The Complexing Tendencies of Cyanocobalamin With Inorganic Compounds*

Heteromolybdates and Heavy Metal Chlorides

By RUTH N. HAVEMEYER† and TAKERU HIGUCHI

The interactions of vitamin B_{12} with numerous inorganic compounds have been investigated. Nine of the compounds studied yielded slightly soluble adducts with cyanocobalamin. These were phosphotungstic and phosphomolybdic acid, the sodium, nickel, and manganese salts of phospho-12-molybdic acid, and the chlorides of gold, platinum, and palladium. Several of the reagents caused a change in the absorption spectrum of the vitamin in the system. This effect was found to be a function of the concentration of the reagent, but independent of hydrogen ion concentration.

THE ROLE OF COMPLEX FORMATION of cyanocobalamin (vitamin B_{12}) as affects its absorption in the gastrointestinal tract has received much attention. There have been reports of the apparent interaction tendencies of the vitamin with mucoproteins, sugar alcohols, peptides, and other organic compounds (1–4).

In earlier work from these laboratories, studies were carried out on the association tendencies of vitamin B_{12} with several classes of organic compounds. Szulczewski and Higuchi (5) studied the complex formation with sugars, sugar alcohols, and phenolic compounds. Mouri and Higuchi (6) interacted the vitamin with various carboxylic acids, amines, amides, and diols. The investigation reported at the present time is concerned with the binding of the vitamin with heteropoly acids and their salts, together with its interactions with some metal chlorides.

Studies of this nature were considered of pharmaceutical interest from two standpoints. It was hoped firstly that they may provide some insight into the influence of various additives upon the rate and efficiency of absorption of the vitamin when taken orally. Secondly, it was thought that some dosage form of the depot type might be based on the discovery of some extremely insoluble complexes formed from cyanocobalamin. It would be pharmacologically desirable to have available dosage forms of vitamin B_{12} which would permit its slow and controlled release into the system. One possible way to realize this might be to form slowly soluble molecular addition compounds, or complexes, suitable for parenteral use.

Such interactions may reasonably be expected. The vitamin B_{12} molecule (Fig. 1) includes several groups which might conceivably act as complexing sites; these are the amide functions, the orthophosphate moiety, the cyano-cobalt system, and the nucleotide portion of the molecule. Interactions of some of these functional types are well-known. For example, orthophosphates complex strongly with ferric ion (7), and simple amides such as acetamide and benzamide form crystalline addition compounds, which can be isolated from aqueous solution, with gold and platinum chlorides (8, 9). Many inorganic compounds function as protein precipitants (10, 11), and some of these may well be expected to act similarly upon the cyanocobalamin structure.

Since poorly soluble or slowly soluble adducts were desired, some substances of high molecular weight were employed as the complexing agents. Other compounds, such as gold chloride, were

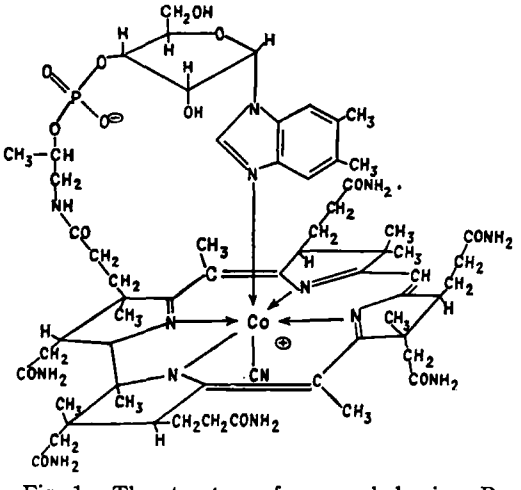

Fig. 1.—The structure of cyanocobalamin. Reprinted by permission, from p. 19 of "Vitamin B-12 und Intrinsic Factor," H. C. Heinrich, editor.

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chosen on the basis of known interactions with pertinent functional groups. Still others were selected for study in an attempt to separate out specific ion effects. In addition, such studies may yield some information as to the sites of interaction.

EXPERIMENTAL

Reagents.—*Heteromolybdates.*—Sodium-phospho-12-molybdate, sodium-silico-12-molybdate, sodium-2-phospho-18-molybdate, Climax Molybdenum Co.; phosphotungstic acid, Eastman Kodak; phosphomolybdic acid, Merck and Co.; manganese-phospho-12-molybdate, nickel-phospho-12-molybdate.

