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

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1. The concentrations of the oxidized and reduced substrates of the lactate-, β-hydroxybutyrate- and glutamate-dehydrogenase systems were measured in rat livers freeze-clamped as soon as possible after death. The substrates of these dehydrogenases are likely to be in equilibrium with free NAD^+ and NADH, and the ratio of the free dinucleotides can be calculated from the measured concentrations of the substrates and the equilibrium constants (Holzer, Schultz & Lynen, 1956; Bücher & Klingenberg, 1958). The lactate-dehydrogenase system reflects the $[NAD^+]/[NADH]$ ratio in the cytoplasm, the β-hydroxybutyrate dehydrogenase that in the mitochondrial cristae and the glutamate dehydrogenase that in the mitochondrial matrix. 2. The equilibrium constants of lactate dehydrogenase (EC 1.1.1.27), β-hydroxybutyrate dehydrogenase (EC 1.1.1.30) and malate dehydrogenase (EC 1.1.1.37) were redetermined for near-physiological conditions ($38°$; $I\,0.25$). 3. The mean $[NAD^+]/[NADH]$ ratio of rat-liver cytoplasm was calculated as 725 (pH 7.0) in well-fed rats, 528 in starved rats and 208 in alloxan-diabetic rats. 4. The $[NAD^+]/[NADH]$ ratio for the mitochondrial matrix and cristae gave virtually identical values in the same metabolic state. This indicates that β-hydroxybutyrate dehydrogenase and glutamate dehydrogenase share a common pool of dinucleotide. 5. The mean $[NAD^+]/[NADH]$ ratio within the liver mitochondria of well-fed rats was about 8. It fell to about 5 in starvation and rose to about 10 in alloxan-diabetes. 6. The $[NAD^+]/[NADH]$ ratios of cytoplasm and mitochondria are thus greatly different and do not necessarily move in parallel when the metabolic state of the liver changes. 7. The ratios found for the free dinucleotides differ greatly from those recorded for the total dinucleotides because much more NADH than NAD^+ is protein-bound. 8. The bearing of these findings on various problems, including the following, is discussed: the number of NAD^+–NADH pools in liver cells; the applicability of the method to tissues other than liver; the transhydrogenase activity of glutamate dehydrogenase; the physiological significance of the difference of the redox states of mitochondria and cytoplasm; aspects of the regulation of the redox state of cell compartments; the steady-state concentration of mitochondrial oxaloacetate; the relations between the redox state of cell compartments and ketosis.

The ratio of the concentrations of free NAD^+ and NADH (referred to below as the $[NAD^+]/[NADH]$ ratio) at the site of oxidoreductions is of importance because it bears on the metabolic behaviour of oxidizable and reducible substrates. Direct measurements of the tissue content of NAD^+ and NADH do not supply the required information: they fail to differentiate between the free and bound nucleotides and they give no information on the distribution of the nucleotides between the various cell compartments, which is known to be uneven (Borst, 1963). The latter difficulty cannot be dealt with by the usual methods of tissue fractionation because the redox state of the nucleotides is liable to undergo rapid changes during the process of fractionation. Both difficulties may be overcome (Holzer, Schultz & Lynen, 1956; Bücher & Klingenberg, 1958) by measuring the ratio of the concentrations of the oxidized and reduced metabolites of suitable NAD-linked dehydrogenase systems that are located in different cell compartments and, on account of their high activity, are in

equilibrium or near-equilibrium with the nucleotides, according to the equation:

$$\frac{[\text{Oxidized substrate}][\text{NADH}]}{[\text{Reduced substrate}][\text{NAD}^+]} = K$$

If K is known, the [NAD$^+$]/[NADH] ratio can be calculated from the concentrations of the substrates. Hohorst, Kreutz & Bücher (1959) showed that there are three NAD-linked dehydrogenases (lactate dehydrogenase, α-glycerophosphate dehydrogenase and malate dehydrogenase) that, under certain conditions, give the same value for the [NAD$^+$]/[NADH] ratio. This is convincing evidence that all three dehydrogenases do in fact establish equilibria between their substrates and the free nucleotides in the cell compartment where the dehydrogenases are located, i.e. the cytoplasm. This conclusion is further borne out by the fact that the same [lactate]/[pyruvate] ratios are found in fluids that readily exchange solutes with the cytoplasm, such as the blood (Huckabee, 1958) and the perfusion medium used in experiments on isolated rat liver (Schimassek, 1963). Bücher & Rüssmann (1963) have further pointed out that of the three dehydrogenase systems mentioned the lactate–pyruvate system most readily gives reliable values for the cytoplasmic [NAD$^+$]/[NADH] ratio under a variety of conditions. The activity of the α-glycerophosphate-dehydrogenase system is not always sufficiently high to establish equilibrium and the malate dehydrogenase is not exclusively located in the cytoplasm.

The use of the β-hydroxybutyrate–acetoacetate system for the assessment of the [NAD$^+$]/[NADH] ratio within the mitochondria was first discussed by Klingenberg & Häfen (1963) and by Borst (1963). The system is readily reversible and in rat liver its activity is high enough to maintain equilibrium. The dehydrogenase is insoluble and located in the mitochondrial cristae (Lehninger, Sudduth & Wise, 1960).

Most of the other mitochondrial NAD-linked dehydrogenase systems are unsuitable for the assay of the [NAD$^+$]/[NADH] ratio, some because their activity is too low to establish an equilibrium and others because there are analytical obstacles in the determination of the concentration of reactants, as with lipoamide dehydrogenase, a component of the systems oxidizing pyruvate and α-oxoglutarate. Another system satisfactory in rat liver is glutamate dehydrogenase (EC 1.4.1.3). The enzyme is exclusively located in the matrix of the mitochondria (Delbrück, Schimassek, Bartsch & Bücher, 1959) and its activity is high. The equilibrium, however, has the disadvantage of being dependent on the concentration of NH$_4^+$; hence three reactants must be determined. In spite of this disadvantage, glutamate dehydrogenase appeared to be the best

system of the mitochondrial matrix for the present work.

On the basis of these considerations the concentrations of the substrates of the lactate-, β-hydroxybutyrate- and glutamate-dehydrogenase systems were measured in the livers of rats as quickly as possible after death. The livers were deep-cooled within seconds with aluminium blocks by the method of Wollenberger, Ristau & Schoffa (1960) and the data obtained therefore reflect conditions in vivo. Although the analyses were carried out on the whole tissue, they may be taken to indicate the redox state of the NAD couple at the site of three enzymes, i.e. the cytoplasm, a locus in the mitochondrial cristae and the mitochondrial matrix respectively. This assumption is justified if the distribution of the metabolites within the cell is even. There is no evidence indicating that this is not the case for the substrates of lactate dehydrogenase and β-hydroxybutyrate dehydrogenase, but it is uncertain whether the components of the glutamate-dehydrogenase system are evenly distributed between mitochondria and the rest of the tissue.

