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The Redox State of Free Nicotinamide–Adenine Dinucleotide Phosphate in the Cytoplasm of Rat Liver

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1. The concentrations of the oxidized and reduced substrates of the 'malic' enzyme (EC 1.1.1.40) and isocitrate dehydrogenase (EC 1.1.1.42) were measured in freeze-clamped rat livers. By assuming that the reactants of these dehydrogenase systems are at equilibrium in the cytoplasm the [free $NADP^+$]/[free NADPH] ratio was calculated. The justification of the assumption is discussed. 2. The values of this ratio obtained under different nutritional conditions (well-fed, 48hr.-starved, fed with a low-carbohydrate diet, fed with a high-sucrose diet) were all of the same order of magnitude although characteristic changes occurred on varying the diet. The value of the ratio fell on starvation and on feeding with the low-carbohydrate diet and rose slightly on feeding with the high-sucrose diet. 3. The mean values of the ratio were calculated to be between 0·001 and 0·015, which is about 100000 times lower than the values of the cytoplasmic [free NAD^+]/[free NADH] ratio. 4. The differences in the redox state of the two nicotinamide–adenine dinucleotide couples can be explained on a simple physicochemical basis. The differences are the result of equilibria that are determined by the equilibrium constants of a number of highly active readily reversible dehydrogenases and transaminases and the concentrations of the substrates and products of these enzymes. 5. The decisive feature is the fact that the NAD and NADP couples share substrates. This sharing provides a link between the redox states of the two couples. 6. The application of the method of calculation to data published by Kraupp, Adler-Kastner, Niessner & Plank (1967), Goldberg, Passonneau & Lowry (1966) and Kauffman, Brown, Passonneau & Lowry (1968) shows that the redox states of the NAD and NADP couples in cardiac-muscle cytoplasm and in mouse-brain cytoplasm are of the same order as those in rat liver. 7. The determination of the equilibrium constant at 38°, pH 7·0 and I 0·25 (required for the calculation of the [free $NADP^+$]/[free NADPH] ratio), gave a value of $3{\cdot}44 \times 10^{-2}$M for the 'malic' enzyme (with CO_2 rather than HCO_3^- as the reactant) and a value of $1{\cdot}98 \times 10^{-2}\,M^{-1}$ for glutathione reductase.

This paper is concerned with the determination in rat liver of the value of the [free $NADP^+$]/[free NADPH] ratio where 'free' refers to that fraction of the dinucleotide which is not bound to protein. It is this fraction that determines the directions of reactions in which the dinucleotides are participants and the free-energy changes of these reactions (Holzer, Schultz & Lynen, 1956).

In a previous paper (Williamson, Lund & Krebs, 1967*a*) the [free NAD^+]/[free NADH] ratio was assayed in the mitochondria and cytoplasm of rat liver by measuring the ratios of the concentrations of the oxidized and reduced metabolites of suitable NAD-linked dehydrogenase systems. If these systems are in equilibrium the above ratio can be calculated from the equilibrium condition

$$\frac{[\text{oxidized substrate}][\text{NADH}]}{[\text{reduced substrate}][\text{NAD}^+]} = K \qquad (1)$$

as proposed by Holzer *et al.* (1956) and Bücher & Klingenberg (1958).

To apply these principles to the NADP couple, the following conditions must be fulfilled: (1) the NADP-linked dehydrogenase must be active in one cell compartment only; (2) the activity of the enzyme must be sufficiently high to establish near-equilibrium in the system:

$$\text{substrate} + \text{NADP}^+ \rightleftharpoons \text{oxidized substrate} + \text{NADPH} + \text{H}^+$$

(3) the equilibrium constant of the system must be known for the temperature and ionic strength of

Table 1. *Activity of rat liver enzymes bearing on the redox state of the nicotinamide nucleotides*

The values are μmoles of substrate removed/min./g. of liver (fresh wt.) at 25° and pH 7·4 under the assay conditions and are given as means ± S.E.M. of the numbers (given in parentheses) of determinations. The values for glutamate dehydrogenase are taken from Table 9 of Williamson *et al.* (1967*a*), those for glutathione reductase from Rall & Lehninger (1952). The other values are new measurements.

	Enzyme activities of livers of rats kept on various diets			
Enzyme	Standard diet	Low-carbohydrate diet	Starvation for 48 hr.	High-sucrose diet
Isocitrate dehydrogenase	22·4 ± 0·88 (6)	20·5 ± 0·26 (6)	20·2 ± 1·15 (3)	
Glutathione reductase	7·0			
6-Phosphogluconate dehydrogenase	2·84 ± 0·16 (3)	2·31 ± 0·25 (3)		
Glucose 6-phosphate dehydrogenase	1·42 ± 0·11 (8)	1·18 ± 0·11 (8)	0·86 ± 0·4 (3)	9·82 ± 1·31 (9)
'Malic' enzyme	1·27 ± 0·12 (8)	0·54 ± 0·04 (6)	0·78 ± 0·6 (7)	6·33 ± 1·02 (9)
Glutamate–oxaloacetate transaminase	86·0 ± 2·7 (6)	145 ± 11·7 (5)		94·0 ± 5·8 (6)
Glutamate–pyruvate transaminase	5·3 ± 0·46 (5)	5·6 ± 0·80 (5)		2·4 ± 0·52 (6)
Malate dehydrogenase	398 ± 12 (6)	404 ± 10 (6)		
Lactate dehydrogenase	247 ± 30 (6)	312 ± 39 (6)		
Glutamate dehydrogenase	118 ± 8·6 (4)			

the tissue; (4) it must be possible to determine accurately the concentrations of 'substrate' and 'oxidized substrate'.

There are five dehydrogenase systems in rat liver cytoplasm that might be expected to fulfil these requirements: *threo*-D_s-isocitrate–NADP oxidoreductase (decarboxylating) (EC 1.1.1.42) [referred to below as isocitrate dehydrogenase (NADP)], glutathione reductase (EC 1.6.4.2), D-glucose 6-phosphate–NADP oxidoreductase (EC 1.1.1.49) (referred to below as glucose 6-phosphate dehydrogenase), 6-phospho-D-gluconate–NADP oxidoreductase (EC 1.1.1.43) (referred to below as 6-phosphogluconate dehydrogenase) and L-malate–NADP oxidoreductase (decarboxylating) (EC 1.1.1.40) (referred to below as 'malic' enzyme). The activities of these, and of other enzymes that bear on the redox state of the nicotinamide–adenine dinucleotides, under optimum assay conditions at pH 7·4 and 25° are shown in Table 1. Three of the cytoplasmic NADP-linked dehydrogenases were unsuitable for the determination of the [free $NADP^+$]/[free NADPH] ratio at the time when the experiments were carrried out. It has so far not proved practicable to avoid an oxidation of GSH during protein precipitation, and, as the physiological concentration of GSSG in the tissues is very low, an oxidation of only 5% of the GSH can increase the concentration of GSSG by several orders of magnitude. With glucose 6-phosphate dehydrogenase difficulties arise from the fact that the primary product of the dehydrogenation is the unstable δ-lactone of 6-phosphogluconate. The use of the combined lactonase and glucose 6-phosphate dehydrogenase is at present impracticable because the combined equilibrium constant is not accurately known. Until very recently this also applied to the 6-phosphogluconate dehydrogenase, but work by Villet & Dalziel (1969) and by Kauffman, Brown, Passonneau & Lowry (1968) has now supplied information on the equilibrium constant of this dehydrogenase as well as on the determination of the relevant metabolites. This system may therefore be used in the future for the estimation of the redox state of the NADP couple in the cytoplasm, though technical difficulties are considerable because of the low concentrations of the reactants.

