Nicotinamide Adenine Dinucleotide (NAD) Content of Liver with Hemorrhagic Shock[1] (37656)

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An earlier report by Loiselle and Denstedt (1) has shown a progressive conversion of the pyridine nucleotides to the reduced form in the livers of rats during shock. This shift was more pronounced in mitochondria than in the cytoplasmic fraction. Work from our laboratory has shown that nicotinamide adenine dinucleotide (NAD)-linked substrates for oxidative phosphorylation are the more sensitive substrates for detecting changes in the functional capability of mitochondria of livers of rats in hemorrhagic shock (2). These studies suggest alteration in the pyridine nucleotide redox state and/or mitochondrial contents in shock. In addition to the mitochondrial enzymes which require NAD as a cofactor for cellular respiration, there are numerous cytoplasmic enzymes which require NAD (3). The nicotinamide adenine dinucleotides are also involved in fatty acid and steroid metabolism and in the excretory functions which involve conjugation (4, 5). Thus the amount as well as the redox state of nicotinamide adenine dinucleotides might be extremely important to cellular function. It semed desirable, therefore, to determine whether the levels of these compounds in the liver of animals were altered by hemorrhagic shock and, if changes took place, could they be reversed by restoring the blood and fluid volume of the animal.

Methods. Male, albino rats of the Holtzman strain weighing 200–300 g were used. All animals were fasted 18 to 20 hr and operated in the morning. The animals were given ether anesthesia and both femoral arteries were cannulated, one for bleeding into a glass syringe reservoir and the other for measurement of arterial blood pressure. They were restrained in a supine position. After the animals had awakened, hemorrhagic shock was produced by bleeding to a mean arterial pressure of 40 mm Hg which was maintained by withdrawing or giving blood. Details of the procedure have been reported previously (2).

Six categories of animals were studied: (a) Unbled controls: Nine animals were anesthetized, cannulated and allowed to awaken, but were not bled. They were killed 1 hr after recovery from anesthesia. (b) Shock, Stage I: Eight animals were bled to a mean arterial pressure of 40 mm Hg and maintained until the pressure stabilized with no further withdrawal of blood and sacrificed at that time (about 0.5 hr). (c) Shock, Stage II: Eight animals were bled, maintained at a mean arterial pressure of 40 mm Hg and sacrificed when 25% of the shed blood was given back to the animal to maintain the hypotension (about 1 hr). (d) Stage II-Treated: Eight animals were bled and maintained at 40 mm Hg arterial pressure as above. When 25% of the shed blood was given back, the animals were treated by giving the remaining shed blood plus Ringer's lactate solution with 5% dextrose in a volume equal to 50% of the maximum volume of the shed blood. These animals were sacrificed 1 hr after the treatment. (e) Shock, Stage III: Eight animals were maintained at 40 mm Hg arterial pressure until 70% of the shed blood had been returned (about 2 hr) and were sacrificed. (f) Stage III-Treated: Six animals were bled and brought to the 70% blood return stage (Stage III). The remaining blood plus Ring-

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er's lactate with dextrose was reinfused as in the Stage II-Treated group. All animals were sacrificed by decapitation and rapid exsanguination. Portions of the liver were excised immediately.

Approximately 0.4 g of liver was blotted, weighed and quickly transferred to a test tube of hot extraction medium. The method of Spirtes and Eichel (6) for the simultaneous extraction of oxidized (NAD^+) and reduced (NADH) forms of NAD was used. The tissue extracts were heated for 2 min during which the sample was dispersed in 3 ml of medium by use of a polytron homogenizer. After rapid chilling in an iced water bath, the sample volume was adjusted to 7 ml with distilled water and samples were centrifuged at about 35,000*g* for 13 min in a Sorvall RC-2 refrigerated centrifuge. The clear, pale yellow supernatant was kept on ice until assayed for NAD^+ and NADH according to the method of Racker (7).

For the NAD^+ determination the following components were added to the cuvette in order: 1.5 ml liver extract, 0.5 ml 0.1 *M* pyrophosphate buffer (pH 9.0), 0.1 ml of 95% ethanol, 0.18 ml 0.1 *N* NaOH, and water to make a final volume of 2.98 ml. After mixing, the optical density at 340 μm was recorded. Following the addition of 0.02 ml of alcohol dehydrogenase (ADH: 15 units, Sigma) and careful mixing, the density was observed until the reading stabilized for at least 1 min, at which point the reaction was considered complete.

For the determination of NADH a similar procedure was followed except that 0.3 ml of 0.1 *M* K_2HPO_4 buffer (pH 7.1), and 0.1 ml of 10% redistilled acetaldehyde were substituted for pyrophosphate buffer and ethanol, respectively. NaOH was omitted. NAD standards were assayed daily. The absorption coefficient of 6.22×10^6 cm^2/mole was used to calculate the results.

