Equilibrium Relations between the Cytoplasmic Adenine Nucleotide System and Nicotinamide-Adenine Nucleotide System in Rat Liver

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1. The ratio $[ATP]/[ADP][P_i]$, as measured by direct determination of the three components in rat liver, was found in various nutritional states to have approximately the same value as the ratio $[ATP]/[ADP][P_i]$ calculated from the concentrations of lactate, pyruvate, glyceraldehyde phosphate and 3-phosphoglycerate on the assumption that lactate dehydrogenase, glyceraldehyde phosphate dehydrogenase and 3-phosphoglycerate kinase are at near-equilibrium in the liver. This implies that the redox state of the NAD couple in the cytoplasm is linked to, and partially controlled by, the phosphorylation state of the adenine nucleotides. 2. The combined equilibrium constant of the glyceraldehyde 3-phosphate dehydrogenase and 3-phosphoglycerate kinase reactions at 38°C and $I/0.25$, was found to be 5.9×10^{-6} . 3. The fall of the $[NAD^+]/[NADH]$ ratio in starvation and other situations is taken to be the consequence of a primary fall of the $[ATP]/[ADP][HPO_4^{2-}]$ ratio.

It is known (Williamson, Lund & Krebs, 1967; Veech & Krebs, 1969) that the value of the $[free\ NAD^+]/[free\ NADH]$ ratio in the cytoplasm of normal rat liver is about 1000 and that this value is in general, for any given physiological state, a characteristic constant. The present paper is concerned with the question of how the value of this ratio, the 'redox state' of NAD, is controlled. Evidence is presented indicating that the redox state of the cytoplasmic NAD couple is regulated by the phosphorylation state of the cytoplasmic adenine nucleotide system, i.e. the value of the ratio $[ATP]/[ADP][HPO_4^{2-}]$. The controlling link between the phosphorylation state of the adenine nucleotide system and the redox state of the NAD couple is provided by the glyceraldehyde phosphate dehydrogenase and 3-phosphoglycerate kinase reactions. The capacities of the enzymes catalysing this reaction are relatively high and it may be expected that the reactants of this system are at near-equilibrium *in vivo*. At equilibrium the following relation holds:

$$\frac{[NAD^+]}{[NADH]} = \frac{1}{K} \cdot \frac{[3\text{-Phosphoglycerate}]}{[Glyceraldehyde\ 3\text{-phosphate}] \cdot \frac{[ATP]}{[ADP][HPO_4^{2-}]}} \quad (1)$$

where K is the product of the equilibrium constants of the glyceraldehyde phosphate dehydrogenase system and the 3-phosphoglycerate kinase system

at pH 7. K is a constant which can be ascertained by direct measurements, and the concentrations of 3-phosphoglycerate and glyceraldehyde 3-phosphate in the tissue can be determined. The $[NAD^+]/[NADH]$ ratio can be obtained from other cytoplasmic dehydrogenase systems that are at near-equilibrium. Among these, lactate dehydrogenase is the most satisfactory system, because of its high activity and the ease of the determination of lactate and pyruvate. Combining eqn. (2):

$$\frac{[NAD^+]}{[NADH]} = \frac{1}{K_{LDH}} \cdot \frac{[Pyruvate]}{[Lactate]} \quad (2)$$

where K_{LDH} is the equilibrium constant for the lactate dehydrogenase reaction, with eqn. (1), an equation is obtained that can be verified by experiment:

$$\frac{[Pyruvate]}{[Lactate]} = \frac{1}{K'} \cdot \frac{[3\text{-Phosphoglycerate}]}{[Glyceraldehyde\ 3\text{-phosphate}] \cdot \frac{[ATP]}{[ADP][HPO_4^{2-}]}} \quad (3)$$

K' in this equation represents:

$$\frac{K_{GAPDH} \cdot K_{3PGK}}{K_{LDH}}$$

where K_{GAPDH} and K_{3PGK} are the equilibrium constants for the glyceraldehyde phosphate dehydrogenase and 3-phosphoglycerate kinase reactions respectively.

