Interactions of Nicotinamide Adenine Dinucleotide and Its Derivatives with Steroids*

CARL MONDER

From the Department of Biochemistry, Albert Einstein College of Medicine, Yeshiva University, New York

Received December 6, 1962

Cortisol and certain other C_{21} steroids reacted reversibly with NAD^+ at alkaline *p*H values to form products with spectral maxima between 325 and 335 mμ. Analogs of NAD^+ also reacted with cortisol to yield products with spectra resembling those of the corresponding reduced analogs. The rates of reaction depended on concentrations of components, *p*H, and dielectric constant. Varying these factors did not shift the position of the spectral maximum. Formation of product did not involve degradation products of NAD^+ as intermediates. It is concluded that an enolate anion derived from the steroid side chain condensed with the quaternary pyridinium group of NAD^+ in a reversible base-catalyzed addition reaction.

It has been suggested that steroids may mediate metabolic processes by influencing the activities of pyridine nucleotide–dependent enzymes including, among others, glutamic acid dehydrogenase (Yielding and Tomkins, 1960a), aldehyde dehydrogenase (Maxwell and Topper, 1961), transhydrogenases (Talalay and Williams-Ashman, 1960; Hagerman and Villee, 1959), and NADH oxidases (Jensen and Neuhard, 1961); or, by being themselves oxidized or reduced, thus influence the ratio of reduced to oxidized nucleotide (Yielding and Tomkins, 1960b). In addition, NAD^+ and $NADP^+$ are essential cofactors in the metabolic interconversion of many steroids (Engel and Langer, 1961).

It is not clear how steroids and pyridine nucleotides interact in biological systems although some *in vitro* evidence exists for weak secondary bonding (Munck *et al.*, 1957). The present paper describes addition compounds resulting from interactions between certain 21 carbon steroids and NAD^+ and its analogs.

Materials and Methods

NAD^+, $NADP^+$, and thio-NAD^+ were purchased from the Sigma Chemical Company, St. Louis, Mo. Other analogs were obtained from Mann Research Laboratories, New York. Adenosine diphosphate ribose was a product of Pabst Laboratories, Milwaukee, Wisc. NAD^+, $NADP^+$, and NADH were reported to be at least 96% pure, and nicotinamide mononucleotide was 94% pure. Samples of 1-methyl-3-carbamidopyridinium iodide and 1-methyl-4-carbamidopyridinium perchlorate were gifts of Dr. Edward M. Kosower. The iodide salt was converted to the perchlorate salt by precipitating silver iodide with aqueous silver perchlorate, followed by passing the solution through an ion exchange resin (IR-45) column in the perchlorate form (Kosower and Bauer, 1960).

Reference samples of corticosteroids, obtained from the United States Pharmacopeia or from Dextran Chemicals Inc., New York, were chromatographically homogeneous in Bush system B_4 (Bush, 1952). Other steroids were obtained from various commercial sources. Phosphate, carbonate-bicarbonate, and glycine buffers were prepared as described by Gomori (Colowick and Kaplan, 1955), and *tert*-butylamine buffer, 0.2 M, by adjusting an aqueous solution to the appropriate *p*H with hydrochloric acid. Kinetic studies were performed spectrophotometrically using the Beckman Model DU instrument maintained at 27° ± 1° with a constant temperature attachment. Absorption spectra were obtained with the Cary Model 14 recording spectrophotometer, or the Bausch and Lomb Spectronic Model 505 spectrophotometer.

NAD^+ was measured quantitatively as its cyanide addition product (Colowick *et al.*, 1951) and NADH was measured by its ability to reduce oxaloacetate, acetaldehyde, and pyruvate in the presence of malate, alcohol, and lactate dehydrogenases, respectively. The crystalline enzymes used were obtained from Mann Research Laboratories Inc.

Results

At *p*H values above 10, cortisol and NAD^+ reacted in aqueous solution to yield a product with an optical absorption spectrum having a broad maximum between 325 and 335 mμ (Fig. 1). At the same *p*H, NADH had a sharp maximum at 340 mμ, which was not altered by the addition of cortisol. The spectral characteristics of the reaction product in the lower ultraviolet region could not be determined because of the high degree of absorption of both steroid and nucleotide at wavelengths below 280 mμ. Possible formation of NADH was tested by the ability of the product to participate in dehydrogenase catalyzed reactions. Cortisol and NAD^+, each at 10^{-3} M, were allowed to react at *p*H 11.5 until no further absorbancy change occurred at 330 mμ (about 20 minutes); reaction product was tested for NADH in the lactate, malate, and alcohol dehydrogenase systems at *p*H 7.6. Under the conditions of the assay a 10% conversion of NAD^+ to NADH would have been detected. Table I shows that the reaction product did not catalyze substrate reductions by itself and inhibited the dehydrogenases when NADH was present. The possibility that cortisol and NAD^+ reacted to yield only NADH was therefore eliminated. The lactate dehydrogenase system was inhibited 35% with added NADH in the presence of levels of reaction product which inhibited alcohol and malate dehydrogenases 67% and 82%, respectively. It may be calculated that a 10% yield of NADH due to reaction of cortisol with NAD would have been detected in the lactate dehydrogenase system under these conditions. NAD^+ treated at *p*H 11.5 in the absence of cortisol did not affect the lactate and alcohol dehydrogenase systems appreciably, but inhibited the malate dehydrogenase about 45%, while cortisol controls treated in the same way inhibited malate dehydrogenase activity and had no effect on lactate and alcohol dehydrogenases. Inhibition by the product was therefore not caused by either of the reactants separately, and was probably due to their interaction.

