The Ionization of Vitamin B_{12} Factor B*

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A comparison of thermodynamic data for the reactions of vitamin B_{12} and its derivatives with those for the corresponding reactions of simpler Co^{III} complexes provides useful criteria for assessing the influence exerted by the complicated molecular structure of the vitamin on the reactivity of the central metal ion. It is hoped that the vitamin B_{12} complexes may serve as model systems for Buchanan's B_{12} enzyme and the hemoproteins, in which reactivities are expected to be influenced further by neighboring groups on the protein. The present study deals with the ionization of vitamin B_{12} factor B. Values of the ionization constant were obtained at various ionic strengths and temperatures. A plot of pK versus $\sqrt{I}$ yields a limiting slope, in agreement with the known change in charge type. The trends observed in the enthalpies and entropies for the ionization of a number of metal complexes are correlated empirically with the net charge and gross structure of the complexes.

A comparison of thermodynamic data for the reactions of vitamin B_{12} and its derivatives with those for the corresponding reactions of simpler Co^{III} complexes provides a useful tool for assessing the influence of the complicated corrinoid ring structure on the reactivity of the central metal ion.

Under ordinary conditions in aqueous solution the four nitrogens of the corrinoid ring are bound rigidly to the metal ion and only the two ligands *trans* to each other and perpendicular to the ring can be varied. The interpretation of thermodynamic data for the B_{12} complexes is therefore less complicated than for simpler inorganic complexes, in which extensive hydrolysis and isomerization may occur, or for the hemoproteins, in which effects of neighboring groups of the protein may be considerable.

In the following study the ionization of vitamin B_{12} factor B (Fig. 1) was investigated:

$$[\text{NC-Co}^{III}\text{-OH}_2]^+ \rightleftharpoons [\text{NC-Co}^{III}\text{-OH}]^0 + H^+ \quad (1)$$

or in a schematic notation:

$$BOH_2^+ \rightleftharpoons BOH + H^+ \quad (2)$$

An operational ionization constant is defined:

$$pK_{obs} = \log [BOH_2^+]/[BOH] + pH \quad (3)$$

where $[BOH_2^+]$, [BOH], and pH are the experimentally observed quantities. At moderate ionic strengths ($I \leq 10^{-2}$) an activity coefficient of unity is assumed for the neutral species BOH. The observed pH approximates $-\log a_{H^+}$ within the experimental error (Bates, 1962). Under these conditions pK_{obs} is related to the thermodynamic constant by the expression

$$pK_{obs}' = pK^0 - \log \gamma_{BOH_2^+} \quad (4)$$

Values of pK_{obs} were obtained at various temperatures and ionic strengths. A plot of pK_{obs} versus $\sqrt{I}$ gave a limiting slope in agreement with the expected change in charge type.

Values of ΔH and ΔS^0 are compared with data on simple inorganic metal complexes and hemoproteins. The effects of charge type and nature of the substituents are discussed.

Experimental

Crystalline vitamin B_{12} factor B was obtained through the courtesy of Dr. Karl Folkers of Merck and Co., Inc. An approximately 3×10^{-4} M stock solution was made up with conductivity water. Such a solution proved to be spectrophotometrically stable over a 4-month period. Stock solutions prepared from different lots of the factor behaved identically in the following experiments. It was therefore thought unnecessary to perform further purifications on the solid material.

A saturated NaOH solution, centrifuged to remove carbonate precipitates, was diluted with CO_2-free conductivity water to prepare stock solutions of approximately 0.01 N. All NaOH solutions were standardized against potassium hydrogen phthalate (National Bureau of Standards standard sample) with phenolphthalein used as an indicator and stored in polyethylene containers provided with an ascarite CO_2 trap.

Sample solutions containing approximately 4×10^{-5} M factor B and $0.5 - 5 \times 10^{-3}$ N NaOH were made up to the desired ionic strength with reagent-grade NaCl.

The Beckman E-2 Electrode was used in conjunction with the Model GS Beckman pH meter. A series of buffer standards, Beckman pH 10.00 buffer, and solutions of 0.01 M Na_2CO_3, 0.025 M Na_2CO_3, and 0.05 M Na_2CO_3 (Bates *et al.*, 1950) were used to standardize and check the internal consistency of the pH meter over the range of pH, ionic strength, and temperature employed.