The attempts to measure the redox state of cell compartments sprang from the concept (Wieland & Löffler, 1963; Löffler, Matschinsky & Wieland, 1965) that the redox state of the NAD$^+$–NADH system in rat liver may play a role in ketogenesis by controlling the steady-state concentration of oxaloacetate. If, for one reason or another, the [NAD$^+$]/[NADH] ratio fell, the [oxaloacetate]/[malate] ratio would also fall, and if the sum of malate plus oxaloacetate remained constant the concentration of oxaloacetate would decrease. Lack of oxaloacetate would direct acetyl-CoA from the tricarboxylic acid cycle to ketone-body formation. Experiments supporting this view have been reported by Hohorst, Kreutz, Reim & Hübener (1961) and by Wieland & Löffler (1963). However, the interpretation of the findings is not clear-cut. Unless the redox states of the various compartments move in parallel (which is not necessarily the case) the data allow no conclusions on the redox state of a particular compartment.

MATERIALS AND METHODS

Rats. Male rats of the Wistar strain weighing 120–160 g. were used. Alloxan-diabetes was induced by the intravenous injection of recrystallized alloxan monohydrate (70 mg./kg.) in 0·3 ml. of 0·9 % NaCl under ether anaesthesia. The alloxan-treated animals had free access to food and water. They were killed 48 hr. after injection and at this time the concentration of blood glucose was in excess of 30 mM and that of blood ketone bodies (acetoacetate plus hydroxybutyrate) was 8–20 mM.

Reagents. NAD$^+$ and NADH were obtained from Boehringer Corporation, London, W. 5. Florisil (60–100., mesh) was obtained from Koch–Light Laboratories Ltd.,

Colnbrook, Bucks. β-Hydroxybutyrate dehydrogenase was prepared by the method of Williamson, Mellanby & Krebs (1962) and purified further on DEAE-Sephadex. It had a specific activity of 7 units/mg. and contained about 1% of malate dehydrogenase (relative to the β-hydroxybutyrate-dehydrogenase activity), but no detectable lactate dehydrogenase. Lactate dehydrogenase and glutamate dehydrogenase were obtained from Boehringer Corporation, London.

Blood. Blood samples were obtained from the tail vein by the method described by Williamson & Wilson (1965).

Treatment of liver. The rats were killed by dislocation of the neck. The liver was rapidly removed and pressed between metal clamps previously cooled in liquid N_2 (Wollenberger *et al.* 1960). The average time between dislocation of the neck and deep-freezing the tissue was 10 sec. The frozen liver was pulverized in a mortar to a fine powder, with frequent additions of liquid N_2. The powder was transferred to a weighed plastic centrifuge tube containing 2 ml. of frozen 30% (w/v) $HClO_4$. After a rapid reweighing, the tissue (1–2 g.) was mixed with the $HClO_4$, care being taken that no thawing occurred. Ice-cold distilled water (5 ml.) was added and the mixture immediately homogenized in the centrifuge tube with a glass pestle, driven by a low-speed motor. This was continued for about 2 min. until thawing was complete. Protein was removed by centrifugation in the cold at 30000g for 10 min. The supernatant fluid was adjusted to pH 5–6 with 20% (w/v) KOH and, after standing for 30 min. in the cold, the precipitate of $KClO_4$ was centrifuged off. The yellow supernatant fluid was then shaken for 30 sec. with Florisil (0·1 g./ml.). This treatment removed flavines from the solution and decreased the slow non-enzymic oxidation of NADH observed with untreated samples, while the recovery of the metabolites determined was not affected. The Florisil was removed by centrifugation and the supernatant fluid was used for the analyses.

Determination of metabolites. Lactate and pyruvate were determined by the method of Hohorst *et al.* (1959), β-hydroxybutyrate and acetoacetate were determined by the method of Williamson *et al.* (1962) and α-oxoglutarate was determined by the method of Bergmeyer & Bernt (1963). Pyruvate, α-oxoglutarate and acetoacetate were determined in the same cuvette by successive addition of lactate dehydrogenase, glutamate dehydrogenase and β-hydroxy-butyrate dehydrogenase. After preliminary separation of the amino acid fraction on Amberlite IR-120 (H$^+$ form), glutamate was determined by the method of Bernt & Bergmeyer (1963).

Ammonia was determined with glutamate dehydrogenase (Kirsten, Gerez & Kirsten, 1963) after preliminary separation on an ion-exchange resin. The liver extract (2–3 ml.) was placed on a column (1 cm. × 5 cm.) of Amberlite IR-120 that had been previously washed with 10 ml. portions of 4 M-NaCl and deionized water. The resin was then washed with about 20 ml. of deionized water and the ammonia eluted with 10 ml. of 4 M-NaCl. A suitable portion of the eluate was taken for the enzymic determination. This preliminary separation of ammonia was necessary because of the appreciable blank oxidation of NADH with untreated liver extract. In experiments where ammonia was to be determined, deionized water was used for the preparation of the reagents and for rinsing the apparatus.

Measurement of the equilibrium constants of dehydrogenase

systems at different ionic strengths. The equilibrium constant of the lactate-dehydrogenase system:

$$K = \frac{[\text{Pyruvate}][\text{NADH}][\text{H}^+]}{[\text{Lactate}][\text{NAD}^+]}$$

was measured by adding crystalline lactate dehydrogenase to known amounts of L-lactate and of NAD$^+$ and determining spectrophotometrically the concentration of NADH at equilibrium. The amount of pyruvate was equivalent to that of NADH. This is expected theoretically and was on several occasions confirmed experimentally by measuring pyruvate by the method of Bücher, Czok, Lamprecht & Latzko (1963). The amounts of lactate and NAD$^+$ in the equilibrium mixture were obtained by difference. L-Lactic acid (crystalline) was neutralized with KOH and its concentration was determined by the method of Hohorst (1963). Fresh solutions were prepared every few days. The concentration of the NAD$^+$ stock solution, freshly prepared, was determined by the method of Klingenberg (1963). The ionic strength was varied by changing the final concentration of the potassium phosphate buffer, pH 7·12–7·22, between 12·5 and 100 mM. [H$^+$] was measured at the end of the reaction electrically at 20° or 25° as it was impracticable to measure it at 38°. The value for 38° was obtained by using the correction factors $-0·04$ (20°) and $-0·02$ (25°) (see Clark, 1960).

The stock solutions from which the reaction mixture was prepared were 0·25 M-potassium phosphate buffer, 0·01 M-NAD$^+$, 0·1 M-L-lactate and CO_2-free water. The final concentration of NAD$^+$ was about 0·3 mM. The final concentration of L-lactate was varied between 1·96 and 9·8 mM.

The spectrophotometric measurements were made at 340 mμ in a Unicam SP.500 spectrophotometer at 38°. Cuvettes of either 1 cm. or 4 cm. light-path were used. An initial reading was taken against a water blank after allowing the cuvette contents to reach 38°. The reaction was started by the addition of 5 μl. (25 μg.) of lactate dehydrogenase (Boehringer muscle type)/3 ml. of reaction mixture. Decreasing the amount of enzyme ten- or 100-fold had no effect on the values obtained. When a constant reading was reached, usually after about 10 min., the extinction was recorded and corrected for the initial reading and a reagent blank, i.e. the increase in extinction due to the addition of the enzyme to the reaction mixture with lactate omitted. The molar extinction coefficient of NADH was taken to be $6·22 \times 10^3$.

