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SHORT COMMUNICATIONS

The effect of ethanol and cyanide on NAD/$NADH_2$ ratios in rat liver

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THE REDOX state of a cell is one of the important factors determining which metabolic reactions may take place at a given time. A useful parameter in describing the redox state is the ratio of oxidised to reduced nicotinamide-adenine dinucleotide. For example, there is evidence of a correlation between this ratio, the rate of gluconeogenesis and fatty acid availability in rat liver. Similarly, although the levels of adenosine phosphates, fatty acyl CoA and other metabolites are thought to be important in the control of tricarboxylic acid cycle activity,[1] it has also been suggested that the intramitochondrial NAD/$NADH_2$ ratio may be a significant influence.[2] Knowledge of the compartmentation of reducing potential between the cytoplasm and mitochondria is essential in determining the role which it plays in the control of the cell's metabolism.

Bücher and Klingenberg[3] introduced the idea that the ratio of free NAD to free $NADH_2$ in various cell compartments could be calculated from the concentrations of the substrates of certain NAD-linked dehydrogenases of high activity. The equation

$$K = \frac{\text{(Oxidised substrate) (NADH}_2\text{)}}{\text{(Reduced substrate) (NAD)}}$$

gives the relationship between the pyridine nucleotide ratio and the equilibrium constant for the enzyme-catalysed reaction. It was pointed out by Williamson, Lund and Krebs[4] that for the cytoplasmic NAD/$NADH_2$ ratio, the pyruvate-lactate couple gives the most reliable results, whereas for the ratio in the mitochondria, the substrates of 3-hydroxybutyrate dehydrogenase (D-3-Hydroxybutyrate: NAD oxidoreductase, E.C.1.1.1.30) and of glutamic dehydrogenase (L-Glutamate: NAD oxidoreductase, (deaminating), E.C.1.4.1.2) yield similar results. Using this principle these workers determined the effect of starvation and diabetes on the cytoplasmic and mitochondrial NAD/$NADH_2$ ratios.

The present communication describes the effects of ethanol and cyanide on these ratios in rat liver. Ethanol oxidation is known to decrease the NAD/$NADH_2$ ratio in the whole cell,[5] but the compartmentation of this effect has not previously been studied. The first step in the oxidation is the formation of acetaldehyde, catalysed by alcohol dehydrogenase (Alcohol : NAD oxidoreductase, E.C.1.1.1.1); acetaldehyde is further oxidised to acetate in a reaction catalysed by aldehyde dehydrogenase (Aldehyde : NAD oxidoreductase, E.C.1.2.1.3). As both these NAD-linked enzymes are located predominantly in the cytoplasm, the primary effect of the oxidation of ethanol should be to decrease the cytoplasmic NAD/$NADH_2$ ratio. The oxidation might also have the secondary consequence of lowering the ratio in the mitochondria; this would depend on the efficiency of the shuttle system which transfers reducing potential generated in the cytoplasm into the mitochondria, and on the ability of the mitochondrial electron transport system to reoxidise the $NADH_2$ in the mitochondria.

Since cyanide acts on the electron transport system to inhibit the reoxidation of $NADH_2$, the primary effect of cyanide administration should be a lowering of the mitochondrial NAD/$NADH_2$ ratio in the liver. If this inhibition were sufficient, a secondary result might be the accumulation of $NADH_2$ in the cytoplasm causing a decrease in the cytoplasmic NAD/$NADH_2$ ratio. Such experiments might give some indirect indication of the importance of the mitochondria in maintaining cytoplasmic redox potentials.

EXPERIMENTAL

Male albino rats (Wistar, Castle Hill), 7–8 weeks old and weighing 200–250 g, were fed *ad libitum* on standard laboratory diet (Drug Houses of Australia Ltd., Sydney). Ethanol, cyanide and water were

administered by stomach tube, ethanol (E. Merck, A.G. Darmstadt) as 25% (w/v) aqueous solution (2·5g/kg), and potassium cyanide as 2 mM aqueous solution (1·25 mg/kg; the lethal dose was found to be about 2·0 mg/kg body wt.). Rats receiving ethanol and cyanide were given both dissolved in 2 ml distilled water. Control animals received 2 ml water. Rats were killed by dislocation of the neck 45 min after tube-feeding, and their livers were rapidly removed and freeze-pressed between two metal blocks at the temperature of liquid nitrogen. Each liver was subsequently ground, deproteinised in 30% perchloric acid, and analysed for substrates. Ammonia,[6] glutamate,[7] and 2-oxoglutarate[8] were determined with glutamic dehydrogenase. Lactate and pyruvate were determined enzymically by the method of Hohorst, Kreutz and Bücher.[9] Ethanol determinations were performed by the method of Kaplan and Ciotti.[10]

