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The content of NADH in rat skeletal muscle at rest and after cyanide poisoning

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The concentration of NADH was determined a high-oxidative mucle (soleus) and a high-glycolytic muscle (extensor digitorum longus, EDL) from resting rats. The NADH content of freeze-clamped control muscles was 0.35 ± 0.04 (mean $\pm$ S.D.) and 0.31 ± 0.04 mmol/kg dry wt. in EDL and soleus respectively, and increased to peak values of 0.58 ± 0.05 (EDL) and 0.87 ± 0.10 (soleus) after 10 min of NaCN treatment. The [lactate]/[pyruvate] ratio, which was not significantly changed in soleus and increased only slightly in EDL after NaCN incubation, shows that only minor changes occurred in the cytosolic NADH concentration. Provided that the major part of muscle NADH is located in the mitochondria it can be calculated that the mitochondrial NADH content in skeletal muscle at rest is about 36 (soleus) and 60% (EDL) of the anoxic value, respectively. These results are in contrast with previous studies with the surface-fluorescence technique, where mitochondrial NAD appeared to be almost completely reduced in resting skeletal muscle.

INTRODUCTION

The nicotinamide nucleotides occupy key positions in intermediary metabolism. The redox state of the NAD couple of isolated mitochondria in different metabolic states has been measured by spectrophotometric and fluorimetric techniques. From the early studies it appeared as though NAD was completely reduced when the mitochondria were in State 4 (Chance & Williams, 1955). Later studies, however, showed that the fluorescence (i.e. the NADH concentration) of isolated mitochondria could be increased further by the addition of succinate (Chance & Hollunger, 1961*a*) and during anoxia, i.e. State 5 (Jöbsis & Duffield, 1967; Sugano *et al.*, 1974).

The redox state of the nicotinamide nucleotides in intact muscle tissue has previously been measured by recording the fluorescence from the surface of the muscle (Chance & Jöbsis, 1959; Jöbsis & Duffield, 1967; Jöbsis & Stainsby, 1968; Paddle, 1985). From these studies it appears that mitochondrial NAD is nearly completely reduced in resting muscle.

Quantitative data on the NADH content in tissue are, however, scarce and can be currently obtained only by direct chemical analysis. The inability of reflectance fluorimetry to provide quantitative estimates of NADH has been discussed by Henriksson *et al.* (1986). Chemical determination of NADH and NAD^+ has been performed on heart muscle during aerobic and anaerobic conditions (Chance *et al.*, 1965), but skeletal msucle has not been studied thoroughly in this respect. The aim of the present investigation was to determine (by chemical analysis) the NADH and NAD^+ contents of resting skeletal muscle under aerobic and anaerobic conditions. An additional aim was to compare the NADH content of a high-oxidative muscle, soleus, with that of a high-glycolytic muscle, extensor digitorum longus (EDL).

MATERIALS AND METHODS

Experimental

Adult rats of the Sprague–Dawley strain (approx. 300 g) were anaesthetized with an intraperitoneal injection of pentobarbital sodium (50 mg/kg body wt.). Soleus and EDL muscles were carefully dissected out, leaving the blood supply to the muscles intact. Muscles were either freeze-clamped *in situ* with tongs pre-cooled in liquid N_2 or incubated *in vitro* in a Ringer solution containing NaCN. The ionic composition of the incubation solution was (in mmol/l): Na^+ 150, K^+ 4, Ca^{2+} 2.3, Cl^- 156 and CN^- 3. The temperature was 25 °C and the muscles were incubated for 10 or 20 min and thereafter frozen in liquid N_2. NaCN, which inhibits cytochrome oxidase, was used to block the utilization of O_2 and thus to mimic anaerobic conditions. The advantage of using NaCN instead of incubating the muscle in an anaerobic milieu is that the former treatment does not necessitate depletion of the local O_2 store (which could take considerable time when the energy turnover is low, as is the case in resting muscle) and is thus expected to exert its effect more rapidly.

