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The Effects of Coenzymes and Substrates on the Rate of Zinc Exchange in Horse Liver Alcohol Dehydrogenase*

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Received March 18, 1964

The rate of exchange of the two zinc atoms of horse liver–alcohol dehydrogenase has been determined by equilibrium dialysis. The isotopically labeled enzyme was exposed to stable zinc ions, and the displacement of ^{65}Zn was measured. Coenzyme, coenzyme moieties, substrates, or substrate homologs alone did not affect the rates of exchange. However, DPN(H), AMP, and ADP ribose *in combination with* substrates or substrate homologs greatly retard the exchange rates. DPNH paired with hexanamide, isobutyramide, or acetamide, and DPN^+ paired with acetate or hydroxylamine were the most effective couples in this regard. *N*-Methylnicotinamide alone or combined with substrates or substrate homologs was completely ineffective in blocking exchange, supporting the view that this moiety is not mandatory for the formation of the enzyme-coenzyme complex (Li and Vallee, 1963; 1964). Some of the substrate homologs which retard exchange, in conjunction with the coenzymes, lack functional groups for binding to the metal atom. The association constants of zinc-DPN(H) complexes have been measured by the ion-exchange method of Schubert (1956). The constants are lower by orders of magnitudes than those of the respective enzyme-coenzyme complexes. Hence interaction of the coenzymes with other, as yet unidentified groups of the apoenzyme must add significantly to the stability of the enzyme-coenzyme complexes.

Recent studies of the mechanism of action of equine liver–alcohol dehydrogenase [(LADH)$^{65}Zn_2$][1] in our laboratory have focused on the binding of coenzymes and substrates, and on the role of zinc in these interactions (Vallee and Coombs, 1959; Vallee *et al.*, 1959; Ulmer *et al.*, 1961; Li *et al.*, 1963). The unique optical properties of the liver-alcohol dehydrogenase–coenzyme complex have permitted direct and detailed examination of the mechanism of coenzyme binding by a variety of approaches (Boyer and Theorell, 1956; Kaplan, 1960; Ulmer *et al.*, 1961; Li *et al.*, 1962; Li and Vallee, 1963, 1964). In contrast, the interaction of substrates and their homologs, which lack suitable chromophoric groups, has had to be studied indirectly, by virtue of their effects on the kinetics of the enzymatic reaction and on the optical properties of the bound coenzyme at equilibrium (Winer and Theorell, 1960; Theorell and McKinley-McKee, 1961; Ulmer *et al.*, 1961).

We have reported that the two firmly bound zinc atoms at the active centers of liver-alcohol dehydrogenase can be exchanged for zinc-65 by equilibrium dialysis (Druyan and Vallee, 1962). When exposed to stable zinc ions, [(LADH)$^{65}Zn_2$] undergoes isotopic exchange, stable Zn^{2+} displacing $^{65}Zn^{2+}$. The rate of exchange measures the reactivity of the zinc atoms at the active sites of the enzyme.

The stability constant of the zinc-enzyme complex is one of the factors which determines the rate at which Zn^{2+} exchanges with $^{65}Zn^{2+}$. However, the binding of coenzymes, coenzyme moieties, substrates, and substrate homologs to the zinc atom or to sites in its proximity would also be expected to influence the rate of exchange. The present study demonstrates that DPN^+, DPNH, and other specific coenzyme moieties, in combination with substrate and substrate homologs, markedly retard isotopic exchange. The stability constants of the zinc-coenzyme (moiety) complexes have also been described.

Materials and Methods

Crystalline horse liver—alcohol dehydrogenase was obtained from C. F. Boehringer und Soehne, Mannheim, W. Germany, and [(LADH)$^{65}Zn_2$] was prepared as described (Druyan and Vallee, 1962). Protein concentration was measured spectrophotometrically at 280 mμ, using an absorbance of 0.455 mg^{-1} cm^2 (Bonnichsen, 1950). The concentration of DPNH

* This investigation was supported in part by the Howard Hughes Medical Institute and by a Public Health Service grant (HE-07297) from the National Institutes of Health.

