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Reactions of Cyanocobalamin and Aquocobalamin with Proteins.* (22268)

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It now appears probable that absorption, transport, storage and function of cobalamins are accomplished by vitamin-protein complex formation. Although there is ample evidence that cobalamins are bound by proteinaceous substances present in gastric and duodenal secretions, blood serum, liver tissue, and milk there is relatively little information concerning the nature of bonds involved in the binding reactions. Cooley and co-workers(1) postulated that cobalamin-protein reactions may occur via cobalichrome formation analogous to the formation of ammonia and histidine cobalichromes. Van der Zant and Underkofler(2) presented evidence that suggests participation of both cobalt and substituted benzimidazole of cobalamins in cobalamin-gastric mucosal extract reaction. Absorption spectrum of purified cyanocobalamin-gastric protein complex, reported by Wijmenga and co-workers(3), indicates that cyano-group may still be present in the complex. Gregory and Holdsworth(4) reported the isolation of a similar cobalamin-protein complex from sow's milk and presented evidence indicating that the cyano-group is not displaced in the formation of this complex, and that the complex will not accept a second cyano-group. The latter authors made an extensive study of this binding reaction and concluded that tyrosyl residues of proteins but not sulfhydryl or amino groups, may participate. This investigation presents further

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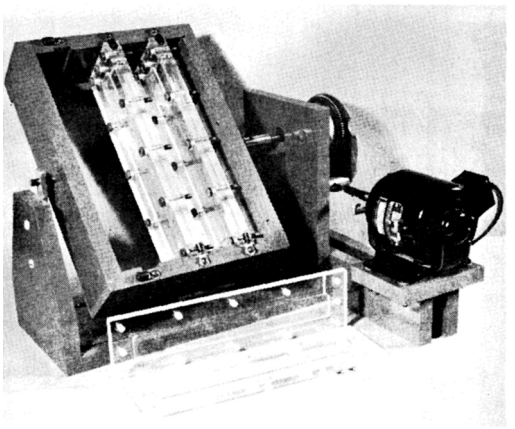

FIG. 1. Dialysis cells and rotation apparatus.

evidence concerning mode of linkage involved in cobalamin-protein reactions.

Dialysis method. Obvious inconsistencies in bacterial inhibition(5,6) and bacterial uptake(7) methods for differentiating free and bound cobalamins led to adoption of a dialytic procedure and radioisotope assay. Initial trials using dialysis bags and dialyzing to equilibrium or exhaustion, indicated that these methods were time-consuming and mechanically awkward. By standardizing the volume, membrane area and agitation a short-term or partial dialysis method was devised. Three identical Plexiglas dialysis cells were used. Each cell half contained a milled-out channel of about 30 ml capacity and a filling hole. The membrane, Visking dialysis tubing slit along one edge, was stretched between the two sections, separating and sealing the channels when cell halves were bolted together. Cells and rotation apparatus (3 r.p.m.) are shown in Fig. 1. In use, 25 ml of buffered protein-Co^{60}-labeled cobalamin mixture were dialyzed against equal volume of the same buffer for 4 hours in the dark at room temperature. Both solutions were then subjected to radio assay using a G-M liquid counting detector. Concentration of protein was adjusted to bind about half of the labeled cobalamin present. Using dialysis rate of free cobalamin, as determined by omitting protein from one of the 3 cells, the amount of cobalamin rendered non-dialyzable by a given amount of protein was calculated. Radioactive cobalamins were derived from a Co^{60}-labeled preparation (225 μc/mg cobalamin) obtained from Merck and Co. on AEC allocation. Cyanocobalamin (vit. B_{12}) was prepared by treatment of an aqueous solution of this preparation with an excess of NaCN followed by nitrogen aeration in the dark at pH 6.5. Aquocobalamin (vit. B_{12b}) was prepared by simultaneous illumination and nitrogen aeration of an aqueous solution of this preparation at pH 5.5. All subsequent operations using these preparations were carried out under conditions of minimum light. Dialysis rate of vit. B_{12} followed Fick's diffusion law, that is, directly proportional to concentration differential across membrane at constant temperature and membrane area, and was unaffected by buffer type or concentration, or pH over the range 2 to 10. Dialysis rate of vit. B_{12b} was less straightforward, being complicated by adsorption by the cellophane membrane, and perhaps dimerization, the effect being more prominent in media of low ionic strength. By using 0.2 M phosphate buffer reproducible dialysis of vit. B_{12b} was obtained at observed rate approaching that of vit. B_{12}.

Results. Using the partial dialysis method, it was found that many proteins bind vit. B_{12b} to a much greater extent than vit. B_{12}, as indicated in Table I. Although not hitherto

TABLE I. Binding of Vit. B_{12} and B_{12b} by Proteins.

Protein preparation*	Bound cobalamin, mμg/mg N†		
	B_{12}, pH 6.6	B_{12b}, pH 6.6	B_{12b}, pH 4.0
Whole hemolysed blood	0	3.8	—
Hemolysed red cells	0	3.0	—
Plasma	0	5.0	.7
Plasma, heat denatured‡	0	5.8	—
Albumin (Armour)	0	3.0	.7
Lysozyme, 2× crystallized	0	0	—
Lysozyme, alkali denatured§	0	3.7	—
Ovalbumin	0	3.7	—
Trypsin, 2× crystallized	0	2.5	.5
Pepsin, " "	0	5.1	—
Gastric mucosal extract	340	340	320

* Preparations contained 1 mg N/ml (0.013 mg N/ml for gastric mucosal extract) and 7.6 mμg cobalamin/ml in 0.2 M phosphate buffer. Blood preparations were of bovine origin.