Spectrophotometric measurements were made with a Beckman Model DU Spectrophotometer at the 553 $m\mu$ maximum of the basic form (George *et al.*, 1960) and a constant slit width of 0.04 mm. Matched 1-cm cuvets were used. The absorption of the two species followed Beer's law. The absorption of the acid species stayed constant over the range of temperature ($0 - 33°$) and ionic strength ($0.5 - 0.001$) investigated. In alkaline solutions an irreversible decrease in the optical density was observed with time, which may be attributed to aggregation phenomena. Therefore all measurements relating to the ionization equilibrium had to be extrapolated to the time of mixing. The most reliable values for 100% reaction were obtained in a strongly alkaline solution at 0°, where the basic form is relatively stable. The spectrum of BOH at an ionic strength of unity was found to be independent of temperature within the accuracy of the extrapolation technique.

All preparations of samples and subsequent measurements were performed under nitrogen to eliminate the absorption of atmospheric carbon dioxide. The samples were thermostated within a maximum temperature range of 0.2°.

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TABLE I

A SAMPLE RUN CONDUCTED AT $I = 0.05$ AND $T = 23.6 \pm 0.1°$

Reference solutions were (1) acidic factor B containing 1 ml of a 3×10^{-4} M stock solution of factor B in water and 5 ml of a 0.06 M NaCl solution, and (2) alkaline factor B containing 1 ml of stock solution and 5 ml of a 1.2 N NaOH solution. Sample solutions contained 1 ml of factor B stock and 5 ml of a NaOH–NaCl stock solution of $I = 0.06$ and final NaOH concentrations quoted below.

NaOH × 10^3	$pH_{meas.}$	$D_{meas.}$	% BOH	pK_{obs}
Ref. soln. 1	~6	0.143	<1	—
Ref. soln. 2	>13.5	0.382	>99	—
0.61	10.73	0.231	33.9	11.00
1.02	10.89	0.249	44.4	10.99
1.22	10.96_5	0.259	48.5	10.99
1.35	10.99	0.259	48.5	11.01_5
1.52	11.07	0.275	55.2	10.98
1.86	11.26	0.295	63.6	11.02
			Mean pK_{obs} =	11.00 ± 0.02

The sample run shown in Table I will serve as an illustration of the experimental technique and the precision of the calculated equilibrium constants.

RESULTS

Linear extrapolation of a plot of pK_{obs} vs. $\sqrt{I}$ at 25° (Fig. 2) gave $pK_0 = 10.95 \pm 0.02$, *i.e.*, $\Delta F^0 = 15.01 \pm 0.02$ kcal/mole. The limiting slope of +0.53 is in good agreement with the value of +0.51 predicted by the simplified Debye-Hückel limiting law for a +1 charged ion (equation 4). A plot of pK_{obs} versus $1/T$ at $I = 0.1$ (Fig. 3) yielded $\Delta H = 11.0 \pm 0.6$ kcal/mole. The entropy change of the ionization $\Delta S^0 = -13 \pm 2$ eu was calculated from ΔF^0 and ΔH.

DISCUSSION

An attempt has been made previously by George *et al.* (1960) to ascertain the influence of the complicated chelate structure of the hemoproteins by comparing the thermodynamic data for a number of reactions with the corresponding values for the parent Fe^{III} hexaquo ion. In all the cases the enthalpy changes for the hemoprotein reactions are considerably more favorable and the corresponding entropy changes less favorable. A comparison of the data for the ionization reaction shows factor B occupying an inte mediate position between the Co^{III} and Fe^{III} hexaquo ions on the one hand and the ferrihemoprotein hydrates on the other. The available data on the enthalpies and entropies of ionization of low spin cobaltic complexes and high spin ferric complexes of various charge types and of other hexacoordinated metal ions in aqueous solution are summarized in Table II. Although the necessary thermodynamic data for quantitative structural correlations are lacking for coordination complexes, a number of observations can be made on the basis of the data in Table II, and further insight can be gained from the more extensive data available for the ionization of organic compounds.

1. Certain trends may be expected on electrostatic grounds alone. Reduction of the net charge of the metal complex by the substitution of negative ligands results in successively less favorable entropy changes for the ionization reaction as charge cancellation and the accompanying desolvation become less important.

