Effect of the Component Parts of Nicotinamide Adenine Dinucleotide ($NAD^{\oplus}$) as Inhibitors of Lactic Dehydrogenase

By ALBERT J. FORLANO

Kinetic experiments were used to determine the K_m value for $NAD^{\oplus}$ and the K_i values for the component parts of $NAD^{\oplus}$ functioning as inhibitors of the coenzyme. The inhibitory powers of these substances progressively increased as the structure of the inhibitors more closely resembled that of the complete coenzyme. The increasing degree of inhibition appears to be due to more complete binding at the active site of LDH as the structure of the inhibitor approaches the structure of $NAD^{\oplus}$ and effectively prevents the normal binding of the coenzyme.

LACTIC ACID DEHYDROGENASE (LDH), an enzyme found in animal cells, catalyzes the reaction:

$$\text{L(+) lactate} + NAD^{\oplus} \underset{\longleftarrow}{\overset{\text{LDH}}{\longrightarrow}} \text{pyruvate} + NADH + H^{\oplus} \quad \text{Reaction } 1$$

LDH from rat muscle is specific for L(+)-lactate; however, D(−)-lactate is neither a substrate for LDH nor is it a competitive inhibitor of the normal substrate (1). With reference to the coenzyme portion of LDH one finds that $NAD^{\oplus}$ is approximately 170 times more effective than $NADP^{\oplus}$ for oxidizing lactate (2). Winer and Schwert (3) determined the Michaelis constant (K_m) for $NAD^{\oplus}$ in the reversible reaction *1* over the pH range 5.58 to 10.20. The values ranged from 45.0×10^{-5} moles/L. (pH 5.58) to 5.4×10^{-5} moles/L. (pH 8.59) to 61.0×10^{-5} moles/L. (pH 10.20). The K_m for $NAD^{\oplus}$ in reaction *1* exhibited a minimum at pH 8.7 (3).

The mechanism of LDH inhibition is not well understood. Green and Brosteaux (4) first observed that anions, presumably as competitive inhibitors of lactate binding, decreased the activity of LDH. Kubowitz and Ott (1) showed that high concentrations of pyruvate acted as an inhibitor of LDH. Since pyruvate can act as a competitive inhibitor of reaction *1*, it was decided that the forward reaction would be facilitated if the pyruvate was tied up as soon as it was formed. In the present study semicarbazide hydrochloride was added to the reaction mixture to form the semicarbazone of pyruvic acid and thus ensure maximal forward reaction.

A study of the competitive inhibitors resembling the component parts of $NAD^{\oplus}$ showed that ADP ribose and diadenylic acid are potent enzymatic inhibitors, whereas AMP, ADP, IMP, nicotinamide mononucleotide, nicotinamide, and adenosine were rated as weak inhibitors (5).

The literature appears to have a number of studies involving the inhibitory properties of the component parts of $NAD^{\oplus}$; however, many of these studies were performed by different workers under different experimental conditions. Since there appears to be a correlation between the chemical similarity of $NAD^{\oplus}$ inhibitors and degree of inhibition, it was decided to make a systematic study of the major single components of $NAD^{\oplus}$, followed by combinations of the single components joined together by covalent bonds. The degree of inhibition by these substances in terms of K_i values was determined under the same experimental conditions as the K_m value for $NAD^{\oplus}$.

EXPERIMENTAL

Materials—Lithium lactate (51.1% L-isomer), β-diphosphopyridine nucleotide ($NAD^{\oplus}$), lactic dehydrogenase type I, 70,000 units/mg. protein, all from Sigma Chemical Co.; niacinamide U.S.P.; D(−)-ribose, Eastman Red Label; adenine hemisulfate; 1 H_2O grade B, adenosine-5′-monophosphate morpholidate, grade B; adenosine CFP, the latter three from California Biochemicals; K_2HPO_4, Baker reagent; adenosine-5′-diphosphate sodium salt, ribose-5′-phosphate barium salt, and adenosine-3′-phosphate, the last three from Schwartz Laboratories; Tris, Mann Biochemicals.

