fold in the $[\text{NADH}_c]^2/[\text{NAD}_c^+]^2$ ratio. Thus the large rise in $[\alpha\text{-glycerophosphate}]$ and the large fall in $[3\text{-phosphoglycerate}]$ are dictated by the very large changes in the redox and phosphorylation states induced by ethanol. The important point is that $\alpha\text{-glycerophosphate}$ concentration, as well as many other metabolite concentrations, is determined largely by the redox and phosphorylation states themselves according to simple thermodynamic laws and not as the result of changes in enzymic activity.

Table 5. Calculated and measured α -glycerophosphate concentrations in freeze-clamped liver of starved rats after ethanol administration

Values of [α -glycerophosphate] are calculated from eqn. (7) by using the values of $[\text{NAD}^+]/[\text{NADH}_2]$ and $[\text{ATP}_c]/[\text{ADP}_c][\text{HPO}_4^{2-}]$ from Table 2 and [3-phosphoglycerate] from Table 1. The measured [α -glycerophosphate] is taken from Table 1.

Time (min)	[α -Glycerophosphate] ($\mu\text{mol/g}$)	
	Calc.	Measured
0	0.24	0.23
5	2.78	1.36
10	0.86	1.45
15	0.68	0.98
30	0.71	1.14

We thank Professor H. A. Krebs, F.R.S., for his helpful criticisms of this work, which was begun in his laboratory, and Dr. H. Weil-Malherbe for reviewing the manuscript.

References

- Adam, H. (1963) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., ed.), pp. 573–576, Verlag Chemie, Weinheim
- Bergmeyer, H. U. & Bernt, E. (1963) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., ed.), pp. 324–327, Verlag Chemie, Weinheim
- Bernt, E. & Bergmeyer, H. U. (1963) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., ed.), pp. 384–388, Verlag Chemie, Weinheim
- Black, W. J. (1966) *Can. J. Biochem.* **44**, 1301–1317
- Blair, J. McD. (1970) *Eur. J. Biochem.* **13**, 384–390
- Blanchaer, M. C. (1965) *Can. J. Biochem.* **43**, 17–24
- Bücher, Th. & Hohorst, H. J. (1963) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., ed.), pp. 246–252, Verlag Chemie, Weinheim
- Bücher, Th. & Rüssman, W. (1964) *Angew. Chem. Int. Ed. Engl.* **3**, 426–439
- Bücher, Th., Czok, R., Lamprecht, W. & Latsko, E. (1963) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., ed.), pp. 253–259, Verlag Chemie, Weinheim
- Cleland, W. W. (1963) *Biochim. Biophys. Acta* **67**, 104–137
- Czok, R. & Eckert, L. (1963) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., ed.), pp. 224–228, Verlag Chemie, Weinheim
- Eggleson, L. V. & Hems, R. (1952) *Biochem. J.* **52**, 156–160
- Forsander, O., Mäenpää, P. H. & Salaspuro, M. P. (1965a) *Acta Chem. Scand.* **19**, 1770–1771
- Forsander, O., Riihää, N., Salaspuro, M. P. & Mäenpää, P. H. (1965b) *Biochem. J.* **94**, 259–265
- Gruber, W. & Moellering, H. (1966) *Biochem. Z.* **346**, 85–88
- Hawkins, R. D., Kalant, H. & Khanna, J. M. (1966) *Can. J. Physiol. Pharmacol.* **44**, 241–257
- Hems, R., Ross, B. D., Berry, M. N. & Krebs, H. A. (1966) *Biochem. J.* **101**, 284–292
- Hohorst, H. J. (1963a) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., ed.), pp. 134–138, Verlag Chemie, Weinheim
- Hohorst, H. J. (1963b) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., ed.), pp. 215–219, Verlag Chemie, Weinheim
- Hohorst, H. J. (1963c) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., ed.), pp. 266–270, Verlag Chemie, Weinheim
- Hohorst, H. J. (1963d) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., ed.), pp. 328–332, Verlag Chemie, Weinheim
- Hohorst, H. J., Kreutz, F. H. & Bücher, Th. (1959) *Biochem. Z.* **332**, 18–46
- Hsu, R. Y., Lardy, H. A. & Cleland, W. W. (1967) *J. Biol. Chem.* **242**, 5315–5322
- Krebs, H. A. (1953) *Biochem. J.* **54**, 78–82
- Krebs, H. A. (1968) *Advan. Enzyme Regul.* **6**, 467–480
- Krebs, H. A. & Perkins, J. R. (1970) *Biochem. J.* **118**, 635–644
- Krebs, H. A. & Veech, R. L. (1969) in *The Energy Level and Metabolic Control in Mitochondria* (Papa, S., Tager, J. M., Quagliariello, E. & Slater, E. C., eds.), pp. 329–382, Adriatica Editrice, Bari
- Lamprecht, W. & Trautschold, I. (1963) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., ed.), pp. 543–551, Verlag Chemie, Weinheim
- Lieber, C. S. (1967) in *Biochemical Factors in Alcoholism* (Maickel, R. P., ed.), pp. 167–183, Pergamon Press, Oxford
- Lieber, C. S. & DeCarli, L. M. (1970) *J. Biol. Chem.* **245**, 2505–2512
- Lindros, K. O. (1970) *Eur. J. Biochem.* **13**, 111–116
- Martin, J. B. & Doty, D. M. (1949) *Anal. Chem.* **21**, 965–967
- Ochoa, S. (1948) *J. Biol. Chem.* **174**, 133–157
- Rawat, A. K. (1968) *Eur. J. Biochem.* **6**, 585–592
- Reboucas, G. & Isselbacher, K. J. (1961) *J. Clin. Invest.* **40**, 1355–1362
- Rose, I. A. (1968) *Proc. Nat. Acad. Sci. U.S.A.* **61**, 1079–1086
- Rüssman, W. (1967) Ph.D. Dissertation, University of Munich
- Salaspuro, M. P. & Mäenpää, P. H. (1966) *Biochem. J.* **100**, 768–774

- Stubbs, M., Veech, R. L. & Krebs, H. A. (1972) *Biochem. J.* **126**, 59–65
- Veech, R. L. (1968) D. Phil. Dissertation, University of Oxford
- Veech, R. L. & Mehlman, M. (1972) in *Energy Metabolism and the Regulation of Metabolic Processes in Mitochondria* (Mehlman, M. A. & Hanson, R. W., eds.), Academic Press, New York, in the press
- Veech, R. L., Eggleston, L. V. & Krebs, H. A. (1969a) *Biochem. J.* **115**, 609–618
- Veech, R. L., Rajiman, L., Dalziel, K. & Krebs, H. A. (1969b) *Biochem. J.* **115**, 837–842
- Veech, R. L., Rajiman, L. & Krebs, H. A. (1970) *Biochem. J.* **117**, 499–503
- Williamson, D. H., Mellanby, J. & Krebs, H. A. (1962) *Biochem. J.* **82**, 90–96
- Williamson, D. H., Lund, P. & Krebs, H. A. (1967) *Biochem. J.* **103**, 514–527
- Williamson, J. R., Scholz, R., Browning, E. T., Thurman, R. G. & Fukami, M. (1969) *J. Biol. Chem.* **244**, 5044–5054
- Wollenberger, A., Ristau, O. & Schoffa, G. (1960) *Pflügers Arch. Gesamte Physiol.* **270**, 399–412