NICOTINAMIDE ADENINE DINUCLEOTIDE (NAD) AND REDUCED NAD IN LIVING AND CHILL-STORED DYING MUSCLE OF COD, GADUS CALLARIAS*

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These adenine nucleotides are of key significance in energy relations in many tissues. Little is known about the concentrations and turnover of the compounds in fish muscle operating under varying physiological conditions, or about any changes consequent upon lysis and anaerobiosis in dying muscle. In this paper, we describe the bases of previously reported long-term changes1) 2) 3) in terms of an enzymic cleavage of the nucleotide. We discuss, also, the effects of exercise on both the concentrations of dinucleotide at death and on short-term changes thereafter. These are shown to relate to changes in the ratios of other redox pairs, as studied by Burt and Stroud4) in our laboratory, and (as NAD+/NADH ratios) to ATP concentrations. From earlier investigations on the concentrations of ATP and lactate in cod muscle at the point of rigor5) it seems reasonable to conclude that changes in NAD are related indirectly to the development of rigor mortis.

1. Nicotinamide adenine dinucleotide glycohydrolase (EC 3.2.2.5) activity (NAD+-ase)

This is readily extractable into water and dilute salt solutions and remains in solution after centrifuging for 15 min. at 120,000 g (0°C). A typical pH-activity curve of such a preparation is illustrated in Fig. 1.

![Fig. 1. Effect of pH on NAD+-ase activity. Crude dialysed preparation; incubation 20 hr., 0°C; 10 mg protein; 1.5 mg NAD+; buffer, 0.1 M: total volume 5 ml. Buffers: acetate-veronal, acetate-acetate, glycine-NaOH. NAD+ destruction measured as change in 325 mµ absorption in the presence of CN–6).](image)

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Confirmation of the position of the splitting of the molecule has been obtained by the ion-exchange chromatography of deproteinised incubates (Fig. 2). Analysis of the large peak separated from incubates containing enzyme that had not been denatured by prior boiling yielded the base: phosphate: ribose ratio expected of adenosine 5'-diphosphate ribose. Chromatography of the water washings from columns after the initial placement of mononucleotides and dinucleotides showed the presence of both nicotinamide and nicotinic acid.

Unlike mammalian NAD+-ases, the cod muscle system is not inhibited to any
appreciable degree by one of the products of the reaction, nicotinamide. However, the crude system is very unstable under many of the conditions of conventional enzyme treatments. For instance, dialysis results in considerable loss of activity—as a consequence of denaturation rather than the removal of a cofactor; and freeze-drying largely inactivates the enzyme.

Some success in concentrating the system without loss of activity has been obtained by batchwise manipulation with Sephadex. Column chromatography on such material has separated crude preparations into two peaks of DPN-ase activity, one lying to each side of the main peak of protein concentration (Fig. 3).

2. Changes in NAD\(^+\) and NADH during exercise and within a short period of death.

The techniques for the sampling of living muscle and for exercising fish are described by Burt and Stroud\(^4\) in their paper in this symposium. Common experimental fish were used in these studies. Biopsy samples were removed as chilled cores by high speed drilling and frozen rapidly. ‘Rested’ fish, as described below, were killed with a minimum of struggling after a prolonged period of acclimatization in an aquarium.

Frozen material was extracted differentially for NAD\(^+\) and NADH by the procedure of Villee\(^9\). Extracts were then analysed in a linked alcohol: NAD oxidoreductase (EC 1.1.1.1): phenazine: 2,6-dichlorophenylindophenol system based essentially on that of Slater et al.\(^10\). Adenosine 5′-triphosphate (ATP) was extracted into cold perchloric acid and analysed in a linked automatic ATP: d-hexose-6-phosphotransferase (EC 2.7.1.1.): d-glucose-6-phosphate: NADP oxidoreductase (EC 1.1.1.49) system\(^5\).

