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Control of the Redox State of the Nicotinamide–Adenine Dinucleotide Couple in Rat Liver Cytoplasm

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1. A study has been made of the ability of rat liver *in vivo* to maintain equilibrium in the combined glyceraldehyde 3-phosphate dehydrogenase, 3-phosphoglycerate kinase and lactate dehydrogenase reactions, i.e. in the system:

$$\frac{[\text{ATP}]}{[\text{ADP}][\text{HPO}_4{}^{2-}]}=\frac{[\text{pyruvate}]}{[\text{lactate}]}\cdot\frac{[\text{glyceraldehyde 3-phosphate}]}{[\text{3-phosphoglycerate}]}\cdot K$$

Attempts were made to upset equilibrium. The [lactate]/[pyruvate] ratio was rapidly changed by injection of ethanol or crotyl alcohol, and the value of [ATP]/[ADP] [$HPO_4{}^{2-}$] was rapidly changed by injection of ethionine or carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone. 2. The concentrations of the metabolites occurring in the above equation were measured in freeze-clamped liver. 3. Although the injected agents caused large changes in the concentrations of the individual components, near-equilibrium in the system was maintained, as indicated by the fact that the value of [ATP]/[ADP][$HPO_4{}^{2-}$], referred to as the phosphorylation state of the adenine nucleotides, measured directly agreed with the value calculated for equilibrium conditions from the above equation. 4. The results are discussed and taken to confirm that the order of magnitude of the value of the redox state of the cytoplasmic NAD couple in rat liver is controlled by the phosphorylation state of the adenine nucleotide system.

The redox state of the NAD couple is linked to the phosphorylation state of the adenine nucleotide system (i.e. the ratio [ATP]/[ADP] [$HPO_4{}^{2-}$]) by the 3-phosphoglycerate kinase and glyceraldehyde 3-phosphate dehydrogenase reactions (Veech *et al.*, 1970; Krebs & Veech, 1970). At equilibrium the following relation holds:

$$\frac{[\text{NAD}^+]}{[\text{NADH}]}=\frac{[\text{ATP}]}{[\text{ADP}][\text{HPO}_4{}^{2-}]}\cdot\frac{[\text{3-phosphoglycerate}]}{[\text{glyceraldehyde 3-phosphate}]}\cdot\frac{1}{K} \tag{1}$$

where K is the product of the equilibrium constants of the glyceraldehyde 3-phosphate dehydrogenase system and the 3-phosphoglycerate kinase system at pH 7.0. By combining eqn. (1) with the equilibrium equation for lactate dehydrogenase a relation is obtained that can be tested experimentally:

$$\frac{[\text{ATP}]}{[\text{ADP}][\text{HPO}_4{}^{2-}]}=\frac{[\text{pyruvate}]}{[\text{lactate}]}\cdot\frac{[\text{glyceraldehyde 3-phosphate}]}{[\text{3-phosphoglycerate}]}\cdot K \tag{2}$$

where K represents the product of the equilibrium constants of the glyceraldehyde 3-phosphate dehydrogenase and the 3-phosphoglycerate kinase reactions divided by the equilibrium constant of the lactate dehydrogenase reaction. The calculation of the phosphorylation state from K and measurements of the concentrations in freeze-clamped rat liver of lactate, pyruvate, glyceraldehyde 3-phosphate and 3-phosphoglycerate gave values that agreed reasonably well under various dietary conditions with the value obtained from direct determinations of ATP, ADP and P_i. This agreement indicates that the components of lactate dehydrogenase, glyceraldehyde 3-phosphate dehydrogenase and 3-phosphoglycerate kinase systems are near equilibrium *in vivo*, and further that the redox state of the cytoplasmic NAD couple is controlled by the phosphorylation state of the adenine nucleotide system.

The previous work (Veech *et al.*, 1970) was carried

out on rat livers from normal, starved and specially dieted rats (high-sucrose, high-glucose and high-fat diets). The present experiments were designed to test whether near-equilibrium in the relation expressed by eqn. (2) is maintained when the phosphorylation state is upset by injection of ethionine or carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone or when the redox state is upset by the injection of ethanol or crotyl alcohol.

Experimental

Rats

Female rats of the Wistar strain weighing about 200g were used except in the ethanol experiments, where rats of the Sprague–Dawley strain were used. All were starved for 48h.

Reagents

Standard analytical grade laboratory reagents were obtained from British Drug Houses Ltd., Poole, Dorset, U.K. All enzymes were obtained from Boehringer Corp. (London) Ltd., London W.5, U.K. Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone was a gift from Dr. P. G. Heytler, E.I. Du Pont de Nemours and Co. (Inc.), Central Research Department, Experimental Station, Wilmington, Del., U.S.A.