The ionic strength of each reaction mixture [including that of the $(NH_4)_2SO_4$ in the enzyme preparation] was calculated from the formula $I = 0·5\Sigma c_i z_i^2$, where c_i is the molarity of the ion and z_i its valency.

The equilibrium constant of the β-hydroxybutyrate-dehydrogenase system was measured by the same principle. Crystalline or highly purified D-β-hydroxybutyrate dehydrogenase was added to known amounts of DL-β-hydroxybutyrate and of NAD$^+$ and the concentration of NADH$_2$ at equilibrium was measured spectrophotometrically either at 366 mμ in an Eppendorf photometer, or at 340 mμ in a Unicam SP.500 spectrophotometer, in 1 cm. cuvettes. The formation of acetoacetate was equivalent to that of NADH, as confirmed by direct determination of acetoacetate by the method of Mellanby & Williamson (1963). The D-β-hydroxybutyrate concentration of the stock solution of DL-β-hydroxybutyrate was determined by

the method of Williamson & Mellanby (1963). The D-β-hydroxybutyrate dehydrogenase, free of NADH oxidase, was prepared from *Rhodopseudomonas spheroides* by the method of Bergmeyer, Gawehn, Klotzsch, Krebs & Williamson (1967). Phosphate buffers of approx. pH 6·8 and 7·2 were prepared by mixing 0·25M-K_2HPO_4 and 0·25M-KH_2PO_4 in the proportions 60:40 and 72:28 respectively. The ionic strength of the former buffer was adjusted to that of the latter by the addition of KCl. The final buffer concentrations varied between 12·5 and 100mM, and the concentrations of D-β-hydroxybutyrate between 0·074 and 0·78mM. The concentration of NAD^+ was kept at about 0·3mM. The reaction was started by the addition of 5μl. of β-hydroxybutyrate-dehydrogenase solution. A constant reading was usually obtained after 15min. Other details were as described for the lactate-dehydrogenase system. The molar extinction coefficient of NADH at 366mμ, which is temperature-dependent (Hohorst, 1956), was taken to be $3·14 \times 10^3$ at 38°.

RESULTS

Effect of starvation and of alloxan-diabetes on the components of redox systems. Table 1 shows the concentrations of the substrates of the three chosen dehydrogenase systems in the livers of well-fed, starved (48hr.) and alloxan-diabetic rats. Some of the differences between these three conditions were as expected. Thus the concentrations of total ketone bodies rose tenfold in starvation and over 50-fold in diabetes above the concentration in the liver of well-fed rats. The concentrations of lactate and pyruvate both fell in starvation, the latter more than the former. Another striking change in the liver of diabetic rats was a major fall in the concentrations of glutamate and α-oxoglutarate, and a rise in the concentration of ammonia.

There were distinct differences between the three metabolic states with respect to the [reduced substrate]/[oxidized substrate] ratios of the three dehydrogenase systems. The [lactate]/[pyruvate] ratio was raised slightly in starvation and considerably (3·5-fold) in diabetes, as reported by Hohorst *et al.* (1961) and by Wieland & Löffler (1963). Unexpectedly, the changes in the mitochondrial dehydrogenase systems did not always parallel those of the cytoplasmic system. In diabetes there was a decrease in the ratios of the two mitochondrial systems, in contrast with the increase in the ratio of the cytoplasmic system. In starvation, on the other hand, all three systems moved in the same direction to approximately the same extent.

Equilibrium constants of the lactate-, β-hydroxybutyrate- and glutamate-dehydrogenase systems. The values reported in the literature for the equilibrium constants of the β-hydroxybutyrate- and glutamate-dehydrogenase systems (Krebs, Mellanby & Williamson, 1962; Shuster & Doudoroff, 1963; Olson & Anfinsen, 1953) were measured at temperatures and ionic strengths different from those of

Table 1. *Concentrations of the substrates of NAD-linked dehydrogenase systems in livers of well-fed, starved and alloxan-diabetic rats*

The blood glucose concentration of the diabetic rats was above 30mM. The concentration of the metabolites are expressed as μmoles/g. fresh wt. (mean ± S.E.M.). For experimental details see the text.

State of animals	No. of observations	[Lactate]	[Pyruvate]	[Glutamate]	[α-Oxoglutarate]	[NH4+]	[β-Hydroxybutyrate]	[Acetoacetate]	[Lactate]/[pyruvate]	[Glutamate]/[oxoglutarate] × [NH4+]	[β-Hydroxybutyrate]/[acetoacetate]
Well-fed	7	1·82±0·33	0·130±0·043	2·41±0·29	0·145±0·013	0·47±0·15	0·144±0·048	0·055±0·02	12·5	35	2·6
Starved (48hr.)	23	0·78±0·40	0·047±0·024	2·64±0·65	0·086±0·032	0·56±0·25	1·79±0·83	0·50±0·24	17	55	3·6
Alloxan-diabetic	12	2·37±0·19	0·054±0·015	0·96±0·31	0·045±0·008	0·86±0·22	7·78±3·26	3·75±2·08	44	25	2·1

liver tissue, and for the lactate-dehydrogenase reaction there are inconsistencies between available values (Hohorst, 1960; Hakala, Glaid & Schwert, 1956). The values for the lactate- and β-hydroxy-butyrate-dehydrogenase systems were therefore redetermined for 38° and I 0·25. The corresponding value for glutamate dehydrogenase was measured by P. C. Engel & K. Dalziel (personal communication). A difficulty is the uncertainty of the ionic strength of animal tissues and their compartments. For the purpose of the present work it is assumed that the ionic strength of the liver is 0·25, as suggested by Hohorst (1960). To evaluate the importance of errors arising from this assumption, the constants were measured over a wide range of ionic strengths.

The equilibrium constants for the lactate- and β-hydroxybutyrate-dehydrogenase systems at 38° and different ionic strengths are given in Tables 2 and 3. The K values for the lactate dehydrogenase are twice those given by Hohorst (1960) but are in good agreement with the values expected from the results of Hakala et al. (1956). The effect of the ionic strength proved to be relatively small for both lactate dehydrogenase and β-hydroxybutyrate dehydrogenase, but is larger for glutamate dehydrogenase. (P. C. Engel & K. Dalziel, personal communication.)

For the purpose of the subsequent calculations the pH of the tissue was assumed to be 7·0. Any error in this assumption will affect the [NAD+]/[NADH] ratios derived from all three enzyme systems equally. The concentration of water was assumed to be unity, in accordance with the convention of the standard state for a solvent. The equilibrium constant of glutamate dehydrogenase is therefore defined here by:

$$K = \frac{[\alpha\text{-Oxoglutarate}][NADH][NH_4^+]}{[Glutamate][NAD^+]}$$

With these assumptions the values of K for the three systems at 38° and I 0·25 are shown in Table 4.