Analytical methods

The muscle samples were freeze-dried, freed from connective tissue, powdered and divided into two portions, of which one was extracted with KOH, neutralized with HCl and assayed for NADH with a bioluminescent method (Sahlin, 1983). The other portion of the muscle powder was extracted with $HClO_4$ (0.5 M), neutralized with $KHCO_3$ (2.2 M) and assayed for NAD^+, lactate and pyruvate with NADH-coupled enzymic methods (Bergmeyer, 1974). Metabolite contents are expressed as mmol/kg dry wt. and can be converted into mmol/kg wet wt. by dividing by 4.3 (water content = 77%). Data are presented as means $\pm$ S.D., and

Abbreviation used: EDL, extensor digitorum longus.

statistical significance was tested with the Student t test for unpaired values.

RESULTS

The NADH contents of freeze-clamped EDL and soleus muscles were similar (Fig. 1 and Table 1). After 10 min incubation in NaCN, NADH increased in both muscle types (Fig. 1). The increase in NADH was, however, more pronounced in soleus than in EDL. When the incubation time was extended to 20 min, the muscle NADH value was similar to that after 10 min incubation. Incubation for 10 min is thus sufficient for obtaining a peak value of NADH, which most likely corresponds to complete reduction of the mitochondrial NAD (see the Discussion section). NAD^+ decreased after NaCN incubation in both soleus and EDL (Table 1). In EDL the decrease in NAD^+ was similar to the increase in NADH, and thus the sum of NAD^+ and NADH remained constant. In soleus the decrease in NAD^+ was slightly less than the increase in NADH.

[Lactate], [pyruvate] and [lactate]/[pyruvate] ratio increased slightly after NaCN treatment in EDL, but were not significantly altered in soleus (Table 1).

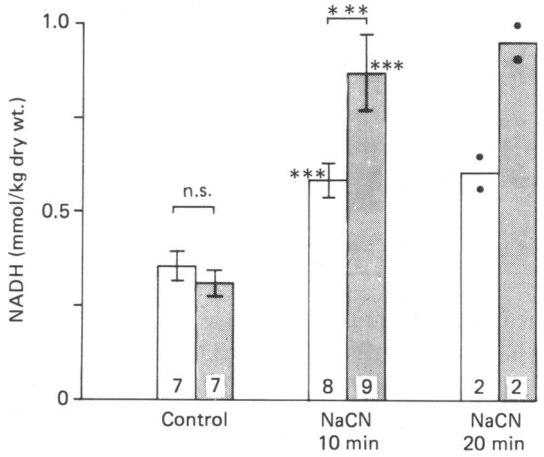

Fig. 1. Effect of NaCN on the concentration of NADH in skeletal muscle from rats at rest

Values are means ± S.D. for the numbers of observations given within the bars. Statistical significance was tested by the unpaired Student t test: ***$P < 0.001$; n.s., $P > 0.05$). □, EDL; ▨, soleus.

DISCUSSION

It is well known that at least two separate pools of the NAD couple exist in the cell (mitochondrial and cytosolic). No reliable method is, however, available whereby the mitochondrial NADH and NAD^+ concentrations *in vivo* can be analytically measured. This is because the fractionation of the tissue is likely to affect the redox state within the mitochondria (Jacobson & Kaplan, 1957). An alternative approach is to calculate [NADH]/[NAD^+] from enzymic equilibrium reactions localized in the specific compartment. Cytosolic [NADH]/[NAD^+] can by this method be calculated from the lactate dehydrogenase reaction, and mitochondrial [NADH]/[NAD^+] has in liver tissue been calculated from the glutamate dehydrogenase reaction. By this method it has been shown in liver tissue that the NAD couple is much more reduced in the mitochondria than in the cytosol (Willamson *et al.*, 1967).