It is shown below that the agreement between the $[ATP]/[ADP][P_i]$ value obtained from this equation and from direct measurements of the concentrations of ATP, ADP and P_i in liver under four different nutritional conditions is satisfactory, and justifies the assumption that in rat liver glyceraldehyde phosphate dehydrogenase and 3-phosphoglycerate kinase maintain near-equilibrium concentrations of their substrates and thereby control the redox state of the NAD couple in the cytoplasm.

EXPERIMENTAL

Rats. Male rats of the Wistar strain weighing about 100g were used.

Diets. The normal diet and high-sucrose diet were the same as those described by Veech, Eggleston & Krebs (1969). The high-glucose diet consisted of 75% glucose, 22.5% light white casein (British Drug Houses Ltd., Poole, Dorset), 2% McCollum's salt mixture, 0.45% vitamin mixture and 0.05% choline chloride.

The low-carbohydrate high-fat diet consisted of 66% hydrogenated peanut oil margarine, 33% light white casein, McCollum's salt mixture, vitamin mixture and 0.01% choline chloride. During the first day or two on this diet the animals ate little, and their stomachs were sometimes empty at the time of killing. After 2 or 3 days the rats accepted the diet, and for this reason one group was maintained on it for 7 days.

Reagents. The reagents were the same as those described by Williamson *et al.* (1967) and Veech *et al.* (1969) with the following additions. DL-Glyceraldehyde-3-phosphate was obtained as the diethylacetal barium salt from Boehringer Corp. (London) Ltd., London W.5, U.K. The salt was hydrolysed according to the following modification of the method of Ballou & Fischer (1955). Diethylacetal barium DL-glyceraldehyde 3-phosphate (82mg) was placed in 10ml of water and added to approx. 2ml of Dowex 50 (H^+ form) and stirred for about 5min, the pH being below 2. The mixture was then spun and the supernatant decanted. The supernatant was left to stand at a pH below 2 for 5 days at room temperature in a stoppered tube, and was then stored at $-18^\circ C$. It was stable for at least 5 months.

Determination of metabolites in liver. Rats were killed by dislocation of the neck and the livers were removed within 10s and clamped between aluminium blocks cooled in liquid N_2 . The further treatment was as described by Veech *et al.* (1969).

The methods used for the determination of metabolites were as follows: pyruvate and lactate by the method of Hohorst, Kreutz & Bücher (1959); 3-phosphoglycerate by the method of Czok & Eckert (1963); ATP by the method of Lamprecht & Trautschold (1963); ADP and AMP by the method of Adam (1963) with the substitution of 0.2M-tris-HCl buffer, pH 7.5, for 0.2M-triethanolamine buffer; dihydroxyacetone phosphate by the method of Bücher & Hohorst (1963); α -glycerophosphate by the method of Hohorst (1963); P_i by the method of Martin & Doty (1949).

The assay of glyceraldehyde 3-phosphate was adapted from Warburg & Christian (1939) and Racker (1957) for

use on the Beckman ratio fluorimeter. To each 2ml cuvette was added 0.01ml of 0.03M-NAD $^+$, 0.025ml of 0.01M-EDTA (disodium salt), 0.10ml of 0.1M-sodium arsenate and 0.34ml of 0.2M-sodium pyrophosphate, pH 8.8. Neutralized $HClO_4$ extract of liver (1–1.5ml) was then added, and water if necessary to bring the volume to 2ml. After stable fluorimeter readings were obtained, 0.005ml of a crystalline suspension of glyceraldehyde phosphate dehydrogenase containing 0.05mg of enzyme was then added, mixed, and the fluorescence measured 2min after addition of enzyme when the reaction was completed; there was no drift. The fluorimeter was calibrated against an external standard of 0.2ml of 10 μ M-D-glyceraldehyde 3-phosphate in 2ml. The standards were prepared by previous assay of more concentrated solutions by using a similar procedure on the Zeiss PMQII spectrophotometer and diluted for fluorimetric use. Standards were stable for several months at $-18^\circ C$. The degree of quenching occurring in the liver extracts was low and reasonably constant and the routine use of internal standards proved unnecessary.

The recoveries of metabolites added to frozen liver powder in concentrations approximating to endogenous values were on average as follows (four separate determinations): pyruvate, 107%; lactate, 90%; 3-phosphoglycerate, 99.5%; glyceraldehyde 3-phosphate, 94%; ATP, 91%; ADP, 98%; AMP, 103%; P_i , 103%.