Calculation of [NAD+]/[NADH] *ratios.* The data of Tables 1 and 4 were used for the calculation of the [NAD+]/[NADH] ratios according to the equation:

$$\frac{[NAD^+]}{[NADH]} = \frac{[Oxidized\ substrate]}{[Reduced\ substrate]} \times \frac{1}{K}$$

In applying this equation to glutamate dehydrogenase, two assumptions are involved that could affect the value of the ratio from this enzyme. The first is that the water concentration in the tissue is the same as that in the solutions in which the equilibrium constant was measured. If the water concentration is in fact significantly lower in the tissue, the calculated [NAD+]/[NADH] ratio for this enzyme will be too large. The second assumption arises from the fact that three substrates are involved in the glutamate-dehydrogenase

Table 2. *Effect of ionic strength on the equilibrium constant of lactate dehydrogenase at 38°*

The ranges of the ionic strength given in the first column are due to the ranges of lactate concentrations tested. For the procedure see the text (Materials and Methods section). The values for K are means ($\pm$ s.e.m.), with numbers of observations in parentheses.

I	Concn. of L-lactate (mM)	$K = \dfrac{[\text{pyruvate}][NADH][H^+]}{[\text{lactate}][NAD^+]}$ (M)
0·251–0·265	1·63–9·8	$1\cdot110\ (\pm0\cdot017)\times10^{-11}$ (12)
0·135–0·140	1·63–6·5	$1\cdot023\ (\pm0\cdot017)\times10^{-11}$ (5)
0·074–0·079	1·63–6·5	$0\cdot967\ (\pm0\cdot013)\times10^{-11}$ (3)
0·043–0·048	1·63–6·5	$0\cdot936\ (\pm0\cdot013)\times10^{-11}$ (3)

Table 3. *Effect of ionic strength on the equilibrium constant of D-β-hydroxybutyrate dehydrogenase at 38°*

For the procedure see the text (Materials and Methods section). The values for K are means ($\pm$ s.e.m.), with numbers of observations in parentheses.

I	Range of pH	Concn. of D-β-hydroxybutyrate (mM)	$K = \dfrac{[\text{acetoacetate}][NADH][H^+]}{[\beta\text{-hydroxybutyrate}][NAD^+]}$ (M)
0·255	6·84–7·16	0·074–0·78	$4\cdot94\ (\pm0\cdot086)\times10^{-9}$ (25)
0·134	6·89–7·19	0·074–0·78	$4\cdot80\ (\pm0\cdot017)\times10^{-9}$ (16)
0·073	6·94–7·24	0·074–0·78	$4\cdot56\ (\pm0\cdot16)\ \times10^{-9}$ (11)
0·042	6·95–7·24	0·074–0·78	$3\cdot83\ (\pm0\cdot26)\ \times10^{-9}$ (11)

reaction. This means that the ratio [α-oxogluta-rate][NH$_4^+$]/[glutamate] is not dimensionless, in contrast with the corresponding terms for the lactate dehydrogenase and β-hydroxybutyrate dehydro-genase. Experimentally the ratio is derived from measurements of μmoles/g. wet wt. of tissue and these are therefore the units of the ratio. The assumption is made that this is equal to μmoles/ml., or mM. Neither of these assumptions is required in the calculations for lactate dehydrogenase and β-hydroxybutyrate dehydrogenase, since water is not a reactant in these systems and the concentration ratio derived from tissue analysis is dimensionless. However, the error introduced by these assumptions is not likely to be large.

The results of the calculations are shown in Table 5. The values obtained for the lactate-dehydrogenase system, indicating the [NAD$^+$]/[NADH] ratios in the cytoplasm in livers of well-fed, starved and alloxan-diabetic rats, essentially confirm those of previous investigators (Hohorst et al. 1961; Thielmann, Frunder, Richter & Börnig, 1960; Wieland & Löffler, 1963), except that the higher value for the equilibrium constant decreases the ratios to about half the value arrived at previously. They confirm that the cytoplasm of livers of the starved and especially of the alloxan-diabetic rats is in a more reduced state than that of the liver of well-fed normal rats. The most remarkable result, surprising at first sight, is the

finding that the glutamate- and β-hydroxybuty-rate-dehydrogenase systems give, within the limits of error, the same values. This implies that the redox states of the mitochondrial matrix and the cristae are identical. The ratios are very much lower than those for the cytoplasm; they differ by about 100-fold in the livers of well-fed liver and normal starved rats and by about 20-fold in the liver of diabetic rats. Another important result already referred to is the demonstration that the changes caused by alloxan-diabetes in the cytoplasm and the two other compartments are not parallel: the [NAD$^+$]/[NADH] ratio fell to less than one-third in the cytoplasm and increased by about one-third in the other compartments.

The fact that the calculations for glutamate- and β-hydroxybutyrate-dehydrogenase systems lead to the same values for the [NAD$^+$]/[NADH] ratio implies that the substrates of these two dehydro-genases are in equilibrium with the same NAD$^+$–NADH pool. The validity of this conclusion can be checked by other calculations and experiments that do not rely on separate experimental values for the equilibrium constants. The combination of the equations for the equilibrium constants of the glutamate- and β-hydroxybutyrate-dehydrogenase systems shows that the following expression should be constant, if the components are in equilibrium:

$$\frac{[\beta\text{-Hydroxybutyrate}][\text{oxoglutarate}][\text{NH}_4^+]}{[\text{Acetoacetate}][\text{glutamate}]} \quad (1)$$

The test using the values given in Table 1 shows that this is the case: the values of expression (1) for the livers of well-fed, starved and alloxan-diabetic rats were 7.3×10^{-2}mM, 6.6×10^{-2}mM and 8.4×10^{-2} mM respectively. This test involves no assumptions on the ionic strength of the tissue, on the value of the equilibrium constants or on the pH of the tissue; the constancy of expression (1) may therefore be taken as conclusive proof of a joint NAD$^+$–NADH pool for the two dehydrogenase systems.

The constant value obtained for expression (1) is an experimental observation. The value can also

Table 4. *Equilibrium constants of lactate-, β-hydroxybutyrate- and glutamate-dehydrogenase (NAD) systems*

The values are those used for the calculation of the [NAD$^+$]/[NADH] ratio and refer to 38°, pH 7·0 and I 0·25. The concentration of water is taken to be unity.

	K
Lactate dehydrogenase	1.11×10^{-4}
β-Hydroxybutyrate dehydrogenase	4.93×10^{-2}
Glutamate dehydrogenase	3.87×10^{-3} mM

Table 5. *Calculation of [NAD$^+$]/[NADH] ratios in rat liver from the concentrations of the oxidants and reductants of the lactate-, glutamate- and β-hydroxybutyrate-dehydrogenase systems*

The calculations are based on the values given in Tables 1 and 4; pH is assumed to be 7·0.

	[NAD$^+$]/[NADH] ratio calculated from		
State of animals	Lactate-dehydrogenase system (cytoplasm)	Glutamate-dehydrogenase system (mitochondrial matrix)	β-Hydroxybutyrate-dehydrogenase system (mitochondrial cristae)
Well-fed	725	7·3	7·8
Starved	528	4·7	5·6
Alloxan-diabetic	208	10·8	9·6

Table 6. *Effect of NH_4^+ on the concentrations of the substrates of NAD-linked dehydrogenase systems in rat liver*

Rats starved for 44–48 hr. were given an intramuscular injection of 1·0 ml. of 0·9% NaCl or M-NH₄Cl and killed after 5–15 min. The concentrations of metabolites are expressed as μmoles/g. fresh wt. (means ± S.E.M.).