As the activity of glutamate dehydrogenase is very low in skeletal muscle (Williamson *et al.*, 1967), mitochondrial [NADH]/[NAD^+] in this tissue cannot be estimated by this method. The free NADH concentration in the cytosol can, however, be estimated from the lactate dehydrogenase reaction (pyruvate + NADH + H^+ ↔ lactate + NAD^+), assuming this to be near equilibrium (Williamson *et al.*, 1967). When cytosolic [NADH]/[NAD^+] is calculated from the lactate dehydrogenase reaction, it is assumed that the tissue contents of lactate and pyruvate reflect the [lactate]/[pyruvate] ratio within the cytosol. This is reasonable, since the extracellular fluid within the muscle contributes only about 13 and 16% of the total muscle water *in vivo* (Sahlin *et al.*, 1978) and in incubated isolated tissue (Roos, 1975) respectively, and since the [lactate]/[pyruvate] ratio in the extracellular fluid has not only been shown to reflect the cytosolic [lactate]/[pyruvate] ratio but in fact is also used as an index of the cytosolic redox state both in liver (Bücher *et al.*, 1972) and in muscle (Keul *et al.*, 1972).

From the present data it can be calculated that the free NADH concentration in the cytosol accounts for only a small fraction (0.5–1.5%) of the analytically determined NADH {$K_{eq.} = 1.11 \times 10^{-4}$ M (Williamson *et al.*, 1967); pH = 7.0; [NAD^+] in cytosol = 0.9 × total [NAD^+]}. From these calculations it appears likely that the major part of the cellular NADH is localized within the mitochondria in skeletal muscle.

Incubation of the muscle in NaCN resulted in only a small increase in the [lactate]/[pyruvate] ratio (Table 1),

Table 1. Effect of NaCN on the concentration of metabolites in skeletal muscle from rats at rest

Values are means ± S.D., in mmol/kg dry wt. Statistical significance was tested by the unpaired Student t test: ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$; n.s., $P > 0.05$).

	EDL			Soleus		
	Control	NaCN	Control versus NaCN	Control	NaCN	Control versus NaCN
No. of rats . . .	7	8		7	9	
NADH	0.35 ± 0.04	0.58 ± 0.05	***	0.31 ± 0.04	0.87 ± 0.10	***
NAD^+	2.44 ± 0.12	2.15 ± 0.13	**	2.25 ± 0.17	2.00 ± 0.13	**
Lactate	6.7 ± 2.2	14.8 ± 4.3	**	5.7 ± 2.4	7.9 ± 1.8	n.s.
Pyruvate	0.33 ± 0.06	0.44 ± 0.08	*	0.43 ± 0.21	0.40 ± 0.07	n.s.
[Lactate]/[pyruvate]	20.7 ± 6.6	33.3 ± 7.3	**	14.6 ± 5.9	19.8 ± 3.6	n.s.

whereas the analytically determined NADH increased 2–3-fold, which shows that the increase in NADH occurred within the mitochondria. This is in accordance with results from human skeletal muscle, where 20 min of ischaemia resulted in an approx. 2-fold increase in NADH, whereas the [lactate]/[pyruvate] ratio remained unchanged (Sahlin, 1983) and where exercise to exhaustion resulted in an NADH concentration similar to that after 20 min of ischaemia, although [lactate] and the [lactate]/[pyruvate] ratio increased to very high values (Sahlin, 1985; Henriksson *et al.*, 1986), and thus both cytosolic and mitochondrial NAD should be reduced to a large extent.