The first five compounds were recrystallized from hot water. The last two were not commercially available and were synthesized by the method of Arnfeld, as follows (12): Freshly precipitated nickelous (or manganous) phosphate was suspended in hot water. To this was added, in small portions, the stoichiometric quantity of molybdenum trioxide which had been recently heated to glowing in a porcelain crucible. The suspension was heated gently and small quantities of water were added until all of the solids dissolved. This solution was concentrated over sulfuric acid and yielded greenish-yellow crystals. The first crystals to separate, those of the phospho-12-molybdic acid, were filtered off in order to separate them from the salt of the 9-acid, which are the last to separate. An aqueous solution of the 12-acid salt, when treated with ammonium or potassium chloride, yields a precipitate. A solution of the 9-acid salt shows no reaction with either of these reagents.

Chlorides.—Aluminum, ammonium, barium, cadmium, cobaltous, cupric, ferric, hexamino-cobalt, lithium, lead, manganous, mercuric, nickelous, sodium, zinc, auric, platinic, palladous, and potassium gold chlorides.

All but the last four compounds were recrystallized from hot water. These four chlorides were used as supplied by the manufacturers; they were not recrystallized because of their hygroscopicity and because of the small quantities available.

Procedure.—The method of study was phase solubility analysis, using a water bath thermostatted at 25 ± 0.05°. The method and the apparatus have been described previously (13, 14).

The crystalline vitamin B_{12} was weighed into 2-cc. vials and sufficient water was added to give a concentration of 20 mg. B_{12}/Gm. water. Sufficient reagent was then added to give the desired concentrations. After a twenty-four-hour equilibrium period, the supernatant liquid was withdrawn through medium-porosity sintered-glass disks. These aliquots were placed in tared 10-ml. volumetric flasks, and the weights of the aliquots were determined. The diluted samples were analyzed for B_{12} content by measuring their absorbance at 550 mμ, using the Cary recording spectrophotometer model 11MS.

All solutions containing B_{12}, in free or combined form, were saved for recovery of the vitamin. The pooled solutions were first lyophilized, and the dry powder obtained was redissolved to give a concentrated solution. This concentrate was then treated by the method of Bernhauer and Friedrich (15) to obtain a purified aqueous solution, and the crystalline vitamin was finally precipitated by addition of 90% acetone. The acetone used was first purified by refluxing it for several hours with potassium permanganate and potassium carbonate, and was then distilled. The reclaimed vitamin was checked for purity spectrophotometrically.

Sample Calculation to Determine the B_{12} Content of the Diluted Aliquots.—(*a*) (Absorbance) (volume of final soln./absorptivity) = mg. B_{12}/10 ml., (*b*) (mg. B_{12}/10 ml.) (1/weight of aliquot) = mg. B_{12}/Gm. (*c*) (mg. B_{12}/Gm.) ÷ (molecular weight of B_{12}) = 10^{-6} mole B_{12}/Gm.

RESULTS

Heteromolybdates.—Figure 2 shows the curves for those interactions which resulted in only an increase in the solubility of cyanocobalamin; Figs. 3 and 4 give the complexing curves for those compounds which interacted with B_{12} to give an insoluble form.

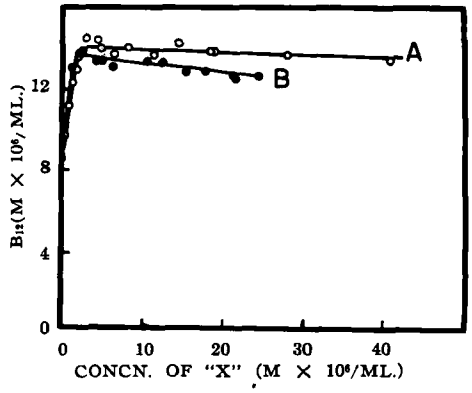


Fig. 2.—A, sodium-silico-12-molybdate; B, sodium-2-phospho-18-molybdate.