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[1] Abbreviation used in this work: [(LADH)$^{65}Zn_2$], liver-alcohol dehydrogenase.

was determined from the known molar absorbance (Kaplan, 1960). Ethanol (95%) was purified by passage over a Dowex-50 column. Sodium acetate, acetamide, dimethylformamide, formamide, hexanamide, and hydroxylamine were reagent grade chemicals. To remove contaminating zinc ions, solutions of these reagents, dissolved in 0.1 M sodium succinate buffer, *p*H 6.0, were extracted three times with 0.01% dithizone. Reagent grade acetaldehyde was distilled at 20° immediately prior to use. *N*-Methyl nicotinamide and urea were recrystallized three times from metal-free water to remove contaminating metals (Hoch *et al.*, 1960). Ionic zinc was prepared by dissolving the metal (Johnson Matthey, "Spec-Pure") in metal-free hydrochloric acid. Dialyses were performed in precleaned (Hughes and Klotz, 1956) cellulose casings (Visking Co.). The purification of water and cleaning of glassware have been described (Vallee and Hoch, 1955).

The rate of exchange of Zn^{2+} with $^{65}Zn^{2+}$ in [(LADH)^{65}-Zn_2] was determined by placing $^{65}Zn^{2+}$-labeled liver-alcohol dehydrogenase into a dialysis bag, and by measuring the displacement of the radioactive isotope (10,000–20,000 cpm/ml) during dialysis against stable zinc ions. In a typical experiment, 1.0 ml of 5×10^{-5} M [(LADH)$^{65}Zn_2$] and 1.0 ml of buffer (blank) were dialyzed against 50 ml of 0.1 M succinate, *p*H 6.0, and the coenzyme, substrate, or both, in appropriate concentrations, were added to the dialysate. Gentle agitation of all dialyses was provided by a rotating platform. After equilibrating radioactive enzyme with substrate or coenzyme or both for 4 hours, isotopic exchange was initiated by the addition of 5×10^{-5} M stable Zn^{2+} to the dialysate. Radioactivity in the dialysis bags containing enzyme and buffer, respectively, was measured as a function of time of dialysis. Since stable zinc progressively displaces $^{65}Zn^{2+}$ from [(LADH)$^{65}Zn_2$] into the dialysate, the number of counts in the dialysis bag containing only buffer was subtracted from that of the dialysis bag containing the enzyme, in order to obtain the actual number of counts remaining in the enzyme. Prior to counting, the dialysis bags were rinsed with cold fresh buffer and placed in a clean test tube, and then radioactivity was counted in a well-type scintillation detector (Tracerlab). The rates of exchange are first order, and for comparison, the rate is expressed as $t_{1/2}$, the time required for one-half the initial $^{65}Zn^{2+}$ to be displaced from the radioactive enzymes.

The association constants of zinc with coenzyme and coenzyme moieties were determined by the ion-exchange method of Schubert (1956). High-specific-radioactivity $^{65}Zn^{2+}$ (Oak Ridge National Laboratories) was used without carrier dilution. Dowex-50-X2 resin (Bio-Rad) was prepared with alternating washes of HCl and NaOH. Solutions were buffered with barbital at *p*H 8.0, and NaCl served as the supporting electrolyte. Both reagents were extracted with dithizone prior to use. Dowex 50 resin (40 mg) was added to 20 ml of 0.001 M barbital–0.1 M NaCl containing 50,000–100,000 cpm $^{65}Zn^{2+}$ per ml, while the concentration of coenzyme or coenzyme moiety was varied. The samples were equilibrated with the resin for 3 hours at 3° in a motor-driven shaker (60 cycles/min). Under these conditions, equilibrium between the aqueous and resin phases was attained within 1 hour. The partition coefficient, λ, expressing the distribution of $^{65}Zn^{2+}$ between aqueous and resin phases at equilibrium, is determined by measuring the initial radioactivity remaining in solution at equilibrium according to the relationship $(^{65}Zn_{I,a}{}^{2+}) = {}^{65}Zn_{E,a}{}^{2+}) + (^{65}Zn_{E,r}{}^{2+})$, where the subscripts I and E refer to the initial distribution of $^{65}Zn^{2+}$ and that attained at equilibrium, respectively, and where a and r identify the aqueous and resin phases. The association constants for a given ligand, L, in the presence of buffer, B, are derived as follows:

$$K_a = \left[\frac{(\lambda_0/\lambda_L) - 1}{L^n}\right]$$

where

K_a = apparent association constant

$\lambda_0 = \dfrac{^{65}Zn^{2+} - \text{resin}}{^{65}Zn^{2+} - \text{supernatant}}$ in the absence of ligand

