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THE OXIDOREDUCTION STATE OF FREE NAD(P) AND MASS-ACTION RATIO OF TOTAL NICOTINAMIDE NUCLEOTIDES IN ISOLATED RAT-LIVER MITOCHONDRIA

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SUMMARY

1. The oxidoreduction state of free NAD(P) in isolated rat-liver mitochondria, calculated from the glutamate and β-hydroxybutyrate redox couples according to the metabolite indicator method, has been compared with the mass-action ratio of the oxidized and reduced nicotinamide nucleotides determined after extraction ('total' NAD(P)).

2. The oxidoreduction state of free NAD, as calculated from the β-hydroxybutyrate couple, was found to be slightly more oxidized than the value given by the mass-action ratio of total NAD, corresponding to an apparent difference in midpoint potential of about 10 mV.

3. The nicotinamide nucleotide pool in equilibrium with the substrates of glutamate dehydrogenase was found to be at approximately the same oxidoreduction state as free NAD (calculated from the β-hydroxybutyrate couple) and the value given by the mass-action ratio of total NAD. Under these conditions the mass-action ratio of total NADP was at a value corresponding to a 10–50 times more reduced state than that of total NAD; this difference may be due to the action of the energy-linked transhydrogenase.

4. Addition of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (+oligomycin) causes an equilibration of the mass-action ratios of total NAD and NADP, but has little or no effect on the oxidoreduction state of the free nicotinamide nucleotides in equilibrium with glutamate and β-hydroxybutyrate dehydrogenase.

5. The results are discussed in relation to the nicotinamide nucleotide specificity of glutamate dehydrogenase in rat-liver mitochondria.

INTRODUCTION

Williamson *et al.*[1] were the first to apply the metabolite indicator method[2] for the calculation of the oxidoreduction state of the mitochondrial NAD (see ref. 3)

Abbreviations: DMO, 5,5'-dimethyloxazolidine-2,4-dione; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

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and NADP in freeze-clamped rat liver using the total tissue levels of reactants of the β-hydroxybutyrate dehydrogenase (EC 1.1.1.30) and glutamate dehydrogenase (EC 1.4.1.3) reactions, respectively. Both couples were found to be at an approximately equal redox potential and changed in parallel when the mitochondrial redox state was varied by different treatments of the rats[1,4,5]. Although other authors sometimes found slightly different values for the two mitochondrial redox couples[6–8], partly due to differences in the measured tissue level of ammonia[7,9] it was generally observed that changes in the mitochondrial redox state were reflected to an equal extent in the β-hydroxybutyrate couple as in the glutamate couple.

Krebs and coworkers[1,4,5] concluded from their work that glutamate and β-hydroxybutyrate dehydrogenase in rat liver *in vivo* are in equilibrium with the same pool of nicotinamide nucleotides and, since glutamate dehydrogenase can react with both NAD and NADP, that the oxidoreduction state of mitochondrial free NAD is equal to that of free NADP.

In contrast, studies on isolated mitochondria[10,11] demonstrated that under energy-rich conditions the relationship between the oxidoreduction states of NAD and NADP is mainly governed by the energy-linked transhydrogenase reaction, which brings about a preferential reduction of $NADP^+$ by NADH (see ref. 12). Moreover, the studies of Tager and coworkers[13–17] suggested that in isolated mitochondria under energy-rich conditions, glutamate dehydrogenase reacts preferentially with NADP, and not with NAD. The relationship between different redox couples in mitochondria has also been extensively investigated by Klingenberg *et al.*[3,10,18,19].

In the work reported in this paper, the problem was approached by comparing the mass-action ratio of total, extractable NAD and NADP with the oxidoreduction state of the free NAD and NADP, as calculated from the β-hydroxybutyrate and glutamate redox couples in isolated mitochondria.

METHODS

Rat-liver mitochondria were prepared by the method of Hoogeboom[20] as described by Myers and Slater[21].

The standard reaction medium contained 15 mM KCl, 5 mM $MgCl_2$, 2 mM EDTA, 50 mM Tris–HCl, 5 mM P_i, 2 mM arsenite, 5 mM malonate, 2.5 mM ATP, 10 μg rotenone and 25 mM sucrose (derived from the mitochondrial suspension) in a final volume of 2.5 ml. The pH was 7.46–7.72. Further additions are indicated in the legends to the tables and Fig. 1. The reaction temperature was 25 °C.