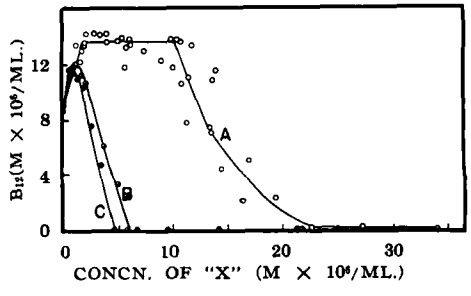


Fig. 3.—A, sodium-phospho-12-molybdate; B, nickel-phospho-12-molybdate; C, manganese-phospho-12-molybdate.

The scatter observed may be the cumulative result of the experimental errors, the principal one of which is probably the weighing error. It is not the result of insufficient equilibration, since the same effect was observed when the equilibrium time was extended to forty-eight hours.

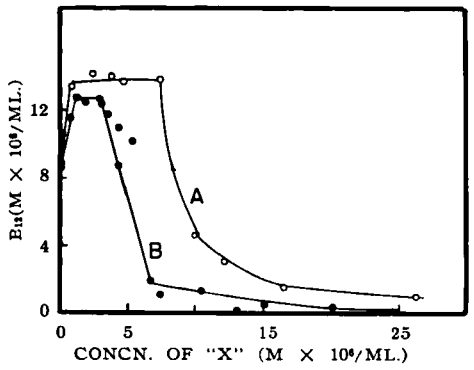


Fig. 4.—A, phosphotungstic acid; B, phosphomolybdic acid.

The data for the compounds which gave insoluble reaction products are given in Table I. The approximate stoichiometries of the solid complexes were calculated in three ways: (*a*) from the initial slope of the descending portion of the curve; (*b*) from the amount of complexing agent necessary to remove all of the vitamin from the system, determined by extrapolating the downcurve of the plots to the *x*-axis. The value, total B_{12}/total agent, is the ratio of the concentration of the vitamin to this value; and (*c*) by spectral analysis, accomplished by calculating the concentration of B_{12} from the absorbance of a solution of 1 mg. of the solid in 25 ml. of water.

Of the three methods, the spectral analysis of the solid is probably the most reliable. Since the other two are based upon calculations from the phase diagrams they have rather large inherent errors in them. The downslopes, for example, are only best-fitting lines by visual (not mathematical) analysis, and in at least one of the curves there is considerable scatter. Also, because of the steepness of the downslopes, it is often difficult to obtain precise numerical values therefrom.

Chlorides.—Of the nineteen chlorides studied, platinic, auric, palladous, and potassium gold chlorides gave insoluble reaction products with cyanocobalamin (Figs. 5 and 6, Table II). Mercuric chloride is the only one which did not cause an initial increase in the solubility of the vitamin (Fig. 7).

Gold, potassium gold, and palladium chlorides caused a shift in the spectrum of B_{12} in the supernatant liquid. In the case of the gold salts, the two maxima at 550 mμ and 520 mμ blended into one maximum at about 525 mμ. This change is a function of concentration of the salt added but is independent of hydrogen ion concentration, as shown by a pH-dependence study using HCl.

With palladous chloride, also, the spectral shift was dependent upon the concentration of the chloride added. However, the spectral change observed in this system was more complicated, several changes in spectrum being noted with changing palladium concentration.

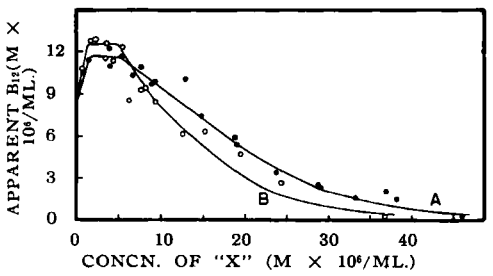


Fig. 5.—A, potassium gold chloride; B, palladium chloride.

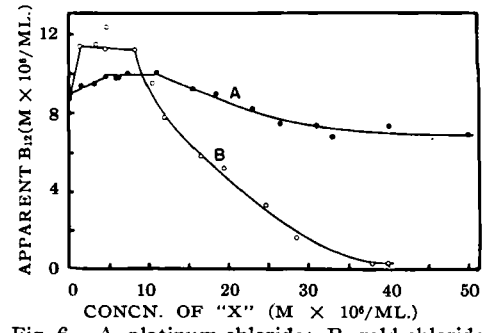


Fig. 6.—A, platinum chloride; B, gold chloride.