There remain the isocitrate dehydrogenase (NADP) (for the assumption that this enzyme can be considered as active in cytoplasm only, see the Discussion section) and the 'malic' enzyme. The concentrations of the substrates of these two dehydrogenase systems can be measured and the activities of the enzymes proved to be high enough to establish equilibrium with the NADP system.

The procedure also depends on whether the determination of the metabolites in the whole tissue reflects their concentration in the cytoplasm. It is likely, though not certain, that in normal liver the concentrations of the metabolites in the two main cell compartments are very similar (see the Discussion section). Since liver cytoplasm constitutes at least two-thirds of the cell volume, an uneven distribution of metabolites between the cytoplasm and other organelles will become significant only when concentration gradients between cytoplasm and organelles are high.

On the basis of these considerations, the concentrations of the reactants of the 'malic' enzyme and isocitrate dehydrogenase were determined in freeze-clamped rat liver, together with those of the lactate and β-hydroxybutyrate systems of normal and starved rats, and of rats given diets rich in sucrose or lacking carbohydrate. The high-

Table 2. *Equilibrium constants of enzyme systems bearing on the redox state of the nicotinamide nucleotides at* pH 7·0, 38° *and* I 0·25

The concentration of water was taken to be unity. Where H^+ is, on balance, a reactant, the equilibrium constant is dependent on pH and in these cases the constant at pH 0 can be obtained by multiplying the value given by 10^{-7}. With 'malic' enzyme, isocitrate dehydrogenase, 6-phosphogluconate dehydrogenase and the transaminases, K is independent of pH. When the constant has units, these are expressed as M. When the concentration of metabolites are expressed as mM, the value of the constant must also be expressed as mM. The constants for the transaminase systems were obtained by new measurements at 38° and I 0·25, but were found to be virtually identical with those reported by Krebs (1953) for 25°.

Enzyme system	Definition of constant	Value of constant	Reference
Isocitrate dehydrogenase (NADP)	$\frac{[\alpha\text{-oxoglutarate}^{2-}][CO_2][NADP]}{[\text{isocitrate}^{3-}][NADP^+]}$	1·17 M	Londesborough & Dalziel (1968)
Glutathione reductase	$\frac{[GSSG][NADPH]}{[GSH]^2[NADP^+]}$	$1{\cdot}98\times10^{-2}$ M^{-1}	Veech (1968)
6-Phosphogluconate dehydrogenase	$\frac{[\text{ribulose 5-phosphate}^{2-}][CO_2][NADPH]}{[\text{6-phosphogluconate}^{3-}][NADP^+]}$	$1{\cdot}72\times10^{-1}$ M	Villet & Dalziel (1969)
'Malic' enzyme	$\frac{[\text{pyruvate}^-][CO_2][NADPH]}{[\text{malate}^{2-}][NADP^+]}$	$3{\cdot}44\times10^{-2}$ M	Veech (1968)
Malate dehydrogenase	$\frac{[\text{oxaloacetate}^{2-}][NADH]}{[\text{malate}^{2-}][NAD^+]}$	$2{\cdot}78\times10^{-5}$	Williamson *et al.* (1967*a*)
Lactate dehydrogenase	$\frac{[\text{pyruvate}^-][NADH]}{[\text{lactate}^-][NAD^+]}$	$1{\cdot}11\times10^{-4}$	Williamson *et al.* (1967*a*)
β-Hydroxybutyrate dehydrogenase	$\frac{[\text{acetoacetate}^-][NADH]}{[\beta\text{-hydroxybutyrate}^-][NAD^+]}$	$4{\cdot}93\times10^{-2}$	Williamson *et al.* (1967*a*)
Glutamate dehydrogenase (NAD)	$\frac{[\alpha\text{-oxoglutarate}^{2-}][NH_4^+][NADH]}{[\text{glutamate}^-][NAD^+]}$	$3{\cdot}87\times10^{-6}$ M	Engel & Dalziel (1967)
Glutamate dehydrogenase (NADP)	$\frac{[\alpha\text{-oxoglutarate}^{2-}][NH_4^+][NADPH]}{[\text{glutamate}^-][NADP^+]}$	$2{\cdot}49\times10^{-6}$ M	Engel & Dalziel (1967)
Glutamate–oxaloacetate transaminase	$\frac{[\alpha\text{-oxoglutarate}^{2-}][\text{aspartate}^-]}{[\text{glutamate}^-][\text{oxaloacetate}^{2-}]}$	6·61	H. A. Krebs & M. Stubbs (unpublished work)
Glutamate–pyruvate transaminase	$\frac{[\alpha\text{-oxoglutarate}^{2-}][\text{alanine}]}{[\text{glutamate}^-][\text{pyruvate}^-]}$	1·52	Brosnan (1968)

sucrose diet was lipogenic; it caused the liver to be loaded with excess of carbohydrate, which was converted into fat. The low-carbohydrate diet was glucogenic and promoted gluconeogenesis from amino acids and glycerol. For the calculation of the redox state of the NADP couple it was also necessary to measure the equilibrium constant of the 'malic' enzyme system at 38° and ionic strength 0·25. The values of the constants used in this paper are given in Table 2.

EXPERIMENTAL

In general the materials and methods were the same as those used by Williamson *et al.* (1967*a*) with the following modifications and additions.

Diets. The 'standard diet' was Oxo Breeding Diet pellets; 'high-sucrose diet' consisted of 70% sucrose, 20% technical grade casein, 5% arachis oil and 5% McCollum's salt mixture; 'low-carbohydrate diet' consisted of 66% margarine, 33% casein and 1% McCollum's salt mixture.

Rats. Wistar albino rats weighing 180–200g. were used.

Reagents. Isocitrate dehydrogenase, citrate lyase, glucose 6-phosphate, $NADP^+$ and NADPH were obtained from Boehringer Corp. (London) Ltd., London W.5, and L-malic acid was from Koch–Light Laboratories Ltd., Colnbrook, Bucks. Monopotassium dihydrogen *threo*-D_s-isocitrate was prepared from leaves of *Sedum praealtum* Sol. by the method of Vickery & Wilson (1960). Its purity, measured enzymically, was 98·8% (w/w). The main impurity was L-malate.