Incubation and quenching procedure

Unless otherwise indicated, incubations were carried out in round-bottomed tubes (diameter 25 mM) in a Dubnoff shaker. Reactions were stopped by centrifugation–filtration through silicone oil according to the following procedure. 1–3 min before the time of quenching a 1.8-ml sample of the reaction mixture was transferred to a 3-ml plastic centrifuge tube on top of a layer of 0.6 ml silicone oil (density 1.05), separating the reaction mixture from a layer of 0.5–0.6 ml 14% $HClO_4$. Centrifugation was carried out in the MSE 'Mistral' centrifuge (rotor No. 69102) for 2 min at 16 000 rev./min at room temperature. Maximum speed was

reached after 15–30 s. After centrifugation the supernatant was removed with a syringe and either kept at room temperature for determination of the pH (see below) or immediately acidified with $HClO_4$ (final concentration 3.5%). The mitochondrial extract was removed from the centrifuge tube with a syringe and Tris–HCl buffer (pH 7.4) or P_i buffer (pH 7.4) was added to a final concentration of 0.05 M. The acidified supernatants and mitochondrial extracts were neutralized with KOH. $KClO_4$ was removed in the cold.

For the determination of the reduced nicotinamide nucleotides a further 0.5 ml of the reaction mixture was treated with 0.25 ml 1 M KOH in alcohol and neutralized with a buffer containing 0.5 M triethanolamine–HCl, 0.4 M KH_2PO_4 and 0.1 M K_2HPO_4 (see ref. 22).

The intramitochondrial pH was calculated from the distribution of 5,5′-[^{14}C]dimethyloxazolidine-2,4-dione (DMO) across the mitochondrial membrane as described earlier[23]. The pH of the supernatant after centrifugation was taken as pH_{out} during the reaction. It was measured in a temperature-controlled vessel (1 ml), provided with a combined pH electrode (Electrofact, Type 7 GR 241), connected to an electrometer (Electronic Instruments Ltd, pH unit, Types C 33 B2 *plus* 33 B2).

Intramitochondrial metabolite concentrations were calculated from the concentrations measured enzymically in the mitochondrial extracts after correction for the amounts present in the sucrose-permeable space. For the determination of the volume of the mitochondrial pellet ^3H_2O (0.2 μCi/ml) was included in the reaction mixture. The volume of the sucrose-permeable space was obtained from parallel incubations where [^{14}C]sucrose (0.1 μCi/ml) was added.

Determination of specific activities

Separation of [^{14}C]glutamate and α-[^{14}C]oxoglutarate was performed on a Dowex-1-formate (Bio-Rad AG 1-X8) column as described by Lanoue *et al.*[24]. Fractions of 2 ml were collected and radioactivity was counted in 100-μl samples. The recovery of radioactivity was 80–120%.

Specific activities in β-hydroxy[^{14}C]butyrate and [^{14}C]acetoacetate were determined by the following procedure. The supernatant obtained after centrifugation of the reaction mixture was acidified (see above) and divided into two parts. One part was neutralized to pH 7.0, treated with excess NADH and β-hydroxybutyrate dehydrogenase for 60 min, and reacidified with $HClO_4$ (final concentration 3.5%). The other part was not neutralized. The samples were incubated for 3 min in a boiling water bath (control determinations showed that more than 95% of the acetoacetate was removed by this procedure). After neutralization, the β-hydroxybutyrate concentration and the radioactivity in the samples was measured. After correction for L-β-hydroxybutyrate, radioactivity in acetoacetate was calculated from the difference between samples treated with β-hydroxybutyrate dehydrogenase and untreated samples.

Specific activities were calculated as the ratio of total radioactivity for each compound and the total amount in the samples as measured enzymically.

Radioactivity was measured as described by Harris and Van Dam[25].

Assays

Glutamate, β-hydroxybutyrate, α-oxoglutarate, ammonia, $NAD(P)^+$ and

NAD(P)H were determined enzymically by the methods described in ref. 26, using either a Zeiss PMQ 4 spectrophotometer or an Aminco-Chance dual-wavelength spectrophotometer (λ 350–375 nm). Before the assay of acetoacetate and α-oxoglutarate in mitochondrial extracts, the samples were treated with Florisil (50 mg/ml) to remove 'NADH oxidase' activity. Florisil was removed by centrifugation.

Protein was determined by the biuret method as described by Cleland and Slater[27].

Chemicals

[2-^{14}C]DMO was obtained from New England Nuclear as a solution in ethyl acetate. Before use the ethyl acetate was evaporated and [^{14}C]DMO was dissolved in water. Other ^{14}C-labelled compounds were obtained from the Radiochemical Centre (Amersham), and $^{3}H_2O$ from Philips Duphar (Netherlands). Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) was a gift from E. J. DuPont de Nemours and Co., and oligomycin from Upjohn Chemical Company. Florisil (60–100 mesh) was obtained from Koch–Light Laboratories (England).