* Aided by grants to Dr. Abraham White from the American Cancer Society (P68D); the National Institutes of Health, U. S. Public Health Service (CY 3154(C5)); and the National Science Foundation (G-18960).

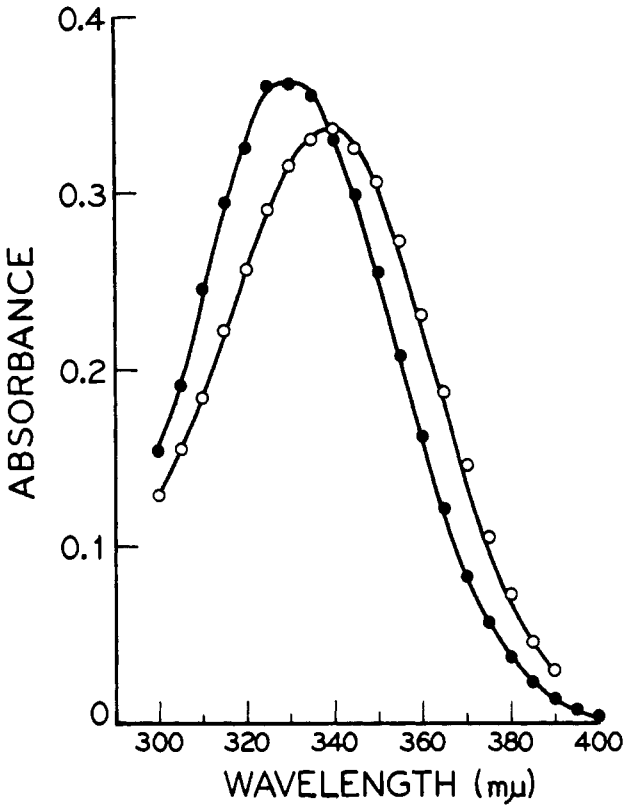


Fig. 1.—Comparison of absorption spectra of cortisol-NAD (solid circles) and NADH (open circles). NAD^+, 9.1×10^{-4} M; cortisol, 2.8×10^{-3} M; 0.2 M *tert*-butylamine buffer, *p*H 11.4. Absorbancy values of the complex were read in quartz cuvets having a 10-mm light path against NAD^+ and cortisol alone using a Beckman Model DU spectrophotometer. Concentration of NADH was 5.4×10^{-5} M.

TABLE I

ANALYSIS OF CORTISOL-NAD REACTION FOR NADH FORMATION

Reduced pyridine nucleotide was determined in systems containing either 0.3 μmole oxalacetate and 0.1 μg crystalline pig heart malic dehydrogenase, 0.7 μmole potassium pyruvate and 25 μg crystalline rabbit muscle lactic dehydrogenase, or 10 μmoles acetaldehyde and 20 μg crystalline beef liver alcohol dehydrogenase. In designated experiments, 0.02 μmole NADH were added. Cortisol-NAD adduct was prepared by allowing cortisol and NAD^+, each at 10^{-3} M, to react at *p*H 11.5 until no further absorbancy change occurred at 330 mμ (about 20 minutes); 0.1 ml of the reaction product was added to the reaction mixture as indicated. Controls contained cortisol or NAD^+ alone at the same concentrations. Each system contained 240 μmoles potassium phosphate, *p*H 7.6. Final volume was 3.0 ml. Reaction was initiated with enzyme after 3 minutes prior incubation of the other components at 30°. Rate of change of absorbancy at 340 mμ was constant for at least 3 minutes.

Reactant	Alcohol Dehydrogenase	Malate Dehydrogenase	Lactate Dehydrogenase
	Δ Absorbancy at 340 mμ/min		
NADH	0.012	0.022	0.020
Cortisol-NAD	0.000	0.000	0.000
Cortisol-NAD + NADH	0.004	0.004	0.013
NAD^+ + NADH	0.011	0.012	0.021
Cortisol + NADH	0.014	0.015	0.020
No addition	0.000	0.000	0.000

TABLE II

REACTION OF STEROIDS WITH NICOTINAMIDE ADENINE DINUCLEOTIDE

Reaction mixture contained 11 μmoles steroid and 5.5 μmoles NAD^+ in 3 ml 33% ethanol at *p*H 11.5 (0.16 M carbonate-bicarbonate buffer). After starting reaction with NAD^+, absorbancy values were read at 15-second intervals at 340 mμ. Velocity calculated from absorbancy changes during initial 30 seconds. Temperature 27° ± 1°.