Measurement of the combined equilibrium constants of glyceraldehyde 3-phosphate dehydrogenase and 3-phosphoglycerate kinase systems. The enzymes used for the determination of the equilibrium constants were a crystalline 3-phosphoglycerate kinase preparation from yeast and a crystalline glyceraldehyde phosphate dehydrogenase from rabbit muscle. The initial substrate concentrations in the incubation mixture were determined as follows: NAD $^+$ by the method of Klingenberg (1963); ATP, ADP and 3-phosphoglycerate as described above. The extinction coefficient of NADH at 340nm was taken as 6.22×10^6 cm 2 /mol (Horecker & Kornberg, 1948). The initial P_i concentration was known from the amounts of K_2HPO_4 and KH_2PO_4 added. The ionic strength was calculated according to the formula $I = 0.5 \sum c_i z_i^2$ where c_i is the molarity of the ion and z_i its valency. Adjustment of I to 0.25 was made with KCl. $MgCl_2$ (0.6mM) was present in all determinations of the equilibrium constant. The amounts of total magnesium were between 50% and 100% greater than the sum of ATP and ADP present. This range was chosen in order to approximate intracellular concentrations. Thus most of the ATP and ADP was present as $MgATP^{2-}$ and $MgADP^-$. The concentration of HPO_4^{2-} was calculated from the total phosphate concentration, the pH measured at $25^\circ C$ and corrected to $38^\circ C$ (Clark, 1960) and the second dissociation constant of phosphoric acid.

The final concentration of ATP, ADP, 3-phosphoglycerate and glyceraldehyde 3-phosphate at equilibrium were calculated from the measured initial concentrations and the measured change in NADH concentration on the basis of the stoichiometry of eqn. (1). The measurements were made in a thermostatically controlled cell holder fitted in a Beckman DU spectrophotometer modified with a Hilger-Gilford Reaction Kinetic Outfit. The reaction was started by adding between 0.2 and 0.05mg each of glyceraldehyde 3-phosphate dehydrogenase and

3-phosphoglycerate kinase. The reaction was complete in a maximum of 8 min.

RESULTS

Combined equilibrium constant of glyceraldehyde 3-phosphate dehydrogenase and 3-phosphoglycerate kinase reactions. The constant was measured at *I* 0.25 and 38°C with different initial substrate concentrations and by approaching equilibrium from both sides. The range of substrate concentrations used is shown in Table 1. Consistent results were obtained for the combined equilibrium constant defined as:

$$K = \frac{[\text{MgATP}^{2-}][\text{NADH}][3\text{-phosphoglycerate}^{3-}][\text{H}^+]}{[\text{MgADP}^-][\text{HPO}_4^{2-}][\text{NAD}^+][\text{glyceraldehyde 3-phosphate}^{2-}]} \quad (4)$$

the mean being 5.9×10^{-6} , S.E.M. = $\pm 1.24 \times 10^{-6}$ (25 observations). The calculation of the concentrations at equilibrium implies the assumption that no significant amounts of 1,3-diphosphoglycerate accumulated at equilibrium. If significant amounts had accumulated they would have varied with the experimental conditions and the results would have been less consistent. This may be regarded as evidence in justifying the assumption.

Individual values for the equilibrium constants of the two enzymes have been reported by Meyerhof & Oesper (1947) and Cori, Velick & Cori (1950) for glyceraldehyde phosphate dehydrogenase and by Bücher (1947) for 3-phosphoglycerate kinase. Combining the best estimates (see Burton & Wilson, 1953), a value of 24.5×10^{-6} is obtained for K' , but the measurements were made at room temperature, ionic strength lower and undefined total magnesium

concentrations. The difference by the factor 4 between this and the present value may be explained by the different experimental conditions.