Treatment of liver. The treatment of the freeze-clamped liver was modified to avoid peroxidative destruction of labile metabolites (see Lemberg, 1942; Lemberg & Legge, 1949). This destruction is catalysed by oxyhaemoglobin and is therefore inhibited by CO. After the liver had been ground into a powder under ordinary liquid N_2 another portion of liquid N_2 through which CO had been bubbled for 10min. was added. This was allowed to evaporate at the temperature of solid CO_2. This treatment with CO was repeated once. The frozen powder (2g.) was then transferred to a cold weighed centrifuge tube and 7·8ml. of cold 3·5% (w/v) $HClO_4$ and 0·2ml. of 0·1M-diethyldithiocarbamate were added before the mixture was homogenized with a glass pestle. Further treatment of the tissue was as described by Williamson *et al.* (1967*a*).

Determination of metabolites. Malate was determined

in the same cuvette as lactate by adding malate dehydrogenase first. Citrate was determined with citrate lyase (Gruber & Moellering, 1966). Isocitrate was determined by modifying the spectrophotometric method of Siebert (1963) for use with a Beckman ratio fluorimeter. The cuvettes contained 0·2–0·5ml. of deproteinized and neutralized tissue extract, 0·5ml. of 0·2M-tris–HCl buffer, pH 7·4, 0·02ml. of 5mM-$NADP^+$, 0·02ml. of 20mM-$MnSO_4$, and water to a final volume of 2ml. After the readings had become constant 0·005ml. of isocitrate dehydrogenase solution (0·05mg. of enzyme) was added to the lip of each cuvette and the cuvettes were rapidly mixed. Readings were then taken promptly because a reoxidation of NADPH tends to occur, and the assay must therefore be completed rapidly. The instrument was calibrated by measuring the fluorescence of NADPH generated on the addition of a standard solution of *threo*-D_s-isocitrate to the assay mixture. Each tissue sample was first assayed against an external sample containing a known amount of isocitrate. Next the tissue samples were reassayed after the addition of an internal isocitrate standard to correct for any quenching. The fluorimeter was repeatedly standardized during an analytic run and from day to day against a glass standard.

The CO_2 concentration of the liver was taken to be the same as that of the superior vena cava. The pressure of CO_2 in blood or aqueous solutions was determined with a Radiometer gas analyser and a CO_2 electrode. Blood samples were collected under light ether anaesthesia from the vena cava at the entrance of the hepatic veins. The CO_2 concentration was calculated on the assumption that $\alpha^{38}_{CO_2}$ is 0·523 (Hastings & Sendroy, 1925) and that the mole volume of CO_2 is 22·26l. (Van Slyke, Hastings, Hiller & Sendroy, 1928). $NADP^+$ was determined by the method of Klingenberg (1963). The CO_2 concentration of the gas mixture was determined either manometrically (Krebs, 1930) or with the Radiometer gas analyser.

For recovery tests, solutions containing known amounts of metabolites (0·025–0·5μmole/g. of liver) were placed in a centrifuge tube and frozen solid. Next a weighed sample of previously assayed frozen liver was added and mixed with the frozen metabolite solution. Recovery in single determinations was accurate within 10%. Metabolite concentrations are expressed as μmoles/g. fresh wt.

Assay of enzyme activities. Fresh rat livers were cooled to 2–4°, homogenized with 4 parts (v/w) of hypo-osmotic medium and stored in a number of small tubes at −20° to avoid repeated thawing and refreezing of any one sample. The medium was either water or 1mM-EDTA; these media showed no disadvantages over the more complex media used by other workers (e.g. Shonk & Boxer, 1964).

The assays were carried out at pH 7·4 (tris–HCl or potassium phosphate buffers) and at 25°. The concentrations of substrates and cofactors were chosen to give maximum activities. In some assays where higher concentrations of tissue homogenate were required the blank oxidation of NADH or NADPH was greatly diminished by the addition of cyanide (KCN, neutralized by HCl, 0·2mM final concentration). In more diluted homogenate, where blanks were small, it was unnecessary to add HCN. In all cases an extra cuvette was used for corrections. Unless otherwise stated the enzymes were assayed by spectrophotometric methods at 340 or 366nm. similar to those used by Shonk & Boxer (1964). The main differences concerned the homogenizing media and buffers, and that in the assay of triose phosphate isomerase 2mM-DL-glyceraldehyde 3-phosphate was used as substrate. Isocitrate dehydrogenase (NADP) was assayed by the method of Plaut (1962), 'malic' enzyme by the method of Fitch & Chaikoff (1959) with 1mM-L-malate and tris buffer; glutamate–oxaloacetate transaminase and glutamate–pyruvate transaminase by the methods of Bergmeyer & Bernt (1963*a*,*b*).

Enzyme activities are expressed as μmoles of substrate removed/min./g. fresh wt.

Equilibrium constants of enzyme systems are abbreviated as follows:

K_{ME}	for 'malic' enzyme
K_{IDH}	for isocitrate dehydrogenase (NADP)
$K_{GDH(NADP)}$	for glutamate dehydrogenase (NADP)
$K_{GDH(NAD)}$	for glutamate dehydrogenase (NAD)
K_{LDH}	for lactate dehydrogenase
K_{MDH}	for malate dehydrogenase
K_{GOT}	for glutamate–oxaloacetate transaminase
K_{GPT}	for glutamate–pyruvate transaminase

RESULTS

Metabolite concentrations in freeze-clamped rat liver. The results of the measurements of the components of NADP-linked dehydrogenase systems in six different nutritional states are shown in Table 3, together with the components of the lactate dehydrogenase and β-hydroxybutyrate dehydrogenase systems in order to have data for the NADP and NAD couples from the same liver. The measurements on the NAD-linked systems confirm and supplement those of Williamson *et al.* (1967*a*).

The various dietary changes did not significantly alter the concentration of malate. The concentration of lactate fell significantly on starvation and on feeding with the low-carbohydrate diet. Pyruvate usually moved in the same direction as lactate but to a greater extent than lactate. When the high-sucrose diet was given for 3 days, however, lactate and pyruvate concentrations moved in opposite directions. Pyruvate and α-oxoglutarate concentrations rose above the normal values after 3 days of feeding with the high-sucrose diet, but fell slightly below normal after 5 days of feeding with the same diet. Citrate and isocitrate concentrations tended to rise on feeding with the high-sucrose diet after both 3 and 5 days. On feeding with the low-carbohydrate diet a significant rise of citrate and isocitrate concentrations occurred, in contrast with a fall in the concentration of α-oxoglutarate. The concentrations of ketone bodies, as expected, rose on starvation and on feeding with the low-carbohydrate diet.

$[NADP^+]/[NADPH]$ and $[NAD^+]/[NADH]$ ratios in the cytoplasm of rat liver. The results of the calculations of the free dinucleotide ratios based on the data of Table 3 are given in Table 4. The methods of calculation are shown in Table 5. The

Table 3. *Concentrations of metabolites in freeze-clamped rat liver in different nutritional states*

The concentrations are expressed as μmoles/g. fresh wt. and are given as means ± S.E.M. of the numbers of observations indicated. The *P* values refer to the significance of the difference between the normal well-fed state and the other conditions tested.