Steroids	Relative Velocity (Cortisol = 100)
17α-Hydroxy Ketol Side Chain	
Pregnane-3α,17α,21-triol-11,20-dione-21-acetate	125
9α-Fluoro-16α-hydroxy-Δ¹-cortisol (triamcinolone)	119
4-Pregnene-11β,17α,21-triol-3,20-dione(cortisol)	100
16α-Hydroxy-9α-fluorocortisol	88
Δ¹-Cortisol	87
9α-Fluorocortisol	86
4-Pregnene-17α,21-diol-3,11,20-trione (cortisone)	78
4-Pregnene-17α,21-diol-3,20-dione	74
16α-Methyl-4-pregnene-17α,21-diol-3,20-dione	27
17-Deoxy Ketol Side Chain	
4-Pregnene-21-ol-3,20-dione (11-deoxycorticosterone)	47
4-Pregnene-21-ol-3,20-dione-21-acetate	49
4-Pregnene-11β,21-diol-3,20-dione (corticosterone)	44
Other Steroids	
4-Androstene-17β-ol-3-one (testosterone)	0
19-Nortestosterone	4
17α-Methyltestosterone	0
17α-Ethyl-19-nortestosterone (Nilevar)	11
5α-Androstan-3α-ol-17-one (androsterone)	0
1,3,5(10)-Estratriene-3-ol-17-one (estrone)	0
1,3,5(10)-Estratriene-3,17β-diol (estradiol)	0
4-Pregnene-3,20-dione (progesterone)	0
17α-Hydroxyprogesterone	0

Steroid Specificity.—The reaction appeared to be specific for steroids containing a 17-deoxyketol or 17-hydroxyketol side chain (Table II). The lack of reaction of NAD^+ with progesterone and 17α-hydroxyprogesterone in contrast with the rapid rate of reaction with 11-deoxycorticosterone and 17α-hydroxy-11-deoxycorticosterone indicated that an alcohol group at carbon 21 was essential for product formation. Acetylation of the 21-hydroxyl group did not diminish the rate of reaction. Androgens and estrogens did not react under these conditions, although 17α-ethyl-19-nortestosterone (Nilevar) produced a small increase in absorption. The reason for the slower reaction rates of steroids containing a 17-deoxyketol side chain in contrast with those containing the 17α-hydroxyketol side chain is not clear; a possible explanation for the slow rate of reaction of 16α-methyl-17α-hydroxy-11-deoxycorticosterone may be that the 16α-methyl group interferes with the solvation of the side chain, thereby suppressing formation of the reacting steroid anion.

Nucleotide Specificity.—Table III summarizes some properties of the products of reaction of cortisol with a number of nicotinamide adenine dinucleotide analogs and other nucleotide derivatives. A pyridine ring containing a quaternary pyridinium nitrogen was essential for reaction. Each derivative showed a characteristic absorption maximum which was, with the exception of thionicotinamide adenine dinucleotide, lower than that of the corresponding dihydro compound. Molar absorbancy indices, estimated by the method of Foster *et al.* (1953), were consistently lower than the corresponding values for the dihydro deriva-

TABLE III

REACTION OF NUCLEOTIDES WITH CORTISOL

Values of association constants K and molar absorbancy indices a_M were obtained by plotting absorbancy over cortisol concentration (A/b) against absorbancy (A) as described by Foster *et al.* (1953). Nucleotide levels were 5×10^{-4} M. Cortisol concentration was varied from 0.011 to 0.0018 M. Solutions were buffered at *p*H 11.8 at 26° in carbonate-bicarbonate (CO_3) or *tert*-butylamine (TBA) buffers.

	Condensation Product				Dihydro Derivative		
Nucleotide	max mμ	K liters × mol^{-1}	$a_M \times 10^{-3}$	Buffer	max mμ	$a_M \times 10^{-3}$	Reference
NAD$^+$	333	59	4.3	CO_3	340	6.2	Horecker *et al.* (1948)
3-Acetylpyridine AD$^+$	350	—	—	CO_3, TBA	363	9.1	Siegel *et al.* (1959)
Deamino-NAD$^+$ (nicotinamide hypoxanthine dinucleotide)	328	58	4.0	CO_3	338	6.2	Siegel *et al.* (1959)
3-Pyridinealdehyde AD$^+$	350	182	3.1	TBA	358	9.3	Siegel *et al.* (1959)
3-Pyridinealdehyde hypoxanthine dinucleotide	350	106	2.7	TBA	357	9.4	Siegel *et al.* (1959)
Thionicotinamide AD$^+$	405	81	6.1	TBA	400	11.3	Kaplan (1960)
NADP$^+$	330	40	5.3	CO_3	340	6.2	Horecker *et al.* (1948)
NADH		No reaction		CO_3, TBA			
Nicotinamide mononucleotide	330	65	1.9	CO_3			
Adenosine diphosphate ribose		No reaction[a]		TBA			
Adenosine-5′-phosphate		No reaction		CO_3, TBA			
Adenosine		No reaction		CO_3, TBA			
Nicotinamide		No reaction		CO_3, TBA			
Nicotinic acid		No reaction		CO_3, TBA			

[a] At 2.8×10^{-3} M adenosine diphosphate ribose, reaction velocity in presence of cortisol was 0.020 absorbancy units per minute at 340 mμ. No spectral maximum was observed.