Injection of agents

Injections were given intraperitoneally under ether anaesthesia. Details of dose are given in the tables. Rats were killed by dislocation of the neck and the livers were removed within 10s and freeze-clamped (Wollenberger *et al.*, 1960). Further treatment of the tissue was as described by Williamson *et al.* (1967). As some variations in control values were found when experiments were performed at intervals of several months, each series of experiments were compared with control animals analysed at the same time.

Determination of metabolites

Metabolites were determined as follows: lactate and pyruvate by the method of Hohorst *et al.* (1959); 3-phosphoglycerate by the method of Czok & Eckert (1963); ATP by the method of Lamprecht & Trautschold (1963); ADP by the method of Adam (1963); dihydroxyacetone phosphate by the method of Bücher & Hohorst (1963); P_i by the method of Martin & Doty (1949); 3-hydroxybutyrate and acetoacetate by the method of Williamson *et al.* (1962). The concentration of glyceraldehyde 3-phosphate was calculated from that of dihydroxyacetone phosphate assuming a [dihydroxyacetone phosphate]/[glyceraldehyde 3-phosphate] ratio of 9.3 (Veech *et al.*, 1969). The concentration of HPO_4^{2-} was taken to be 60% of the total P_i.

Results

Effect of ethionine

Injection of ethionine decreases [ATP] in the liver by the formation of *S*-adenosylethionine at a high rate (Shull, 1962; Farber *et al.*, 1964). [ADP] and [P_i] also decreased (Table 1). The [lactate]/[pyruvate] ratio increased 2.5-fold in the ethionine-treated animals, but there was no significant change in the [glyceraldehyde 3-phosphate]/[3-phosphoglycerate] ratio.

The calculated and measured [ATP]/[ADP][HPO_4^{2-}] are in reasonably good agreement (Table 1), considering the large changes in [ATP] and [ADP] and in the [ATP]/[ADP] ratio. The latter fell from 1.51 to 0.76 on injection of ethionine. As there was very little change in the [glyceraldehyde 3-phosphate]/[3-phosphoglycerate] ratio it follows that a change in the [lactate]/[pyruvate] ratio compensates for the change in [ATP]/[ADP][HPO_4^{2-}] in eqn. (2).

Effect of ethanol

Ethanol is known to increase the [lactate]/[pyruvate] ratio (see Forsander *et al.*, 1958; Smith & Newman, 1959; Reboucas & Isselbacher, 1961; Field *et al.*, 1963; Isselbacher & Greenberger, 1964*a,b*; Freinkel *et al.*, 1965; Lieber, 1967). In the experiments recorded in Table 2, this ratio rose from 14.2 to 45.3. This increase was almost entirely due to a rise in [lactate]. The [glyceraldehyde 3-phosphate]/[3-phosphoglycerate] ratio was also increased by ethanol. In this case the increase was caused by a fall in [3-phosphoglycerate]. Although [ATP] was unchanged, [ADP] and [P_i] decreased in the ethanol-treated animals.

In spite of major changes in most of the components in eqn. (2), the agreement between the calculated and measured phosphorylation state is satisfactory and indicates near-equilibrium in this system.

Effect of crotyl alcohol

Crotyl alcohol reacts with liver alcohol dehydrogenase at about the same rate as ethanol, but the crotonaldehyde formed, unlike acetaldehyde, is not a substrate of liver aldehyde dehydrogenase (Williamson, 1967).

The change in [lactate]/[pyruvate] ratio was the same with ethanol and crotyl alcohol (Tables 2 and

Table 1. *Effect of* L-*ethionine on the metabolite content of rat liver, the redox state of the NAD couple and the phosphorylation state of the adenine nucleotide system*

The rats were killed and the livers freeze-clamped 6h after intraperitoneal injection of 0.75mg of L-ethionine/g body wt. The rats were starved for 48h before the injection. Control animals were injected with 0.9% NaCl. Concentrations are expressed in μmol/g fresh wt., as means±S.D. with the numbers of observations in parentheses. The calculated:

$$\frac{[\text{ATP}]}{[\text{ADP}][\text{HPO}_4^{2-}]}=\frac{[\text{pyruvate}]}{[\text{lactate}]}\cdot\frac{[\text{glyceraldehyde 3-phosphate}]}{[\text{3-phosphoglycerate}]}\cdot K$$

where $K = 53\times10^4$ (pH7.0). The [NAD$^+$]/[NADH] ratios are calculated from the means of [lactate]/[pyruvate] and [3-hydroxybutyrate]/[acetoacetate] ratios (see Williamson *et al.*, 1967). The *P* values were calculated on the basis of Student's *t* test.