RESULTS

General considerations

A calculation of the oxidoreduction state of free NAD(P) from the β-hydroxybutyrate and glutamate couples according to Eqns 1 and 2:

$$\frac{NAD^+}{NADH} = \frac{[H^+]}{K_{HBDH}} \cdot \frac{[\text{acetoacetate}]}{[\beta\text{-hydroxybutyrate}]} \quad (1)$$

$$\frac{NAD(P)^+}{NAD(P)H} = \frac{[H^+]}{K_{GLDH}} \cdot \frac{[\alpha\text{-oxoglutarate}]\,[\text{ammonia}]}{[\text{glutamate}]} \quad (2)$$

(K_{HBDH} and K_{GLDH} are the equilibrium constants of the β-hydroxybutyrate and glutamate dehydrogenase reactions, respectively) requires a knowledge of the intramitochondrial pH and the equilibrium constants of the reactions. The former was calculated from the distribution of [^{14}C]DMO (for a discussion on the applicability of the DMO method, see refs 9 and 28). The equilibrium constants at 25 °C, ionic strength 0.25, are given in Table I (the ionic strength of the mitochondrial matrix space was assumed to be 0.25; *cf.* ref. 29). The equilibrium constant of the β-hydroxybutyrate dehydrogenase reaction was calculated from the data of Williamson *et al.*[1,30], assuming that the variation of the equilibrium constant with the ionic strength at 25 °C was similar to that at 38 °C (ref. 1). The equilibrium constants of the glutamate dehydrogenase reaction were obtained from ref. 31.

The calculation of the glutamate couple was complicated by the fact that considerable amounts of metabolically inactive ammonia (3–4 nmoles/mg protein) were found in mitochondrial extracts even at extremely low levels of ammonia in the incubation medium (*cf.* refs 32 and 33). This ammonia could not be used for glutamate or citrulline synthesis (Renooij, W. and Hoek, J. B., unpublished). Thus, the measured internal concentration of ammonia can not be used in the calculation of the glutamate couple. The intramitochondrial concentration of free ammonia was calculated on the assumption that its equilibrium distribution across the

TABLE I

EQUILIBRIUM CONSTANTS OF GLUTAMATE AND β-HYDROXYBUTYRATE DEHYDROGENASES AT 25 °C AND IONIC STRENGTH 0.25

Calculated from the data of refs 1, 30 and 31.

Enzyme	*Definition of constant*	*K*
Glutamate dehydrogenase (NAD-linked)	$\frac{\alpha\text{-oxoglutarate}\cdot NH_4^+\cdot NADH\cdot H^+}{\text{glutamate}\cdot NAD^+}$	$7.3\cdot10^{-14}$ M²
Glutamate dehydrogenase (NADP-linked)	$\frac{\alpha\text{-oxoglutarate}\cdot NH_4^+\cdot NADPH\cdot H^+}{\text{glutamate}\cdot NADP^+}$	$4.8\cdot10^{-14}$ M²
β-Hydroxybutyrate dehydrogenase	$\frac{\text{acetoacetate}\cdot NADH\cdot H^+}{\beta\text{-hydroxybutyrate}\cdot NAD^+}$	$1.83\cdot10^{-9}$ M

mitochondrial membrane is related to the pH difference between the intra- and extramitochondrial water (*cf.* refs 9 and 34).

$$[NH_4^+]_{in} = [NH_4^+]_{out}\cdot 10^{pH_{out}-pH_{in}} \quad (3)$$

Although in experiments with isolated mitochondria the establishment of a 'near-equilibrium' state (an essential condition for the metabolite-indicator method[1,2,4]) can be promoted by the addition of different metabolic inhibitors, it may be difficult to assess if, indeed, equilibrium has been approached. In the first place, the concentrations of the metabolites may be so far below the K_m of the enzymes that no detectable change occurs in time , even though the mass-action ratio deviates considerably from the equilibrium constant. Secondly, some of the inhibitors may directly or indirectly affect the activity of the dehydrogenases (*cf.* refs 35 and 36). In the experiments reported in this paper, rotenone was added to inhibit electron transport through the respiratory chain and arsenite to inhibit the oxidation of α-oxoglutarate. Malonate was present to maintain a free permeability of the mitochondrial membrane to α-oxoglutarate[27]. ATP (2.5 mM) served as a source of energy.

In the experiment of Fig. 1 mitochondria were incubated under these reaction conditions and the reduction level of the nicotinamide nucleotides was changed by addition of the substrates of β-hydroxybutyrate and glutamate dehydrogenases, either separately (Traces A and B) or in combination (Trace C). The rapid changes observed demonstrate that both dehydrogenases are active under these conditions.

Relationship between free and total NAD and NADP

In Tables II and III the results of two parallel experiments are presented. Mitochondria were incubated in the standard reaction medium with the substrates of either β-hydroxybutyrate dehydrogenase (Table II) or glutamate dehydrogenase (Table III). The intra- and extramitochondrial concentrations of all substrates as well as oxidized and reduced NAD(P) were determinated after different reaction times as described under Methods. The addition of different ratios of the metabolites