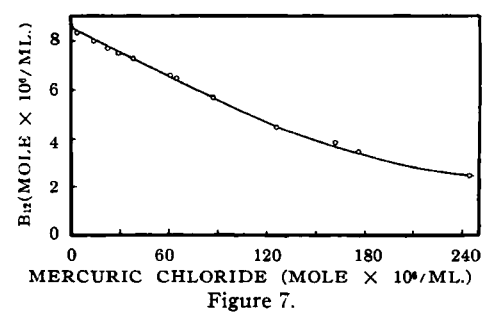


Figure 7.

TABLE I—THE HETEROMOLYBDATES

Reagent	Approximate Molar Ratio (B_{12}:Agent) of Solid Complex Method of Calculation: Downslope	Total B_{12} / Total Agent	Spectral Analysis
Na-phospho-12-molybdate	10:3 (?)	1:1	1:1
Mn-phospho-12-molybdate	7:2	7:2	6:1
Ni-phospho-12-molybdate	3:1	5:2	5:2
Phosphomolybdic acid	2.7:1	2:1	1.7:1
Phosphotungstic acid	6:1 (?)	8:5 (?)	4:1

DISCUSSION

Heteromolybdates.—The heteropoly anions are composed of molybdenum oxide groups which surround a central atom such as potassium, tungsten, or silicon. For example, in the phospho-12-molybdates, such as $Na_3(PMo_{12}O_{40})$, there is a PO_4 tetrahedron surrounded by twelve MoO_6 octahedra (Fig. 8). These twelve octahedra are joined together by the sharing of oxygen atoms at the corners. In the hetero-12-molybdates, there are twelve molybdenum atoms to one central atom; in the hetero-2,18-molybdates, there are eighteen molybdenum atoms to two central atoms. The structures of the two complex acids used are similar; each consists of a

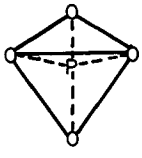
TABLE II.—THE TRANSITION METAL CHLORIDES

Reagent	% Change in B_{12} Solubility[a]	Approx. Stoichiometry of Solid Complex (B_{12}:Agent) Method of Calculation: Downslope of Plot	Total B_{12} / Total Agent	Spectral Analysis of Precipitate	Upper limit of Reagent Concn. (10^{-6} Mole/ml.)
$HAuCl_4$	−100	3:2	1:1	incompletely soluble[b]	..
H_2PtCl_6	− 16	1:7	1:5	2:3	..
$KAuCl_4$	−100	2:5	1:2	incompletely soluble	..
$PdCl_2$	−100	1:1	4:5	insoluble[c]	..
$HgCl_2$	− 71[d]	..	..	..	..
$PbCl_2$	+0.6	..	..	..	30
NaCl	+ 4	..	..	..	5480
LiCl	− 9	..	..	..	90
$CoCl_2.6H_2O$	+ 15	..	..	..	44
$NiCl_2.6H_2O$	+ 15	..	..	..	49
$CdCl_2$	+ 15	..	..	..	114
$MnCl_2.4H_2O$	+ 18	..	..	..	50
$CuCl_2.2H_2O$	+ 20	..	..	..	74
$BaCl_2$	+ 23	..	..	..	115
$ZnCl_2$	+ 30	..	..	..	110
NH_4Cl	+ 30	..	..	..	656
$AlCl_3$	+ 30	..	..	..	46
$[Co(NH_3)_6]Cl_3$	+ 53	..	..	..	173
$FeCl_3.6H_2O$	+ 65	..	..	..	58

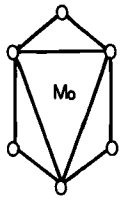
[a] + Indicates increase in solubility and − indicates decrease in solubility, calculated at reagent concentration in column 6. [b] One milligram incompletely soluble in 25 ml. of water. [c] One milligram insoluble in 25 ml. water. [d] See Fig. 7.

PO_4 tetrahedron surrounded by six XO_6 octahedra, where X is molybdenum or tungsten. These compounds are generally highly hydrated and may contain as many as forty molecules of water. Some of these water molecules are intimately bound in the crystal lattices and others are apparently zeolytic, i. e., they are lost on heating because they are not part of the crystal structure (16, 17).