Metabolite or metabolite ratio	Control (4)	Ethionine-treated (5)	*P*
Lactate	0.28 ±0.02	0.85 ±0.45	<0.05
Pyruvate	0.021 ±0.006	0.028 ±0.008	>0.1
[Lactate]/[Pyruvate]	12.6 ±1.72	31.3 ±8.44	<0.005
Glyceraldehyde 3-phosphate	0.0014±0.0005	0.0013±0.0004	>0.1
3-Phosphoglycerate	0.12 ±0.03	0.18 ±0.05	<0.05
[Glyceraldehyde 3-phosphate]/[3-Phosphoglycerate]	0.011 ±0.003	0.008 ±0.004	>0.1
3-Hydroxybutyrate	1.90 ±0.24	1.46 ±0.41	>0.1
Acetoacetate	0.71 ±0.13	0.47 ±0.11	<0.025
[3-Hydroxybutyrate]/[Acetoacetate]	2.70 ±0.32	3.06 ±0.30	<0.05
ATP	2.15 ±0.11	0.45 ±0.10	<0.001
ADP	1.43 ±0.17	0.59 ±0.06	<0.001
P_i	4.83 ±0.61	3.98 ±0.36	<0.05
[NAD$^+$]/[NADH] in cytoplasm	715	288	
[NAD$^+$]/[NADH] in mitochondria	7.15	6.63	
[ATP]/[ADP][HPO$_4^{2-}$] measured	533	319	
[ATP]/[ADP][HPO$_4^{2-}$] calculated	408	143	

3) but there were major differences between the effects of the two alcohols on the [glyceraldehyde 3-phosphate]/[3-phosphoglycerate] ratio and on [P_i] values. The latter rose from 4.39 to 5.92 with crotyl alcohol whereas it fell with ethanol. Ethanol increased the [glyceraldehyde 3-phosphate]/[3-phosphoglycerate] ratio sixfold but there was no significant change in this ratio with crotyl alcohol. In spite of major changes brought about by crotyl alcohol the agreement between the calculated and measured phosphorylation state was good.

Effect of carbonyl cyanide p-trifluoromethoxyphenylhydrazone

This compound, as an uncoupler of oxidative phosphorylation, is expected to decrease the value of the phosphorylation state of the adenine nucleotide system. The effects of the inhibitor on concentrations of metabolites showed considerable quantitative variations but they were always of the same kind. These variations were due to the fact that the effects of the inhibitor were very rapid. Thus [ATP] fell to about one-quarter within 2–3min. With the dose given (5mg) the animals went into tetanic spasm and died within 5min. The upset of cellular organization evidently proceeds very rapidly and at the time of freeze-clamping (2–3min after intraperitoneal injection) the degree of disorganization was liable to differ from experiment to experiment. A representative experiment is shown in Table 4.

Both [lactate] and [pyruvate] increased on injection of the inhibitor and the [lactate]/[pyruvate] ratio increased from 13.2 to 23.5. [3-Phosphoglycerate] increased threefold but [glyceraldehyde 3-phosphate] hardly altered. [ATP] fell fourfold, [ADP] did not change and [P_i] increased twofold, causing a 7.6-fold decrease in the [ATP]/[ADP][HPO$_4^{2-}$].

Although the changes in some of the components of eqn. (2) are greater than in any other situation tested the calculated and measured phosphorylation states agree remarkably well.

Table 2. *Effect of ethanol on the metabolite content of rat liver, the redox state of the NAD couple and the phosphorylation state of the adenine nucleotide system*

The rats were killed 30min after intraperitoneal injection of 2ml of 1M-ethanol. The concentrations are expressed in μmol/g fresh wt., as means±S.E.M. with the numbers of observations in parentheses. The ratios are calculated as described in Table 1.

