Pyridine Coenzymes. VII. Catalysis of the Hydration of the Reduced Form by Iodoaromatic Compounds*

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ABSTRACT: Several aromatics related to thyroxine have been found to catalyze the hydration of 1-benzyl-1,4-dihydronicotinamide, a model for the reduced pyridine nucleotides; the systems containing the natural coenzyme reacted much slower. Some of these catalysts are the most active hitherto reported for this hydration reaction, except for the hydronium ion. From their anomalous position in the Brönsted plot, from activation parameters and nuclear magnetic resonance data it is inferred that: (i) general acid catalysis is involved and (ii) the carbonium ion formed in the rate-determining step is concomitantly stabilized by complexation with the conjugate base of the catalyst. Iodinated casein was somewhat active as a catalyst, the efficiency being hampered by an unfavorable entropy of activation. Thyroglobulin had a greater activity upon the coenzymes than would have its diiodotyrosyl and thyroxyl groups if they were in the free form.

The hydration of the reduced pyridine coenzymes and of their models (Stock *et al.*, 1961; Johnston *et al.*, 1963; Alivisatos *et al.*, 1965; Anderson *et al.*, 1965) is important in connection with the formation of the so called NADH-X (Chaykin *et al.*, 1956; Hilvers *et al.*, 1966), a possible participant in oxidative phosphorylation (Barltrop *et al.*, 1963).

We shall report catalysis of the hydration of the reduced pyridine coenzymes and of their model, 1-benzyl-1,4-dihydronicotinamide, by compounds related to thyroxine. Such catalysts are, on a molar basis, the most efficient hitherto reported for these hydrations, except for the hydronium ion. This finding acquires additional interest in connection with the role that iodoaromatic compounds may play in the oxidation of the reduced pyridine coenzymes as shown in the next paper (Silva Araujo and Cilento, 1969).

Materials and Methods

1-Benzyl-1,4-dihydronicotinamide (mp 123°; Mauzerall and Westheimer, 1955), 3,5-dichloro-4-hydroxybenzoic acid (mp 263°; Gray and Jones, 1954), and 3,5-dibromo-4-hydroxybenzoic acid (mp 278°; Pope and Wood, 1912) were prepared according to the literature. *o*-Bromophenol, *o*-iodophenol, 2,6-dibromophenol, 4-hydroxybenzoic acid and its 3,5-diiodo derivative, DL-tyrosine, and cysteine hydrochloride were all from Eastman Organic Chemicals; L-3-iodotyrosine, L-3,5-dibromotyrosine, and L-3,5-diiodotyrosine were from Aldrich Chemical Co. The following compounds from Sigma Chemical Co. were used without further purification: Tris, NADH, NADPH, thyronine, 3,5-diiodothyronine, 3,5-dibromothyronine, 3,5,3′-triiodothyronine, thyroxine, and bovine thyroglobulin. Glucose-1-phosphoric acid (potassium salt) was from Nutritional Biochemicals Co.

The purity of the compounds was checked spectrally and, whenever possible, by the melting point.

Tyrosine, 3-iodotyrosine, 3,5-diiodotyrosine, and 3,5-dibromotyrosine were recrystallized from aqueous acetic acid and 3,5-diiodo-4-hydroxybenzoic acid from aqueous acetone.

Iodinated casein was prepared according to Reinecke and Turner (1943).

Kinetic runs were made in a Beckman D. U. spectrophotometer; the temperature was kept constant at 24 ± 1° by circulating water from a water bath through a thermospacer fitted in the cell compartment of the spectrophotometer. Stoppered cells of 1-cm optical path and 3-ml capacity were used.

Solutions of the dihydronicotinamide and of the catalyst were made in 0.25 M Tris buffer; whenever necessary, the pH of the catalyst solution was brought to the desired value. The reaction was started by adding the catalyst solution to that of the dihydronicotinamide; a control run was made at the same time in order to follow the "uncatalyzed" reaction. The initial concentration of the dihydronicotinamide was usually about 1×10^{-4} M. The reaction was followed for no more than 5 hr, by the disappearance of the long-wavelength absorption band of the dihydronicotinamide and whenever possible, by the development of the new ultraviolet absorption (280–290 mμ). For most of the catalyzed reactions, spectra were also run as a function of time; the spectral curves were as expected for the hydrated product.