State of animal ...	Well-fed, standard diet	Starved for 48 hr.	Fed with high-sucrose diet for 3 days	Fed with high-sucrose diet for 5 days	Fed with low-carbohydrate diet for 3 days	Fed with low-carbohydrate diet for 5 days
No. of observations ...	(12)	(8)	(8)	(8)	(12)	(8)
[L-Malate]	0·307 ± 0·023	0·375 ± 0·047 *P* > 0·10	0·366 ± 0·047 *P* > 0·10	0·298 ± 0·049 *P* > 0·10	0·411 ± 0·028 *P* > 0·10	0·400 ± 0·030 *P* > 0·10
[L-Lactate]	0·828 ± 0·104	0·330 ± 0·032 *P* < 0·01	0·788 ± 0·059 *P* > 0·10	0·566 ± 0·072 *P* > 0·10	0·281 ± 0·027 *P* < 0·001	0·447 ± 0·068 *P* < 0·05
[Pyruvate]	0·107 ± 0·015	0·021 ± 0·002 *P* < 0·001	0·159 ± 0·017 *P* > 0·10	0·080 ± 0·017 *P* > 0·10	0·016 ± 0·004 *P* < 0·001	0·022 ± 1·008 *P* < 0·001
[α-Oxoglutarate]	0·225 ± 0·009	0·058 ± 0·002 *P* < 0·001	0·270 ± 0·013 *P* > 0·10	0·183 ± 0·027 *P* > 0·10	0·085 ± 0·005 *P* < 0·01	0·092 ± 0·005 *P* < 0·01
[Acetoacetate]	0·055 ± 0·006	0·349 ± 0·022 *P* < 0·001	0·030 ± 0·0019 *P* > 0·10	0·089 ± 0·030 *P* > 0·10	0·203 ± 0·036 *P* < 0·001	0·102 ± 1·011 *P* < 0·001
[β-Hydroxybutyrate]	0·144 ± 0·014	1·219 ± 0·094 *P* < 0·001	0·091 ± 0·0066 *P* > 0·10	0·350 ± 0·100 *P* > 0·10	1·006 ± 0·159 *P* < 0·001	0·513 ± 0·023 *P* < 0·001
[Isocitrate]	0·020 ± 0·031	0·013 ± 0·001 *P* > 0·10	0·026 ± 0·002 *P* > 0·10	0·021 ± 0·002 *P* > 0·10	0·032 ± 0·004 *P* < 0·01	0·043 ± 0·010 *P* < 0·02
[Citrate]	0·294 ± 0·017	0·259 ± 0·020 *P* > 0·10	0·443 ± 0·023 *P* > 0·10	0·446 ± 0·038 *P* > 0·10	0·572 ± 0·063 *P* < 0·001	0·766 ± 0·120 *P* < 0·01

Table 4. $[NAD^+]/[NADH]$ *and* $[NADP^+]/[NADPH]$ *ratios in rat liver calculated from the values given in Table* 3

The values are given as means ± S.E.M. of the numbers of observations indicated. pH is taken to be 7·0 and $[CO_2]$ 1·16 μmoles/g. wet wt. of liver. For the method of calculation see Table 5. The *P* values refer to the difference between the normal well-fed state and the test diet.

		Cytoplasm			Mitochondria
	No. of observations	[NAD+]/[NADH] calculated from lactate dehydrogenase	[NADP+]/[NADPH] calculated from 'malic' enzyme	[NADP+]/[NADPH] calculated from isocitrate dehydrogenase	[NAD+]/[NADH] calculated from β-hydroxybutyrate dehydrogenase
Well-fed, standard diet	12	1164 ± 218	0·0118 ± 0·0019	0·0101 ± 0·0008	7·74 ± 1·10
Starved for 48 hr.	8	564 ± 81 *P* < 0·05	0·00186 ± 0·00031 *P* < 0·05	0·00442 ± 0·0003 *P* < 0·001	5·48 ± 0·53 *P* < 0·10
Fed with high-sucrose diet for 3 days	8	1820 ± 237 *P* > 0·10	0·0146 ± 0·0024 *P* > 0·1	0·0105 ± 0·0012 *P* > 0·1	6·71 ± 0·64 *P* > 0·10
Fed with high-sucrose diet for 5 days	8	1270 ± 319 *P* > 0·10	0·00907 ± 0·0025 *P* > 0·1	0·00870 ± 0·0014 *P* > 0·1	5·14 ± 1·13 *P* > 0·10
Fed with low-carbohydrate diet for 3 days	12	526 ± 126 *P* < 0·02	0·00134 ± 0·0003 *P* < 0·001	0·00264 ± 0·00035 *P* < 0·001	4·09 ± 0·97 *P* < 0·02
Fed with low-carbohydrate diet for 5 days	8	443 ± 69 *P* < 0·02	0·00187 ± 0·00015 *P* < 0·001	0·00212 ± 0·00049 *P* < 0·001	4·02 ± 0·46 *P* < 0·02

findings should be considered in conjunction with those reported by Williamson *et al.* (1967*a*), which showed that: (*a*) glutamate dehydrogenase and β-hydroxybutyrate dehydrogenase give virtually the same values for the [NAD+]/[NADH] ratios, which indicates that the two enzymes are in equilibrium with the same mitochondrial coenzyme pool, although glutamate dehydrogenase is

Table 5. *Formulae used for calculation of [NADP+]/[NADPH] and [NAD+]/[NADH] ratios at* pH 7·0

The CO_2 concentration was taken to be 1·16 mM.

$$\frac{[NAD^+]}{[NADH]}\text{(cytoplasm)} = \frac{[\text{pyruvate}]}{[\text{lactate}]} \cdot \frac{1}{1{\cdot}11 \times 10^{-4}}$$

$$\frac{[NADP^+]}{[NADPH]}\text{(cytoplasm)} = \frac{[\text{pyruvate}]}{[\text{malate}]} \cdot \frac{1{\cdot}16 \times 10^{-3}}{3{\cdot}44 \times 10^{-2}}$$

$$\frac{[NADP^+]}{[NADPH]}\text{(cytoplasm)} = \frac{[\alpha\text{-oxoglutarate}]}{[\text{isocitrate}]} \cdot \frac{1{\cdot}16 \times 10^{-3}}{1{\cdot}17}$$

$$\frac{[NAD^+]}{[NADH]}\text{(mitochondria)} = \frac{[\text{acetoacetate}]}{[\beta\text{-hydroxybutyrate}]} \cdot \frac{1}{4{\cdot}93 \times 10^{-2}}$$

located in the matrix and β-hydroxybutyrate dehydrogenase in the cristae; (*b*) the cytoplasmic and mitochondrial ratios for the NAD couple differ by about 100-fold, the mitochondrial ratio being the more reduced one.