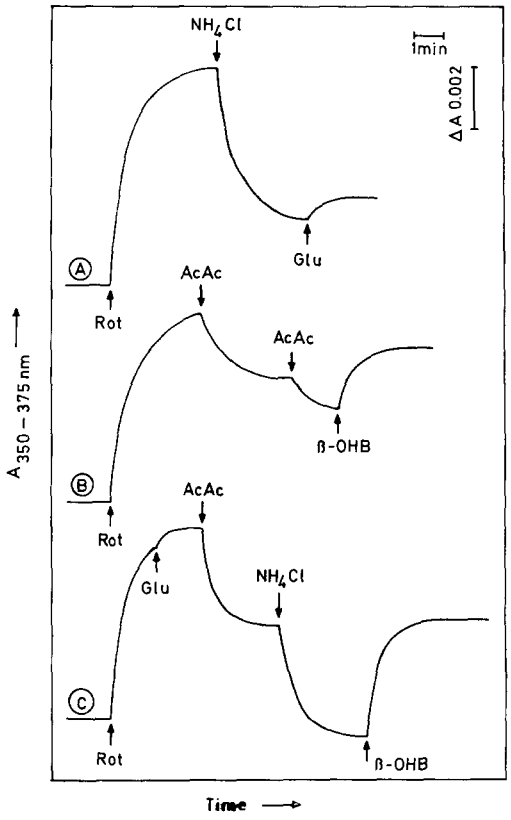

Fig. 1. Changes in the reduction level of intramitochondrial nicotinamide nucleotides. The reaction medium contained the standard components (without rotenone), *plus* 2 mM α-oxoglutarate and 1.0 mg mitochondrial protein in a final volume of 3 ml. Rotenone (Rot) (2 μg/ml), acetoacetate (AcAc) (1 mM), β-hydroxybutyrate (β-OHB) (3.5 mM), NH_4Cl (0.8 mM) and glutamate (Glu) (3.2 mM) were added as indicated. The reactions were performed in a cuvette at room temperature. Changes in absorbance (wavelength pair 350–375 nm) were directly monitored in an Aminco-Chance dual-wavelength spectrophotometer.

brought about corresponding variations in the mass-action ratio of the total nicotinamide nucleotides (Expts A and B in Tables II and III). The results obtained after 10- and 20-min reaction time were not significantly different, suggesting that both dehydrogenase couples had attained a condition that can be regarded as near equilibrium. The mass-action ratio of total NADP gave a more reduced value than that of total NAD, suggesting that the energy-linked transhydrogenase was operative under these conditions.

The oxidoreduction of free NAD, calculated with both the intra- and extramitochondrial ratio of acetoacetate to β-hydroxybutyrate (Table II) was found to give a slightly more oxidized value than that indicated by the mass-action ratio of total NAD. The difference observed in this experiment between the internal and external concentration ratio of acetoacetate to β-hydroxybutyrate was not consistently found (*cf.* Tables IV–VI). It may be caused mainly by technical com-

TABLE II

THE OXIDOREDUCTION STATE OF THE β-HYDROXYBUTYRATE REDOX COUPLE AND THE MASS-ACTION RATIO OF TOTAL NAD AND NADP IN ISOLATED RAT-LIVER MITOCHONDRIA

The reaction medium contained the standard components *plus* 10.0 mg mitochondrial protein. ^3H_2O and either [^{14}C]DMO or [^{14}C]sucrose were added in parallel incubations. After a 3-min preincubation, 0.38 mM acetoacetate *plus* 1.60 mM β-hydroxybutyrate (Expt A), or 1.17 mM acetoacetate *plus* 0.56 mM β-hydroxybutyrate (Expt B) were added. Quenching of the reactions and further treatment of the samples as described under Methods. Samples obtained from two parallel incubations were pooled. Abbreviations: AcAc, acetoacetate; β-OHB, β-hydroxybutyrate.

Expt	*Reaction time (min)*	*pH*		*AcAc/β-OHB*		*NAD$^+$/NADH*			*NADP$^+$/NADPH total*
		In	*Out*	*In*	*Out*	*Total*	*From AcAc/β-OHB*		
							In	*Out*	
A	0	7.73	7.46	—	0.25	0.7	—	—	0.08
	10	7.50	7.45	0.19	0.37	2.2	3.3	6.4	0.05
	20	7.52	7.44	0.21	0.37	1.9	3.5	6.1	0.09
B	0	7.73	7.46	—	2.1	0.7	—	—	0.08
	10	7.53	7.45	1.0	2.9	11	16	46	0.08
	20	7.47	7.44	1.0	3.2	20	19	59	0.11

TABLE III

THE OXIDOREDUCTION STATE OF THE GLUTAMATE REDOX COUPLE AND THE MASS-ACTION RATIO OF TOTAL NAD AND NADP IN ISOLATED RAT-LIVER MITOCHONDRIA

Reaction conditions were the same as described in the legend to Table II. After a 3-min preincubation, either 0.18 mM α-oxoglutarate, 0.20 mM NH_4Cl and 1.94 mM glutamate (Expt A), or 0.88 mM α-oxoglutarate, 0.75 mM NH_4Cl and 0.53 mM glutamate (Expt B) were added. Abbreviations: α-OG, α-oxoglutarate; Glu, glutamate.