Homogenates of liver from control and Stage III animals were prepared and assayed for NAD hydrolyzing activity. Approximately 0.5 g liver was blotted free of blood and homogenized in a Teflon–glass tissue grinder in 20 ml of cold sucrose (250 m*M*), Tris–HCl (18 m*M*), pH 7.4, medium. The resulting supernatant, after centrifuging at 500*g*, for 10 min and 20,000*g*, for 20 min, was incubated in 0.8 ml of buffered (pH 7.4) medium containing 300 nmoles β-NAD (Sigma) for 10 min at 37°. The reaction was stopped by plunging the tubes into an iced water bath. Two and two-tenths ml of a glycine (0.1 *M*; pH 9.5) nicotinamide (0.02 *M*) and ethanol (0.5 *M*) mixture was added. The mixture was immediately transferred to a cuvette. An initial reading was taken at 340 μm. Fifteen units (0.02 ml) of ADH were added and a final reading was taken when the reaction had reached completion. The amount of NAD remaining was calculated as above. Each assay contained from 0.8 to 1.3 mg of protein. Protein was assayed by the method of Lowry *et al.* (8).

Results. NAD content of the liver of rats in shock decreased rapidly and progressively as the duration of shock increased. The total NAD was decreased 30% in the Stage I group, about 0.5 hr from the initiation of hemorrhage, and 56% in Stage III shock, about 2 hr (Table I). The oxidized form ap-

TABLE I. Total Nicotinamide Adenine Dinucleotide (NAD) Content of Rat Liver with Hemorrhagic Shock.[a]

	Total[b] NAD (nmole/g wet tissue)			
	Control	Shock		
		Stage I	Stage II	Stage III
Untreated	843 ± 49 (9)	592 ± 61[d] (8)	563 ± 38[d] (8)	458 ± 59[d] (8)
Treated[c]	—	—	746 ± 39[d] (8)	784 ± 34 (6)

[a] Values are means ± SEM of numbers of rats given in parentheses.

[b] Oxidized and reduced forms of NAD were analyzed separately.

[c] Treated animals were given shed blood plus Ringer's lactate in a volume equal to 50% of maximum shed blood. See text for details of treatment procedure.

[d] $p < 0.05$ when compared with control group.

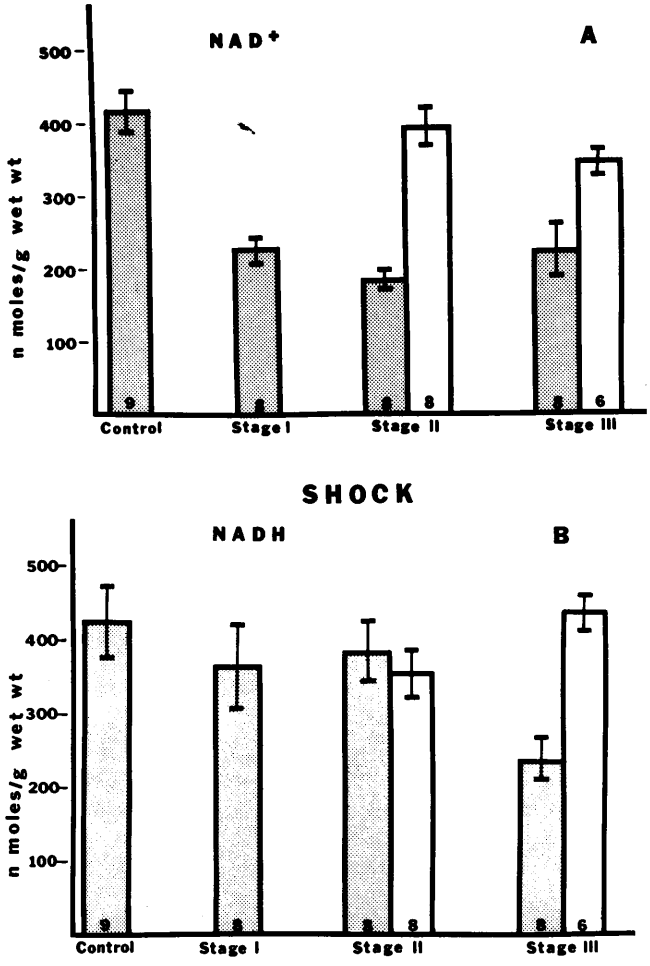

FIG. 1. Rat liver NAD (A) and NADH (B) content with hemorrhagic shock: shaded bars represent untreated animals; unshaded are those treated with blood and Ringer's lactate. Each bar represents the mean ± (SEM) obtained from duplicate determinations made on the number of animals shown at the base of the bar.

peared to be rapidly decreased as a result of hemorrhagic shock (Fig. 1A); whereas the concentration of the reduced form did not show a significant change from the control level until Stage III shock was reached (Fig. 1B). Reinfusion of the remaining blood and of Ringer's lactate resulted in a return to normal values for both NAD^+ and NADH (Fig. 1) in animals in Stage II as well as Stage III shock. Table II shows the changes in the NAD^+/NADH ratio with the various stages of shock. In the initial stages of shock there was a shift to the reduced form (NADH). The ratio returned toward normal in the late stage; however, the total NAD level was far below that of control. The animals in Stage II shock showed a return to control NAD^+/NADH ratio at 1 hr after reinfusion of blood and Ringer's lactate.