Kinetics. The catalyzed hydration of 1,4-dihydronicotinamides strictly follows pseudo-first-order kinetics. The observed rate constant, k, corresponds to

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$$k = k_{[H^+]}[H^+] + k_w[H_2O] + k_{[Tris,H^+]}[Tris, H^+] + k_{cat}[cat]$$

To obtain the value of $k_{cat}[cat]$, runs were made without the catalyst and the readings subtracted from those for the catalyzed process. This procedure is only justified when the catalyst exists in low concentration and is very active. This is valid in several cases, but not in all. Accordingly, data for some catalysts are only approximate.

Values of $k_{cat}[cat]$ were obtained from the slope of the linear plot of log $(A_t - A_\infty)$ *vs.* t, with a computer programmed for the method of least squares.

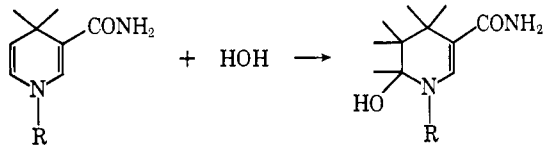
Activation energies, E_A, were obtained from the slope $-E_A/2.303R$ in the plot of log k against $1/T$. The enthalpy of activation, $\Delta H^\ddagger$, was obtained by subtracting RT from E_A. The entropy of activation, $\Delta S^\ddagger$, was calculated with the formula

$$\Delta S^\ddagger = 2.303R(\log k - \log 5.665 \times 10^{10} T) + E_A/T$$

where k is expressed in the unit of sec^{-1}.

Results

The addition of compounds related to thyroxine to a solution of NADH or of its model, 1-benzyl-1,4-dihydronicotinamide, in 0.25 M Tris buffer at pH values near neutrality results in the disappearance of the long-wavelength band of the dihydronicotinamide and appearance of a new, stronger band with maximum at 280 mμ, in the case of the coenzyme, and 290 mμ for the model. These spectral changes are characteristic of hydration of dihydronicotinamides at the 5,6-double bond, more generally of addition across this bond

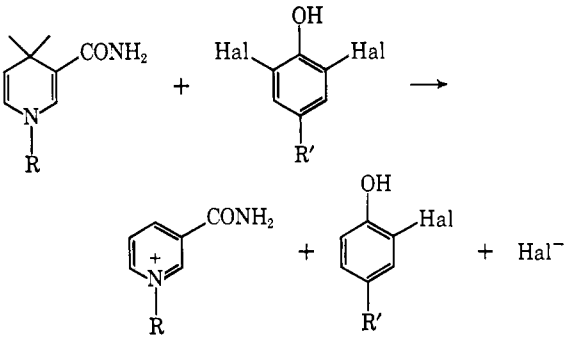


That the compounds act as catalysts is shown by the disappearance of the dihydronicotinamide to an extent much larger than that expected from the addition of the catalyst.

In many cases the ratios $\Delta D_{280}/-\Delta D_{340}$ and $\Delta D_{290}/-\Delta D_{355}$ indicate that hydration of the 5,6-double bond was practically the only reaction taking place; a representative case is shown in Figure 1. The other conceivable process, autoxidation of the dihydronicotinamide (Silva Araujo and Cilento, 1969), was manometrically excluded.

A further concomitant possibility, albeit a remote one, the oxidation of the dihydronicotinamide by the catalyst suggested by a similar reaction with aliphatic halogen derivatives (Kurz *et al.*, 1961) was ruled out by lack of conductometric evidence of ion generation in catalysis by dibromophenol and by the failure to isolate the pyridinium salt with the Dowex 50X-4 cationic exchange resin, a routine procedure in these laboratories.