KINETIC PROCEDURES

$NAD^{\oplus}$ and the inhibitors were assayed for their effect on LDH in the following manner. The solutions of reagents were added to the spectrophotometer cells in the specified order: 1.0 ml. lithium lactate solution, 0.10 ml. semicarbazide HCl solution, varying amounts of the $NAD^{\oplus}$ solution (as indicated in Table I) to give different concentrations of the $NAD^{\oplus}$ and enough 0.1 *M* Tris buffer (pH 8.8) to make a total volume of 3.10 ml. In the case of the inhibited solutions the specified amount of inhibitor (Table I) was also added and the volume was brought up to 3.10 ml. with 0.1 *M* Tris buffer (pH 8.8). Then 0.03 ml. of the enzyme solution (absorbance 280 mμ = 0.050), which was prepared fresh daily, was added last from a microsyringe. The tubes were rapidly shaken and placed into a Gilford model 2000 multiple sample absorbance recorder equipped with a Beckman DU monochromater. The increases in absorbance at 340 mμ were determined every 20 sec. at 25°. Since the increase in absorbance at 340 mμ is a function of the conversion of $NAD^{\oplus} \rightarrow NADH$ which occurs when the lactate is oxidized, the absorbance increase was used as a means of determining the reaction rate. Inhibitors and combinations of inhibitors were handled in the same fashion. The K_m for $NAD^{\oplus}$ was determined at the different concentrations specified in Table I in the absence of inhibitors and all other conditions remained constant. In the K_i determinations the amount of inhibitor or combinations of inhibitors remained constant and the $NAD^{\oplus}$ concentration was varied as specified in Table I. The final concentrations of the inhibitors in the reaction mixture are given in Table II.

RESULTS

The change in absorbance at 340 mμ *versus* time was followed over a period of 15 min. There was an initial reaction rate that appeared to be maximal which subsequently tapered off. The slope of the initial plot of absorbance *versus* time was utilized in the calculation of the Michaelis constant (K_m) and the inhibitor constants (K_i). The calculation for K_m was based on Eq. 1 from White *et al.* (6) utilizing the

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TABLE I—REAGENT CONCENTRATION AND AMOUNTS USED IN REACTION VOLUME OF 3.1 ml.[a]

Reagent	Vol., ml.
0.240 *M* Lithium lactate	1.00
0.384 m*M* $NAD^{\oplus}$	0.05, 0.10, 0.20, 0.40, 0.60, 0.80, 1.00
0.050 *M* Semicarbazide HCl	0.10
3.840 *M* Niacinamide	0.10
3.840 *M* D(−)-Ribose	0.75
0.019 *M* Adenine	1.00
3.840 *M* K_2HPO_4	1.00
0.041 *M* Adenosine-5′-phosphate	0.20
0.100 *M* Adenosine-5′-diphosphate	0.10
0.050 *M* Ribose-5′-phosphate	1.00
0.033 *M* Adenosine	1.00
0.100 *M* Adenosine-3′-phosphate	0.20
0.100 *M* Tris buffer pH 8.8	

[a] All the solutions were prepared in 0.1 *M* Tris buffer. The differences in concentrations were due to differences in solubilities of these reagents. Maximal solubility was desired wherever possible.

Lineweaver-Burk modification of the Michaelis-Menten equation:

$$\frac{1}{v} = \frac{1}{V_{max.}} + \frac{K_m}{V_{max.}} \cdot \frac{1}{[S]} \qquad \text{(Eq. 1)}$$

where $[S]$ = substrate concentration $[NAD^{\oplus}]$, v = reaction velocity at $[S]$, $V_{max.}$ = maximal velocity, and K_m = Michaelis constant for $NAD^{\oplus}$. If a plot of $1/v$ *versus* $1/[S]$ is made, the slope of the line is equal to $K_m/V_{max.}$ and the y intercept equals $1/V_{max.}$; consequently K_m can easily be calculated. The K_m value for $NAD^{\oplus}$ determined in the absence of inhibitors is found in Table II. The calculation of the K_i value utilized Eq. 2 from White *et al.* (6):

$$\frac{1}{v} = \frac{K_m}{V_{max.}}\left(1 + \frac{[I]}{K_i}\right)\frac{1}{[S]} + \frac{1}{V_{max.}} \qquad \text{(Eq. 2)}$$

where $[I]$ represents the concentration of inhibitor, and K_i is the dissociation constant for the enzyme inhibitor complex EI. The K_i is analogous to the K_m value which is equal to the concentration of substrate present at $^1/_2$ the numerical maximal velocity. Consequently, the smaller the value of K_i, the more effective the inhibitor. The reaction velocities obtained in the presence of the inhibitors were used for the calculation of the K_i values. A plot of $1/v$ *versus* $1/[S]$ (S = $[NAD^{\oplus}]$) in the presence of a constant amount of inhibitor was made and the slope of the line was equal to $(K_m/V_{max.})(1 + [I]/K_i)$. Recall that the value of $(K_m/V_{max.})$ is the same as for the uninhibited reaction. The inhibited plots also have the same ordinate value $(1/V_{max.})$ as the uninhibited reaction because these agents function as competitive inhibitors of $NAD^{\oplus}$. The K_i values are presented in Table II.