Alcohol dehydrogenase was used to determine the amount of exogenous NAD remaining after exposure to NAD hydrolyzing enzymes in liver homogenate. This method detects the action of either NAD-glycohydrolase and/or NAD-pyrophosphorylase in lowering the levels of tissue NAD. The total

TABLE II. NAD^+:NADH Ratios in Rat Liver with Hemorrhagic Shock.[a]

	Control	Shock		
		Stage I	Stage II	Stage III
Untreated	1.10 ± 0.15 (9)	0.72 ± 0.12 (8)	0.54 ± 0.08[c] (8)	0.96 ± 0.17 (8)
Treated[b]	—	—	1.20 ± 0.17 (8)	0.80 ± 0.04 (6)

[a] Values are means ± SEM of numbers of rats given in parentheses.

[b] Treated animals were given shed blood plus Ringer's lactate in a volume equal to 50% of maximum shed blood. See text for details of treatment procedure.

[c] $p < 0.05$ when compared with control group.

NADase activity of liver homogenates prepared from animals in Stage III shock was not different from control values. The values in the control and Stage III groups, respectively, were: 78 ± 8.7 (nmoles NAD hydrolyzed/mg protein in 10 min) and 80 ± 8.8.

Discussion. This study indicates that there is a decrease in the total nicotinamide adenine dinucleotides in liver in shock with a concomitant shift toward the reduced form lowering the NAD^+/NADH ratio. The decrease in NAD^+ with shock in liver homogenates is in agreement with the findings of Loiselle and Denstedt (1). The experimental shock model employed by Loiselle and Denstedt (1) was, however, quite different from the model employed in this study. Whereas we monitored the degree of shock by following mean arterial blood pressure and maintained hypotension by slow reinfusion of varying amounts of shed blood, the above authors merely allowed animals to bleed and determined the stage of shock by observing respiratory alterations. As nearly as it could be ascertained, the "irreversible shock" stage of Loiselle and Denstedt (1) would be comparable to the beginning of take back of shed blood in our shock model. The results of treatment of animals with blood and Ringer's lactate in this study indicate that the levels of NAD are rapidly restored to normal by replenishment of circulatory volume. This occurs even after treatment of animals in late shock (70% blood return) when the mortality would be expected to approach 100% in spite of treatment. Thus, decreased NAD is not likely to be related to eventual death of an animal in hemorrhagic shock after treatment.

To account for the decrease in NAD content of liver from shock rats three alternatives can be considered: (a) the enzymic cleavage of NAD could be increased as has been shown to occur in fasting (9), in infection (10) and in response to alkylating agents (11); (b) the cell membranes could have become more permeable to NAD as has been reported by Gallagher and Rees (12) in liver poisoned with carbon tetrachloride; or (c) there could be decreased synthesis of NAD. In this study, we have not detected any increase with shock in NADase activity as determined in the liver homogenates. However, it is possible that studies with liver slices or with perfusion of labeled NAD might reveal differences in activity. The other two possibilities have not been studied.

It is possible that the NAD-linked enzyme systems are adversely affected early in shock by the altered ratio of NAD^+ to NADH and then in the later stages by the low NAD content of the liver. Greenbaum, Gumaa and McLean (13) have suggested that control of the pentose phosphate cycle might well be through a shift in the ratio of NAD^+ to NADH. This concept of mass action control has also been suggested from studies on fat cells (14), and on erythrocytes (15).

Alterations in membrane permeability in shock have been suggested by measurements of membrane potential differences (16) and by the changes in ion transport capability (17). This might be compatible with the concept that NAD and cytoplasmic enzymes "leak" through the liver cell membrane in carbon tetrachloride-poisoned rats (12, 18). It, therefore, would be of interest to know if the lowered levels of NAD found in liver in shock might be another index of cell membrane alteration.

Summary. Nicotinamide adenine dinucleotides were determined in the liver of rats subjected to three stages of hemorrhagic shock and treatment. Total nucleotide content was decreased in all stages. The oxidized form, NAD^+, appeared to decrease more rapidly than the reduced form, NADH; however this was likely the result of a shift from oxidized to reduced state due to decreased tissue perfusion. Increased enzymic degradation of NAD did not appear to be the mechanism whereby NAD content was lowered; NADase activity of shock liver was not different from that of control liver. These results also indicate that the decreased NAD content in shock is restored to normal by nonspecific treatment by replenishment of circulatory volume.

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