$\lambda_L = \dfrac{^{65}Zn^{2+} - \text{resin}}{^{65}Zn^{2+} - \text{supernatant}}$ in the presence of ligand

L = ligand concentration

n = moles of ligand per mole of zinc

$$K_c = K_a\,[1 + (B)^n(K_B)]$$

where

K_c = association constant, corrected for buffer

B = buffer concentration

n = moles buffer per mole zinc

K_B = association constant of the buffer

Values for n are obtained by curve fitting. K_a (or K_B) are essentially invariant over wide ranges of ligand or buffer ion concentrations when the correct value, n, for the molar ratios of $^{65}Zn^{2+}$ to ligand is chosen. Thus, for example, at barbital concentrations of 1.0, 3.0, 6.0, and 8.0×10^{-3} M, zinc and barbital form a 1:1 complex ($n = 1$); the association constant, K_B, is 114 ± 26.

The coefficient of variation for these measurements ranges between 10 and 25% when the standard deviation is calculated from duplicate measurements at each ligand concentration studied.

Results

When [(LADH)$^{65}Zn_2$] is dialyzed against 0.1 M succinate buffer at *p*H 6.0, no $^{65}Zn^{2+}$ is lost from the enzyme over a 40-hour period. The addition of stoichiometric concentrations of zinc, however, displaces $^{65}Zn^{2+}$ from the enzyme at a first-order rate; the half-life of the exchange is 20 ± 4 hours.

The addition of catalytically active coenzymes, which bind firmly to liver-alcohol dehydrogenase at, or near its zinc atoms, might be expected to alter the rate of exchange. However, the addition of 5×10^{-3} M DPN^+ or 5×10^{-4} M DPNH to the dialysate, concentrations at which the coenzyme-binding sites are saturated 95% or more (Yonetani, 1963), result in only a slight increase in the half-life for $Zn^{2+} \rightleftharpoons {}^{65}Zn^{2+}$ exchange (Table I); binding of the coenzyme alone at the active sites does not result in a significant alteration of the exchangeability of zinc.

The chemical identity of the sites at which substrates bind to the enzyme are not known. Aliphatic amides, aliphatic acids, and hydroxylamine, all of which serve as catalytically inactive substrate homologs, are thought to bind at the same sites at which the true substrates interact (Kaplan and Ciotti, 1954; Winer and Theorell, 1960). Like DPN^+ and DPNH, 0.1 M ethanol, acetate, acetamide, hydroxylamine, isobutyramide, and 0.01 M hexanamide affect the rate of zinc exchange little or not at all.

In sharp contrast to the effects of coenzyme or substrate (homolog) alone, joint addition of coenzyme and substrate (nomolog) markedly retards the rate of exchange (Table I). As a function of the coenzyme-substrate (homolog) pair added the half-life for zinc exchange is increased from 2.5- to 11.5-fold. Among these, DPNH paired either with acetamide, isobutyr-

TABLE I

RETARDATION OF EXCHANGE OF Zn^{2+} FOR $^{65}Zn^{2+}$ IN [(LADH)$^{65}Zn_2$][a] BY COENZYME AND/OR SUBSTRATE (HOMOLOG)

Coenzyme	Substrate	Substrate	Half-Life (hours)
0		0	20
DPN$^+$ 5×10^{-3} M		0	27
DPNH 5×10^{-4} M		0	26
0	Acetate	0.1 M	33
	Acetamide		20
	Ethanol		26
	Hydroxylamine		20
	Isobutyramide		25
	Hexanamide[a]	0.01 M	26
DPN$^+$ 5×10^{-3} M	Acetaldehyde	0.1 M	22
	Acetate		163
	Hydroxylamine		110
	Isobutyramide		55
DPNH 5×10^{-4} M	Ethanol	0.1 M	69
	Hydroxylamine		53
	Acetamide		110
	Isobutyramide		169
	Hexanamide[b]	0.01 M	231

[a] [(LADH)$^{65}Zn_2$] = Zn^{2+} = 5×10^{-5} M. [b] Employed at lower concentrations because of limited solubility.

amide, or hexanamide, and DPN$^+$ paired with acetate or hydroxylamine are most effective in retarding the exchange. DPN$^+$ paired with ethanol, and DPNH paired with acetaldehyde could not be examined since both substrates are catalyzed under these conditions.