2. The triply charged cobaltic ammine complexes show a definite trend toward less favorable values of the free energy of ionization with the successive sub-

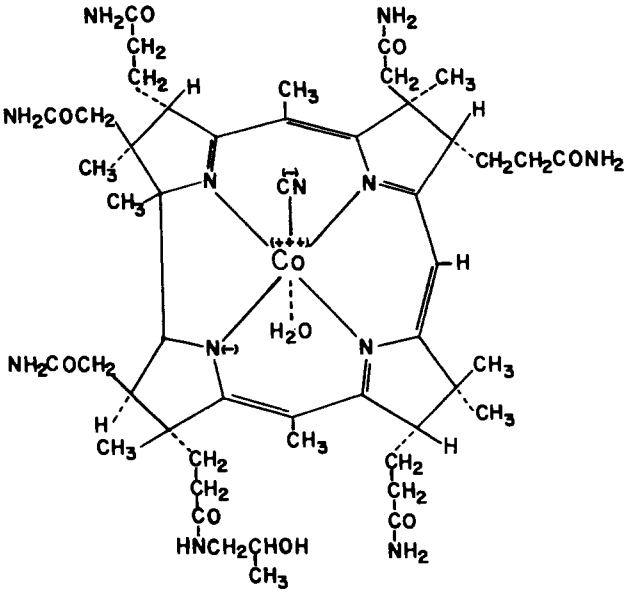

FIG. 1.—The structure of vitamin B_{12} factor B.

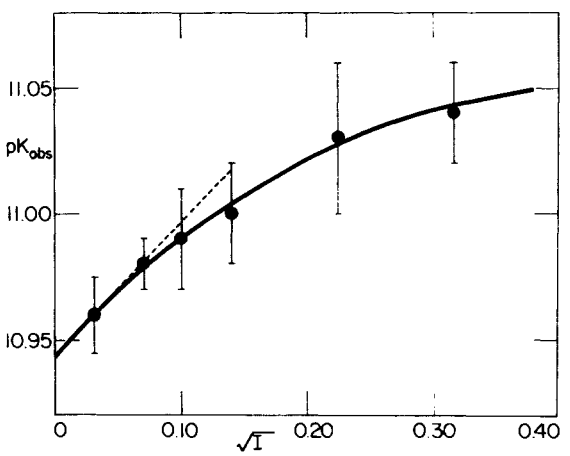

FIG. 2.—The variation of pK_{obs} (equation 4) with $\sqrt{I}$ at 25°.

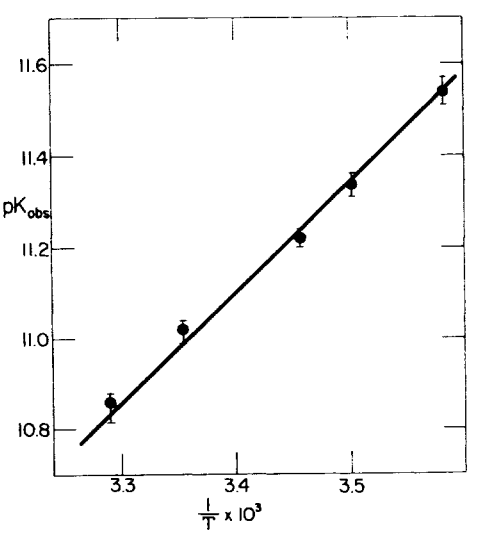

FIG. 3.—A plot of pK_{obs} versus $1/T$ at an ionic strength of 0.1.