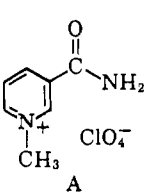
tives. The values for the association constants show that in each case the reactions were readily reversible. Reproducible molar absorbancy index and association constant could not be obtained for acetyl pyridine adenine dinucleotide and only the spectral maximum of this nucleotide is recorded. For each nucleotide a linear relationship was obtained when absorbancy of complex was plotted against absorbancy of complex divided by cortisol concentration, consistent with a reversible reaction involving one molecule of cortisol with one of nucleotide. Adenine derivatives, including fragments derived from NAD$^+$ and containing no nicotinamide, did not react with cortisol. Nicotinamide, nicotinic acid, and NADH, each of which contains a tertiary ring nitrogen, were also not reactive.

The possibility that the reaction involved alkaline degradation products of cortisol or NAD$^+$ was examined. Reaction rate decreased when NAD$^+$ was kept at *p*H 11.5 for intervals up to two hours before adding cortisol. During this time, in *tert*-butylamine buffer, NAD$^+$ (measured by reaction with cyanide (Colowick *et al.*, 1951)), decreased 53%. The primary products of the degradation of NAD$^+$ at *p*H 11.5 , nicotinamide and adenosine diphosphate ribose (Kaplan *et al.*, 1951), did not react with cortisol (see Table III), and therefore were not involved in the reaction. Cortisol was stable at *p*H 11.5 for at least 15 minutes. It was concluded that the reaction between cortisol and NAD$^+$ involved these compounds and not degradation products.

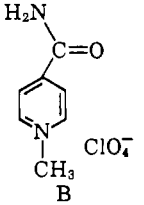
Effect of varying pH and concentrations of reactants.— The slope of the line in Figure 2, in which *p*H is plotted against the logarithm of the initial reaction rate of cortisol with NAD$^+$, is 1.0, indicating a direct relationship between the reaction of cortisol with NAD$^+$ and hydrogen ion concentration. The results were similar in carbonate-bicarbonate buffers, or in unbuffered systems adjusted to the desired *p*H with sodium hydroxide.

The rate of absorbancy change was directly proportional to cortisol concentration when NAD$^+$ concentration was constant at all levels of nucleotide examined. However, the reaction velocity did not vary with NAD$^+$ concentration except at very low levels of nucleotide (Fig. 3).

Nature of the reaction between cortisol and NAD$^+$.— Recent evidence indicates that pyridine nucleotides may form charge-transfer complexes with a number of low molecular weight substances such as indoles (Cilento and Tedeschi, 1961; Alivisatos *et al.*, 1961) or phosphate (Ungar and Alivisatos, 1961). Reaction of NAD$^+$ with other substances, such as cyanide (San Pietro, 1955) or dihydroxyacetone (Burton and Kaplan, 1954), appears to result in the formation of addition compounds with localized bonds on the nicotinamide grouping. Experiments were therefore designed to determine whether the reaction between cortisol and NAD$^+$ yields a charge-transfer complex or an addition compound.[1] The reactions of cortisol with two NAD$^+$ analogs, 1-methyl-3-carbamidopyridinium perchlorate (A) and 1-methyl-4-carbamidopyridinium perchlorate (B) were studied.



A



B

If a charge-transfer complex were formed reaction of cortisol with both analogs would be observed spectrophotometrically, with a characteristic absorption spectrum for each complex. In forming an addition compound, cortisol might be expected to react only with the 3-carboxamide analog. Transitional charge-transfer intermediates would yield with both analogs transitory maxima in the visible region of the spectrum. Figure 4 shows that cortisol reacted only with the 3-carboxamide isomer. Measurement of the reaction at various wavelengths in the interval 300–400 mμ during the first 30 seconds of reaction revealed no transient

[1] The author is indebted to Dr. Edward M. Kosower for suggesting this experiment.