Treatment	No. of observations	[Lactate]	[Pyruvate]	[Glutamate]	[α-Oxo-glutarate]	[NH₄⁺]	[β-Hydroxy-butyrate]	[Acetoacetate]	[Lactate]/[pyruvate]	[Glutamate]/[oxoglutarate] ×[NH₄⁺]	[β-Hydroxybuty-rate]/[aceto-acetate]
NaCl	14	0·46±0·13	0·028±0·003	2·22±0·36	0·065±0·017	0·56±0·25	1·98±0·54	0·71±0·28	16·5	62	2·8
NH₄Cl	11	0·70±0·33	0·037±0·010	2·70±0·67	0·035±0·011	1·81±0·32	1·82±0·65	1·04±0·32	19	43	1·8

be obtained by calculations because it is the ratio of the equilibrium constants of the glutamate- and β-hydroxybutyrate-dehydrogenase systems. By using the data given in Table 4 one obtains:

$$\frac{K_{\text{glutamate (NAD)}}}{K_{\beta\text{-hydroxybutyrate}}} = \frac{3\cdot87\times10^{-3}\text{mM}}{4\cdot93\times10^{-2}} = 7\cdot9\times10^{-2}\text{mM}$$

The agreement between this theoretical value and the observed values ($6\cdot6\times10^{-2}$–$8\cdot4\times10^{-2}$mM) is excellent.

Effect of injection of ammonium chloride on liver dehydrogenase systems. The existence of an equilibrium between the components of the glutamate- and β-hydroxybutyrate-dehydrogenase systems was checked by another series of experiments in which an attempt was made to change the equilibrium in the living rat by injection of a solution of ammonium chloride. A rise in the concentration of NH_4^+ in the liver is expected to affect both dehydrogenase systems, if they are in equilibrium. The rats were killed 5–15 min. after the intramuscular injection. The analysis of the liver (Table 6) shows an approximately threefold increase of the NH_4^+ concentration. The other changes were as expected. There was a large fall in the concentration of α-oxoglutarate, a slight decrease in the concentration of β-hydroxybutyrate and an increase in that of acetoacetate. The ratios [glutamate]/[α-oxoglutarate] [NH₄⁺] and [β-hydroxybutyrate]/[aceto-acetate] changed in parallel and the values for expression (1) were approximately equal after injection of ammonium chloride and in the sodium chloride control. The values ($4\cdot2\times10^{-2}$ and $4\cdot6\times10^{-2}$mM) are somewhat lower than those obtained in the series recorded in Table 1. Whether this is within the limits of error or connected with the injection of a salt solution remains to be clarified.

DISCUSSION

Simplifying assumptions. The calculations of the [NAD⁺]/[NADH] ratio (Table 5) and the discussion below are based on a few simplifying assumptions that are correct only to a first approximation. They are: (a) that the pH of both mitochondria and cytoplasm is 7·0; (b) that the ionic strength of both mitochondria and cytoplasm is 0·25; (c) that the concentrations of the substrates of the dehydrogenases concerned are even throughout the tissue; (d) with glutamate dehydrogenase, that μmoles/g. wet wt. of tissue are equal to m-moles/l. and that the water concentration of the tissue is the same as that in the solutions in which the equilibrium constants were determined.

In the absence of precise information on these points assumptions were necessary. It is in fact likely that there are pH differences between

cytoplasm and mitochondria and even within the mitochondria. It is also probable that there are concentration gradients of substrates of dehydrogenases. However, it can be said with confidence that any correction factors that might have to be introduced would be of a minor kind and would not affect the nature of the conclusions drawn from the data.

Common NAD⁺–NADH pool of glutamate dehydrogenase and β-hydroxybutyrate dehydrogenase. Although β-hydroxybutyrate dehydrogenase is insoluble and located in the cristae, in contrast with glutamate dehydrogenase, which is soluble and located in the matrix, a common NAD^+–NADH pool would be expected if the dinucleotides behaved (as is usually the case) as coenzymes rather than as prosthetic groups (see Dixon & Zerfas, 1940; Dixon & Webb, 1964). A coenzyme, unlike a prosthetic group, is necessarily dissociated from the enzyme after each cycle of reaction, and would thus not be fixed to the site of the enzyme but would be free to mix with the coenzyme molecules reacting with other dehydrogenases. The cristae have been regarded as a space where reactions can take place (Klingenberg & Pfaff, 1966), but the joint pool indicates that β-hydroxybutyrate dehydrogenase is located in the cristae in such a way as to combine only with the nicotinamide–adenine nucleotides of the matrix and not with those of other compartments.

Assay of mitochondrial [NAD⁺]/[NADH] ratio. The common pool of coenzymes implies that both β-hydroxybutyrate dehydrogenase and glutamate dehydrogenase are suitable for assaying the intramitochondrial [NAD⁺]/[NADH] ratio. The former is more convenient because the concentrations of the two substrates can be measured enzymatically. A disadvantage of the glutamate-dehydrogenase system, already mentioned, is the

need to determine NH_4^+, a cell constituent that may undergo rapid post-mortem increases (Brown, Duda, Korkes & Handler, 1957) and is not easy to estimate. The validity of the assay of the intramitochondrial [NAD⁺]/[NADH] ratio depends on a sufficiently high activity of the enzyme system to establish equilibrium. As Tables 7, 8 and 9 show, the activities of β-hydroxybutyrate dehydrogenase and glutamate dehydrogenase vary greatly from tissue to tissue and are exceptionally high in rat liver. No specific statement can be made about the activity required to maintain equilibrium, as this depends on the turnover of the substrates. Evidence of a state of equilibrium is needed for each tissue and each experimental condition before the principles used in the present work can be applied. A potential

Table 8. *β-Hydroxybutyrate-dehydrogenase activity of liver of various species*

The results are those of Lehninger *et al.* (1960).

Species	NADH formed (μmoles/hr./g. wet wt. of tissue at 20°)
Rat	1141
Guinea pig	71
Rabbit	203
Dog	256
Rhesus monkey	310
Mouse	792
Cat	325

Table 9. *Glutamate-dehydrogenase (NAD) activity of rat tissues at 25°*

The data refer to well-fed male rats weighing about 300 g. The tissue was homogenized with 9 parts of ice-cold water in a MSE overhead-drive homogenizer. The homogenate was treated with ultrasound for 1 min. and centrifuged at 38000g for 30 min. The enzyme assay was carried out by a modification of the method of Schmidt (1963) in the presence of NADH and 1·6 mм-ADP, which activates the enzyme (Tomkins, Yielding, Talal & Curran, 1963).

Tissue	NADH oxidized (μmoles/g. wet wt. of tissue/hr.)
Brain cortex	930 ± 70 (3)
Brain medulla	910 ± 45 (3)
Liver	7080 ± 519 (4)
Heart	322 ± 26 (4)
Leg muscle	34 ± 5 (4)
Diaphragm	67 ± 15 (4)
Testis	119 ± 19 (4)
Spleen	281 ± 43 (3)
Lung	320 ± 20 (3)
Kidney cortex	3720 ± 238 (5)

Table 7. *β-Hydroxybutyrate-dehydrogenase activity in rat tissues*

The results are those of Lehninger *et al.* (1960).