Expt	*Reaction time (min)*	*pH*		*α-OG·NH$_3$/Glu (μM)*		*NAD$^+$/NADH*			*NADP$^+$/NADPH*		
		In	*Out*	*In*	*Out*	*Total*	*From α-OG·NH$_3$/Glu*		*Total*	*From α-OG·NH$_3$/Glu*	
							In	*Out*		*In*	*Out*
A	0	7.73	7.46	—	19.4	0.7	—	—	0.08	—	—
	10	7.53	7.44	15	6.6	5.8	6.1	2.7	0.09	9.2	4.1
	20	7.49	7.42	14	6.9	5.8	6.2	3.1	0.11	9.4	4.7
B	0	7.73	7.46	—	1240	0.7	—	—	0.08	—	—
	10	7.62	7.44	740	710	>25	240	230	0.3	370	350
	20	7.54	7.42	340	550	>25	130	220	1.2	200	330

plications in the determination of the intramitochondrial concentrations, especially of β-hydroxybutyrate.

Using the equilibrium constants of both the NAD- and the NADP-linked reaction, the oxidoreduction states indicated by the intra- and extramitochondrial

TABLE IV

TIME COURSE OF CHANGES IN THE OXIDOREDUCTION STATE OF FREE AND MASS-ACTION RATIO OF TOTAL NICOTINAMIDE NUCLEOTIDES IN ISOLATED RAT-LIVER MITOCHONDRIA

The incubation medium contained the standard components, *plus* 1 mM aminooxyacetate and 13.0 mg mitochondrial protein. Four parallel incubations were run for each reaction time, one of which contained ^3H_2O and [^{14}C]DMO, and another ^3H_2O and [^{14}C]sucrose. After a 3.5-min preincubation, the reactions were started by addition of glutamate (2 mM), α-oxoglutarate (0.4 mM) and acetoacetate (0.7 mM). At $t = 1.5$ min 10 μl of either DL-β-hydroxy[^{14}C]butyrate (specific activity 100 μCi/ml) or α-[^{14}C]oxoglutarate (specific activity 100 μCi/ml) was added to the incubations without [^{14}C]DMO or [^{14}C]sucrose. Further treatment as described under Methods. For the determination of the intramitochondrial concentrations the mitochondrial extracts of the four parallel incubations were pooled. Abbreviations: AcAc, acetoacetate; α-OG, α-oxoglutarate; β-OHB, β-hydroxyl butyrate.

Reaction time (min)	*pH*		*AcAc/β-OHB*		*α-OG·NH₃/Glu (μM)*		*NAD⁺/NADH*			*NADP⁺/NADPH*			*Spec. act. β-OHB/ Spec. act. AcAc*	*Spec. act. α-OG/ Spec. act. Glu*
	In	*Out*	*In*	*Out*	*In*	*Out*	*Total*	*From AcAc/β-OHB*		*Total*	*From α-OG·NH₃/Glu*			
								In	*Out*		*In*	*Out*		
0	8.08	7.72	—	25	—	23	2.0	—	—	0.07	—	—		
1.5	8.15	7.76	6.0	28	47	11	11	23	110	0.63	7.7	1.6	> 1000	75
7.5	8.13	7.75	7.3	13	65	13	4.0	29	52	0.71	10	2.0	—	16
13.5	8.12	7.76	5.8	6.2	80	20	15	24	26	1.0	13	3.2	2.5	—
19.5	8.12	7.76	7.4	7.7	92	15	14	30	32	1.2	15	2.6	1.1	2.3
25.5	8.08	7.76	3.2	5.5	150	34	14	15	25	1.2	26	5.8	2.0	1.2

concentrations of components of the glutamate couple were compared with the mass-action ratios of total NAD and NADP (Table III). It appears that the oxidoreduction state of the nicotinamide nucleotide pool reacting in the glutamate dehydrogenase reaction is very close to that indicated by the mass-action ratio of total NAD, and much more oxidized than that indicated by total NADP.

In the experiment shown in Table IV the relationship between the glutamate and β-hydroxybutyrate couples was investigated under conditions where the substrates of both dehydrogenase reactions were added together. After a 3.5-min preincubation in the standard medium *plus* 1 mM aminooxyacetate (an inhibitor of aspartate transaminase (EC 2.6.1.1)[38]) glutamate, α-oxoglutarate and acetoactate were added. In parallel incubations α-[^{14}C]oxoglutarate or β-hydroxy[^{14}C]butyrate were added in order to compare the changes in specific activities of α-oxoglutarate and glutamate, and of β-hydroxybutyrate and acetoacetate, respectively. (The equilibration of label between α-oxoglutarate and glutamate *via* aspartate transaminase was prevented by the preincubation with aminooxyacetate.)