DISCUSSION

The K_m value of 8.52×10^{-5} *M* determined at pH 8.8 in this study compares favorably with the literature value of Winer and Schwert (3) who found a value of 5.4×10^{-5} *M* at pH 8.59 and 12.0×10^{-5} *M* at pH 8.96. Since the K_i values were determined with the same enzyme preparation, reagents, *etc.*, as the K_m value, there should be a good degree of correlation with each other.

The data in Table II indicate that none of the inhibitors or combination of inhibitors was more firmly bound to LDH than $NAD^{\oplus}$. The high K_i values for ribose and phosphate indicate that these portions individually were weakly bound; however, when they were joined by a covalent bond as in the case of ribose-5′-phosphate, the K_i value dropped sharply indicating much stronger binding than either of the component portions alone. Adenine itself had a moderately high K_i value, however, when it was joined covalently to ribose-5′-phosphate to yield adenosine-5′-phosphate, the K_i of the nucleotide was about $^1/_2$ that of the ribose-5′-phosphate. This is a good indication that adenine is a necessary binding portion of the coenzyme. When an additional phosphate was joined covalently to AMP to yield ADP, the K_i of ADP dropped to $^1/_3$ the value for AMP, indicating the additional attraction afforded by the second covalently bonded phosphate. The increased binding ability contributed by the extra phosphate is understandable because phosphate groups are ionized at this pH and could easily form ionic bonds with some positive group at the active site of LDH. Niacinamide, which is not a potent

TABLE II—K_m FOR $NAD^{\oplus}$ AND K_i VALUES IN THE LDH CATALYZED CONVERSION OF LACTATE TO PYRUVATE IN 0.1 *M* TRIS BUFFER AT pH 8.8 at 25° C.

Substance	K_m or K_i (moles/L)	Concn. of Inhibitor in Reaction Mixture, moles/L.	Ratio $K_i \times 10^{-2}/K_m$
$NAD^{\oplus}$	8.52×10^{-5}	...	...
D(−)-Ribose	23.68	0.93	2780.00
Niacinamide	7.08×10^{-2}	0.12	8.31
Adenine	4.51×10^{-2}	6.19×10^{-3}	5.30
Niacinamide + adenine[a]	6.36×10^{-2}	$0.12 + 6.19 \times 10^{-3}$	7.47
Phosphate	1.80	1.24	212.00
Adenosine-5′-PO_4	4.57×10^{-3}	2.60×10^{-3}	0.54
Adenosine-5′-$DiPO_4$	1.48×10^{-3}	3.22×10^{-3}	0.17
Adenosine-5′-$DiPO_4$ + niacinamide[a]	5.21×10^{-4}	$3.22 \times 10^{-3} + 0.123$	0.06
Adenosine-5′-$DiPO_4$ + niacinamide + ribose[a]	4.67×10^{-4}	$3.22 \times 10^{-3} + 0.123 + 0.929$	0.06
D(−)-Ribose-5′-PO_4	1.07×10^{-2}	1.61×10^{-2}	1.26
Adenosine	1.56×10^{-2}	1.07×10^{-2}	1.83
Adenosine-3′-PO_4	7.65×10^{-3}	6.45×10^{-3}	0.90

[a] The calculations of these K_i values are based solely upon the molar concentration of the first inhibitor even though the other inhibitors were present.

inhibitor of NAD⊕, potentiated the inhibitory powers of ADP when used in physical combination indicating that another one of the points that NAD⊕ uses for attachment to the LDH had been partially blocked. The same was true for the mixture of ADP with niacinamide and D(−)-ribose.

The K_i for adenosine was fairly high; however, if a phosphate group was joined to the 5′ position covalently to yield the adenosine-5′-PO_4 the K_i decreased to $^1/_3$ of its value. The placement of a covalently bonded phosphate in the 3′ position of adenosine reduced the K_i of adenosine to only $^1/_2$ of its original value. This indicates that the phosphate of a nucleotide is a required portion for bonding to the enzyme; in addition there are steric requirements for maximum binding since both isomers do not have equal K_i values.