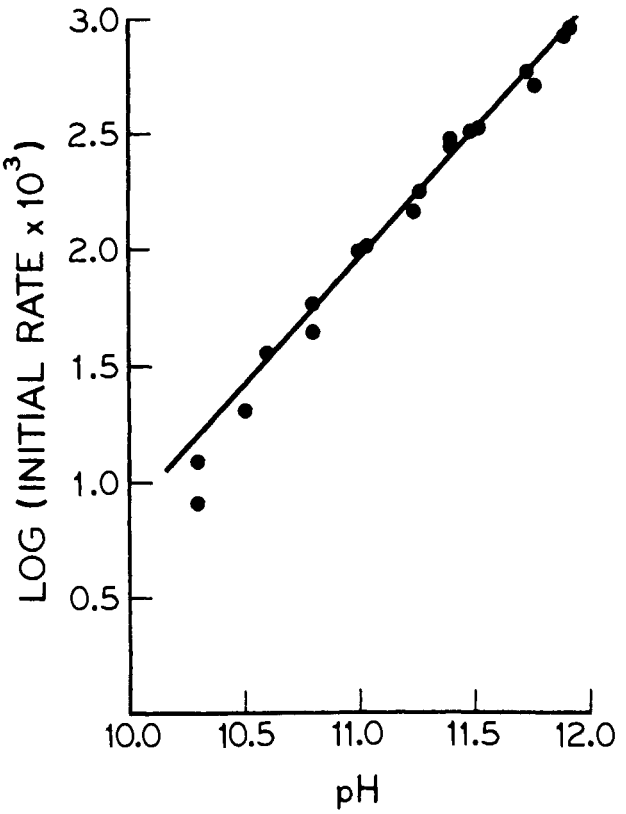


Fig. 2.—Effect of hydrogen ion concentration on reaction of cortisol with NAD$^+$. Reaction mixtures contained 3.7 × 10^{-3} M cortisol, 1.8 × 10^{-3} NAD$^+$, and 3.3 × 10^{-2} M carbonate-bicarbonate buffer. Velocities above *p*H 11.5 obtained in unbuffered systems containing NaOH. Initial rate determined from absorbancy increase at 335 mμ during first 15 seconds of reaction.

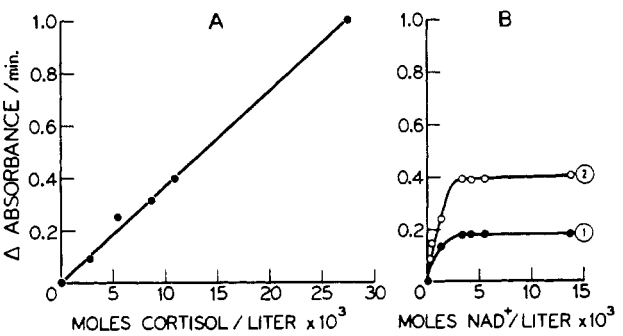


Fig. 3.—*A*. Influence of cortisol concentration on rate of reaction. Reaction mixture contained 2.7 × 10^{-3} M NAD$^+$, 3.3 × 10^{-2} M carbonate buffer. Cortisol was added in 1 ml ethanol. Final volume 3.0 ml; final *p*H, 11.8. Initial velocity was measured at 335 mμ in quartz cuvets of 10-mm light path during first 30 seconds of reaction. *B*. Influence of NAD$^+$ concentration on rate of reaction. Cortisol concentration was 5.5 μmoles/ml (curve 1), and 11 μmoles/ml (curve 2). Other conditions as in 3A.

increase in absorbancy, indicating no detectable formation of a charge-transfer intermediate. These experiments were also performed with 10-fold higher concentrations of the two 1-methyl nicotinamide analogs with similar results. When dihydroxyacetone was substituted for cortisol, reaction with 1-methyl-3-carbamidopyridinium perchlorate yielded a product with a spectral maximum at 376 mμ. There was no detectable reaction with the 4-carbamido isomer.

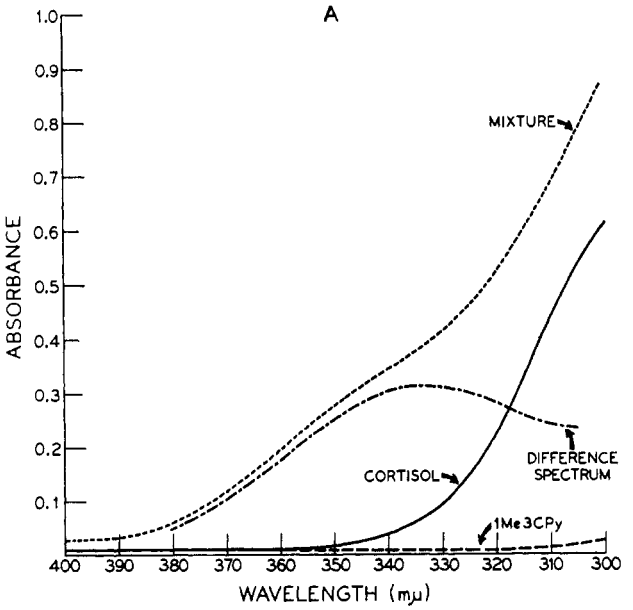


Fig. 4A.—Reaction of cortisol with 3-carbamidopyridinium perchlorate (1Me3CPy). Cortisol, 2.77 μmoles in 1 ml 59% aqueous ethanol, 1Me3CPy 0.9 μmoles, sodium carbonate, 67 μmoles. Final volume, 3.0 ml; *p*H 11.5. Spectra were obtained when no further absorbancy increase at 335 mμ was observed (165 minutes). - - - -, cortisol + 1Me3CPy; ———, cortisol; — — — —, 1Me3CPy; — - — - — -, difference spectrum.