The results on the [NADP+]/[NADPH] ratio (Table 4) show good agreement between the values obtained in the cytoplasm from the 'malic' enzyme system and the isocitrate dehydrogenase system. The values are all of the same order of magnitude and this order is about 10^5 times lower than that of the cytoplasmic [NAD+]/[NADH] ratio, as has been anticipated by Bücher & Klingenberg (1958) and by Bücher & Rüssmann (1964). The agreement between the values given by the 'malic' enzyme and the isocitrate dehydrogenase is very close in liver from well-fed rats and from rats on the high-sucrose diet. It is less close in liver from starved rats and from rats on the low-carbohydrate diet, though the ratios calculated from the 'malic' enzyme system were at most 2·4-fold lower than those obtained from the isocitrate dehydrogenase system. In several other series of experiments the value calculated from the 'malic' enzyme system was only 1·3-fold lower. Since comparisons are made on the basis of four measurements, the differences are not significant ($P > 0{\cdot}1$).

DISCUSSION

Validity of assumptions. The approach used in this work involves a series of assumptions about the compartmentation of dehydrogenases and metabolites that at best are reasonable approximations. The consistency of the results obtained, especially the agreement between the values of the redox states calculated from the 'malic' enzyme and isocitrate dehydrogenase systems, suggests that the approximations are in fact closer to reality (in the present context) than could be anticipated. We therefore think it justifiable to assume that the concentrations of metabolites measured in the whole liver are close to the cytoplasmic concentration, that the concentrations of the reactants of the NADP-linked isocitrate dehydrogenase in the liver reflect those in equilibrium with the cytoplasmic enzyme, and that the activity of the mitochondrial NADP-linked isocitrate dehydrogenase can be neglected in our calculations. In view of the relative sizes of the cytoplasmic and mitochondrial compartments the approximate validity of the assumptions is in fact understandable. Since the nucleus and the external mitochondrial membrane are readily permeable to small molecules, the relevant compartmentation is that between the mitochondrial matrix and the rest of the cell. According to Werkheiser & Bartley (1957), Klingenberg & Pfaff (1966) and Tipton (1967) about half the mitochondrial space is readily permeable to sucrose, and the metabolite concentrations in this 'external' space are likely to be the same as those of the cytoplasm. As the mitochondria occupy about 20% of the liver volume the matrix space constitutes only about 10%. Hence considerable variations of mitochondrial metabolite concentrations are possible without affecting the total tissue concentrations in a major way. If the metabolite concentrations were twice those of the cytoplasm or zero, the difference between the whole-liver concentrations and the cytoplasmic concentrations would be no more than 10%.

Analogous considerations hold for the NADP-linked isocitrate dehydrogenase that is known to be present also in the mitochondria. What is not known is whether the mitochondrial enzyme is sufficiently active to establish equilibrium; the flux rate through isocitrate in the mitochondria, because of the tricarboxylic acid cycle, is probably very much higher than in the cytoplasm. In any case the position is similar to that of malate dehydrogenase, which is present in both cytoplasm and mitochondria, but many measurements have shown that the concentrations of malate and oxaloacetate in the whole liver are those expected for the equilibrium in the cytoplasm: the [free NAD+]/[free NADH] ratio calculated from the malate dehydrogenase systems is the same as those calculated from the lactate dehydrogenase and L-glycerol 3-phosphate–NAD oxidoreductase

systems, both exclusively located in the cytoplasm (Bücher & Klingenberg, 1958; Hohorst, Kreutz & Bücher, 1959; Wieland & Löffler, 1963; Williamson, Kreisberg & Felts, 1966).

Although there are observations that apparently contradict the assumptions made in this work, many of these are derived from work on isolated mitochondria. Thus mitochondria suspended in an unphysiological environment can create major concentration gradients of di- and tri-carboxylic acids, but whether this occurs *in situ* in the normal liver is not known.

Origin of the differences of the redox states of the NAD and NADP couples in the cytoplasm. A striking result of the measurements is the observation of the large (100000-fold) difference between the values for the [NAD^+]/[NADH] and [$NADP^+$]/[NADPH] ratios in the cytoplasm. Against this 100000-fold difference the variations in the values of the [$NADP^+$]/[NADPH] ratio obtained under some conditions with 'malic' enzyme and isocitrate dehydrogenase are negligible. These findings raise the question of how the differences arise and how they are maintained. The following considerations throw light on this problem.

When a NAD-linked and a NADP-linked dehydrogenase system, together with coenzymes and substrates (e.g. lactate dehydrogenase and glucose 6-phosphate dehydrogenase) are present in a solution, the redox states of the two couples are expected to be independent of one another unless they share a common reactant. The 'malic' enzyme and lactate dehydrogenase share pyruvate as a common reactant, and when equilibrium exists in such a mixture the differences in the redox states of the NAD and NADP couples are no longer independent, but fixed by the equilibrium constants of the two systems and the concentrations of the substrates. The following equations hold at equilibrium.

$$[\text{pyruvate}] = K_{\text{LDH}} \times \frac{[\text{lactate}][\text{NAD}^+]}{[\text{NADH}][\text{H}^+]}$$

$$[\text{pyruvate}] = K_{\text{ME}} \times \frac{[\text{malate}][\text{NADP}^+]}{[\text{NADPH}][\text{CO}_2]}$$

Combination gives:

$$\frac{[\text{lactate}][\text{NAD}^+][\text{NADPH}][\text{CO}_2]}{[\text{malate}][\text{NADH}][\text{NADP}^+][\text{H}^+]} = \frac{K_{\text{ME}}}{K_{\text{LDH}}}$$

or

$$\frac{[\text{NAD}^+]}{[\text{NADH}]} = \frac{[\text{NADP}^+]}{[\text{NADPH}]} \cdot \frac{K_{\text{ME}}}{K_{\text{LDH}}} \cdot \frac{[\text{malate}][\text{H}^+]}{[\text{lactate}][\text{CO}_2]}$$

Substitution in this equation of the values of the constants (derived from Table 2) and the substrate concentrations for liver from well-fed rats (Table 3) gives:

$$\frac{[\text{NAD}^+]/[\text{NADH}]}{[\text{NADP}^+]/[\text{NADPH}]} = \frac{K_{\text{ME}}}{K_{\text{LDH}}} \cdot \frac{[\text{malate}][\text{H}^+]}{[\text{lactate}][\text{CO}_2]}$$

$$= \frac{3{\cdot}44 \times 10^{-2}\,\text{M}}{1{\cdot}11 \times 10^{-11}\,\text{M}} \cdot \frac{[3{\cdot}07 \times 10^{-4}\,\text{M}][10^{-7}\,\text{M}]}{[8{\cdot}28 \times 10^{-4}\,\text{M}][1{\cdot}16 \times 10^{-3}\,\text{M}]}$$

$$= 3{\cdot}1 \times 10^9 \times 3{\cdot}2 \times 10^{-5} = 9{\cdot}91 \times 10^4 \qquad (2)$$

(Because in some cases [H^+] is a reactant, whereas in others it is not, [H^+] has been included, for the purpose of the present considerations, in the definition of the equilibrium constants. It should be noted that the constants given in Table 2 are those at pH 7 and the values used here are the constants at pH 0. As shown below this clarifies some characteristics of the equilibrium systems).

