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Effects of pH on the Intra- and Extramitochondrial Nicotinamide Adenine Dinucleotide Systems

H. I. D. THIEDEN, S. E. HANSEN and J. DICH

Department of Biochemistry A, University of Copenhagen, Juliane Maries vej 30, DK-2100 Copenhagen Ø, Denmark

Rat liver slices were incubated in a fructose-containing medium at different pH for one hour at 37°C, and the metabolites lactate, pyruvate, β-hydroxybutyrate, acetoacetate, dihydroxyacetone phosphate, and glycerol-1-phosphate were determined at the end of the incubation period. The changes in the concentrations of lactate and pyruvate could be explained as a direct effect of $[H^+]$, whereas the changes in the intramitochondrial metabolites β-hydroxybutyrate and acetoacetate could be explained as effects which are secondary to changes in cytoplasmic $[NAD^+]/[NADH]$ ratio. The glycerophosphate dehydrogenase system does not seem to be in equilibrium with the cytoplasmic $NAD^+ - NADH$ pool.

It is generally accepted, and used in calculations, that the hydrogen ion concentration, $[H^+]$, in the cell under normal conditions is nearly constant.[1–4] In experiments *in vitro*, however, when homogenates, liver slices, or the perfused livers are used, changes in $[H^+]$ during the time of observation often occur. A change in pH from 7.4 to 7.2, which may be considered moderate, gives rise to an increase in the $[H^+]$ of about 60 %.

Such changes in $[H^+]$ would be expected to affect the concentration ratios of metabolites involved in reactions in which also H^+ takes part. The purpose of this investigation was to determine in rat liver slices incubated with a modified Robinson buffer, the effect of the $[H^+]$ on the following redox systems: (1) pyruvate/lactate, (2) dihydroxyacetone phosphate/glycerol-1-phosphate, and (3) β-hydroxybutyrate/acetoacetate.

MATERIALS AND METHODS

Female rats of the Wistar strain weighing about 200 g were used. All rats were fed *ad libitum*. The animals were killed by dislocation of the neck. Livers were rapidly removed and placed on ice. Slices 0.3 mm thick were cut with a McIlwain-Buddle tissue chopper and transferred to prepared Erlenmeyer flasks, containing 10 ml of cold incubation medium. The incubation medium was a modified Robinson buffer. Constituents in the buffer were the usual [5] except that the solution of NaCl contained 40 mM tris(hydroxy-

methyl)aminomethane adjusted to the pH used, the total molarity being 0.154 M. Ca^{2+} concentration was 1.3 mM.

Fructose (11 mM) was used as substrate as this is known to produce sufficiently high concentration of pyruvate to allow the estimation of pyruvate and of the [lactate]/[pyruvate] ratio with reasonable accuracy. Each Erlenmeyer flask contained 0.5 g of tissue. All flasks were gassed with oxygen for 5 min at the beginning of the incubation and again for 1 min after 30 min of incubation. The total incubation time was 60 min. The flasks were shaken in a thermostatically controlled water bath at 37°C.

As pH decreases during the incubation a separate set of experiments were performed in a pH-stat (Radiometer, Copenhagen). pH was kept constant by continuous titration with NaOH of the acid produced. The same medium as above was used, but O_2 was bubbled through during the whole period (1 h) to ensure oxygenation and stirring.

The concentrations of metabolites were determined at the end of the incubation period. Determinations of lactate,[6] pyruvate,[7] acetoacetate,[8] and β-hydroxybutyrate [9] were made on the medium after deproteinization with 1 M perchloric acid, whereas the determinations of dihydroxyacetone phosphate [10] and glycerol-1-phosphate [11a] were made on the slices after homogenization.

All reagents used were analytical grade. The enzymes and cofactors were obtained from Boehringer Corp. (Mannheim, Germany).

RESULTS

1. *pH in the medium during incubation.* In experiments in which pH was not kept constant, a decrease in pH was seen during the time of incubation. Most of the decrease occurred during the first 10 min of incubation. The change was only small in experiments with low initial pH, possibly due to a higher buffer capacity of the medium or a lower production of acid equivalents. When in the following a decrease or an increase in $[H^+]$ is mentioned, this does only refer to differences in final pH-values, not changes, if any, during the period of incubation.