TABLE II
THERMODYNAMIC CONSTANTS OF THE IONIZATION REACTIONS OF METAL COMPLEXES

Metal Complex	ΔF^0 (kcal/mole)	ΔH (kcal/mole)	ΔS^0 (eu)	Ionic Strength	Temp. (°C)	Reference
$Fe(H_2O)_6^{3+}$	3.0	10.4	25	I → O	25	Milburn, 1956
$Co(H_2O)_6^{3+}$	2.5	10	25	1.0 ($NaClO_4$)	25	Sutcliffe and Weber, 1956
$Co(NH_3)_2(H_2O)_4^{3+}$	4.5	—	—	I → O	15	Brønsted and Volqvartz, 1928
$Co(NH_3)_3(H_2O)_3^{3+}$	6.2	—	—	I → O	15	Brønsted and Volqvartz, 1928
$Co(NH_3)_4(H_2O)_2^{3+}$	6.9	—	—	I → O	15	Brønsted and Volqvartz, 1928
$Co(NH_3)_5(H_2O)^{3+}$	7.5	—	—	I → O	15	Brønsted and Volqvartz, 1928
$Co(NH_3)_4(H_2O)(OH)^{2+}$	10.2	—	—	Variable	25	Lamb and Damon, 1937
$Tl^{+}_{(aq.)}$	18.0	13.8	−14	O correction	25	Bell and George, 1953
Factor B	15.0	11.0	−13	I → O	25	This paper
Myoglobin (horse heart)	12.2	5.8	−22	I → O	25	George and Hanania, 1952
Hemoglobin (horse erythrocytes)	12.0	3.9	−27	I → O	25	George and Hanania, 1953
Hemoglobin (*Chironomus plumosus*)	11.1	3.8	−24	I → O	25	Scheler and Fischbach, 1958
Leghemoglobin (soy bean)	11.7	2.4	−31	I → O	25	George *et al.*, 1959

stitution of ammonia for the coordinated water in the parent aquo ion. In the absence of reliable ΔH and ΔS^0 data for the ionization of these complexes, one may postulate that the introduction of a more polar ligand (OH^-) into the weak field of the hexaquo ion would produce a more favorable enthalpy of ionization than the same ligand introduced into the already much stronger field of the ammine substituted complex. When the ammine ligands are replaced by highly conjugated nitrogenous bases, the ligand field would be expected to increase further and the enthalpy of ionization would become correspondingly less favorable. In general this ligand effect decreases as the formal charge on the metal ion decreases. The strength of the ligand fields in the +1 charged complexes of Table II increases in the order Tl^+ < factor B < hemoprotein hydrates. However the trend in the enthalpies of ionization is opposite to that predicted from the above ligand field considerations.

3. Sizable contributions from neighboring groups on the protein may be ruled out, since the significant differences among the protein moieties of myoglobin and the three hemoglobins do not seem to lead to large differences in the ionization data.

4. Allowance for the partial conversion to the low spin form of Fe^{III} in the ferrihemoprotein hydrates can account for a maximum of −2 kcal/mole in the observed enthalpy term and −7 eu in the entropy term (George *et al.*, 1961).

Despite the intrinsic differences between the metals in the thallous aquo ion, factor B, and the ferrihemoprotein hydrates, it is nevertheless of interest to see whether any important contributions to the ionization process are likely to originate in the specific corrinoid and porphyrin ring systems.

5. Thermodynamic criteria of a general nature can be based on the extensive data that are available for the ionization of a great variety of organic compounds. The entropy and enthalpy values are distinct for each charge type, which will be referred to in terms of the change in charge accompanying the ionization, *i.e.*, +1 → 0, and 0 → −1. For +1 → 0 ionizations, *e.g.*, the ionization of primary and secondary aliphatic amines, the amino acids, and others, the enthalpies of ionization range between 10.5 and 14 kcal/mole and the entropies between −1 and −10 eu. For 0 → −1 ionizations, *e.g.*, the ionization of aliphatic, α-hydroxy, α-keto, α-halo, and aromatic carboxylic acids, the amino-acids, and the first ionization of the phosphoric acids, the enthalpies range between −2 and +2 kcal/mole and the entropies generally between −6 and −26 eu. A typical range of enthalpies and entropies of members of an homologous series, *e.g.*, primary aliphatic amines, is 1.5 kcal/mole and 4 eu respectively:

6. Significant overlap of the enthalpy values of the two charge types occurs only among the +1 → 0 ionizations of the conjugated and aromatic nitrogen compounds, the substituted imidazoles and pyridines, and the 0 → −1 ionizations of the substituted phenols and hydroquinones. In every homologous series, from partially saturated ring compounds to highly conjugated and aromatic members, the trend in the enthalpy of ionization, from values characteristic of the charge type to large deviations, closely parallels the extent of delocalization of the π electrons in the uncharged species. Thus the loss in resonance energy upon the introduction of a charge in the ionization process, and the corresponding gain on charge removal, could be responsible for the large unfavorable deviations of the enthalpies of the 0 → −1 ionizations and the large favorable deviations of the +1 → 0 ionizations.