It follows from Table 4 that in the normal liver:

$$\frac{[\text{NAD}^+]/[\text{NADH}]}{[\text{NADP}^+]/[\text{NADPH}]} = \frac{1164}{0{\cdot}01175} = 99100 \qquad (3)$$

(with the 'malic' enzyme system) or

$$\frac{[\text{NAD}^+]/[\text{NADH}]}{[\text{NADP}^+]/[\text{NADPH}]} = \frac{1164}{0{\cdot}0101} = 115000 \qquad (4)$$

(with the isocitrate dehydrogenase system).

The agreement between the results obtained by eqn. (2) and by eqn. (3) when the 'malic' enzyme values are used is not to be regarded as proof confirming experimental findings by theoretical predictions because the two calculations are based on the same constants and the same concentration measurements. The derivation of eqn. (2) is intended to show that the large differences in the redox state of the NAD and NADP couples present nothing miraculous in the sense that no special energy-driven forces acting directly on the couples, or spatial separation, is required to bring them about. They arise partly from the differences in the equilibrium constants of the two reactions sharing pyruvate, and partly from the concentrations of metabolites. Thus in eqn. (2) the factor containing the equilibrium constant has a value of $3{\cdot}1 \times 10^9$, whereas the factor containing the metabolite concentrations has a value of $3{\cdot}2 \times 10^{-5}$.

The broad agreement between the outcome of the calculations according to eqns. (3) and (4) when in eqn. (4) the value for isocitrate dehydrogenase is substituted (115000 and 99100) rests on independent procedures and therefore supports the conclusions about the differences in the redox states of the two cytoplasmic nicotinamide nucleotide couples. Further confirmatory proof is provided by the following independent considerations, involving again different enzyme systems.

'Malic' enzyme and lactate dehydrogenase are not the only systems in the cytoplasm that establish

and maintain the differences in the redox states of the NAD and NADP couples. When NAD-linked and NADP-linked dehydrogenases do not share a reactant they can be linked by a third reaction. Such a three-reaction system is provided in the cytoplasm by the NADP-linked isocitrate dehydrogenase, malate dehydrogenase and glutamate–oxaloacetate transaminase. The equilibrium equations of the three reactions are:

$$\frac{[\alpha\text{-oxoglutarate}][CO_2][NADPH]}{[\text{isocitrate}][NADP^+]} = K_{IDH(NADP)} \quad (5)$$

$$\frac{[\alpha\text{-oxoglutarate}][\text{aspartate}]}{[\text{glutamate}][\text{oxaloacetate}]} = K_{GOT} \quad (6)$$

$$\frac{[\text{oxaloacetate}][NADH]}{[\text{malate}][NAD^+]} = K_{MDH} \quad (7)$$

Division of eqn. (5) by eqns. (6) and (7) eliminates common intermediates:

$$\frac{[\text{glutamate}][\text{malate}][CO_2][NADPH][NAD^+]}{[\text{isocitrate}][\text{aspartate}][NADP^+][NADH]} = \frac{K_{IDH(NADP)}}{K_{GOT}.K_{MDH}}$$

or

$$\frac{[NAD^+]}{[NADH]} = \frac{[NADP^+]}{[NADPH]}.\frac{[\text{isocitrate}][\text{aspartate}]}{[\text{glutamate}][\text{malate}][CO_2]} \times \frac{K_{IDH(NADP)}}{K_{GOT}.K_{MDH}} \quad (8)$$

Substitution in eqn. (8) by the constants recorded in Table 2, by the substrate concentrations recorded for liver from well-fed rats in Table 4, by 2·4mM for [glutamate] and by 0·74mM for [aspartate] (from Williamson, Lopes-Vieira & Walker, 1967*b*), gives:

$$\frac{[NAD^+]}{[NADH]} = \frac{[NADP^+]}{[NADPH]}.\frac{0{\cdot}02\text{mM} \times 0{\cdot}74\text{mM}}{2{\cdot}4\text{mM} \times 0{\cdot}31\text{mM} \times 1{\cdot}16\text{mM}} \times \frac{1{\cdot}17 \times 10^3\text{mM}}{6{\cdot}61 \times 2{\cdot}78 \times 10^{-5}} \quad (9)$$

or

$$\frac{[NAD^+]/[NADH]}{[NADP^+]/[NADPH]} = 109000$$

Analogously, glutamate–pyruvate transaminase provides a link between lactate dehydrogenase and isocitrate dehydrogenase (NADP) in rat liver cytoplasm. Rearranging the equilibrium equations for the three enzymes gives:

$$[\text{pyruvate}] = \frac{[NAD^+]}{[NADH]}.[\text{lactate}].K_{LDH} \quad (10)$$

$$[\text{pyruvate}] = \frac{[\text{alanine}][\text{oxoglutarate}]}{[\text{glutamate}]}.\frac{1}{K_{GPT}} \quad (11)$$

$$[\text{oxoglutarate}] = \frac{[\text{isocitrate}][NADP^+]}{[CO_2][NADPH]} \times K_{IDH(NADP)} \quad (12)$$

Elimination from eqn. (11) of [pyruvate] by eqn. (10) and of [oxoglutarate] by eqn. (12) gives:

$$\frac{[NAD^+]}{[NADH]} = \frac{[NADP^+]}{[NADPH]}.\frac{[\text{isocitrate}][\text{alanine}]}{[\text{glutamate}][CO_2][\text{lactate}]} \times \frac{K_{IDH(NADP)}}{K_{LDH}.K_{GPT}}$$

Substitution of values used above, and for glutamate and alanine values obtained from the same liver samples, gives:

$$\frac{[NAD^+]}{[NADH]} = \frac{[NADP^+]}{[NADPH]}.\frac{0{\cdot}02\text{mM} \times 1{\cdot}07\text{mM}}{2{\cdot}05\text{mM} \times 1{\cdot}16\text{mM} \times 0{\cdot}84\text{mM}} \times \frac{1{\cdot}17 \times 10^3\text{mM}}{1{\cdot}11 \times 10^{-4} \times 1{\cdot}51}$$

or

$$\frac{[NAD^+]/[NADH]}{[NADP^+]/[NADPH]} = 75000$$

The facts just discussed can also be expressed by saying that the reduction of $NADP^+$ in the cytoplasm is achieved by coupled reactions in which the coupling is not brought about by shared coenzymes (as is the case in many coupled oxidoreductions) but by shared substrates. The three coupled systems discussed above may be written as follows:

$$\begin{aligned} \text{malate} + NADP^+ &\rightleftharpoons \text{pyruvate} + CO_2 + NADPH \\ \text{pyruvate} + NADH + H^+ &\rightleftharpoons \text{lactate} + NAD^+ \end{aligned}$$

$$\text{Sum:} \begin{cases} \text{malate} + H^+ \rightleftharpoons \text{lactate} + CO_2 \\ NADP^+ + NADH \rightleftharpoons NADPH + NAD^+ \end{cases}$$

and

$$\begin{aligned} \text{isocitrate} + NADP^+ &\rightleftharpoons \alpha\text{-oxoglutarate} + NADPH + CO_2 \\ \alpha\text{-oxoglutarate} + \text{aspartate} &\rightleftharpoons \text{oxaloacetate} + \text{glutamate} \\ \text{oxaloacetate} + NADH + H^+ &\rightleftharpoons \text{malate} + NAD^+ \end{aligned}$$

$$\text{Sum:} \begin{cases} \text{isocitrate} + \text{aspartate} + H^+ \rightleftharpoons \text{glutamate} + \text{malate} + CO_2 \\ NADP^+ + NADH \rightleftharpoons NADPH + NAD^+ \end{cases}$$

and

$$\begin{aligned} \text{pyruvate} + NADH + H^+ &\rightleftharpoons \text{lactate} + NAD^+ \\ \text{alanine} + \text{oxoglutarate} &\rightleftharpoons \text{pyruvate} + \text{glutamate} \\ \text{isocitrate} + NADP^+ &\rightleftharpoons \text{oxoglutarate} + CO_2 + NADPH \end{aligned}$$

$$\text{Sum:} \begin{cases} \text{isocitrate} + \text{alanine} + H^+ \rightleftharpoons \text{glutamate} + \text{lactate} + CO_2 \\ NADP^+ + NADH \rightleftharpoons NADPH + NAD^+ \end{cases}$$