Groups of the purine nucleotide moiety of DPN(H) are apparently crucial for direct binding to the enzyme, while the nicotinamide moiety does not seem essential for this purpose (Li and Vallee, 1963, 1964). Hence the effects of various moieties of DPN(H) on the rate of zinc exchange in the enzyme were examined in a similar manner (Table II). Adenosine, AMP, ADP-ribose, and *N*-methylnicotinamide, all 5×10^{-4} M, do not retard the exchange of Zn^{2+} for $^{65}Zn^{2+}$ significantly. In conjunction with 0.1 M isobutyramide, however, 5×10^{-4} M AMP and ADP-ribose here employed, prolong the half-life of exchange sufficiently to make the measured $t_{1/2}$ comparable to that observed with 5×10^{-3} M DPN and 0.1 M isobutyramide (Table II). In contrast, adenosine and *N*-methylnicotinamide remain ineffective in blocking exchange (Table II). These results support the view that the adenine nucleotide moiety of the coenzyme does, indeed, bind to the enzyme, further localizing its site of interaction to or near the zinc atoms of liver-alcohol dehydrogenase.

TABLE II

RETARDATION OF EXCHANGE OF Zn^{2+} FOR $^{65}Zn^{2+}$ IN [(LADH)$^{65}Zn_2$] BY COENZYME MOIETIES ± ISOBUTYRAMIDE

Coenzyme Moiety[a]	Substrate Homolog	Half-Life (hours)
None	None	20
None	Isobutyramide 0.1 M	25
Adenosine	5×10^{-4} M None	24
AMP		35
ADP-r		33
NMN		20
Adenosine	5×10^{-4} M Isobutyramide 0.1 M	25
AMP		63
ADP-r		58
NMN		20

[a] AMP = adenosine monophosphate, ADP-r = adenosine diphosphate ribose, NMN = *N*-methylnicotinamide.

TABLE III

RETARDATION OF EXCHANGE OF Zn^{2+} FOR $^{65}Zn^{2+}$ IN [(LADH)$^{65}Zn_2$][a] BY SUBSTRATE HOMOLOGS + DPNH

Coenzyme	Substrate Homolog	Substrate	Half-Life (hours)
None	None		20
DPNH 5×10^{-4}	None		26
None	Formamide	0.1 M	[b]
	Acetamide		20
	Isobutyramide		25
	Urea		22
	Dimethylformamide		24
DPNH 5×10^{-4} M	Formamide	0.1 M	41
	Acetamide		110
	Isobutyramide		169
	Dimethylformamide		154
	Urea		31

[a] [(LADH)$^{65}Zn_2$] = Zn^{2+} = 5×10^{-5} M. [b] Does not follow first-order kinetics; exchange is more rapid than control.

The substrate specificity of liver-alcohol dehydrogenase is known to be broad (Winer, 1958); further, the enzyme is inhibited by a variety of aliphatic amides (Winer and Theorell, 1960). Therefore a series of substituted amides was added in conjunction with DPNH to examine the structural requirements for the binding of substrate homologs (Table III). Formamide retards exchange only slightly. Acetamide, which contains a second aliphatic carbon atom, increases the half-life by 70 hours. Urea, the product of substitution of a second amide in the same position, does not exhibit the effect. Dimethylformamide, with two methyl group substituents in the amide function of formamide, markedly retards exchange. Longer-chain aliphatic substitution, as represented by isobutyramide and hexanamide, extends the half-life even further.