The intra- and extramitochondrial ratios of acetoacetate to β-hydroxybutyrate gradually approached each other until, after 13.5 min, a similar value was obtained. The glutamate couple showed a slow oxidation throughout the reaction period, mainly caused by a gradual increase in the concentration of ammonia (not shown). Total nicotinamide nucleotides were oxidized during the initial phase of the reaction and reached a constant level after 13.5 min. Total NADP gave a 10–15 times more reduced value than total NAD. The β-hydroxybutyrate couple again indicated that free NAD was somewhat more oxidized than the value indicated by the mass-action ratio of total NAD. The intramitochondrial glutamate couple initially indicated an oxidoreduction state of free NADP slightly more reduced than that of free NAD, although much more oxidized than that indicated by total NADP. (In this and the following tables only the values calculated with the NADP-dependent equilibrium constant are given.) However, after longer reaction times the glutamate couple became more oxidized until eventually similar values were found for free NAD and NADP. It should be mentioned that if free NAD and free NADP are both in equilibrium with the glutamate dehydrogenase couple, NADP would be expected to be 1.5 times more oxidized than NAD (*cf.* Table I).

The equilibration of ^{14}C label in the two redox couples is given in the last column of Table IV as the ratio of specific activities of β-hydroxybutyrate to acetoacetate and of α-oxoglutarate to glutamate. It appears that in a 13.5-min reaction time the β-hydroxybutyrate–acetoacetate system had approached equilibration (within the limits of accuracy in this experiment*; ratio of specific activities 1.1–2.5). The glutamate–α-oxoglutarate system equilibrated within 20–25 min. Thus, the ratio of specific activities found indicated that the two dehydrogenase reactions were close to equilibrium under conditions where the glutamate and β-hydroxybutyrate couples had reached the same potential.

This conclusion is supported by the experiment shown in Table V, where

* In the β-hydroxybutyrate system there is a relatively high contribution of L-β-hydroxybutyrate to the total activity measured. In the α-oxoglutarate system the α-[^{14}C]oxoglutarate was eluted from the column as a very broad peak (divided over 40–50 fractions). With the activities applied in this experiment the correction for background activity was relatively high. Thus the ratios of specific activities found are probably not significantly different from unity.

TABLE V

THE OXIDOREDUCTION STATE OF FREE AND MASS-ACTION RATIO OF TOTAL NICOTINAMIDE NUCLEOTIDES AT DIFFERENT SUBSTRATE RATIOS OF THE GLUTAMATE AND β-HYDROXYBUTYRATE COUPLES

The incubation medium contained the standard components, *plus* 24.7 mg mitochondrial protein and different combinations of substrates as indicated. ^3H_2O and either [^{14}C]DMO or [^{14}C]sucrose were added in parallel incubations. Reactions were stopped after 12 min as described under Methods. Samples from three parallel incubations were pooled. Abbreviations: AcAc, acetoacetate; β-OHB, β-hydroxybutyrate; α-OG, α-oxoglutarate.

Additions (mM)					*pH*		*AcAc/β-OHB*		*α-OG·NH₃/Glu (μM)*		*NAD⁺/NADH*			*NADP⁺/NADPH*		
AcAc	*β-OHB*	*Glu*	*NH_4Cl*	*α-OG*	*In*	*Out*	*In*	*Out*	*In*	*Out*	*Total*	*From AcAc/β-OHB*		*Total*	*From α-OG·NH₃/Glu*	
												In	*Out*		*In*	*Out*
—	0.75	0.80	—	0.60	8.03	7.68	0.56	0.07	10	19	0.78	2.8	0.35	0.02	1.9	3.7
—	0.75	—	1.31	0.60	8.00	7.64	2.1	1.1	22	21	3.1	11	6.0	0.07	4.6	4.3
0.65	—	0.80	—	0.60	8.05	7.67	7.2	2.3	100	110	5.2	35	11	0.10	19	21
0.25	0.50	—	1.31	0.60	8.10	7.67	8.8	3.5	72	36	6.1	39	15	0.32	12	6.0

TABLE VI

EFFECT OF FCCP *PLUS* OLIGOMYCIN ON THE OXIDOREDUCTION STATE OF FREE AND THE MASS-ACTION RATIO OF TOTAL NICOTINAMIDE NUCLEOTIDES IN ISOLATED RAT-LIVER MITOCHONDRIA

The incubation medium contained the standard components, *plus* 0.46 mM α-oxoglutarate, 0.39 mM NH_4Cl, 1.42 mM glutamate, 0.21 mM acetoacetate, 0.64 mM β-hydroxybutyrate and 15.2 mg mitochondrial protein. Where indicated 4 μM FCCP and 10 μg oligomycin were also present. In parallel incubations ^3H_2O and either [^{14}C]DMO or [^{14}C]sucrose were added. Samples of two parallel incubations were pooled.