2. *Effects of pH on the metabolites of some pyridine-nucleotide linked dehydrogenases.* Both in experiments in which pH was kept constant and in experiments where it decreased during the incubation, increase of $[H^+]$ caused a decrease both in the lactate and pyruvate concentrations, when measured at the end of the incubation. Pyruvate was diminished more than lactate, and consequently the [lactate]/[pyruvate] ratio was increased (Tables 1 and

Table 1. Effects of pH on the concentrations of pyruvate, lactate, dihydroxyacetone phosphate, and glycerol-1-phosphate. Rat liver slices were incubated in buffer with varying pH at 37°C for 1 h. Fructose (11 mM) was used as substrate. The concentrations of lactate and pyruvate are given in nmol/ml incubation medium as means of 8 experiments ± S.E.M. and glycerol-1-phosphate and dihydroxyacetone phosphate in nmol/g wet wt. of liver as means of 8 experiments ± S.E.M. The ratios were determined for each experiment; the S.E.M. then calculated in the usual way.

pH Initial	pH Final	[Pyruvate]	[Lactate]	$\frac{[Lactate]}{[Pyruvate]}$	$\frac{[NAD^+]}{[NADH]}$	[Dihydroxyacetone phosphate]	[Glycerol-1-phosphate]	$\frac{[Glyc\text{-}1\text{-}P]}{[Dihydroxyacetone\ phosphate]}$
8.00	7.31	178 ± 15	1777 ± 130	10.1 ± 0.5	435	109 ± 9	653 ± 45	6.2 ± 0.6
7.60	6.98	105 ± 10	1114 ± 72	10.9 ± 0.9	865	110 ± 5	529 ± 35	4.9 ± 0.4
7.20	6.68	65 ± 9	921 ± 89	16.1 ± 3.0	1166	98 ± 10	451 ± 25	5.2 ± 0.8
6.80	6.48	35 ± 5	616 ± 23	20.7 ± 3.5	1443	97 ± 11	452 ± 16	5.2 ± 0.8
6.40	6.32	19 ± 2	546 ± 35	32.7 ± 6.1	1316	100 ± 8	404 ± 35	4.2 ± 0.5

Table 2. Effects of pH on the concentrations of acetoacetate and β-hydroxybutyrate. The experimental details are as for Table 1. Results are expressed in nmol/ml as means of 5 experiments ±S.E.M.

pH		[Acetoacetate]	[β-Hydroxybutyrate]	$\frac{[\beta\text{-Hydroxybutyrate}]}{[\text{Acetoacetate}]}$
Initial	Final			
8.50	7.82	127±10	112±8	0.91±0.04
8.00	7.38	118±16	100±5	0.96±0.07
7.50	6.95	108±18	78±5	0.79±0.06
7.00	6.62	98 ±21	54±5	0.61±0.06
6.50	6.35	81 ±10	34±3	0.48±0.08

3). In the experiments, where pH was allowed to decrease, the rate of formation of each metabolite during the incubation period has been determined. It was constant with time in the individual experiments, regardless of the decrease in pH.

Also the concentrations of both acetoacetate and β-hydroxybutyrate were lowered when $[H^+]$ in the medium was increased. The ratio of [β-hydroxybutyrate]/[acetoacetate] was unchanged in the pH interval between 7.4 and

Table 3. Effect of constant pH on the concentrations of pyruvate, lactate, β-hydroxybutyrate, and acetoacetate. The experimental details are as for Table 1, except that pH was kept constant by titration and the rat liver slices were constantly oxygenated. The concentrations of lactate and pyruvate, β-hydroxybutyrate and acetoacetate are given in nmol/ml, as means of 7 experiments ±S.E.M.

pH	[Pyruvate]	[Lactate]	$\frac{[\text{Lactate}]}{[\text{Pyruvate}]}$	[Acetoacetate]	[β-Hydroxybutyrate]	[β-Hydroxybutyrate]/[Acetoacetate]
8.0	118±19	1283±139	12.0±1.7	92±16	59±11	0.78±0.18
7.4	103±14	1094±116	12.9±3.0	76±10	49± 6	0.92±0.22
6.4	29±12	440 ±78	28.4±8.0	73± 6	26± 2	0.34±0.04

8.0, but a further decrease in pH resulted in lowering of the ratio mentioned (Tables 2 and 3). The concentration of dihydroxyacetone phosphate was not affected by variations in pH, whereas the glycerol-1-phosphate concentration was slighly decreased by an increase $[H^+]$. Consequently the ratio of [glycerol-1-phosphate]/[dihydroxyacetone phosphate] was also slightly decreased (Table 1).

DISCUSSION

The results presented in this work show, that a variation of pH of the incubation medium produces changes in the ratio between the reduced and the oxidized metabolites in the three pyridine nucleotide linked dehydrogenase

systems: lactate/pyruvate, β-hydroxybutyrate/acetoacetate, and glycerol-1-phosphate/dihydroxyacetone phosphate. In the experiments $[H^+]$ in the incubation medium was primarily changed. A change in the medium will, however, provided that no active counterregulation takes place, give rise to a change in the same direction in the cytoplasm. Thus the observed changes in the ratios of the metabolites are probably due to changes in the cytoplasmic pH.