The equilibrium constants used in the calculations of cytoplasmic and mitochondrial $NAD/NADH_2$ ratios were those given by Williamson *et al.*[4];

for Lactic Dehydrogenase, $K = 1{\cdot}11 \times 10^{-4}$

for Glutamic Dehydrogenase, $K = 3{\cdot}87 \times 10^{-3}$mM,

assuming that the concentration of water is unity, the pH 7·0 and the ionic strength 0·25.

TABLE 1. CONCENTRATIONS OF THE SUBSTRATES OF NAD-LINKED DEHYDROGENASE SYSTEMS IN THE LIVERS OF RATS IN VARIOUS METABOLIC STATES

	Control	Ethanol-Fed	Cyanide-Fed	Ethanol- and Cyanide-Fed
Lactate	0·97 ± 0·11 (7)	1·11 ± 0·12 (7)	0·70 ± 0·03 (8)	0·87 ± 0·13 (5)
Pyruvate	0·030 ± 0·003 (7)	0·002 ± 0·003 (7)	0·047 ± 0·006 (8)	0·039 ± 0·003 (5)
Glutamate	2·36 ± 0·31 (8)	2·93 ± 0·47 (6)	2·21 ± 0·16 (8)	2·45 ± 0·43 (5)
2-Oxoglutarate	0·081 ± 0·014 (8)	0·056 ± 0·011 (6)	0·062 ± 0·012 (8)	0·040 ± 0·010 (5)
Ammonia	0·63 ± 0·03 (8)	0·69 ± 0·05 (6)	0·58 ± 0·05 (8)	0·52 ± 0·03 (5)

The tissue ethanol concentration of ethanol-fed rats was 110 ± 5·5 mg/100 ml, assuming the liver to contain 70% water. The concentrations of metabolites are expressed as μmoles/g wet wt., and are mean values ± S.E.M., with the number of observations in parentheses.

TABLE 2. SUBSTRATE AND COENZYME RATIOS IN THE LIVERS OF RATS IN VARIOUS METABOLIC STATES

	Control	Ethanol-fed	Cyanide-fed	Ethanol- and Cyanide-fed
$\frac{\text{Lactate}}{\text{Pyruvate}}$	32	49	15	22
$\frac{\text{Glutamate}}{\text{2-Oxoglutarate} \cdot NH_4^+}$	46	76	61·5	118
$NAD/NADH_2$ (cytoplasm)	280	184	600	410
$NAD/NADH_2$ (mitochondria)	5·6	3·4	4·2	2·2

Ratios are calculated from the data in Table 1.

DISCUSSION

Table 2 shows that ethanol administration had the anticipated effect of decreasing the cytoplasmic $NAD/NADH_2$ ratio. The fact that the mitochondrial ratio also decreased with ethanol implies that reducing potential was transferred from the cytoplasm to the mitochondria more quickly than it could be utilised by the electron transport system. As expected, cyanide caused a decrease in the mitochondrial $NAD/NADH_2$ ratio when administered alone. When administered with ethanol, it

caused an even greater decrease in the mitochondrial ratio. However, the effect of cyanide on the cytoplasmic system was quite unexpected. Despite the fact that the mitochondria were in a more reduced state, the cytoplasmic NAD/$NADH_2$ ratio actually increased more than 2-fold. In rats fed ethanol and cyanide, the oxidation of ethanol partially counteracted this increase, but the cytoplasmic ratio in the livers of these rats was still almost 50 per cent greater than in the controls. As Table 1 shows, there was also a significant reduction in the sum of the concentrations of lactate and pyruvate in the livers of rats fed cyanide, compared to the controls. A metabolic pathway is postulated to account for these results.