While the thallous aquo ion and factor B ionizations have enthalpies in keeping with their charge type, the ionizations of the ferrihemoprotein hydrates have enthalpies of a similar magnitude to those of the conjugated nitrogen compounds. In factor B and the hemoproteins the ionizing group is conjugated to the ring system by metal-ligand π bonding. The thermodynamic evidence thus lends support to the importance of the effect of the conjugated porphyrin ring system on the affinity of the metal ion in the hemoproteins, a result expected from structural and spectral considerations. The lesser effect observed with factor B stands in agreement with the partially saturated corrinoid structure and the spectral characteristics of the vitamin B_{12} derivatives.

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Genetically Modified Folic Acid Synthesizing Enzymes of Pneumococcus*

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Cell-free extracts have been prepared from eight genetically related sulfanilamide-resistant mutant strains of pneumococcus. The biosynthesis of folic acid has been studied in these extracts, the growth of *Streptococcus faecalis* being used for the assay of folic acid compounds. This biosynthesis is dependent upon an exogenous supply of *p*-aminobenzoic acid. As with the growing bacteria, the cell-free extracts are subject to competitive inhibition by specific *p*-aminobenzoic acid analogs, the resistance patterns being characteristic of the mutant strain used. Differences in temperature sensitivity of the rate of synthesis were also observed; with extracts of some strains, including the wild type, the reaction was effective at temperatures up to 50°, whereas extracts from all strains containing the particular genetic marker F_d were inactive at 50°, and optimal synthesis occurred below 37°. These differences are all attributable to genetically controlled alterations of affinity in the substrate binding groups of one enzyme in the folic acid synthesizing system.

The now classical work of Beadle and Tatum (1941) described mutations having biochemical effects ultimately attributed to the loss of specific enzymatic capacities of the mutant strains. Many of these seem to be due to essentially complete loss of a specific protein from the organism, but less drastic biochemical changes also occur because of genetic mutations resulting in altered physical properties of the proteins (*e.g.*, Maas and Davis, 1952; Horowitz, 1956; Fincham, 1960). These more subtle changes are of interest from both the biochemical and the genetic points of view. An enzyme which has been altered in several ways in different mutants can be used as a tool to study the mode of action of the catalyst. Geneticists have accomplished fine-structure mapping within single genetic loci governing the presence or absence of specific enzymes, and when different alterations within a single enzyme can be recognized in the phenotype, it becomes possible to map the genetic regions determining these alterations.

A highly sulfonamide-resistant mutant strain of pneumococcus, RF29, isolated in 1955 (Hotchkiss and Evans, 1958), has provided a system in which qualitative phenotypic alterations brought about by mutation could be detected and also correlated on a biochemical basis with a genetic analysis of the strains involved.

The genetic analysis of the mutant was accomplished by transformation experiments and showed that the high resistance of the mutant to sulfanilamide was genetically attributable to a complex locus bearing three closely linked but separate subunits (called *a*, *b*, and *d* for convenience). These subunits could be transferred *via* transformation processes either singly or together in any of seven combinations, thereby conferring on the recipient strains quantitatively distinct levels of resistance to sulfanilamide. Thus, for example, a wild recipient strain, which is barely resistant to 4 μg of sulfanilamide/ml, upon incorporating the *a* or *d* region of a DNA donor molecule becomes resistant to about 20 μg or 80 μg of sulfanilamide/ml, respectively. Incorporation of both the *a* and *d* portions of the donor molecule results in a resistance to approximately 400 μg of sulfanilamide/ml in the transformed cells. This fact suggests that the same cell function is being affected cumulatively by each of the genetic units.

According to the classical system described by Woods (1940), *p*-aminobenzoic acid competitively releases the bacteriostatic action of sulfanilamide. Later it was shown (Lascelles and Woods, 1952) that folic acid synthesis by resting bacterial suspensions required *p*-aminobenzoic acid and was inhibited by sulfathiazole. The same relationships hold for all of our pneumococcal strains. It was therefore suggested that the genetic alterations in the pneumococcal strains, phenotypically expressed as a resistance to sulfanilamide, had altered the affinities of an enzymatic system concerned with the synthesis of folic acid and, more specifically, with the reaction involving *p*-aminobenzoic acid as substrate.

Early biochemical investigations of these mutant strains were directed toward observing effects of specific inhibitory analogs of *p*-aminobenzoic acid upon growing cultures. As in the case with sulfanilamide, other analogs gave characteristic different quantitative growth-inhibition patterns; the seven mutant strains differed in their growth responses depending upon how

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