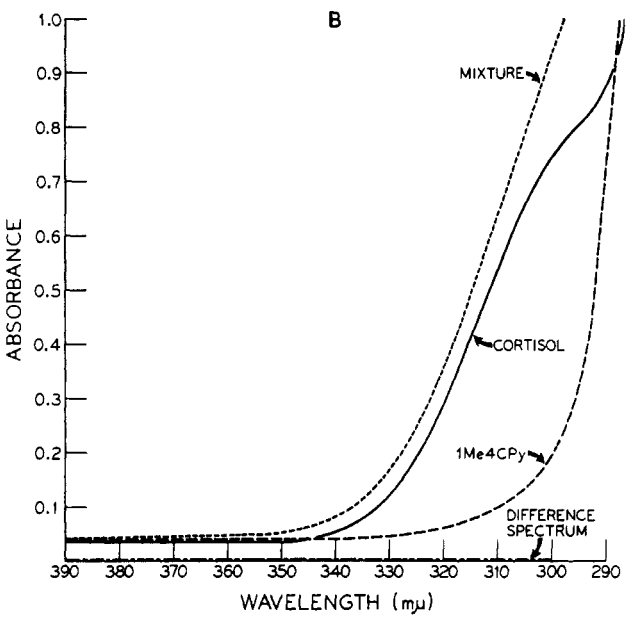


Fig. 4B.—Reaction of cortisol with 4-carbamidopyridinium perchlorate (1Me4CPy). Conditions as described under Figure 4A, - - - -, cortisol + 1Me4CPy; ———, cortisol; — — — —, 1Me4CPy; — - — - — -, difference spectrum.

The product of reaction of cortisol with 1-methyl-3-carbamidopyridinium perchlorate had an absorption spectrum similar to that of the cortisol-NAD condensation product. These results reinforce the previous conclusion that cortisol reacted with the pyridinium ring of NAD$^+$.

Further evidence for the formation of addition product rather than charge-transfer complex was obtained from studies of the effects of solvent polarity

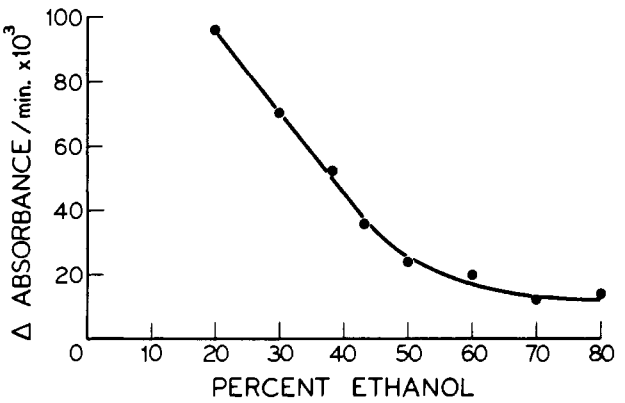


Fig. 5.—Effect of ethanol concentration on reaction of cortisol with NAD$^+$. Cortisol, 5.6 $\times$ 10^{-3} M; NAD$^+$, 1.36 $\times$ 10^{-3} M. Final volume 3.0 ml. *p*H 11.5, 26°. Final absorbancy obtained after subtracting blanks containing no NAD$^+$ or no cortisol.

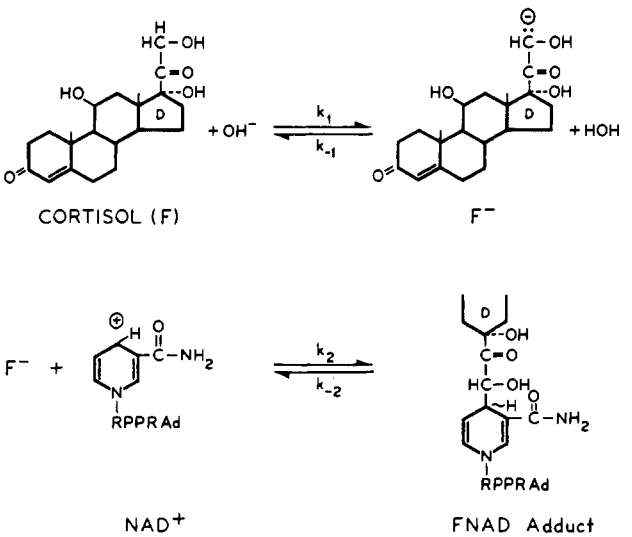


Fig. 6.—Postulated pathway of interaction of cortisol with NAD$^+$.

on the reaction product. The position of the spectral maximum at 333 mμ was not shifted by increasing the ethanol concentration from 20% to 80% or by replacing ethanol by methanol, consistent with the behavior of addition compounds. Increased ethanol concentration caused a progressive decrease in initial velocity (Fig. 5), indicating that the ionization of the steroid side chain was suppressed by the less polar solvent.

Attempts to isolate the cortisol-NAD adduct using the methods described for the dihydroxyacetone complex of NAD$^+$ (Burton *et al.*, 1957) were unrewarding. A preparation was made using cortisol-4-C^{14} but no radioactivity was present in the precipitate, which appeared to contain only alkaline degradation products of NAD$^+$. All of the radioactivity remained in the supernatant fluid apparently as unbound steroid. Attempts to stabilize the reaction product by oxidation with neutral (*p*H 8) or alkaline (*p*H 11) ferricyanide were also unsuccessful.