Although studies with metal-chelating agents have shown that the zinc atoms participate in enzyme-coenzyme binding in some manner (Vallee and Coombs, 1959; Mahler *et al.*, 1962), the contribution of a possible metal-coenzyme bond to the overall stability of the enzyme-coenzyme complex is not known. The failure of the coenzyme to retard zinc exchange significantly suggests that the stability of the zinc-coenzyme complex is low. This is supported experimentally by direct measurements of the association constants of complexes of zinc ions with coenzymes and coenzyme moieties, as evaluated by ion-exchange methods at *p*H 8.0, 3°.

Data and calculations of the association constants for two ligands, AMP and adenosine, are presented in Table IV. For AMP, when $n = 1$, $K_a = 256 \pm 32$ remaining constant over a 4-fold ligand concentration range. When higher values of n are assumed, K_a decreases as the ligand concentration increases. Adenosine apparently does not form a zinc complex under the conditions employed; increasing concentrations of adenosine fail to keep $^{65}Zn^{2+}$ in the aqueous phase (Table IV).

The association constants of the 1:1 complexes of the zinc with AMP, DPN$^+$, and DPNH were measured in this fashion (Table V). Confidence limits for K_a were calculated as described by Snedecor (1946).

TABLE IV

MEASUREMENTS AND CALCULATIONS FOR THE DETERMINATION OF ASSOCIATION CONSTANTS FOR Zn^{2+}-AMP AND Zn^{2+}-ADENOSINE COMPLEXES[a]

L	$^{65}Zn_{E,a}{}^{2+}$	$^{65}Zn_{E,r}{}^{2+}$	λ	$\frac{\lambda_0}{\lambda_L} - 1$	$K_{a(n=1)}$
		AMP			
0	23,603	45,548	2.0811[b]		
	21,392	47,759			
1.1×10^{-3}	25,939	43,212	1.6659	0.2492	226
	26,323	42,828	1.6220	0.2791	253
2.2×10^{-3}	29,817	39,334	1.3191	0.5776	262
	28,594	40,557	1.4183	0.4673	212
2.9×10^{-3}	30,955	38,195	1.2339	0.6866	236
	32,714	36,437	1.1158	0.8684	299
3.7×10^{-3}	34,880	34,271	0.9825	0.1181	302
	34,414	34,737	1.0094	1.0617	286
		Adenosine			
0	23,737	45,789	2.1488[b]		[c]
	21,195	49,331			[c]
1.25×10^{-3}	20,848	49,678	2.3828	−0.0983	[c]
	21,871	48,665	2.2250	−0.0343	[c]
2.5×10^{-3}	20,732	49,794	2.4017	−0.0527	[c]
	21,579	48,947	2.2682	−0.1054	[c]
5.0×10^{-3}	23,461	47,065	2.0060	+0.0711	14
	21,077	49,449	2.3461	−0.0841	[c]
7.5×10^{-3}	21,287	49,239	2.3131	−0.0811	[c]
	21,093	49,433	2.3435	−0.0831	[c]

[a] Upper half of table, Zn-AMP; lower half, Zn-adenosine. L = ligand concentration (moles/liter). $^{65}Zn_{E,a}{}^{2+}$ = cpm/ml of $^{65}Zn^{2+}$ in aqueous phase at equilibrium. $^{65}Zn_{E,r}{}^{2+}$ = cpm/ml of $^{65}Zn^{2+}$ in resin phase at equilibrium. λ = partition coefficient, $^{65}Zn_r{}^{2+}/^{65}Zn_a{}^{2+}$. λ_0 = partition coefficient in absence of ligand. λ_L = partition coefficient in presence of ligand. K_a = association constant. n = number of moles ligand per mole zinc. [b] Value of λ_0. [c] No measurable binding.

TABLE V

ASSOCIATION CONSTANTS OF $^{65}Zn^{2+}$ AND COENZYME (COENZYME MOIETIES) COMPLEXES AS MEASURED BY ION EXCHANGE[a]

Ligand	n	K_a	95% Confidence Limits (K_a)	K_c
Barbital	1	114	53–175	
AMP	1	256	180–332	285
DPN^+	1	25	13– 37	28
DPNH	1	714	459–969	795

[a] n = moles zinc per mole ligand; K_a = association constant, uncorrected for buffer; K_c = association constant, corrected for buffer. For the calculation of K_c, K_B = 114 and n = 1.