In vitro experiments have shown that, in the presence of cyanide, a mixture of glyceraldehyde-3-phosphate and dihydroxyacetone phosphate at physiological pH is converted via methylglyoxal (pyruvaldehyde—CH_3COCHO) to pyruvate.[11] The first step, the dephosphorylation of triose phosphate to form methylglyoxal, appears to be non-enzymic and is independent of cyanide. A proposed mechanism is given in Fig. 1. Cyanide then catalyses the nonenzymic conversion of methylglyoxal to pyruvate, in the proportion 0·5 mole pyruvate formed per mole methylglyoxal. In a reaction

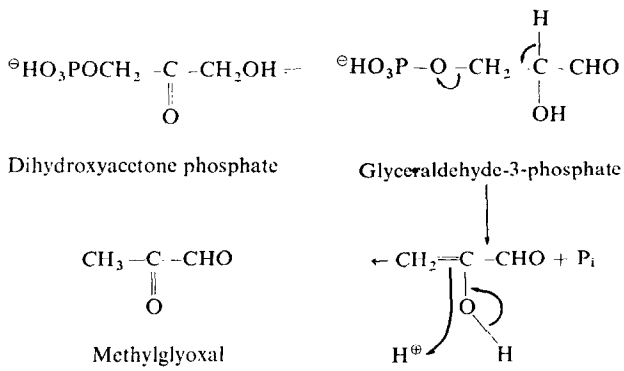

FIG. 1. A proposed mechanism for the conversion of triose phosphates to methylglyoxal.

accompanying this oxidation, the remaining 3-carbon skeletons are reduced and condensed to acetol and other unidentified 6-carbon (or larger) compounds.[12] Methylglyoxal also appears to be a normal intermediary metabolite. For example, as a precursor of glucose it is more than 20 per cent as efficient as lactate.[13] Methyl glyoxalase (S-lactoylglutathione methylglyoxal-lyase (isomerizing), E.C.4.4.1.5.), a two enzyme system, converts methylglyoxal to lactate, but it appears that some or all

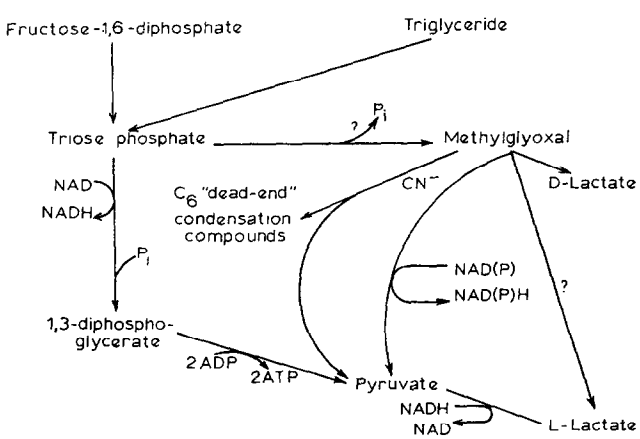

FIG. 2. Alternative pathways of triose phosphate metabolism.

of the product is D-lactate, which cannot be oxidised by lactic dehydrogenase. A more likely fate for methylglyoxal is oxidation to pyruvate.[14] This reaction is catalysed by ketoaldehyde oxidase, which preferentially uses NADP as a coenzyme, with a slightly lower affinity for NAD, which is more abundant in the cell than NADP. Fig. 2 shows that triose phosphate metabolism via methylglyoxal makes the same demands on the cell's pyridine nucleotides as the "normal" glycolytic pathway.

Since ketoaldehyde oxidase is inhibited by cyanide, the extremely efficient non-enzymic dismutation of methylglyoxal should take precedence in the livers of rats fed cyanide. As Fig. 2 shows the metabolism of triose phosphates to L-lactate in this way results in the oxidation of one mole of $NADH_2$ per mole of triose phosphate, and this accounts for the increase in $NAD/NADH_2$ ratio observed in the cytoplasm of liver cells of rats administered cyanide.

This scheme also explains the observed decrease in the sum of the concentrations of lactate and pyruvate in cyanide-fed rats. Assuming that the normal cell lactate level is the balance of the synthesis of lactate and its removal, then the fact that the cyanide pathway converts 50 per cent of the methylglyoxal formed to "dead-end" condensation products should result in decreased lactate formation and a lower cell lactate plus pyruvate concentration.

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Metabolic fate of zoxazolamine in tumor bearing rats

A MARKED reduction in the rate of Pentobarbital and *d*-Amphetamine metabolism in tumor bearing rats and mice even at a very early stage of tumor growth, has been already reported.[1,2] Further support for the hypothesis that an impairment of drug metabolizing microsomal enzymes occurs in tumor bearing animals has been obtained with zoxazolamine, which is transformed by such enzymes in the presence of oxygen and NADPH.[3–5] Male, Sprague–Dawley rats of the average weight of 150 ± 10 g, received s.c. the following transplantable tumors: Walker 256 carcinosarcoma,