Formation of the compound could be demonstrated as a yellow fluorescence under ultraviolet light when 300 μg cortisol in 0.1 ml ethanol were added to 300 μg NAD$^+$ in 0.2 ml 0.1 M carbonate buffer *p*H 12. When the reaction mixture was evaporated to dryness, the fluorescence persisted in the residue.

Discussion

In addition to the well-known property of reversibly combining with hydrogen, N^1-substituted nicotinamide derivatives react with a number of low molecular weight compounds (Kaplan, 1960) including dihydroxyacetone. Since C$_{21}$ steroids with a hydroxy or a deoxyketol side chain may be regarded as derivatives of dihydroxyacetone, it was anticipated and confirmed that similar reactions would occur between such steroids and NAD$^+$. The evidence presented here indicates that these reactions involve the condensation of a carbanion derived from the steroid side chain at *p*H values above 10 with the quaternary pyridinium group of NAD$^+$ or its analogs. Theoretically, the nucleophile may attack the 2, 4, or 6 position of the pyridine ring. Direct evidence for the condensation of acetone derivatives with N^1-substituted pyridinium compounds at the γ-position has been presented (Doering and McEwen, 1951). Burton and Kaplan (1954) have suggested 1,4 addition for the analogous dihydroxyacetone-NAD$^+$ derivative, because the spectrum of the addition product resembled that of NADH. A similar suggestion was made by Kosower (1956) on other grounds. However, the studies of Wallenfels and Schuly (1959) indicate that 1,6 addition of steroid and nucleotide would also be consistent with the spectral data. The lack of reactivity of cortisol with pyridinium derivatives when the pyridine ring was γ-substituted indicates a 1,4 addition of cortisol to NAD$^+$. Attempts to isolate the cortisol-NAD condensation product were unsuccessful apparently because, unlike that of dihydroxyacetone with NAD$^+$ (Burton and Kaplan, 1954), the reaction of cortisol with NAD$^+$ was readily reversible. Therefore direct structural studies on the compound could not be conducted.

The rate of formation of condensation product was directly proportional to the hydroxyl ion concentration, indicating that an ionization step was involved in the reaction. The rate was also proportional to cortisol concentration, but independent of NAD$^+$ concentration over a wide range of nucleotide levels. These results suggest that the over-all rate is dependent on the formation of an anion from cortisol. The mechanism shown in Figure 6 is consistent with these data. Although the reaction as written favors an anion at C-21, an alternative mechanism may be suggested in which the attacking anion is formed at C-20.

On the basis of this mechanism, assuming that $k_{-1} >> k_1$, the rate of formation of product is best described by

$$\frac{d(F\text{NAD})}{dt} = \frac{k_1 k_2 (F_t)(\text{NAD}^+)(\text{OH}^-)}{k_{-1}(\text{H}_2\text{O}) + k_2(\text{NAD}^+)}$$

where $F_t = F^- + F$.

Insufficient data are available to evaluate all the kinetic constants. However, the equation indicates qualitatively that the rate is a linear function of (F_t) and (OH^-) when (NAD^+) is held constant. With variable (NAD^+), the equation is that of a rectangular hyperbola. The analysis is therefore consistent with the results illustrated in Figures 2 and 3.

Decreasing the polarity of the system by addition of ethanol resulted in a decreased ionization of cortisol and a diminished over-all reaction rate. If the addition reaction, k_2, were rate-limiting, lowering the polarity of the medium in this way would accelerate the rate of formation of adduct. Burton and Kaplan (1954) observed that the rate of reaction between dihydroxyacetone and NAD$^+$ increased with decreasing dielectric constant and interpreted their results to

mean that the attack of the dihydroxyacetone anion on the pyridinium ring was rate-limiting.

The reaction of acetone with N-substituted pyridinium derivatives has been reported from several laboratories (Huff, 1947; Kaplan *et al.*, 1951). Since progesterone is a substituted acetone derivative, similar reactivity would be anticipated, but it was not observed. This may be due to the low rate of enolization of progesterone, and the low concentration of steroid used.

The nature of the linkage in the present case is clearly different from that described by Munck *et al.* (1957), who reported the formation of complexes between steroids and pyridine nucleotides in which the attractive forces between the components were of the London–Van der Waals type. It is not known at present whether the reactions described in this paper may contribute to an explanation of the mode of action of certain of the corticosteroids. However, it may be significant that, of the various steroids examined, this model reaction was restricted to steroids with a 17-hydroxy- or 17-deoxyketol side chain.

References

Alivisatos, S. G. A., Ungar, F., Jibril, A., and Mourkides, G. A. (1961), *Biochim. Biophys. Acta 51*, 361.

Burton, R. M., and Kaplan, N. O. (1954), *J. Biol. Chem. 206*, 283.

Burton, R. M., San Pietro, A., and Kaplan, N. O. (1957), *Arch. Biochem. Biophys. 70*, 87.

Bush, I. E. (1952), *Biochem. J. 50*, 370.

Cilento, G., and Tedeschi, P. (1961), *J. Biol. Chem. 236*, 907.

Colowick, S. P., and Kaplan, N. O. (1955), *in* Methods in Enzymology, vol. I, New York, Academic, p. 138.