The hydration of the dihydronicotinamides was found to be independent of the ionic strength. It follows pseudo-first-order kinetics (Figure 2) and depends



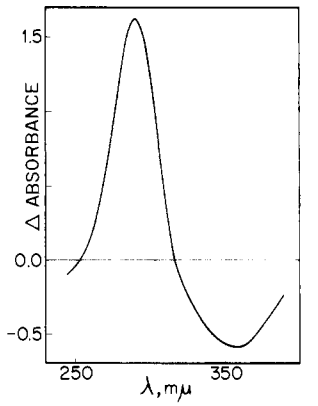


FIGURE 1: Difference spectrum of a 1.36×10^{-4} M solution of 1-benzyl-1,4-dihydronicotinamide in the presence of 5.4×10^{-5} M 3,5-diiodotyrosine at pH 7.14. For each wavelength, the absorbance at zero time was subtracted from that after 21 hr. The value 2.68 for the ratio $\Delta_{290}/\Delta_{355}$ is very close to the theoretical value.

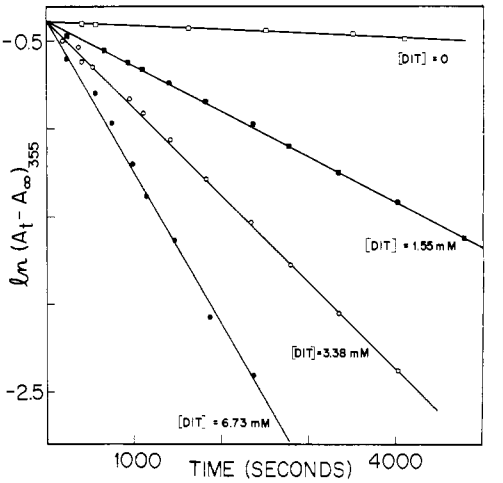


FIGURE 2: Catalytic effect of 3,5-diiodotyrosine at different concentrations upon the disappearance of the long-wavelength band of 1-benzyl-1,4-dihydronicotinamide, at pH 7.00. The rate constants from this experiment are presented in Table I.

linearly upon the catalyst concentration (Table I); therefore, to obtain the second-order rate constant, the pseudo-first-order rate constant should be divided by the catalyst concentration. This raises the question of what form of the catalyst is the active species. The latter appears to be the phenolic form; a representative case is shown in Table II.

Second-order rate constants for the most active systems, those containing dihalogenophenols, are presented in Table III. For less active systems data are collected in Table IV; here the pseudo-first-order rate constants have been divided by the total concentration of the catalyst.

TABLE I: The Effect of the Concentration of 3,5-Diiodotyrosine upon the Rate of Hydration of 1-Benzyl-1,4-dihydronicotinamide at pH 7.00.

Catalyst Concentration			
Total (mM)	Phenolic[a] (mM)	$10^4\ k_{cat}$[cat] (sec^{-1})	k_{cat}[b] ($l\ mol^{-1}\ sec^{-1}$)
1.55	0.29	2.0	0.69
3.38	0.63	4.8	0.76
6.73	1.23	8.4	0.68

[a] Calculated applying the Handerson–Hasselbach equation with the pK_a value, 6.36, taken from Gemmill (1955). [b] For the phenolic form of the catalyst.

In the case of thyroxine, because of its low solubility, even the catalyzed process is still very slow and, therefore, data are only approximate.

Activation parameters are presented in Table V. For comparative purpose, data for cysteine catalysis and for glucose 1-phosphate, which are representative of other classes of active catalysts (S. Schreier, Ph.D. Thesis in preparation, 1968), have also been secured and included in the table. It can be seen that all of the catalysts markedly reduce the activation energy, but 3,5-diiodotyrosine is more active because of a less negative entropy of activation.

No accurate data could be obtained in the case of NADH because even the catalyzed reaction is quite slow. However it seems that the effect of either 3,5-diiodotyrosine or thyroxine is mainly upon the $\Delta S^{\ddagger}$ term.

Clearly, it was of interest to verify if iodinated proteins could, by virtue of their 3,5-diiodotyrosyl groups, act catalytically. On a molar basis, iodinated casein was found to be quite more active than casein upon the hydration of the model coenzyme. Then the pK of the diiodotyrosyl groups was ascertained spectrophotometrically and used for calculating the number of active groups at the pH values of the experiments. The value

TABLE II: The Effect of the pH upon the Rate of the Hydration of 1-Benzyl-1,4-dihydronicotinamide Catalyzed by 3,5-Diiodotyrosine.