Ringold (7) recently theorized that there is an interaction between the quaternary nitrogen of the NAD⊕ and the basic nitrogen of the imidazole ring from histidine at the active site of LDH. With this idea in mind we can see why niacinamide, without a quaternary nitrogen, is not a catalytic species, nor is it very strongly bound to the active site of the enzyme.

One can conclude that the individual components of NAD⊕ are not very strongly bound to LDH, especially in the case of ribose or phosphate alone. However, the amount of inhibition increases as one systematically joins these portions together covalently and the products more closely resemble NAD⊕. This is expected since they become more effective in preventing the complete binding of NAD⊕ to LDH which apparently is a necessary prerequisite for enzymatic activity.

SUMMARY AND CONCLUSIONS

The K_m value for NAD⊕ and the K_i values for the component parts of NAD⊕ as inhibitors of the LDH catalyzed conversion of lactate to pyruvate were determined in 0.1 *M* Tris buffer at pH 8.8.

One finds that the individual component parts of NAD⊕, especially the phosphate and D(−)-ribose, were very poor inhibitors of the coenzyme; however, as the component parts were joined together covalently to make the nucleoside, nucleotide, nucleotide phosphate, *etc.*, their inhibitory powers increased because of greater binding at the active site thus preventing the normal binding of NAD⊕ to LDH.

REFERENCES

(1) Kubowitz, F., and Ott, P., *Biochem. Z.*, **314**, 94(1943).
(2) Mehler, A. H., Kornberg, A., Grisolia, S., and Ochoa, S., *J. Biol. Chem.*, **174**, 961(1948).
(3) Winer, A. D., and Schwert, G. W., *ibid.*, **231**, 1065 (1958).
(4) Green, D. E., and Brosteaux, J., *Biochem. J.*, **30**, 1489 (1936).
(5) Baker, B. R., and Sachdev, A. S., *J. Pharm. Sci.*, **52**, 933(1963).
(6) White, A., Chandler, P., and Smith, E., "Principles of Biochemistry," 3rd ed., McGraw-Hill Book Co., Inc., New York, N. Y., pp. 223–236.
(7) Ringold, H. J., *Nature*, **210**, 535(1966).

Alterations in Hyaluronidase Activity and Serum Protein Electrophoretic Patterns After Chronic Heat Stress

By M. M. CLAY* and M. W. ADLER

The effects of a hot environment upon plasma proteins and dermal hyaluronidase spreading activity was studied in rats. Hyaluronidase activity decreased after 1 day of heat, increased after 4 and 16 days, and again decreased after 48 days. Swimming for 1.5 hr. after each period of heat stress resulted in inhibition of hyaluronidase spreading activity with the exception of the 48-day period when activity increased. Swimming caused adrenal gland hypertrophy in unstressed but not in heat stressed rats. The relative concentration of plasma albumin increased during heat stress.

WITTERSFORS *et al.* (1, 2) have reported hypoalbuminemia with increased extravascular albumin distribution in some patients with gastric carcinoma. The authors suggested increased vascular permeability as a mechanism for loss of circulating albumin. Enhanced as well as diminished dermal connective tissue permeability to colloidal particles has been reported after stress and cortisone administration by this laboratory (3–5). The above work and that of Menkin (6) show a role for glucocorticoid hormones in regulation of vascular as well as connective tissue permeability.

The authors studied the effects of stress upon circulating proteins of rats. They also attempted to determine whether changes in serum proteins accompanied changes in dermal connective tissue permeability. For the first study environmental heat was selected as the stress and intradermally injected India ink and hyaluronidase–India ink were selected as indicators of connective tissue permeability to colloidal particles.

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* Present address: College of Pharmacy, University of Houston, Houston, TX 77004

EXPERIMENTAL

Male albino Wistar strain rats initially weighing 90–120 Gm. were used. Food and tap water were supplied *ad libitum* at all times. Unless otherwise indicated, the rats were maintained at 24 ± 2°

Animal Treatment—Five groups of 24 rats each were maintained at 32 ± 2° for 1, 4, 16, 32, or 48 days in a ventilated semidark chamber. Each group was then divided into three equal subgroups. Dermal permeability was assayed 2 hr. and 24 hr. after removal from the heat in groups *A* and *B*, respectively. Twenty-four hours after heat stress, the remaining rats of group *C* were forced to swim for 1.5 hr. in water at 25 ± 1°. After swimming,