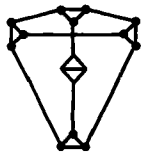
The molecular weights of the heteromolybdates range from 2,000 to 4,000. Because of the complexity of these anions, there is still much confusion as to their structures and bond types. Another complication is that in solution there is an equilibrium between several of the anionic species.



THE PO_4 TETRAHEDRON



THE MoO_6 OCTAHEDRON



THE PHOSPHO-12-MOLYBDATE MOLECULE

EACH ● REPRESENTS ONE MoO_6 OCTAHEDRON. HENCE, THERE ARE 12 MoO_6 GROUPS:1 PO_4 GROUP

Figure 8.

From the complexing data, it would appear that for the several compounds studied, the ratio of 12 Mo: 1 P in the structure of the heteromolybdate is required to obtain an insoluble or slowly soluble adduct with the vitamin.

Chlorides.—The first chlorides studied were those of gold and platinum, since it was known that they react with acetamide groups (8, 9). The results of these interactions with vitamin B_{12} led to the investigation of other chlorides.

The spectrum change observed as a result of the interactions of the vitamin with gold and potassium gold chlorides is similar to that reported by Kaczka, *et al.*, on work done with various B_{12} analogs, in which the cyanide group coordinated to the cobalt in the B_{12} molecule was replaced with hydroxide, chloride, bromide, sulfide, sulfate, nitro, and cyanate groups (18). Hence, it is possible that cyanocobalamin is no longer present in solution. Possible causes of such spectral shifts may be replacement of the cyanide by other anions, interaction of the cation with the molecule in such a way as to disturb the conjugated bond system which is responsible for the characteristic absorption spectrum of vitamin B_{12}, or oxidation-reduction reactions due to the presence of the auric ion. The shifted peak obtained in this study, 525 mμ, could conceivably correspond to that of cyanatocobalamin (18).

A photometric study was undertaken in which a saturated solution of gold chloride or palladium chloride was added dropwise to a rapidly stirred vitamin B_{12} solution. The titration flask was connected to a Corex cell (19), and the spectrum of the resultant solution was recorded after each addition of the metal chloride. In each case, the reaction which resulted in the changing spectrum was found to be time dependent.

The spectral changes observed in the palladium chloride-cyanocobalamin system seem to indicate that there are several complex species forming.

We were unable to determine isosbestic points in those samples in which the agent was present in concentrations low enough to form soluble species, and it may be that at all reasonable concentrations of palladium chloride there are more than two species in equilibrium. Since the palladium salt used, palladous chloride, is not palladium in its highest oxidation state, a more complex oxidation-reduction reaction may be occurring. It may be for this reason that the spectral shift is not constant.

The infrared spectra of several of the insoluble reaction products were inconclusive as regards the nature of the reactions taking place.

The decrease in solubility observed with mercuric chloride and vitamin B_{12} may be the result of a salting out effect or a medium effect upon the absorption spectrum. The latter is perhaps a more feasible explanation, since the concentrations of mercuric chloride used were very low. It seems reasonable to expect that if there were complex formation between these compounds, then an initial plateau region would result until all of the solid excess B_{12} in the system had reacted.

The majority of the metal chlorides employed in this study resulted in an increase in the solubility of the cyanocobalamin (Table II), but without a subsequent decrease. These compounds may be of value as additives to aid in the increased absorption of vitamin B_{12} preparations, or they may prove to be useful in the stabilization of the vitamin in solution.

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Antioxidants. The Microdetermination of Hydroquinone*

By MOHAMED Z. BARAKAT, SAAD K. SHEHAB, and ABDEL MAKSOUD ABDALLA

A new titrimetric method for the microdetermination of hydroquinone is described. This method is recommended for the assay of fats and oils, photographic developers, and ether anesthetics. The assay is carried out within limits of 0.5 to 2 mg. of hydroquinone. The procedure is quite simple and rapid but yet shows relatively high accuracy over the suggested range. The experimental error does not exceed ±2 per cent.

HYDROQUINONE is an effective antioxidant for fats and oils (1–5). In tropical districts, hydroquinone is used as a preservative for ether anesthetics (6). Furthermore, hydroquinone is a prominent constituent in photographic developers (7). Hence, our interest in the microdetermination of hydroquinone.

Previously, various methods have been reported (8–12) for the determination of hydroquinone. Of these, the titrimetric methods are used extensively because of their rapidity and simplicity. So far as we are aware, certain de-

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