	Catalyst Concentration			
pH	Total (mM)	Phenolic[a] (mM)	$10^4\ k_{cat}$[cat] (sec^{-1})	k_{cat}[a] ($l\ mol^{-1}\ sec^{-1}$)
6.16	0.85	0.52	4.3	0.83
6.67	0.88	0.29	2.8	0.96
6.85	1.28	0.31	2.3	0.74
6.99	0.88	0.17	1.3	0.76

[a] As in Table I.

TABLE III: Rate Constants for the Hydration of 1-Benzyl-1,4-dihydronicotinamide and NADH Catalyzed by the Phenolic Form of Halogenated Phenols.

		k_{cat} ($l\ mol^{-1}\ sec^{-1}$)	
Catalyst	pK_a	Benzyldihydronicotinamide	NADH[a]
2,6-Dibromophenol	6.36[b]	0.33	
3,5-Dichloro-4-hydroxybenzoic acid	6.1[c]	0.26	
3,5-Dibromo-4-hydroxybenzoic acid	6.1[d]	0.30	
3,5-Diiodo-4-hydroxybenzoic acid	6.1[c]	0.55	5×10^{-3}
L-3,5-Dibromotyrosine	6.36[e]	0.41	6×10^{-3}
L-3,5-Diiodotyrosine	6.36[e]	0.73	9×10^{-3}
Thyroxine	6.73[e]	0.75	
Cysteine[f]		0.004	
Glucose 1-phosphate[f]	6.13[g]	0.017	

[a] The values are less accurate because the reactions were slow. [b] Taken as equal to that of 3,5-dibromotyrosine. [c] Taken as equal to that of the dibromo analog. [d] Ibne-Rasa (1962). [e] Gemmill (1955). [f] Included for comparative purposes. [g] At 30° reported by Cori *et al.* (1937).

TABLE IV: Rate Constants for the Hydration of 1-Benzyl-1,4-dihydronicotinamide and NADH Catalyzed by Various Phenolic Compounds.

Catalyst	pH	$10^3 k_{cat}$[a] (l mol^{-1} sec^{-1})	
		Benzyldihydro-nicotinamide	NADH
Tyrosine	6.92	0.8	
	7.82	0.8	
	10.11	0.6	
o-Bromophenol	7.20	6.1	
o-Iodophenol	7.10	6.4	
p-Hydroxybenzoic acid	6.92	1.4	0.03
L-3-Iodotyrosine	6.80	13	
	7.03	17	
	7.12	—	0.26
	7.69	8.6	
Thyronine	6.82	3.3	
	7.01	2.8	0.18
	7.92	3.6	
3,5-Dibromothyronine	6.85	28	
	7.16	26	
	7.74	24	
3,5-Diiodothyronine	7.15	78	
3,5,3′-Triiodothyronine	7.74	78	7.8
	6.76	151	

[a] Based on the total concentration of the catalyst.

obtained for the apparent pK is 7.6 and does not depend upon the ionic strength; this value is close to that for thyroglobulin (Edelhoch, 1962). It turned out that the diiodotyrosyl groups in the protein are less active than the free amino acid. Because of solubility limitations the effect of the dihydronicotinamide concentration upon the rate could not be investigated.

The influence of pH on the catalysis by iodocasein was also studied. When account was taken of the relative concentration of the diiodotyrosil groups in the phenolic form, constant values were obtained in the range pH 8.5–7.0. At lower pH values the control reaction becomes serious; despite this fact it appears that the catalyzed process becomes markedly faster (Figure 3). This may be due to a more active diiodotyrosyl group, partly because of a lower pK_a or because of a new conformation of the protein, perhaps as a result of protonation of an imidazole group.

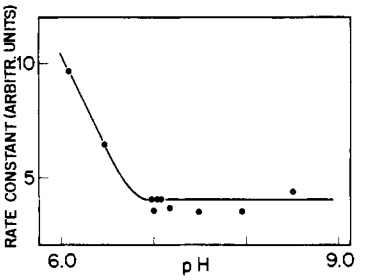


FIGURE 3: The effect of the pH upon the rate constant of the hydration of 1-benzyl-1,4-dihydronicotinamide catalyzed by iodocasein.