Concentration of the substrates of glyceraldehyde phosphate dehydrogenase, 3-phosphoglycerate kinase and lactate dehydrogenase in rat liver in different nutritional states. The results of the determination of metabolites in rat liver in five different nutritional states are given in Table 2. The nutritional state had marked effects on most of the metabolites. [Pyruvate] fell in starvation to 18% and on the low-carbohydrate diet to about 25% of the normal value, whereas on the high-sucrose and high-glucose diets it rose to 280% and 170% of the

normal value respectively. [Lactate] fell to 47% of the normal value on starvation, and to 29% and 25% on feeding with the low-carbohydrate diet for 3 and 7 days respectively; it rose to 170% of the normal on sucrose feeding. There was no significant change of [lactate] on the glucose diet. [Glyceraldehyde 3-phosphate] fell on starvation and the low-carbohydrate diet to 69–85% of normal, and rose to 170% on sucrose feeding; there was only a small rise on glucose feeding. [ATP] did not change on starvation or low-carbohydrate feeding for 3 days, but there was a significant rise on the high-sucrose and glucose diets, and on the low-carbohydrate diet after 7 days. [ADP] varied somewhat independently of [ATP], rising on starvation (whereas [ATP] remained constant) and on the low-carbohydrate diet, falling slightly on sucrose feeding (whereas [ATP] rose), and marginally rising on glucose feeding (when [ATP] rose markedly). [AMP] showed yet another pattern. It rose to 130% on starvation, but fell to 49% and 57% on sucrose and glucose feeding respectively, and to 77% on low-carbohydrate feeding for 7 days. [P_i] changed roughly in the same way as [AMP]; there was a rise on starvation, a fall on the sucrose diet, but no change on the glucose and low-carbohydrate diets. The sum of the concentrations of the three adenine nucleotides was not much affected by the different nutritional conditions.

Table 1. *Range of initial and final concentration of reactants used in the determination of the combined equilibrium constant of the glyceraldehyde phosphate dehydrogenase and 3-phosphoglycerate kinase reactions*

The concentrations are given in mM. The ionic strength was 0.25 and temperature was 38°C. The MgCl_2 concentration was 0.6 mM. The final pH varied between 6.88 and 7.02.

	Initial concn.	Final concn.
ATP	0.012–0.24	0.059–0.132
ADP	0.161–0.312	0.262–0.428
HPO_4^{2-}	20.0–40.0	20.5–42.6
3-Phosphoglycerate	0.057–1.77	1.07–1.65
Glyceraldehyde 3-phosphate	0–0.146	0.087–0.12
NAD^+	0–0.148	0.12–0.807
NADH	0–0.173	0.0442–0.0873

DISCUSSION

Comparison of the measured and calculated phosphorylation states of the adenine nucleotides. The measured values for the ratio $[\text{ATP}]/[\text{ADP}][\text{HPO}_4^{2-}]$ show satisfactory agreement with the calculated values in the five nutritional conditions, although the actual concentrations of the metabolites vary

Table 2. *Measured and calculated values for the phosphorylation state of the adenine nucleotides ([ATP]/[ADP][HPO₄²⁻]) in rat liver cytoplasm*

The metabolite concentrations are given as means $\pm$ S.E.M. expressed in nmol/g fresh wt. The concentration of HPO₄²⁻ at pH 7.0 was taken to be 60% of the total P_i. The phosphorylation state of the adenine nucleotides was calculated according to the formula:

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

where $K = 53 \times 10^4$ (at pH 7.0). The values of the ratios are the means of the ratios calculated for each individual animal.