DISCUSSION

The formation of an enzyme-coenzyme-substrate complex has been inferred from kinetic studies. (Theorell and Chance, 1951; Vallee *et al.*, 1959; Theorell and McKinley-McKee, 1961). The existence of such complexes has been substantiated further by the effects of substrate homologs on the optical properties of the enzyme-coenzyme complex. Thus fluorescence of the enzyme-DPNH complex is enhanced on addition of substrate homologs (Winer and Theorell, 1960). Similarly, in studies of optical rotatory dispersion, acetamide increases the amplitude, but not the breadth, of the Cotton effect of the horse liver–alcohol dehydrogenase–DPNH complex (Ulmer *et al.*, 1961). However, in both instances the evidence for binding of the substrate to the active center of the enzyme is indirect, since the optical parameters under observation are generated by the coenzyme moiety of the complex, and since the physicochemical basis for the manifestations accompanying the addition of substrate homologs is not apparent.

The alteration in the capacity of the two zinc atoms of the apoenzyme to exchange introduces a new experimental approach for the evaluation of binding of coenzymes, coenzyme moieties, substrates, and substrate homologs. In contrast to the optical methods, the isotopic exchange technique focuses on changes induced at the active site of the enzyme protein, rather than those affecting the bound coenzyme. This approach is therefore specifically directed at interactions pertaining to the catalytic site of the enzyme. Furthermore, since $Zn^{2+} \rightleftharpoons {}^{65}Zn^{2+}$ exchange is sensitive only to conditions in which substrate and coenzyme are added jointly, the isotopic exchange method provides additional and more direct evidence for the existence of enzyme-coenzyme-substrate complexes, while localizing them at the active, zinc-containing sites of horse liver–alcohol dehydrogenase. The possibility that exchange may involve extrinsic, noncatalytic sites (Vallee, 1955) is effectively excluded by earlier experiments, which demonstrated that virtually all of the functional radioactive zinc in [(LADH)$^{65}Zn_2$] is part of the active centers of the enzyme (Druyan and Vallee, 1962; R. Druyan and B. L. Vallee, paper in preparation).

The coenzymes are known to bind at or near the zinc atoms, since the chelating agent, 1,10-phenanthroline competes with them and abolishes the Cotton effect of the enzyme-DPNH complex (Vallee *et al.*, 1959; Ulmer *et al.*, 1961). There is still ambiguity, however, concerning the sites at which the substrates may bind. Since 1,10-phenanthroline does not compete directly with the substrates, the latter have been thought to interact at sites other than the zinc atoms (Vallee *et al.*, 1959; Mahler *et al.*, 1962), though a number of considerations and additional data have led to other conclusions (Plane and Theorell, 1961). Since the substrates were found to affect the competition of 1,10-phenanthroline with the coenzyme, it was suggested that the substrate may interact with the bound coenzyme at a vicinal site, in a manner which could alter the affinity of the coenzyme for the zinc site indirectly (Vallee *et al.*, 1959). The present data, obtained under equilibrium conditions, lend support to this view,

though neither the present nor previously employed techniques are capable of detecting binary enzyme-substrate complexes directly. The retardation of zinc exchange by some substrates and substrate homologs upon formation of ternary complexes is consistent with the previous observation that increasing concentrations of ethanol, in the presence of DPN, further reduce the accessibility of zinc to 1,10-phenanthroline (Vallee *et al.*, 1959). In analogous fashion, the slight acceleration of isotopic-exchange rates by the DPN-acetaldehyde pair as compared to that observed with DPN alone would seem to correspond to the increased accessibility (or apparent affinity) of the zinc atoms to 1,10-phenanthroline observed in the presence of acetaldehyde (Vallee *et al.*, 1959).

The isotopic-exchange system also permits the examination of the interaction of catalytically inactive coenzyme moieties with liver-alcohol dehydrogenase. Thus, AMP- and ADP-ribose bind to the enzyme and therefore behave similarly to DPN(H) while *N*-methylnicotinamide and adenosine fail to do so. This supports the view that the adenine nucleotide moiety of DPN(H) is crucial for coenzyme binding, consistent with the spectropolarimetric evidence reported by Li and Vallee (1963, 1964). The retardation of $Zn^{2+} \rightleftharpoons {}^{65}Zn^{2+}$ exchange by pairs of substrate homologs and AMP (or ADP-ribose) would furthermore seem to indicate that the nicotinamide moiety of the coenzyme is not essential for ternary complex formation.