Fig. 4 illustrates the effect of exercise on combined concentrations NAD\(^+\) and NADH, and of early post-mortem changes in the exercised fish. Concentrations fell during exercise. Within a short time of death, there was a rapid rise in concentration,
Fig. 5. Effect of chill-storage post-mortem on combined NAD+ and NADH concentrations in the muscles of 'rested' fish.

Fig. 6. Effect of exercise before death and of post-mortem chill-storage, on individual NAD+ and NADH concentration in muscles. For explanation of 'C.S.' etc. see Fig. 4.

Fig. 7. Effects of chill-storage post-mortem on individual NAD+ and NADH concentrations in the muscles of 'rested' fish.
but thereafter there was a steady fall. Although maximal concentrations were somewhat higher (Fig. 5), this sequential rise and fall had its parallel in rested muscle post-mortem. Both NAD⁺ and NADH are concerned in these changes, although in somewhat different time scales. Fig. 6 shows the effects of exercise and post-mortem

![Figure 8](image.png)

**Fig. 8.** Effects of exercise before death, and of post-mortem chill-storage on NAD⁺/NADH ratios in muscles. For explanation of ‘C.S.’ etc., see Fig. 4.

![Figure 9](image.png)

**Fig. 9.** Effects of chill storage post-mortem on NAD⁺/NADH ratios in the muscles of ‘rested’ fish.

storage on the oxidised and reduced forms. Fig. 7 shows post-mortem changes in the muscles of rested fish. In both types of muscle, the increased levels of NADH, relative to concentrations at death, tended to be maintained longer than those of NAD⁺.

In general, NAD⁺/NADH *ratios* tended to instability within the first few hours
of death, but fell thereafter. This fall was more rapid in the muscles of exercised fish than in rested fish (Fig. 8, 9).

Changes in ATP concentrations are illustrated in Figs. 10 and 11. In common with most studies of a similar nature, ATP disappeared more rapidly from exercised muscle.

Fig. 10. Effects of exercise before death, and of post-mortem chill-storage on ATP concentrations in muscles. For explanation of 'C.S.' etc., see Fig. 4.

Fig. 11. Effect of chill-storage post-mortem on ATP concentrations in the muscles of ‘rested’ fish.

Discussion

It is apparent that a number of factors can affect the concentration of NAD+ in the muscles of cod post-mortem. Firstly, it has been established that there are at least two NAD+-ases that can be operative; and there is little doubt that the long-term disappearance of the nucleotide results from their activity. The NAD+-ases of cod muscle differ from those of mammalian tissues in their ready solubility and freedom from inhibition by nicotinamide.

Secondly, it would appear that exercise can alter the proportions of NAD+ to NADH at death. However there is a fairly wide scatter in the data available in this respect, both in these experiments and others. Further experimentation on the time-course of such changes during exercise will be necessary to explain some apparent anomalies, which may be related to variations in blood supply to the tissue. N.P. Thirdly, there are transient increases post-mortem in the combined concentrations of NAD+ and NADH. So far, we have not established the source of this nucleotide.

Fourthly, there are eventual falls in NAD+/NADH ratios. These occur more rapidly in muscle from exercised fish than that from rested fish. To this extent these changes are in somewhat closer accord with the dihydroxyacetone phosphate/glycerophosphate ratios reported for this material by Burt and Stroud, than their
data on the other redox pair, pyruvate/lactate. The ratios of all three pairs agree in their indications considerable changes in the redox status of muscle (at the biochemical level) within a short time of death. Changes in ratios differ, however, by order of magnitude.

Comparison of analyses on rested and exercised muscles indicates a closer relationship between ATP concentrations and NAD+/NADH ratios than between ATP and the absolute concentrations of either the reduced or oxidised cofactor.

From earlier reports on the interrelationships of ATP, lactate, and anaerobiosis e.g.\textsuperscript{5,11} the course of rigor mortis in fish, it would appear from data printed in this paper that more extensive examinations of nicotinamide adenine dinucleotide metabolism in the muscles of different species could be well worthwhile.

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References