Colowick, S. P., Kaplan, N. O., and Ciotti, M. M. (1951), *J. Biol. Chem. 191*, 447.

Doering, W. Von E., and McEwen, W. E. (1951), *J. Am. Chem. Soc. 73*, 2104.

Engel, L. L., and Langer, L. J. (1961), *Ann. Rev. Biochem. 30*, 499.

Foster, R., Hammick, D. L., and Wardley, A. A. (1953), *J. Chem. Soc.*, 3817.

Hagerman, D. D., and Villee, C. A. (1959), *J. Biol. Chem. 234*, 2031.

Horecker, B. L., and Kornberg, A. (1948), *J. Biol. Chem. 175*, 385.

Huff, J. W. (1947), *J. Biol. Chem. 167*, 151.

Jensen, P. K., and Neuhard, J. (1961), *Biochim. Biophys. Acta 52*, 97.

Kaplan, N. O. (1960), *in* The Enzymes, vol. 3, Boyer, P. D., Lardy, H. A., and Myrbäck, K., eds., New York, Academic, p. 105.

Kaplan, N. O., Colowick, S. P., and Barnes, C. C. (1951), *J. Biol. Chem. 191*, 461.

Kosower, E. M. (1956), *J. Am. Chem. Soc. 78*, 3497.

Kosower, E. M., and Bauer, S. W. (1960), *J. Am. Chem. Soc. 82*, 2191.

Maxwell, E. S., and Topper, Y. L. (1961), *J. Biol. Chem. 236*, 1032.

Munck, A., Scott, J. F., and Engel, L. L. (1957), *Biochim. Biophys. Acta 26*, 397.

San Pietro, A. (1955), *J. Biol. Chem. 217*, 579.

Siegel, J. M., Montgomery, G. A., and Bock, R. M. (1959), *Arch. Biochem. Biophys. 82*, 288.

Talalay, P., and Williams-Ashman, H. G. (1960), Recent Progr. Hormone Res. *16*, 1.

Ungar, F., and Alivisatos, S. G. A. (1961), *Biochim. Biophys. Acta 46*, 406.

Wallenfels, K., and Schuly, H. (1959), *Ann. 621*, 106.

Yielding, K. L., and Tomkins, G. M. (1960a), *Proc. Nat. Acad. Sci. U. S. 46*, 1483.

Yielding, K. L., and Tomkins, G. M. (1960b), *Biochim. Biophys. Acta 39*, 348.

Acid-Catalyzed Addition of Water to 1,4-Dihydronicotinamide Derivatives*

CLARE C. JOHNSTON, JAMES L. GARDNER, CLARENCE H. SUELTER, AND DAVID E. METZLER

From the Department of Biochemistry and Biophysics, Iowa State Univerity, Ames

Received February 6, 1963

The acid modification reactions of reduced diphosphopyridine nucleotide and of *N*-propyl-1,4-dihydronicotinamide have been studied spectrophotometrically. The primary reaction is a general acid-catalyzed transformation. Catalysis constants for a series of acids have been measured: phosphate is especially effective in the neutral *p*H range. The primary acid modification product from *N*-propyl-1,4-dihydronicotinamide exhibits a strong absorption band with a peak at 290 mμ, which shifts reversibly, with a pK_a of 2.3, to a low peak at 304 mμ in acid solutions. The product from diphosphopyridine nucleotide behaves similarly except that the peak in acid solution is at 300 mμ and very high and the pK_a is 0.6. The rate of the secondary modification reaction of *N*-propyl-1,4-dihydronicotinamide is *p*H dependent, the protonated and unprotonated forms of the primary product reacting with different velocities.

Reduced diphosphopyridine nucleotide (DPNH)[1] and other 1,4-dihydronicotinamide derivatives (I) undergo a rapid and apparently irreversible alteration in acidic solutions resulting in a shift of the characteristic ultraviolet absorption band in the 340–360 mμ region downward to around 290 mμ (Fig. 1). This primary acid modification product undergoes a slower secondary reaction to form less strongly absorbing compounds. In most studies of the reaction no products have been isolated, but from the acid decomposition of 1-benzyl-3-acetyl-1,4-dihydropyridine (II) Anderson and Berkelhammer (1958) have prepared two crystalline products. The major product was identified as the hydrated compound, III (equation 1), and the second one as a dimer. It appears likely that similar products are formed from the 1,4-dihydronicotinamides, and Segal and Stein (1960) have isolated a crystalline hydrate from the action of acid on 1-benzyl-1,4-dihydronicotinamide. Most of the earlier literature has been reviewed by Anderson and Berkel-

* Journal Paper No. J- 4238 of the Iowa Agricultural and Home Economics Experiment Station Ames, Project No. 1259. This research was supported by grants from the United States Public Health Service (A-1549) and from the National Science Foundation (G-12339). Presented at the 139th meeting of the American Chemical Society in St. Louis, Mo., March, 1961, Abstracts, p. 60.

[1] Abbreviations used in this paper are as follows: DPNH-reduced diphosphopyridine nucleotide; NPrNH, *N*-propyl, 1,4-dihydronicotinamide; DPN, oxidized diphosphopyridine nucleotide.