Although all existent evidence localizes the interaction between coenzymes and apoenzyme at or near the zinc sites the exact manner in which the metal atom might participate in binding the coenzyme is not known. The stability of DPN(H)-zinc complexes in solution and the contribution of the metal atom to the formation of enzyme-coenzyme complexes have been discussed repeatedly, but the various models proposed thus far have not lent themselves to examination by decisive experiments (Vallee *et al.*, 1956). Studies by ionophoresis (Kaye, 1955) or observations of the effect of DPN(H) on the solubility of $^{65}Zn(OH)_2$ (Vallee *et al.*, 1956) failed to demonstrate the formation of zinc-coenzyme complexes. The formation of $Zn(OH)_2$ renders difficult the interpretation of association constants of Zn^{2+}-coenzyme complexes calculated on the basis of potentiometric titrations at alkaline *pH* (Wallenfels and Sund, 1957).

The present studies utilizing the ion-exchange procedure of Schubert (1956), and employing high-specific-activity $^{65}Zn^{2+}$, demonstrate that both DPN^+ and DPNH form 1:1 complexes with zinc, although these association constants are small and differ significantly from those measured for the enzyme-coenzyme complex (Table VI).

It has been suggested that zinc might bind to the phosphate group of the nucleotide (Wallenfels and Sund, 1957). The observation that AMP complexes zinc, while adenosine does not, is consistent with this view. On this basis the markedly weaker binding of adenosine to the enzyme (Li and Vallee, 1963) could be attributed to the lack of a phosphate-zinc bond in this complex. However, this inference is not exclusive of different modes of binding. While zinc-coenzyme bonds may contribute to the overall enzyme-coenzyme binding constant, the zinc bonds are not the sole or even major factors responsible for the formation of the enzyme-coenzyme complex. The interaction of the coenzyme with other, as yet unidentified, groups of the apoenzyme must add significantly to the stability of the complexes.

It is similarly intriguing to consider the possible mode of binding of those substrate (analog) molecules which lack functional groups to coordinate with zinc. The retardation of zinc exchange when these substances are added jointly with a coenzyme does not seem to be related to a direct interaction with the metal; rather, this phenomenon must be related to the binding with other groups of the protein. In an attempt to delineate those groups of the substrates which are essential for binding, as detected by the effect on isotopic exchange, the substrate homolog formamide was modified. Introduction of a second amide group, forming urea, decreased the effectiveness of the substrate homolog in the presence of DPNH, while, addition of one or more aliphatic carbon atoms in the same position, i.e., isobutyramide, or hexanamide, increases it. An unsubstituted amide group is not requisite for binding to the enzyme, however, since dimethylformamide is much more effective in blocking isotopic exchange than is formamide (Table III). Thus substitutions for R_1 and R_2 in the aliphatic amide homolog, $R_1 - \overset{\overset{O}{\|}}{C} - N\begin{matrix}R_2\\R_2\end{matrix}$ decreases binding in the order $C_n(H)_{2n+1} > CH_3 > NH_2$ for the R_1 position and $CH_3 > H$ for the R_2 position. The increased affinity observed on lipophilic substitution is evident and suggests that the interaction of the substrate with the enzyme is of primarily hydrophobic character.

TABLE VI

COMPARISON OF ASSOCIATION CONSTANTS OF ENZYME-COENZYME (MOIETY) COMPLEXES WITH THOSE OF ZINC-COENZYME COMPLEXES

Coenzyme (moiety)	K^a Enzyme-Coenzyme	K^a Zinc-Coenzyme
DPN^+	1.4×10^{4}[b]	2.8×10^{1}
DPNH	2.3×10^{6}[b]	7.95×10^{2}
AMP	7.7×10^{4}[c]	2.85×10^{2}
Adenosine	5×10^{2}[c]	[d]

[a] *K*'s refer to association constants. [b] Measured kinetically at *p*H 8.0 (Theorell *et al.*, 1955). [c] Measured by spectropolarimetry at equilibrium, *p*H 7.5. [d] No measurable binding.