TABLE V: Activation Parameters for the Catalyzed Hydration of 1-Benzyl-1,4-dihydronicotinamide.

Catalyst	E_a (kcal/mole)	$\Delta H^{\ddagger}$ (kcal/mole)	$\Delta S^{\ddagger}$ (eu)
	(21.9)		
3,5-Diiodotyrosine	15.5	14.9	−9.2
Iodocasein	14.7	14.1	−16
Cysteine	16.0	15.4	−19
Glucose 1-phosphate	14.0	13.4	−21

One should inquire why at neutral and alkaline pH values the activity of iodinated casein is lower than that of the free amino acid. From the values of activation parameters (Table V) it appears that the articulation of the transition state is more difficult in the case of the protein, as a more negative $\Delta S^{\ddagger}$ value obtains.

A natural iodinated protein is thyroglobulin. It was found to be no more active upon the model coenzyme than if the diiodotyrosyl and thyroxyl groups were free. The number of those groups was calculated from data of Robbins and Rall (1960). However, preliminary results indicate an enhanced activity for this protein acting upon NADH and NADPH. These findings are

consistent with those of Edelhoch and Perlman (1968) which indicate complete exposition of the diiodotyrosyl and thyroxyl groups.

Discussion

It is likely that the mechanism of hydration by phenolic catalysts involves general acid catalysis. However, the operation of other factors is clearly indicated by the anomalies in the Brönsted plot (Figure 4). In this regard, one may note that on passing from tyrosine to thyronine, and from the latter to its 3,5-diiodo derivative a relatively great increase in catalytic efficiency occurs despite the fact that the three compounds have similar pK value for the phenolic group. It is also striking the increase on passing from 3-iodotyrosine to 3,5,3′-triiodothyronine, a thyroid hormone.

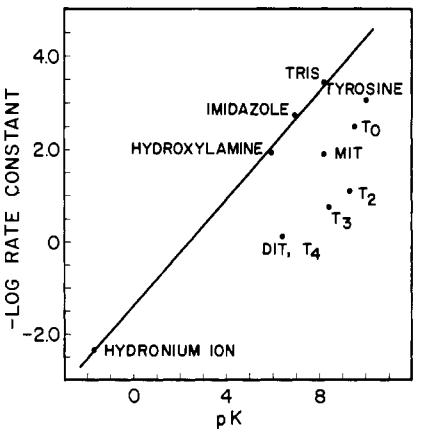


FIGURE 4: "Brönsted plot" of catalytic rate constants against the acid dissociation constant of the catalyst. The line has been driven with the same slope as that in the work of Anderson *et al.* (1965). T_0, thyronine; T_2, 3,5-diiodothyronine; T_3, 3,5,3′-triiodothyronine; T_4, thyroxine; MIT, L-3-iodotyrosine; DIT, L-3,5-diiodotyrosine.

It may be that simultaneously with the rate-determining proton transfer a complex starts forming between the incipient carbonium ion and the phenolate ion, whereby the free energy of activation is further lowered. The carbonium ion formed by proton addition to 1,4-dihydropyridines can form charge-transfer complexes by acting as acceptor (Kosower and Sorensen, 1962); on the other hand an outstanding ability of the diiodophenolic group, and even greater of the diiodophenolate group, to act as donor in charge-transfer complexes was observed by Cilento and Berenholc (1965).

Two points should be stressed in connection with such hypothetical mechanism. First, that it has some analogy with the one proposed by Braude *et al.* (1954) for the quinol-catalyzed hydrogen transfer from 1,4-dihydronaphthalene to quinones. Second, that the extra stabilization by complex formation has probably a large entropic contribution, that is, the catalyst would reduce the extent of ordering of water molecules in the transition state. The latter suggestion is based on the fact that 3,5-diiodotyrosine associates with the pyridinium cation to a greater extent than tyrosine does, mainly because of a less negative entropy (Cilento and Berenholc, 1965). The suggestion is also supported by the more favorable $\Delta S^{\ddagger}$ term in catalysis by 3,5-diiodotyrosine as compared with other catalysts (Table V). The hydrophobic stabilization might make somewhat more difficult the second step, the collapse of the cation in the solvation shell, but presumably not to the extent to make this step rate determining.