In all three cases the equilibrium of the coupled system is far on the right so that the reduction of $NADP^+$ by NADH is extensive. The reaction ceases when $[NAD^+]/[NADH]$ is about $10^5[NADP^+]/[NADPH]$ (depending on substrate concentrations).

Considering the large number of measurements involved the agreement between the results obtained by the different procedures may be regarded as satisfactory. There is thus a multiplicity of interlinked reactions that maintain the redox states of the main nicotinamide nucleotide-linked dehydrogenase systems in the cytoplasm at near equilibrium. Some of the enzymes possess a very high activity; others, like the 'malic' enzyme, are relatively weak. The fact that, in spite of this relative weakness, a near-equilibrium exists is explained by the joint effects of the interlinked enzymes.

To recapitulate, the main participants of the interlinking reactions are the following six reversible systems:

$$\text{lactate} + NAD^+ \rightleftharpoons \text{pyruvate} + NADH + H^+$$

$$\text{malate} + NAD^+ \rightleftharpoons \text{oxaloacetate} + NADH + H^+$$

$$\text{malate} + NADP^+ \rightleftharpoons \text{pyruvate} + CO_2 + NADPH$$

$$\text{isocitrate} + NADP^+ \rightleftharpoons \alpha\text{-oxoglutarate} + CO_2 + NADPH$$

$$\alpha\text{-oxoglutarate} + \text{aspartate} \rightleftharpoons \text{glutamate} + \text{oxaloacetate}$$

$$\alpha\text{-oxoglutarate} + \text{alanine} \rightleftharpoons \text{glutamate} + \text{pyruvate}$$

Oxoglutarate and pyruvate are shared by three of the six reactions and malate, oxaloacetate, glutamate, CO_2, H^+, NAD^+, NADH, $NADP^+$ and NADPH by two each.

Evidently this network of equilibria is made possible only on account of the characteristic coenzyme function of the nicotinamide nucleotides. Without the sharing of reactants interlinking cannot be achieved, and without the detachment of the coenzymes from the enzymes a sharing of the nicotinamide nucleotides between different reactions is not feasible. The emergence of the coenzyme function may therefore be looked upon as an important feature of biochemical evolution.

Redox state of the NADP couple in rat liver mitochondria. The data in Table 4 confirm the previous finding that the value of the $[NAD^+]/[NADH]$ ratio of the mitochondrial NAD couple, as calculated from the concentrations of β-hydroxybutyrate and acetoacetate, is about 100-fold lower than the cytoplasmic ratio. As rat liver contains a highly active glutamate dehydrogenase that can react with both NAD and NADP it is very probable that the $[NADP^+]/[NADPH]$ ratio equals the $[NAD^+]/[NADH]$ ratio times the equilibrium constant of the reaction:

$$NADH + NADP^+ \rightleftharpoons NAD^+ + NADPH$$

which, at 38° and I 0·25, is 1·56. For a detailed discussion of the subject see Krebs & Veech (1969).

Redox states of the nicotinamide nucleotides in tissues other than liver. The principles elaborated for rat liver would be generally applicable to other tissues, if the dehydrogenases are sufficiently active and if the enzyme systems that provide links between the two nicotinamide nucleotide couples are present. This is certain for some of the links but not for all. Thus malate dehydrogenase, glutamate–oxaloacetate transaminase and isocitrate dehydrogenase (NADP) occur in high activity in most metabolically active animal tissues. Glutamate–pyruvate transaminase, which provides a link between lactate dehydrogenase and the isocitrate dehydrogenase, and the 'malic' enzyme, which links the lactate dehydrogenase system to an NADP-linked system, have little or no activity in many tissues. However, the latter two enzymes are secondary links and it follows that the essential cytoplasmic linking enzymes are generally present.

The data published by Kraupp, Adler-Kastner, Niessner & Plank (1967) on rat heart can be used

Table 6. *Redox states of the NAD and NADP couples in the heart of well-fed and starved rats*

The values of the metabolite concentrations used in the calculations are taken from Tables 3–5 of Kraupp *et al.* (1967), except for the value of isocitrate. This was calculated from the measured concentration of citrate on the assumption that there was equilibrium in the aconitase system (see Garland & Randle, 1964).

	Cytoplasm			Mitochondria
State of rat	$[NAD^+]/[NADH]$ calculated from lactate dehydrogenase	$[NADP^+]/[NADPH]$ calculated from 'malic' enzyme	$[NADP^+]/[NADPH]$ calculated from isocitrate dehydrogenase	$[NAD^+]/[NADH]$ calculated from β-hydroxybutyrate dehydrogenase
Fed	640	0·0082	0·0036	3·8
Starved for 48hr.	535	0·0040	0·0025	4·3

for the type of calculations employed in this paper. The results (Table 6) show that the ratios of the free nicotinamide nucleotide couples in rat heart are very similar to those of rat liver. The values of the [NAD^+]/[NADH] ratios are about 100-fold lower in the mitochondria than in the cytoplasm and the values for the [$NADP^+$]/[NADPH] ratios of the cytoplasm about 10^5-fold lower than those of the NAD couple.

Other published data to which the treatment described in this paper can be applied are those of Fig. 1 of Goldberg, Passonneau & Lowry (1966) and Kauffman *et al.* (1968) for three NADP-linked dehydrogenases in mouse brain. The values of the [$NADP^+$]/[NADPH] ratio calculated from isocitrate dehydrogenase, 'malic' enzyme and phosphogluconate dehydrogenase are 0·007, 0·008 and 0·005 respectively. They are thus consistent within the expected limits of the procedure; they are of the same order as those in liver.

Physiological significance of the variations in the redox states of the NAD and NADP couples. In general the changes of the redox states caused by starvation, or by feeding with a high-sucrose diet (which induces lipogenesis) or a low-carbohydrate diet (which induces gluconeogenesis), are not great (Table 4). The redox states of the two couples are evidently maintained by the cell within relatively narrow limits. The fall of [NADPH] occurring on the lipogenic diet, and the rise of [NADPH] occurring in starvation (when lipogenesis ceases), may be visualized as being connected with the variations of the rate of NADPH utilization. It is generally accepted that the main function of NADPH is the provision of reducing equivalents for biosyntheses and that the synthesis of fatty acids from carbohydrate is quantitatively the most important single NADPH-consuming process. It is therefore not unexpected that there is a fall in the steady-state concentration of NADPH when the rate of fat synthesis rises, and an increase when fat synthesis stops.