It is widely accepted that a state of equilibrium or near-equilibrium exists in the cytoplasm of liver cells between the components of the lactate dehydrogenase system. An increase of $[H^+]$ in the medium will consequently result in a shift of this equilibrium (1) towards the left. The result of this

$$\text{lactate} + NAD^+ \rightleftharpoons \text{pyruvate} + NADH + H^+ \quad (1)$$

will be a rise in the [lactate]/[pyruvate] ratio provided that no active counterregulation takes place on the level of the [lactate]/[puryvate] or on the $[NAD^+]/[NADH]$ ratio. In the pH interval between 7.0 and 8.0 the [lactate]/[pyruvate] ratio was constant, but a further decrease in pH resulted in an increased [lactate]/[pyruvate] ratio, a consequence to be expected from the considerations mentioned above. The increase in hydrogen ion concentration by a factor 5 increased the [lactate]/[pyruvate] ratio 2 to 3 times, indicating that the ratio between NAD^+ and NADH is increased to the same extent.

If the components of the lactate dehydrogenase system did not participate in other reactions, stoichiometry should exist between the amount of NADH oxidized to NAD^+ and the amount of pyruvate reduced to lactate. This means that if the concentration of lactate and pyruvate present is much higher than that of the nucleotides, a given change in the ratio between NAD^+ and NADH would correspond to a much smaller change in the ratio between lactate and pyruvate. This could explain why the effect of the changes in hydrogen ion concentration is most pronounced on the $[NAD^+]/[NADH]$ ratio when pH is about 7.4 (Tables 2 and 3). When, however, pH is decreased and the lactate and pyruvate concentration becomes equal to that of the nucleotides (about 0.6 mM [11b]), changes in hydrogen ions will affect both the $[NAD^+]/[NADH]$ and the [lactate]/[pyruvate] ratios to an equal degree.

The experimental results, however, do not provide any possibility to verify these considerations. It is the free NADH, which is supposed to be in equilibrium with other components of the lactate dehydrogenase system and it is not possible from the results to estimate how the distribution between the free and the bound NADH depends on changes in pH.

Considerations analogous to those used on the [lactate]/[puryvate] ratio in the cytoplasm, can be applied to the redox couple β-hydroxybutyrate/acetoacetate, which in the mitochondria is supposed to be in equilibrium with free NAD^+ and NADH [12] according to reaction scheme (2)

$$\beta\text{-hydroxybutyrate} + NAD^+ \rightleftharpoons \text{acetoacetate} + NADH + H^+ \quad (2)$$

Provided changes in pH in the mitochondria are parallel to those in cytoplasm, an increase in $[H^+]$ should displace also this equilibrium to the left and consequently it should increase the [β-hydroxybutyrate]/[aceto-

acetate] ratio. However, no increase was observed. On the contrary the [β-hydroxybutyrate]/[acetoacetate] ratio decreased about 50 %, when pH in the incubation medium was lowered from approximately 7.4 to 6.4 (Tables 2 and 3).

In the following it is taken for granted that the mitochondrial membrane is essentially impermeable for H^+-ions,[13] and it is assumed that there is metabolic communication between the cytoplasmic and mitochondrial NAD^+-NADH pools. The latter assumption is difficult to prove under all metabolic conditions in the liver cell, but several transport systems, which could mediate such a communication, have been proposed.[14a, 14b] In addition direct evidence has been given for the transport of reducing equivalents through the mitochondrial membranes during gluconeogenesis [15] and during ethanol metabolism.[16]

An increase in $[H^+]$ will give rise to an increase of the $[NAD^+]/[NADH]$ ratio in the cytoplasm (calculated results, see Table 1), and this increase will be reflected in the mitochondrial $[NAD^+]/[NADH]$ ratio, provided that no substantial increase in $[H^+]$ in the mitochondira occurs (this requires active transport of H^+-ions out of the mitochondria [3]), and provided also that the $[NAD^+]/[NADH]$ratio is not actively changed by processes inside the mitochondria. An increase in the ratio of$[NAD^+]/[NADH]$ will displace the mitochondrial equilibrium (2) to the right and cause a decrease in the [β-hydroxybutyrate]/[acetoacetate] ratio.

Increased $[H^+]$ caused changes in the [β-hydroxybutyrate]/[acetoacetate] ratio which were opposite to changes in the [lactate]/[pyruvate] ratio. A similar situation where the two ratios are changed in opposite directions is found in alloxan diabetes,[2] and could be caused by the acidosis present.

As mentioned previously the results suggest that pH in mitochondria and cytoplasm is not identical and that it does not exhibit a parallel change. The consequence is that in our experiments it is not possible to calculate the mitochondrial $[NAD^+]/[NADH]$ ratio by determining the concentrations of acetoacetate and β-hydroxybutyrate.

The glycerol-1-phosphate dehydrogenase system does not seem to be in equilibrium with the cytoplasmic NAD^+/NADH pool as assumed by several authors,[17–19] as the ratio of [glycerol-1-phosphate]/[dihydroxyacetone phosphate] decreases with increasing $[H^+]$ in contrast to the [lactate]/[pyruvate] ratio. Bücher [20] and Hohorst [19] have arrived to the same conclusion investigating thyreotoxic liver. This result might be expected from the existence of two separate glycerol-1-phosphate/dihydroxyacetone phopshate pools intra- and extramitochondrially.[21]

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