Metabolite or metabolite ratio	Control (22)	Ethanol-treated (9)
Lactate	0.27 ±0.02	0.77 ±0.08
Pyruvate	0.019 ±0.001	0.017 ±0.001
[Lactate]/[Pyruvate]	14.2	45.3
Glyceraldehyde 3-phosphate	0.0025±0.0001	0.0022±0.0001
3-Phosphoglycerate	0.14 ±0.02	0.022 ±0.003
[Glyceraldehyde 3-phosphate]/[3-Phosphoglycerate]	0.018	0.101
3-Hydroxybutyrate	1.82 ±0.13	1.85 ±0.17
Acetoacetate	0.65 ±0.04	0.24 ±0.02
[3-Hydroxybutyrate]/[Acetoacetate]	2.8	7.7
ATP	2.32 ±0.08	2.28 ±0.05
ADP	1.48 ±0.03	1.14 ±0.04
P_i	3.69 ±0.12	2.88 ±0.10
$[NAD^+]/[NADH]$ in cytoplasm	634	198
$[NAD^+]/[NADH]$ in mitochondria	7.24	2.63
$[ATP]/[ADP][HPO_4^{2-}]$ measured	709	1189
$[ATP]/[ADP][HPO_4^{2-}]$ calculated	658	1181

Mitochondrial redox state

[3-Hydroxybutyrate] and [acetoacetate], indicators of the mitochondrial redox state, were also measured (see Tables 1–4). In three of the four situations when the redox state of the cytoplasmic NAD couple became more reduced, the mitochondrial ratio also changed towards reduction. With ethionine (Table 1) and crotyl alcohol (Table 3) the changes were relatively small (2.7 to 3.06 and 2.49 to 3.04 respectively), but with ethanol (Table 2) there was a 2.8-fold increase in the [3-hydroxybutyrate]/[acetoacetate] ratio (see also Williamson *et al.*, 1969). With carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone there was no change from the control.

Discussion

The experiments indicate that the system represented by eqn. (2) remains at near-equilibrium even when drastic agents suddenly upset either the phosphorylation state (as do carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone and ethionine) or the redox state of the NAD couple (as do ethanol and crotyl alcohol). The evidence for the existence of the near-equilibrium is the fact that the direct measurement of the phosphorylation state and the value calculated from K and the concentrations of the intermediates on the right-hand side of eqn. (2) agree reasonably well.

An analysis of the factors that contribute to the maintenance of the equilibrium shows that the change in the [lactate]/[pyruvate] ratio is the same as the change of the factor $[\text{glyceraldehyde 3-phosphate}][ADP][HPO_4^{2-}]/[\text{3-phosphoglycerate}][ATP]$. Since the phosphorylation state is not constant under the different experimental conditions, it follows that the ratios [lactate]/[pyruvate] and [glyceraldehyde 3-phosphate]/[3-phosphoglycerate] or $[\text{glyceraldehyde 3-phosphate}][HPO_4^{2-}]/[\text{3-phosphoglycerate}]$ do not always move in parallel. This is noteworthy because other cytoplasmic redox systems, e.g. [glycerol 3-phosphate]/[dihydroxyacetone phosphate] are known to change in parallel with the [lactate]/[pyruvate] ratio because they are at equilibrium with the same cytoplasmic NAD^+ pool (Bücher & Klingenberg, 1958).

It further follows from the agreement between the measured and calculated phosphorylation state that the overall values of ATP, ADP and P_i in the liver correspond closely to those of the cytoplasmic compartment. This is to be expected if the greater part of these metabolites is located in the cytoplasmic compartment and those compartments that readily

Table 3. *Effect of crotyl alcohol on the metabolite content of rat liver, the redox state of the NAD couple and the phosphorylation state of the adenine nucleotide system*

The rats were killed and the livers freeze-clamped 15 min after intraperitoneal injection of 1 ml of 5% crotyl alcohol. Control animals were injected with 0.9% NaCl. Concentrations are expressed in μmol/g fresh wt., as means ± S.D. with numbers of observations in parentheses. The ratios are calculated as described in Table 1. The *P* values were calculated on the basis of Student's *t* test.

Metabolite or metabolite ratio	Control (6)	Crotyl alcohol-treated (5)	*P*
Lactate	0.31 ±0.05	1.66 ±0.34	<0.001
Pyruvate	0.026 ±0.006	0.040 ±0.006	<0.0025
[Lactate]/[Pyruvate]	12.2 ±1.46	41.8 ±10.1	<0.001
Glyceraldehyde 3-phosphate	0.0022±0.0006	0.0033±0.0006	>0.1
3-Phosphoglycerate	0.18 ±0.03	0.22 ±0.10	>0.1
[Glyceraldehyde 3-phosphate]/[3-Phosphoglycerate]	0.011 ±0.002	0.018 ±0.014	>0.1
3-Hydroxybutyrate	1.91 ±0.24	1.61 ±0.11	<0.025
Acetoacetate	0.77 ±0.10	0.53 ±0.06	<0.0025
[3-Hydroxybutyrate]/[Acetoacetate]	2.49 ±0.26	3.04 ±0.47	<0.025
ATP	2.24 ±0.22	2.08 ±0.07	>0.1
ADP	1.51 ±0.17	1.74 ±0.22	>0.1
P_i	4.39 ±0.73	5.92 ±0.93	<0.05
$[NAD^+]/[NADH]$ in cytoplasm	738	215	
$[NAD^+]/[NADH]$ in mitochondria	8.14	6.67	
$[ATP]/[ADP][HPO_4^{2-}]$ measured	645	276	
$[ATP]/[ADP][HPO_4^{2-}]$ calculated	488	267	