Tissue	NADH formed (μmoles/hr./g. wet wt. of tissue at 20°)
Brain	58
Liver	1255
Heart	104
Spleen	28
Kidney	162
Skeletal muscle	23
Testis	22
Adrenals	102
Pancreas	30
Thyroid	0

complication in the use of the glutamate-dehydro-genase system for the assay is the possibility of an uneven distribution of glutamate, α-oxoglutarate and NH_4^+ over the cell compartments. However, the fact that the same [NAD$^+$]/[NADH] ratios were obtained for the β-hydroxybutyrate- and glutamate-dehydrogenase systems seems an improbable coincidence and this makes it very likely that the concentration of the components of the glutamate-dehydrogenase system reflect their mitochondrial concentrations.

Number of NAD$^+$–NADH pools in liver cells. If it is correct that cristae and matrix share the same pool and that the external compartment of the mito-chondria freely communicates with the cytoplasm, then there are only two major NAD$^+$–NADH pools in the liver cell (apart from a possible separate pool in the nuclei). There are, it is true, large amounts of enzyme-bound dinucleotides, apart from the free dinucleotides. That this is so becomes obvious when the [NAD$^+$]/[NADH] ratios calculated from the equilibrium concentrations are compared with those obtained by the determination of the total dinucleo-tide contents of the tissue (Table 10). The direct determination gives much lower ratios for both cytoplasm and mitochondria. These differences are due to the fact that the reduced forms of the dinucleotides are more firmly bound than the oxidized forms (Frieden, 1961; Winer, Schwert & Millar, 1959; Raval & Wolfe, 1962). Indeed, most of the cytoplasmic NADH must be protein-bound. However, as the free and bound dinucleotides readily exchange, the bound forms could hardly be regarded as a separate pool.

Transhydrogenase activity of glutamate dehydro-genase. The experiments recorded in Tables 1 and 6 indicate that glutamate dehydrogenase rapidly reacts with NAD in the liver of the living rat. This has been questioned by Klingenberg & Slenczka (1959), Klingenberg & Pette (1962) and Tager & Papa (1965), who came to the conclusion that gluta-mate dehydrogenase of intact liver mitochondria may react with NADP only, in contrast with the isolated enzyme, which reacts with both NAD and NADP. The prompt changes of the concentrations of α-oxoglutarate, glutamate, β-hydroxybutyrate and acetoacetate after injection of ammonium chloride are not compatible with the conclusions of these authors. If glutamate dehydrogenase *in situ* reacts readily with both dinucleotides the NAD and NADP systems in the matrix of liver mitochondria would be in equilibrium. In this case the following relation holds:

$$\frac{[NADP^+]}{[NADPH]} = \frac{[NAD^+]}{[NADH]} \times \frac{K_{\text{glutamate (NAD)}}}{K_{\text{glutamate (NADP)}}}$$

where the subscripts denote the two different equilibrium constants. At 38° and I 0.25 the value for the ratio $K_{\text{glutamate (NAD)}}/K_{\text{glutamate (NADP)}}$ is 1.56 (P. C. Engel & K. Dalziel, personal communication), i.e. very similar to the value 1.47, found by Olson & Anfinsen (1953) at 27°.

If the two systems are in equilibrium, they con-stitute an effective transhydrogenase. The physio-logical significance of this non-energy-requiring transhydrogenation may lie in the storage of a readily available electron donor, in the form of NADPH. The high activity of liver glutamate dehydrogenase and the great binding power of this enzyme for NADH and NADPH is compatible with this concept. When a rapid need for energy arises NADPH could react as a generator of NADH to serve as a substrate for the electron-transport chain. The roles of NADPH and the enzymes generating and removing it could thus be conceived as being analogous to those of creatine phosphate and creatine kinase.

Physiological significance of the differences of the redox state of mitochondria and cytoplasm. The differences between the redox states of the two cell compartments are presumably essential and con-nected with the function of the two compartments. The cytoplasm of the liver cell is the main site of glycolysis and of gluconeogenesis, which includes the transfer of hydrogen from glyceraldehyde phosphate to NAD$^+$ in glycolysis and of the reverse process in gluconeogenesis. The direction of these reactions depends on the redox state of the hydro-gen-carrier systems.

In the mitochondria the main function of the NAD system is to channel hydrogen atoms to the electron-transport chain from the substrates of respiration. To be effective as an energy source the [NAD$^+$]/[NADH] ratio must be below a critical value, if the free-energy change of the transfer of electrons from NADH to flavoprotein is to be large enough for coupling with the synthesis of ATP.

Role of P_i and NH_4^+ in the regulation of the redox state of cell compartments. It is a remarkable fact

Table 10. *Free and bound nicotinamide–adenine dinucleotide in the liver of well-fed rats*

The values for the free dinucleotides are those of Table 5. The values for the total dinucleotides have been calculated from the values given in Table 1 of Glock & McLean (1956) for the soluble fraction and mitochondria.

	Cytoplasm	Mitochondria
[Free NAD$^+$] / [Free NADH]	725	7.6
[Total NAD$^+$] / [Total NADH]	$\frac{359}{50} = 7.2$	$\frac{47}{21} = 2.2$
[Total NADP$^+$] / [Total NADPH]	$\frac{26}{88} = 0.30$	$\frac{3}{90} = 0.033$

that each of the two cell compartments in which the NAD couple is a major metabolic agent contains one powerful dehydrogenase system, the oxidizing and reducing capacity of which is dependent not only on the concentrations of the oxidized and reduced carbon metabolites but also on a third component, the concentration of which is variable. These are the glyceraldehyde phosphate dehydrogenase in which P_i is the third component, and glutamate dehydrogenase in which NH_4^+ is the third component. The concentrations of P_i and NH_4^+ can thus play a major role in the control of the redox state of the two compartments. The concentration of P_i varies mainly as the result of oxidative phosphorylation, the concentration of NH_4^+ mainly as the result of the synthesis and degradation of glutamate and glutamine and, in the liver, the synthesis of urea. The concentrations of both P_i and NH_4^+ are very much higher in the tissues than in the blood plasma (Table 11), which implies that the tissues have mechanisms for accumulating or retaining these ions.

The importance of the concentrations of P_i and NH_4^+ for the redox state is illustrated by the following example. If the equilibrium constant of the glyceraldehyde phosphate dehydrogenase is taken to be 1.5×10^{-8} [this value is based on those of Meyerhof & Oesper (1947) and Burton & Wilson (1953), corrected for I 0.25 and 38°] it follows that in the liver of well-fed rats ([NAD$^+$]/[NADH] 725 and [P_i] 4mM) the ratio [glyceraldehyde phosphate]/ [diphosphoglycerate] in equilibrium with the NAD system is about 2. Thus the concentrations of the oxidized and reduced components are of the same order of magnitude. Higher concentrations of P_i shift the equilibrium in favour of diphosphoglycerate, i.e. glycolysis; lower concentrations of P_i shift it in favour of reduction, i.e. gluconeogenesis, as does a low [NAD$^+$]/[NADH] ratio. In the liver of severely diabetic rats, where the ratio was 208, the [glyceraldehyde phosphate]/[diphosphoglycerate] ratio would be about 8, i.e. in favour of gluconeogenesis. The controlling action of P_i may be

regarded as being superimposed on the control of glycolysis and gluconeogenesis by the redox state of the NAD couple. It ensures that gluconeogenesis occurs only when the source of energy in the form of ATP is available: high ATP concentrations imply low P_i concentrations and vice versa. It should be emphasized that the redox state of the glyceraldehyde 3-phosphate-dehydrogenase system is only one of several control points. It can be a factor in determining the direction of carbohydrate metabolism, i.e. breakdown or synthesis.