In this work, it has been assumed that protonation is the rate-determining step. However, two other mathematically equivalent mechanisms should also be considered. One of them, concerted catalysis involving an *o*-iodo group, is made unlikely by the activity of tyrosine, thyronine, and 3,5-diiodothyronine. The other, equilibrium protonation followed by a rate-determining general base catalyzed reaction with water, could be ruled out using nuclear magnetic resonance data reported by Choi and Alivisatos (1968) for the hydration of dihydronicotinamides catalyzed by phosphate in D_2O. Thus, a preliminary fast protonation step would induce, by exchange, incorporation of deuterium at the carbon to which the proton adds, that is, at C_5. This is not consistent with the fact that all signals due to ring protons decay simultaneously and with the existence of signal from C_5 in the reaction product.

That protonation is indeed rate determining in aqueous media has been, very recently and unequivocally, demonstrated by the observation of a significant isotopic rate effect (Kim and Chaykin, 1968).

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Pyridine Coenzymes. VIII. Autoxidation of the Reduced Form Catalyzed by 4-Amino-2,6-diiodophenol*

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ABSTRACT: The reduced pyridine coenzymes are stoichiometrically and directly oxidized by molecular oxygen to the pyridinium form, under the catalytical effect of protonated 4-amino-2,6-diiodophenol. With the model coenzyme, 1-benzyl-1,4-dihydronicotinamide, two processes take place simultaneously: (i) the direct oxidation and (ii) addition to the 5,6-double bond of the dihydronicotinamide followed by autoxidation of the resulting product. The latter process is more important in phosphate than in Tris buffer and leads to a new cation, besides the pyridinium ion. The catalyst does not shuttle electrons between the dihydronicotinamide or its 5,6 derivatives, and oxygen; it specifically promotes the transfer of an hydride ion or of its elements to the O_2 molecules by preferential stabilization of the transition state through complexation and proton donation.

In the precedent paper (Schreier and Cilento, 1969) it was reported that *o*-halogenophenols, including thyroid hormones, catalyze the hydration of 1,4-dihydronicotinamides. We have now found that DIPAP[1] not only catalyzes solvent addition to 1-benzyl-1,4-dihydronicotinamide, a model coenzyme, but also the autoxidation of the resulting or intermediate products as well as of the model itself. In the case of the natural coenzymes, NADH and NADPH, only the autoxidation occurs with stoichiometric formation of NAD, and, almost so, of NADP. This work joins and significantly expands another line of research, that of nonclassical oxygen activation (Cilento and Zinner, 1966, 1967a–c). The important novel feature is that DIPAP, unlike other nonclassical oxygen activators, does not catalyze the autoxidation of compounds which autoxidize by one electron or H · atom transfer.

The present work appears therefore of considerable significance in connection with the transfer of the H^- ion or of its elements to oxygen.

Materials

DIPAP (mp 220°) was prepared according to Woollett *et al.* (1937), 2,6-diiodo-*p*-benzoquinone (mp 180°) according to Seifert (1883), and 1-benzyl-1,4-dihydronicotinamide (mp 123°) according to Mauzerall and Westheimer (1955). They were pure by both melting and spectral criteria. NADH, NADPH, and D-glucose 6-phosphate (disodium salt) were from Sigma Chemical Co.; the alcohol dehydrogenase and glucose 6-phosphate dehydrogenase from C. F. Bohringer & Soehne. All the other compounds were available in these laboratories from earlier work and were purified once more by routine procedures.

Methods

The reaction has been studied at 25° manometrically by following O_2 uptake in the Warburg apparatus and spectrophotometrically by following the disappearance of the long-wavelength band of the dihydronicotinamide. The solvents were Tris–CH_3OH (1:1 or 1:2, v/v) and phosphate–CH_3OH (1:1, v/v) buffers of 0.1 M concentration. Occasionally acetone was substituted

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† Predoctoral fellow of the "Fundação de Amparo a Pesquisa do Estado de São Paulo."

[1] DIPAP = 4-amino-2,6-diiodophenol.