State of animal (no. of observations)	[Pyruvate]	[Lactate]	[Dihydroxyacetone phosphate]	[α -Glycero- phosphate]	[Glyceraldehyde 3-phosphate]	[3-Phospho- glycerate]	[ATP]	[ADP]
Normal (8)	95 $\pm$ 11	714 $\pm$ 117	27 $\pm$ 2.5	158 $\pm$ 15	2.9 $\pm$ 0.2	359 $\pm$ 22	1950 $\pm$ 64	1130 $\pm$ 28
Starved for 48h (7)	17 $\pm$ 3	336 $\pm$ 65	26 $\pm$ 2	420 $\pm$ 42	2.0 $\pm$ 0.2	316 $\pm$ 44	1730 $\pm$ 99	1610 $\pm$ 39
High-sucrose diet for 3 days (5)	265 $\pm$ 54	1230 $\pm$ 200	52 $\pm$ 6	286 $\pm$ 41	4.9 $\pm$ 0.7	430 $\pm$ 16	2350 $\pm$ 52	950 $\pm$ 35
High glucose diet for 3 days (8)	161 $\pm$ 26	855 $\pm$ 91	33 $\pm$ 3	148 $\pm$ 14	4.0 $\pm$ 0.5	576 $\pm$ 44	2570 $\pm$ 76	1350 $\pm$ 110
Low-carbohydrate high-fat diet for 3 days (8)	25 $\pm$ 3.4	204 $\pm$ 27	14 $\pm$ 0.5	129 $\pm$ 9	2.5 $\pm$ 0.1	288 $\pm$ 29	1920 $\pm$ 58	1590 $\pm$ 29
Low-carbohydrate high-fat diet for 7 days (9)	26 $\pm$ 1.7	179 $\pm$ 14	13 $\pm$ 0.4	135 $\pm$ 12	2.0 $\pm$ 0.1	168 $\pm$ 13	2310 $\pm$ 73	1510 $\pm$ 52
[AMP]	[P _i]	[Lactate] [Pyruvate]	[α -Glycerophosphate] [Dihydroxyacetone phosphate]	[α -Glycerophosphate]/ [Dihydroxyacetone phosphate] [Lactate]/[Pyruvate]		[ATP]/[AMP] [ADP] ²	[ATP] [ADP][HPO ₄ ²⁻]	
							Calculated	Measured
575 $\pm$ 41	5210 $\pm$ 136	7.52	5.85		0.78	0.88	648	551
744 $\pm$ 36	6320 $\pm$ 120	19.8	16.2		0.82	0.50	199	282
279 $\pm$ 17	4610 $\pm$ 122	4.64	5.50		1.2	0.70	1290	874
326 $\pm$ 32	4820 $\pm$ 150	5.31	4.48		0.84	0.46	738	660
640 $\pm$ 34	4890 $\pm$ 340	8.16	9.21		1.1	0.48	612	427
444 $\pm$ 37	4700 $\pm$ 202	6.88	10.4		1.5	0.44	1026	556

considerably. The largest disagreement was in the rats fed on the low-carbohydrate diet for 7 days, where the calculated and measured values differ by the factor 1.8. Considering the large number of measurements and their limited accuracy and the possibility of some deviation from equilibrium, the general agreement between the calculated and measured values for the above ratio can be considered excellent.

The agreement indicates that the glyceraldehyde phosphate dehydrogenase - 3-phosphoglycerate kinase system was at near-equilibrium and that in rat liver the cytoplasmic phosphorylation state can be calculated from determinations of the concentrations of lactate, pyruvate, glyceraldehyde 3-phosphate and 3-phosphoglycerate. The agreement further implies that the cytoplasmic redox state of the NAD couple is closely linked to the phosphorylation state of the adenine nucleotide system.

Instead of eqn. (3) an analogous equation in which [pyruvate]/[lactate] is replaced by [dihydroxyacetone phosphate]/[α -glycerophosphate] can be used because of the approximate constancy, under many conditions, of the ratio:

$$\frac{[\alpha\text{-Glycerophosphate}]/[\text{Dihydroxyacetone phosphate}]}{[\text{Lactate}]/[\text{Pyruvate}]}$$

(see Table 2). However, the value of the equilibrium constant of the α -glycerophosphate dehydrogenase system is not as accurately known as that of the lactate dehydrogenase system, and moreover the former is more likely to be displaced from equilibrium than the latter (Bücher & Rüssmann, 1964).

It is well established that the [lactate]/[pyruvate] ratio rises on starvation (Table 2) and certain other conditions, such as diabetes. These are situations where the rate of gluconeogenesis is high and where the [ATP]/[ADP][P_i] ratio falls, presumably on account of the high ATP requirements of gluconeogenesis. It is thus reasonable to assume that the primary process responsible for the change of the cytoplasmic redox state of the NAD couple is a fall in the phosphorylation state of the adenine nucleotide system that is followed by a change of the [NAD⁺]/[NADH] ratio on account of equilibrium relations between the cytoplasmic adenine nucleotides and nicotinamide nucleotides.

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