Analogous considerations apply to the role of NH_4^+ in the glutamate-dehydrogenase system of the mitochondrial matrix.

Maintenance of separate redox states in mitochondria and cytoplasm. Since both mitochondria and cytoplasm contain highly active NAD-linked malate dehydrogenases, the [NAD$^+$]/[NADH] ratios of the two compartments would be expected to be equal if malate and oxaloacetate readily traversed the mitochondrial membranes. The fact that they are very different in the intact cell indicates that the passage through the membranes of either malate or oxaloacetate or both is too slow to establish equilibrium between the two compartments. Substantial gradients have in fact been observed for many low-molecular-weight solutes such as Ca^{2+}, phosphate, succinate, malate and citrate (Amoore & Bartley, 1958; Amoore, 1958; Chappell & Crofts, 1965; Gamble, 1965).

Steady-state concentration of mitochondrial oxaloacetate. Information on the concentrations of oxaloacetate in cell compartments is of importance because of the key role of oxaloacetate as a metabolite and as an inhibitor. Direct determinations (Löffler & Wieland, 1963) show that overall concentration in the liver is very low (0.006mM in normal liver, 0.002mM in liver of the alloxan-diabetic rat), but these analyses give no information on the distribution between cytoplasm and mitochondria. As the distribution is liable to changes during fractionation it has so far not been possible to obtain reliable analytical values for the concentration in the mitochondria.

The concentration of oxaloacetate within a cell compartment can be calculated if the concentration of malate and the [NAD$^+$]/[NADH] ratio is known and if the malate-dehydrogenase system is in equilibrium. The assumption that this is the case seems permissible on account of the high activity of the enzyme in both cytoplasm and mitochondria. The overall concentration of malate in rat liver is about 0.5mM (Bücher & Klingenberg, 1958). There is no information on a concentration gradient of malate *in vivo* between mitochondria and cytoplasm but it is unlikely that it is greater than, say, 5 in favour of the mitochondria. It is probably smaller. In view of the various uncertainties the calculations

Table 11. *Concentration gradients between liver tissue and blood plasma of NH_4^+ and inorganic phosphate*

For sources see: Kirsten, Gerez & Kirsten (1963) for [NH_4^+] in plasma; Table 1 for [NH_4^+] in liver. The P_i value of liver is based on measurements in this Laboratory of rapidly frozen tissue.

	Concn. in plasma (mM)	Concn. in liver (mM)
P_i	1.2	4
NH_4^+	< 0.004	0.5

Table 12. *Calculation of oxaloacetate concentration in cytoplasm and mitochondria of rat liver at* pH 7·0

The values have been calculated by the formula:

$$[\text{Oxaloacetate}] = [\text{malate}] \times [\text{NAD}^+]/[\text{NADH}] \times K$$

where K is the equilibrium constant of the malate-dehydrogenase system. At 38°, I 0·25 and pH 7·0, K is $2\cdot78 \pm 0\cdot04 \times 10^{-5}$ (22). This value is based on measurements in this Laboratory by R. L. Veech. Hohorst (1960) reported a value of $0\cdot98 \times 10^{-5}$ for 37° and I 0·25. This is very close to values obtained at 25° by Burton & Wilson (1953) and Raval & Wolfe (1962). As it is improbable that there is no effect of the temperature K was redetermined at 38° in phosphate buffer, pH 7·15 and I 0·25. Crystalline pig heart malate dehydrogenase (Boehringer) was used and the method was essentially as described in this paper for the measurement of the equilibrium constant of lactate dehydrogenase. The reasons for the discrepancy between the present value and that of Hohorst (1960) are not clear. The assumed values for malate are near the likely upper and lower limits.

State of animals	Cell compartment	[NAD+]/[NADH] (from Table 5)	[Malate] (assumed) (mM)	[Oxaloacetate] (calculated) (mM)
Well-fed	Cytoplasm	725	0·5	0·01
Well-fed	Mitochondrial matrix	8	0·5	0·00011
Well-fed	Mitochondrial matrix	8	2·5	0·00056
Alloxan-diabetic	Cytoplasm	208	0·5	0·0029
Alloxan-diabetic	Mitochondrial matrix	10	0·5	0·00014
Alloxan-diabetic	Mitochondrial matrix	10	2·5	0·00070

cannot be expected to give a precise result, but even knowledge of the order of magnitude is valuable. The results of the calculations (Table 12) illustrate the great differences of the oxaloacetate concentrations in mitochondria and cytoplasm that are expected if equilibrium within the compartments is established. There is a 91-fold difference in normal liver and a 19-fold difference in liver of the alloxan-diabetic rat. Passive diffusion of oxaloacetate out of the mitochondria would thus not occur as long as malate is the only source of oxaloacetate. The position would be different if the equilibrium were upset by a rapid synthesis of oxaloacetate from pyruvate. In this case most of the oxaloacetate would be reduced to malate. By this process a concentration gradient of malate between mitochondria and cytoplasm would be built up and the malate would diffuse into the cytoplasm. The inference is that the differences in the redox states of mitochondria and cytoplasm automatically 'pump' into the cytoplasm the carbon skeleton of oxaloacetate arising in the mitochondria by the carboxylation of pyruvate.

It also follows that very low concentrations of oxaloacetate are to be postulated in the mitochondria if a formation of oxaloacetate from malate is to take place. As the normal [malate]/[oxaloacetate] ratio in the mitochondria is calculated to be about 3000 the oxaloacetate concentration in equilibrium with malate is below 0·001mM. At higher concentrations the tricarboxylic acid cycle would virtually stop at the stage of malate.

The measured overall concentration of oxaloace-

tate in the liver (Löffler & Wieland, 1963) lies between the values calculated for the cytoplasm and mitochondria. It is nearly two-thirds that calculated for the cytoplasm, which is of the expected order since the cytoplasm contributes about two-thirds of the cell volume.