The concomitant changes in the same direction of the NAD couple can be explained by the links between the two couples. Parallel changes in the redox states of the two couples are expected, but it must be emphasized that the parallelism is not necessarily strict (see Krebs & Veech, 1969).

Glutathione reductase. It was mentioned in the introduction that the glutathione reductase system, though of relatively high activity, is not suitable for the assay of the [$NADP^+$]/[NADPH] ratio, because of the difficulty of measuring the concentration of GSSG. The concentration of GSSG at equilibrium can now be calculated since the values for K:

$$K = \frac{[GSSG][NADPH][H^+]}{[GSH]^2[NADP^+]} \qquad (13)$$

and the [$NADP^+$]/[NADPH] ratio are known. Scott, Duncan & Ekstrand (1963) found $K = 1{\cdot}06 \times 10^{-9}$ at 40°. New measurements (six observations) at 38° and I 0·25 gave a value of $1{\cdot}98\ (\pm 0{\cdot}033) \times 10^{-9}$ (Veech, 1968). Substituting this value, 0·01 for the cytoplasmic [$NADP^+$]/[NADPH] ratio and a normal value of 6mM for [GSH] in eqn. (13), the expected equilibrium concentration at pH 7·0 of GSSG would be 7·1nM. A factor contributing to this low value is that GSH appears in eqn. (13) as the square.

Although it is uncertain whether the glutathione system is at equilibrium (because the rate of reaction of GSSG may be too slow at 1nM) it can be stated with confidence that the physiological concentration of GSSG is extremely low. Experimentally, concentrations as high as 0·5mM are found, but recovery experiments indicate that this is due to peroxidative conversion of GSH into GSSG during deproteinization (Veech, 1968).

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REFERENCES

Bergmeyer, H. U. & Bernt, E. (1963*a*). In *Methods of Enzymatic Analysis*, p. 837. Ed. by Bergmeyer, H. U. New York and London: Academic Press.

Bergmeyer, H. U. & Bernt, E. (1963*b*). In *Methods of Enzymatic Analysis*, p. 846. Ed. by Bergmeyer, H. U. New York and London: Academic Press.

Brosnan, J. T. (1968). D. Phil. Thesis: University of Oxford.

Bücher, Th. & Klingenberg, M. (1958). *Angew. Chem.* **70**, 552.

Bücher, Th. & Rüssmann, W. (1964). *Angew. Chem.* **75**, 881; *Angew. Chem. (int. Ed.)*, **3**, 426.

Engel, P. C. & Dalziel, K. (1967). *Biochem. J.* **105**, 691.

Fitch, W. M. & Chaikoff, I. L. (1959). *J. biol. Chem.* **235**, 554.

Garland, P. B. & Randle, P. J. (1964). *Biochem. J.* **93**, 678.

Goldberg, N. D., Passonneau, J. V. & Lowry, O. H. (1966). *J. biol. Chem.* **241**, 3997.

Gruber, W. & Moellering, H. (1966). *Biochem. Z.* **346**, 85.

Hastings, A. B. & Sendroy, J. (1925). *J. biol. Chem.* **65**, 445.

Hohorst, H. J., Kreutz, F. H. & Bücher, Th. (1959). *Biochem. Z.* **332**, 18.

Holzer, H., Schultz, G. & Lynen, F. (1956). *Biochem. Z.* **328**, 252.

Kauffman, F. C., Brown, J. G., Passonneau, J. V. & Lowry, O. H. (1968). *Fed. Proc.* **27**, 463.

Klingenberg, M. (1963). In *Methods of Enzymatic Analysis*, p. 528. Ed. by Bergmeyer, H. U. New York and London: Academic Press.

Klingenberg, M. & Pfaff, E. (1966). In *Regulation of Metabolic Processes in Mitochondria*, vol. 7, p. 181. Ed. by Tager, J. M., Papa, S., Quagliariello, E. & Slater, E. C. Amsterdam: Elsevier Publishing Co.

Kraupp, O., Adler-Kastner, L., Niessner, H. & Plank, B. (1967). *Europ. J. Biochem.* **2**, 197.

Krebs, H. A. (1930). *Biochem. Z.* **220**, 250.

Krebs, H. A. (1953). *Biochem. J.* **54**, 82.

Krebs, H. A. & Veech, R. L. (1969). In *The Energy Level and Metabolic Control in Mitochondria*, p. 329. Ed. by Papa, S., Tager, J. M., Quagliariello, E. & Slater, E. C. Bari: Adriatica Editrice.

Lemberg, R. (1942). *Aust. J. exp. Biol. med. Sci.* **20**, 111.

Lemberg, R. & Legge, J. W. (1949). *Hematin Compounds and Bile Pigments*, p. 401. New York: Interscience Publishers Inc.

Londesborough, J. C. & Dalziel, K. (1968). *Biochem. J.* **110**, 217.

Plaut, G. W. E. (1962). In *Methods in Enzymology*, vol. 5, p. 645. Ed. by Colowick, S. P. & Kaplan, N. O. New York and London: Academic Press.

Rall, T. W. & Lehninger, A. (1952). *J. biol. Chem.* **194**, 119.

Scott, E. M., Duncan, I. W. & Ekstrand, V. (1963). *J. biol. Chem.* **238**, 3928.

Shonk, C. E. & Boxer, G. E. (1964). *Cancer Res.* **24**, 709.

Siebert, G. (1963). In *Methods of Enzymatic Analysis*, p. 318. Ed. by Bergmeyer H. U. New York and London: Academic Press.

Tipton, K. F. (1967). *Biochim. biophys. Acta*, **135**, 910.

Van Slyke, D. D., Hastings, A. B., Hiller, A. & Sendroy, J. (1928). *J. biol. Chem.* **79**, 769.

Veech, R. L. (1968). D. Phil. Thesis: University of Oxford.

Vickery, H. B. & Wilson, D. G. (1960). In *Biochemical Preparations*, vol. 7, p. 72. Ed. by Lardy, H. A. New York: John Wiley and Sons Inc.

Villet, R. & Dalziel, K. (1969). *Biochem. J.* **115**, 633.

Werkheiser, W. C. & Bartley, W. (1957). *Biochem. J.* **66**, 79.

Wieland, O. & Löffler, G. (1963). *Biochem. Z.* **339**, 204.

Williamson, D. H., Lopes-Vieira, O. & Walker, B. (1967*b*). *Biochem. J.* **104**, 497.

Williamson, D. H., Lund, P. & Krebs, H. A. (1967*a*). *Biochem. J.* **103**, 514.

Williamson, J. R., Kreisberg, R. A. & Felts, P. W. (1966). *Proc. nat. Acad. Sci., Wash.*, **56**, 247.