The detailed physical basis for the blocking of isotope exchange by substrate-coenzyme pairs is not now apparent and must await a more complete understanding of the structure and composition of the active center of liver-alcohol dehydrogenase. The magnitude of the stability constants of the zinc-coenzyme or zinc-substrate complexes does not appear to govern crucially the interaction of the apodehydrogenase with substrate and coenzyme, if the stabilities of the ionic complexes serve as a basis for comparison. Since the association constants for zinc and coenzymes are low, and some of the substrate homolog molecules lack groups known to coordinate metals, it appears more likely that binding of substrate and coenzyme prevents zinc exchange through steric effects rather than through a direct stabilization of the zinc-protein bond. This may be compared to studies of another zinc metalloenzyme, bovine carboxypeptidase A, where substrate binding may prevent access of the metal to its ligand site; but binding of the substrate does not necessarily depend on the presence of the zinc atom (Coleman and Vallee, 1962).

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The 2-Desmethyl Vitamin K_2's. A New Group of Naphthoquinones Isolated from *Hemophilus parainfluenzae**

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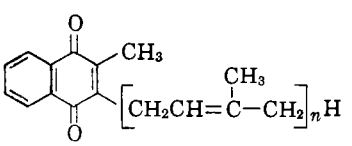
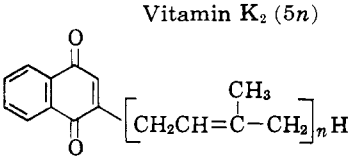
Received March 19, 1964

Three naphthoquinones related to vitamin K_2 have been purified from *Hemophilus parainfluenzae*. Ultraviolet, infrared, and nuclear magnetic resonance spectra are consistent with a structure that differs from vitamin K_2 in that the 2-methyl substituent is replaced with hydrogen; this group of compounds is termed the 2-desmethyl vitamin K_2's. The principal component has a C_{30} polyisoprenoid side chain; lesser amounts of what appear to be the C_{25} and C_{35} isoprenologs have also been detected. A method is described for reversed-phase paper chromatography of these compounds and subsequent detection by ultraviolet absorbance. A reliable technique for reduction of these compounds by KBH_4 is also described.

The isoprenologs of coenzyme Q and vitamin K_2 occur widely in microorganisms. A growing body of evidence suggests a respiratory function for these quinones. Studies on the development and characterization of the electron-transport system in *Hemophilus parainfluenzae* (White and Smith, 1962) and the demonstration of a menadione-requiring auxotroph of a closely related strain (Lev and Reiter, 1962) led us to attempt to characterize the quinones in *H. parainfluenzae*. Examination of the lipid extracts of this organism revealed the presence of naphthoquinones which differed from vitamin K_2 in that the methyl substituent on the quinone ring was replaced with a hydrogen. Three such compounds which differ in the length of their polyisoprenoid side chains have been recognized in *H. parainfluenzae*. A concise nomenclature for these compounds can be based on the system used for the vitamin K_2 homologs. The group of compounds is designated as the 2-desmethyl vitamin K_2's or DMK_2.[1] As in the vitamin K_2 series the number of carbon atoms in the side chains is given for the particular homolog. Thus in *H. parainfluenzae* we have found large amounts of DMK_2 (30) with lesser amounts of DMK_2 (25) and DMK_2 (35).



Vitamin K_2 (5*n*)



DMK_2 (5*n*)

This communication will describe the isolation and characterization of this group of compounds.

* This investigation was supported by research grants (GM-07627 and GM-10285) from the National Institutes of Health, U. S. Public Health Service.

[1] Abbreviations used in this work: DMK_2, the 2-desmethyl derivatives of vitamin K_2; SFQ, substance purified from lipid extracts of as train of *Streptococcus faecalis* (Baum and Dolin, 1963).

EXPERIMENTAL

Growth of Bacteria.—The strain of *H. parainfluenzae* was that utilized previously (White and Smith, 1962). The growth medium contained 2% proteose peptone, 0.5% yeast extract (Difco), 102 mM NaCl, 9 mM KNO_3,