Reaction time (min)	*pH*		*AcAc/β-OHB*		*α-OG·NH₃/Glu (μM)*		*NAD⁺/NADH*			*NADP⁺/NADPH*		
	In	*Out*	*In*	*Out*	*In*	*Out*	*Total*	*From AcAc/β-OHB*		*Total*	*From α-OG·NH₃/Glu*	
								In	*Out*		*In*	*Out*
No additions												
0				0.33		126						
4	7.86	7.44	1.7	0.85	8.8	15	5.3	13	6.4	0.52	2.5	4.3
9	7.83	7.43	2.7	1.0	25	12	4.7	22	8.0	0.44	7.7	3.7
14	7.93	7.43	4.6	1.2	25	10	4.8	29	7.8	0.43	6.1	2.5
FCCP + oligomycin												
0				0.33		126						
4	7.57	7.35	2.4	0.59	29	49	3.3	35	8.7	3.9	16	27
9	7.62	7.37	1.3	0.67	25	45	3.7	17	8.8	4.2	12	44
14	7.55	7.30	2.0	0.70	23	39	3.7	30	11	4.2	12	23

the substrates of glutamate and β-hydroxybutyrate dehydrogenase were added in different combinations, in order to approach the two equilibria from different directions. Irrespective of the combination of substrates added, a relatively constant relationship was found between the ratio of total NAD^+/NADH and that of acetoacetate/β-hydroxybutyrate, especially when the extramitochondrial concentrations of the latter were used. (The deviation of the extramitochondrial substrate ratio to the reduced side, found in the first incubation, could indicate that no equilibrium was reached in this case. This may be related to the extremely low level of the oxidized substrates, acetoacetate and ammonia, under these conditions.) The glutamate couple was found again to indicate an oxidoreduction state of free NADP very close to that of free NAD and the mass-action ratio of total NAD, independent of the combination of substrates added. Total NADP gave a 20–50 times more reduced value than total NAD under all conditions used.

The effect of the mitchondrial energy state on the different redox couples was investigated in the experiment shown in Table VI. All substrates of glutamate and β-hydroxybutyrate dehydrogenase were added simultaneously and the time course of the reaction was followed both in the standard reaction medium (ATP serving as a source of energy) and in the presence of FCCP *plus* oligomycin. Under both conditions an initial oxidation of β-hydroxybutyrate occurred, coupled to a reduction of α-oxoglutarate (*plus* ammonia). No significant changes were found after a 4–9-min reaction time. In the control (Expt A) the usual picture is observed again: the intramitochondrial glutamate couple indicates an oxidoreduction state approximately equal to that of free NAD and that indicated by the mass-action ratio of total NAD, while total NADP gives a much more reduced value. The addition of FCCP (+ oligomycin) caused the mass-action ratio of total NADP to change to the same value as that of total NAD. However, the intramitochondrial glutamate couple became only slightly more oxidized. This small change may indicate a certain degree of interaction with total NADP, but the effect is too small to exclude the possibility that secondary factors are responsible (*e.g.* effects of FCCP on the determination of pH_{in}; see Discussion).

Thus, it must be concluded from this experiment that the oxidoreduction state as calculated from the glutamate couple does not exhibit the energy-dependent changes that are observed in total NADP, and that are related to the action of the energy-linked transhydrogenase.

DISCUSSION

The experiments shown in this paper leave little doubt that under the conditions employed, the near-equilibrium situation is approached closely for the two dehydrogenase reactions studied. Three lines of evidence support this conclusion. (a) A rapidly completed adjustment of the nicotinamide nucleotide oxidoreduction state occurs when the substrate of only one of the dehydrogenases are added, *i.e.* when the net flow of hydrogen through the system is negligible (Figs 1A and 1B, Tables II and III). Measurements done after 10 or 20 min are not significantly different. (*b*) Equilibration of ^{14}C label among the oxidized and reduced substrates was observed when the two redox couples were at the same potential (Table IV). However, this preliminary result should be confirmed under conditions where a

more accurate determination of the specific activities is possible. (*c*) The observed relationship between the redox couples is independent of the direction from which the equilibria are approached (TableV).

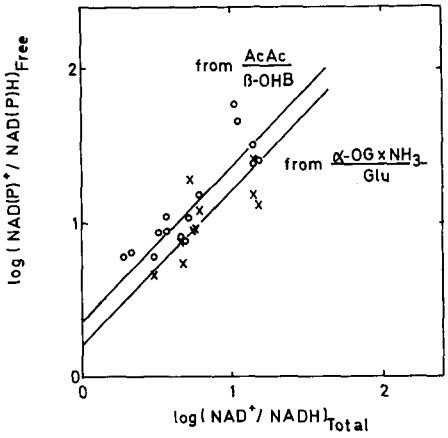

Fig. 2. Relationship between the mass-action ratio of total NAD, and the oxidoreduction state of free NAD(P) as calculated from the β-hydroxybutyrate (β-OHB) and glutamate (Glu) redox couples. The data were taken from Tables II–VI. Only data from incubations where equilibrium had presumably been approached are plotted. ×—×, from α-oxoglutarate (α-OG) × NH_3/Glu; ○—○, from acetoacetate (AcAc)/β-hydroxybutyrate (β-OHB).