Redox state of liver cell compartments and diabetic ketosis. As mentioned at the end of the introduction, a starting point for this investigation was the problem of the role of the redox state of the NAD+–NADH system in ketogenesis. Though the results confirm the earlier observations that the cytoplasm is more reduced in severe ketosis (the average change of the [NAD+]/[NADH] ratio being a decrease of 3·5) there is no parallel change in the mitochondria. On the contrary, the [NAD+]/[NADH] ratio in the mitochondria changed by 20–30% in the direction of oxidation. This implies that any decrease in the concentration of oxaloacetate in the mitochondria cannot be attributed to a shift of the [malate]/[oxaloacetate] ratio in favour of malate. If malate and oxaloacetate were in equilibrium in both cytoplasm and mitochondria, the [malate]/[oxaloacetate] ratio would, in severe alloxan-diabetes, rise substantially in the cytoplasm and fall slightly in the mitochondria. This fall would increase the concentration of oxaloacetate at the site of the tricarboxylic acid cycle, and the increased formation of ketone bodies cannot be a simple consequence of the change of the redox state of the mitochondria. Nevertheless, evidence discussed elsewhere (Krebs, 1966a,b) supports the concept that severe ketosis is caused by a fall in the oxaloacetate concentration of

the liver, but that this fall is due to the rapid conversion of oxaloacetate into phosphopyruvate and subsequently glucose.

Ketogenic effect of NH_4^+. It is known that NH_4^+ causes an increased formation of acetoacetate in liver slices and in other liver preparations (Annau, 1934; Edson, 1935; Recknagel & Potter, 1951; Berry, 1964). If the glutamate- and β-hydroxybutyrate-dehydrogenase systems are in equilibrium so that the components of expression (1) are in equilibrium, the addition of NH_4^+ should decrease the [β-hydroxybutyrate]/[acetoacetate] ratio, i.e. increase the relative concentration of acetoacetate, as shown in Table 6. The [β-hydroxybutyrate]/[acetoacetate] ratio fell from 2·8 to 1·8 when the concentration of NH_4^+ increased 3·2-fold. Recknagel & Potter (1951) explained the ketogenic effect of NH_4^+ by the assumption that NH_4^+ reacted with α-oxoglutarate to form glutamate and thereby blocked the tricarboxylic acid cycle, preventing the formation of oxaloacetate. Both mechanisms are feasible. The shift of the equilibrium would leave the total ketone-body concentration constant whereas a block of the tricarboxylic acid cycle would raise the total ketone-body yield. In the experiments recorded in Table 6 there was no significant increase in the concentration of total ketone bodies although NH_4^+ increased threefold. Experiments by Berry (1964) on liver homogenates with pyruvate as substrate show that in this system both factors contribute to higher rates of acetoacetate formation.

Effect of alloxan-diabetes on the redox state of the mitochondria. The value of the [NAD^+]/[NADH] ratio in the mitochondria of alloxan-diabetic rats was about twice that found in the liver of starved rats. In both conditions fatty acids are the main fuel of respiration, but there is a major difference in respect of the rate of ketone-body production. The steady-state concentration of the ketone bodies was fivefold higher in alloxan-diabetes (Table 1). The high rate of ketone-body formation may have been one of the factors responsible for the greater degree of mitochondrial oxidation in alloxan-diabetes, because the acetoacetate formed primarily constitutes an acceptor for the H atom of NADH and would therefore be expected to raise the [NAD^+]/[NADH] ratio. Another factor may be an increased rate of transfer of reducing equivalents, in the form of malate, from mitochondria to cytoplasm in diabetes connected with the increased rates of gluconeogenesis (Krebs, Gascoyne & Notton, 1967).

Control of redox state of cell compartments. If the sum of [NAD^+] and [NADH] is constant (i.e. if the balance of the synthesis and of the degradation of the dinucleotides is zero, the [NAD^+]/[NADH] ratio is the resultant of the action of the dehydrogenase systems that interconvert NAD^+ and

Table 13. *Major NAD-linked dehydrogenases in cytoplasm and mitochondria of rat liver and their mid-potentials*

'Pyruvate dehydrogenase' and 'α-oxoglutarate dehydrogenase' are listed as lipoamide dehydrogenase. Cytoplasmic isocitrate dehydrogenase is taken to be NADP-linked and therefore omitted. Also omitted are the relatively weak dehydrogenases involved in the degradation of proline, other amino acids and polyols. The mid-potential of glyceraldehyde phosphate dehydrogenase is given for the physiological concentration of P_i (4mM), that of glutamate dehydrogenase for the physiological concentration of NH_4^+ (0·5mM). The values of the mid-potentials are based on the data of Burton (1961), Krebs *et al.* (1962) for β-hydroxybutyrate dehydrogenase and Massey (1960) for lipoamide dehydrogenase (see also Lusty & Singer, 1964).

	Mid-potential (25°; pH 7·0) (v)
Cytoplasmic dehydrogenases	
Lactate dehydrogenase	−0·185
Glyceraldehyde phosphate dehydrogenase	−0·214
α-Glycerophosphate dehydrogenase	−0·192
Malate dehydrogenase	−0·166
Alcohol dehydrogenase	−0·197
Mitochondrial dehydrogenases	
Isocitrate dehydrogenase	−0·375
Malate dehydrogenase	−0·166
Lipoamide dehydrogenase	−0·280
Glutamate dehydrogenase	−0·220
β-Hydroxybutyrate dehydrogenase	−0·266
β-Hydroxy-acyl-CoA dehydrogenase	−0·238

NADH, including the NADH dehydrogenases of the electron-transport chain.

The limits of the [NAD^+]/[NADH] ratio are set by the equilibrium constants of the dehydrogenase systems and the relative concentrations of the reduced and oxidized metabolites. A survey of the main dehydrogenases of the two compartments (Table 13) shows that the mid-potentials of the dehydrogenases of the cytoplasm are less negative than those of most of the mitochondrial systems. This limits the extent of reduction of the cytoplasmic NAD^+ much more narrowly than that of the mitochondria. Moreover, in a mixture of dehydrogenases in equilibrium with a common pool of NAD^+–NADH, the more negative couple reduces the less negative one under standard conditions. Given a supply of the reduced substrate of the more negative couple, the [NAD^+]/[NADH] ratio is therefore bound to fall. Thus a mixture of the substrates of lactate dehydrogenase and β-hydroxybutyrate dehydrogenase with their enzymes and coenzymes is in equilibrium when:

$$\frac{[\text{Lactate}]}{[\text{Pyruvate}]} = \frac{[\beta\text{-hydroxybutyrate}]}{[\text{acetoacetate}]} \times \frac{K_{\beta\text{-hydroxybutyrate}}}{K_{\text{lactate}}}$$

Substituting the values given for K in Table 4, the [lactate]/[pyruvate] ratio is 440 if the ratio [β-hydroxybutyrate]/[acetoacetate] is 1. The presence in the mitochondria of several powerful dehydrogenase systems of a relatively negative potential can therefore explain why the NAD system is in a more reduced state in the mitochondria than in the cytoplasm.

If, then, the redox potential of the dehydrogenases present limits the possible degree of reduction of NAD^+, other factors determine the actual redox state within the permissible range. Among these other factors are: (a) concentrations of reduced and oxidized substrates of dehydrogenases; (b) rate of removal of NADH by the respiratory chain; this in turn is controlled by the concentrations of ADP and P_i and by substrates that join the chain at the flavoprotein level (succinate, fatty acyl-CoA esters); (c) rate of transfer of reducing equivalents between cytoplasm and mitochondria by the malate and α-glycerophosphate shuttles (see Krebs et al. 1967). A detailed attempt to analyse their scope and interplay appears premature.

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