communicate with the cytoplasm. The latter includes the outer mitochondrial space and probably the nucleus. The concentrations of ATP, ADP and P_i in the matrix are different from those of the cytoplasm (Klingenberg *et al.*, 1969) but this is not of major significance in the present context because the matrix space constitutes probably no more than 6% of the total liver space. The mitochondria occupy about 20% of the liver space (Lehninger, 1964) and the matrix is only about 30% of the total mitochondrial space (Pfaff *et al.*, 1968).

The maintenance of equilibrium in reaction (2) implies that the redox state of the NAD couple depends on the phosphorylation state of the adenine nucleotide system, and vice versa. In view of the reversible interrelation, it may be argued that there is no justification in stating that the redox state is controlled by the phosphorylation state; with equal justification, it could be said that the phosphorylation state is controlled by the redox state. However, there are good reasons for assuming that the phosphorylation state is the master factor. The order of magnitude of the phosphorylation state of the cytoplasm is controlled by oxidative phosphorylation and by the translocation of the adenine nucleotides between mitochondria and cytoplasm. Oxidative phosphorylation, because of the obligatory coupling of oxidation and phosphorylation, is so adjusted as to maintain a standard phosphorylation state ($[ATP]/[ADP][HPO_4^{2-}]$) in the cytoplasm of between about 200 and 1000. No explanation has as yet been put forward why in the starved liver the value for the phosphorylation state is lower than in well-fed liver. Factors responsible for variations within the above range may be the rate of ATP consumption and, as the present experiments show, the availability of reducing (or oxidizing) substances in the cell.

Although, then, finer details of the regulation of the cytoplasmic redox state are still unknown, it is reasonable to consider oxidative phosphorylation as the master process controlling the redox state of the NAD couple. The redox state, generally speaking, is therefore a consequence of the phosphorylation state. The reverse sequence of events postulating that changes in the redox state regulate the phosphorylation state is hardly feasible, although the redox state of the NAD couple, by affecting the concentration of the substrates of oxidative phosphorylation (NADH) may, within limits, modify the value of the phosphorylation state.

Table 4. *Effect of carbonyl cyanide p-trifluoromethoxyphenylhydrazone on the content of rat liver metabolites, the redox state of the NAD couple and the phosphorylation state of the adenine nucleotide system*

The rat was killed 2–3 min after intraperitoneal injection of 5 mg of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone. Control animals were injected with 0.9% NaCl. The concentrations are expressed in μmol/g fresh wt. For reasons stated in the text the carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone values are for one experiment. The ratios are calculated as described in Table 1.

Metabolite or metabolite ratio	Control (7)	Carbonyl cyanide *p*-trifluoromethoxy-phenylhydrazone (1)
Lactate	0.30 ±0.06	3.05
Pyruvate	0.027 ±0.011	0.13
[Lactate]/[Pyruvate]	13.2 ±7.1	23.5
Glyceraldehyde 3-phosphate	0.0021 ±0.0006	0.0017
3-Phosphoglycerate	0.185 ±0.029	0.58
[Glyceraldehyde 3-phosphate]/[3-Phosphoglycerate]	0.012 ±0.000	0.0029
ATP	2.33 ±0.26	0.58
ADP	1.69 ±0.18	1.58
P_i	4.70 ±0.76	8.96
3-Hydroxybutyrate	1.85 ±0.19	0.69
Acetoacetate	0.76 ±0.12	0.25
[3-Hydroxybutyrate]/[Acetoacetate]	2.48 ±0.32	2.76
$[NAD^+]/[NADH]$ in cytoplasm	816	386
$[NAD^+]/[NADH]$ in mitochondria	8.61	7.35
$[ATP]/[ADP][HPO_4^{2-}]$ measured	489	66
$[ATP]/[ADP][HPO_4^{2-}]$ calculated	549	68

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