The most constant relationship between the redox potential of the β-hydroxybutyrate couple and that indicated by the mass-action ratio of total NAD was obtained when the extramitochondrial acetoacetate/β-hydroxybutyrate ratio was used (see Fig. 2). From the intercept on the vertical axis the difference in midpoint potential between free NAD and that indicated by the mass-action ratio of total NAD can be calculated to be about 10 mV. This number should, however, be interpreted with caution. In the first place, the present results cannot exclude the possibility that the intramitochondrial ratio of acetoacetate to β-hydroxybutyrate is slightly different from the external one. Secondly, a critical assumption in the determination of the intramitochondrial pH from the distribution of DMO is that the activity coefficient for the DMO anion in the matrix space (f_{in}) is equal to that in the incubation medium (f_{out}). If f_{in} were smaller than f_{out}, the DMO method would result in an overestimation of pH_{in} to the extent of $\log(f_{in}/f_{out})$ (*cf.* refs 9 and 39). Consequently, the oxidoreduction states of free NAD^+/NADH may be higher than those reported in this paper. Thus, the difference in midpoint potential between free and 'total' NAD may be more than 10 mV.

No clear relationship was found between the intra- and extramitochondrial glutamate couple under the conditions used. This is not unexpected, since the distribution of α-oxoglutarate, glumate and ammonia may be influenced by many different factors, such as the concentration of counter anions for exchange–diffusion, the pH difference across the membrane and the mitochondrial energy state. The collected data, shown in Fig. 2, emphasize the close similarity between the redox potentials of the intramitochondrial glutamate couple and the β-hydroxybutyrate couple. The difference in midpoint potential between total NAD (as

indicated by the mass-action ratio) and the pool of nicotinamide nucleotides reacting with glutamate dehydrogenase is calculated to be 6 mV. The small difference in redox potential found between the glutamate and β-hydroxybutyrate couple may be caused by an overestimation of the intramitochondrial pH. This would affect the calculation of the glutamate couple more than the β-hydroxybutyrate couple, since the pH_{in} is involved in the estimation of the internal ammonia concentration (Eqn 3). Alternatively, the observed potential difference between the two redox couples may indicate that the oxidoreduction state of the glutamate couple is influenced to a small extent by interaction with the highly reduced 'total' NADP.

The conclusion that the glutamate and β-hydroxybutyrate redox couples indicate a similar oxidoreduction state of free nicotinamide nucleotides, close to that indicated by the mass-action ratio of total NAD, is in agreement with the observations of Krebs and coworkers[1,4] on freeze-clamped rat liver. However, this finding does not necessarily mean that the energy-linked transhydrogenase is not operative; under these conditions the mass-action ratio of total NADP gives a highly reduced value (Tables II–VI). Thus the question of the occurrence of the energy-linked transhydrogenase *in vivo* remains open.

With respect to the nicotinamide nucleotide specificity of glutamate dehydrogenase in intact rat-liver mitochondria different interpretations are possible. (*a*) Glutamate dehydrogenase may react preferentially with NAD under these conditions and not with NADP. (This does not exclude the possibility that under other conditions the enzyme might react preferentially with NADP.) However, some of the crucial experiments of Papa *et al.*[16], from which it was concluded that glutamate dehydrogenase reacts preferentially with NADP, were performed under very similar conditions. (*b*) If glutamate dehydrogenase can react effectively with NADP under these conditions (irrespective of a reactivity of the enzyme with NAD), it must be concluded that rat-liver mitochondria contain a pool of free NADP that has the same oxidoreduction state as free NAD. This may indicate that the difference in midpoint potential between free NADP and the value given by the mass-action ratio of total NADP is much larger than that between free NAD and the corresponding value of total NAD. Since the effect of FCCP (*plus* oligomycin) on the mass-action ratio of total NADP is not reflected in the glutamate couple (Table VI), the mitochondrial energy state would influence the difference in midpoint potential between the different pools of NADP. Alternatively, it may be envisaged that there is a kinetic distinction between the pool of NADP reacting with glutamate dehydrogenase and that reacting with the transhydrogenase. Glutamate dehydrogenase may be unable to compete effectively with the highly active energy-linked transhydrogenase, and its substrate couple would thus reflect mainly the oxidoreduction state of NAD.

The experiments reported in this paper do not allow a distinction to be made between these alternatives. In a forthcoming paper the interaction between glutamate dehydrogenase and the energy-linked transhydrogenase in rat-liver mitochondria will be discussed further on the basis of a reinvestigation of the nicotinamide nucleotide specificity of glutamate dehydrogenase. In this paper, the relationship between free and total NAD(P) and the energetic implications of the interaction between glutamate dehydrogenase and the energy-linked transhydrogenase will also be discussed.

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