From studies on isolated mitochondria (Chance & Hollunger, 1961*b*) and intact brain tissue (Chance *et al.*, 1964), it has been concluded that the nicotinamide nucleotides of mitochondria become maximally reduced during the transition from State 4 to anaerobiosis. This is also supported by the observed NADH content of anaerobic perfused rat hearts (1.53 mmol/kg dry wt.; Chance *et al.*, 1965), which is similar to the total mitochondrial $NAD^+ + NADH$ content, 6.2 nmol of $NAD^+ + NADH$/mg of mitochondrial protein (LaNoue *et al.*, 1972), and 240 mg of mitochondrial protein/g dry wt. of heart (Idell-Wenger *et al.*, 1978). Klingenberg (1961) has estimated the mitochondrial content of $NAD^+ + NADH$ in a variety of tissues (brain, kidney, liver, heart and unspecified skeletal muscle). When these tissues were made anaerobic, their NADH content became similar to their mitochondrial content of $NAD^+ + NADH$ (Klingenberg, 1961), which thus appears to become completely reduced during anaerobiosis. Since NaCN inhibits cytochrome oxidase and thus the cellular utilization of O_2, the reduction state of the NAD couple of NaCN-incubated muscles should correspond to that of complete anaerobiosis, and thus mitochondrial NAD is expected to be completely reduced. The NAD^+ content of NaCN incubated muscles was 89% of that of control muscles (Table 1), and thus the NAD^+ in NaCN-treated muscles would be confined to the extramitochondrial space, which is reasonable, since this space occupies approx. 90 and 93% of the total cellular volume in soleus and EDL respectively (Eisenberg, 1983).

From the results in Table 1, and assuming that the major part of tissue NADH is confined to the mitochondria, it can be calculated that the mitochondrial NADH content at rest is about 60 and 36% of the anoxic value in EDL and soleus muscles respectively. Thus the mitochondrial nicotinamide nucleotides are more oxidized in soleus muscle than in EDL under resting conditions. This difference is interesting, considering the high oxidative capacity and capillarization of the soleus muscle. Whether the different reduction state of the mitochondrial NAD couple at rest reflects a difference in O_2 availability or a different degree of metabolic control between the two types of muscles is currently not known. In this respect it is noteworthy that the ATP content and the ATP/ADP ratio (calculated from the total issue contents) are lower in soleus (ATP = 19.7 ± 2.3 mmol/kg dry wt.; ATP/ADP = 6.3) than in EDL (ATP = 28.0 ± 1.0 mmol/kg dry wt.; ATP/ADP = 6.3) than in EDL (ATP = 28.0 ± 1.0 mmol/kg dry wt.; ATP/ADP = 9.4; Edström *et al.*, 1982) and that the free ADP concentration as calculated from data on perfused cat muscle, obtained by n.m.r. measurements (Meyer *et al.*, 1985), is higher in soleus than in biceps muscle (a high-glycolytic muscle). Considering that the adenine nucleotides are important regulators of cellular respiration, it was suggested that the intramitochondrial redox potential might be lower in resting soleus muscle (Meyer *et al.*, 1985). The present data are consistent with this hypothesis.

The estimated reduction state of the mitochondrial NAD couple of skeletal muscle can be compared with that of cardiac muscle. From chemical measurements of NADH under aerobic and anaerobic conditions it can be calculated that the mitochondrial NAD couple in rat is about 12% reduced *in vivo* (Chance *et al.*, 1965) and 5.5–13% in the perfused heart (Chance *et al.*, 1965; Meno *et al.*, 1984). Our own data on perfused rabbit heart show a reduction extent of 4.2% (A. Katz, A. Edlund & K. Sahlin, unpublished work), The mitochondrial NAD couple is thus considerably more oxidized in cardiac muscle than in skeletal muscle, which may be partially explained by the constant activity of cardiac muscle. The contractile activity of the heart will induce an increase in ADP, which will stimulate respiration and thus oxidize mitochondrial NADH. This explanation is in accordance with the original theory of Chance & Williams (1955) (State 4–State 3 transition).

In conclusion, the present report shows that the mitochondrial NAD couple of rat soleus and EDL muscles at rest is about 36 and 60% reduced respectively. This is in contrast with previous studies with the surface fluorescence technique, where NAD appeared to be almost completely reduced in resting skeletal muscle. The different extent of reduction of the mitochondrial NAD couple between a high-oxidative muscle and a high-glycolytic muscle may be related to their inherent metabolic characteristics.

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