† Sensitivity ca. 0.2 mμg cobalamin/mg N.

‡ Heated at 100°C for 5 min.

§ Incubated in 0.1 N NaOH at 37°C for 2 hr.

reported, this difference in extent of reaction between the two forms of vitamin can be expected as manifestation of cobalichrome formation. It was found that denaturation increased the vit. B_{12b} binding capacity of plasma and lysozyme. The binding capacity of 3 preparations was markedly decreased in acid media. Unreported spectrophotometric studies(8) indicated that vit. B_{12b}-histidine reaction is similarly depressed in acid media, as expected, and that histidine is the only amino acid giving evidence of cobalichrome formation in neutral solution. Similar compounds such as histamine, imidazole, carnosine, histidyl histidine and pyridine were found to form cobalichromes with vit. B_{12b}.

However, it cannot be inferred that benzimidazole is the only possible vit. B_{12b} binding site, for it was observed(8) that substances such as cellophane, filter paper, heparin, and nucleic acids (DNA and RNA) rendered nondialyzable considerable amounts of vit. B_{12b}. These substances did not bind measurable amounts of vit. B_{12}. The vit. B_{12b} bound by these substances and by the protein preparations was released by treatment with excess of cyanide in neutral solution.

The binding of as much as 5 mμg of vit. B_{12b}/mg of N by plasma is of interest for the physiological level is in the order of only 0.02 mμg of cobalamin/mg of N. Pitney, Beard and Van Loon have reported(9) that naturally occurring cobalamins are present in alpha and beta globulin components and that these components may also bind additional amounts of cobalamins, presumably vit. B_{12}, to a maximum of about 0.05 mμg/mg of N.

The position of bound labeled vit. B_{12} and B_{12b} in bovine serum was determined by subjecting to paper electrophoresis exhaustively dialyzed serum-cobalamin mixtures, and measuring radioactivity of the separate protein components(10). Serum used in this study was found to retain 4.3 mμg vit. B_{12b} or 0.092 mμg vit. B_{12}/mg of N, after exhaustive dialysis. Results presented in Table II indicate that all fractions of serum participated in binding of vit. B_{12b}, whereas only beta and gamma globulin components bound vit. B_{12}.

TABLE II. Position of Bound Co^{60}-Cobalamins in Bovine Serum Fractions Obtained by Paper Electrophoresis.

Preparation	Percent of activity			
	Albumin	α-	β-	γ-globulins
Serum + Co^{60}-B_{12b}	7	28	20	45
" " " -B_{12}	0	0	82	18

An entirely different situation was encountered in studying reaction of cobalamins with swine gastric mucosal extract. This extract consisted of water soluble, 50% alcohol insoluble, nondialyzable components of Ventriculin (Parke-Davis). As shown in Tables I and III, this preparation bound rather large and equal amounts of vit. B_{12} or B_{12b}, and binding of one form of vitamin was essentially blocked by prior excess of the other.

The effect of pH and excess cyanide on

TABLE III. Binding of Vit. B_{12} and B_{12b} by the Gastric Extract (GE).

Components*	Bound labeled cobalamin,† mμg/mg N
GE + Co^{60}-B_{12}	350
GE + excess B_{12b} + Co^{60}-B_{12}‡	0
GE + Co^{60} B_{12b}	350
GE + excess B_{12} + Co^{60}-B_{12b}‡	15

* Preparations contained 0.013 mg N/ml, 7.6 mμg labeled cobalamin/ml, and 2 μg nonlabeled cobalamin/ml, in 0.2 M phosphate buffer of pH 6.6.

† Sensitivity ca. 10 mμg cobalamin/mg N.

‡ Gastric extract and nonlabeled cobalamin allowed to react before adding labeled cobalamin.

TABLE IV. Effect of Hydrogen Ion Concentration and Excess Cyanide on Binding of Vitamin B_{12} by Gastric Extract.

pH*	Bound vit. B_{12}, mμg/mg N	
	No CN^-	Excess CN^-
2	320	—
4	320	—
6	330	—
6.6	340	340
8	320	—
10	330	250
10	340	250†
10	330	200‡
11	220	80
12	90	0

* Preparations contained 0.013 mg N/ml and 7.6 mμg Co^{60}-B_{12}/ml in 0.2 M phosphate buffer.

† Gastric extract and B_{12} allowed to react before adding cyanide. In all others, cyanide and B_{12} mixed before adding gastric extract.

‡ Contained 0.4 mg NaCN per ml. All others contained 0.1 mg NaCN/ml.

TABLE V. Fate of Cyano-Group of Vit. B_{12} in the Vit. B_{12}-Gastric Extract Reaction.

Treatment	Gastric extract	H_2O control
CN^- removed initially by N_2 aeration		
In dark, mμg	86	—
Illuminated, mμg	15	—
Idem	8	5
B_{12} added in dark, μg	4.5	4.5
(CN^- equivalent, mμg)	(87)	(87)
CN^- recovered by N_2 aeration		
In dark, mμg	14	12
Illuminated, mμg	79	83
B_{12} bound,* μg	3.4	—
(CN^- equivalent, mμg)	(66)	—

* Determined by the partial dialysis method.

binding of vit. B_{12} by this extract is shown in Table IV. The reaction was essentially unaffected by pH changes from pH 2 to 10 but was inhibited in more basic solution. Excess cyanide had no effect in neutral solution, but exerted a progressive inhibition above pH 10. The order of addition of excess cyanide and labeled vit. B_{12} to gastric extract did not affect the magnitude of inhibition. Excess cyanide did not affect the dialysis rate of free cobalamin. These data suggest that cobalamin is bound to this protein through the position sought by the second cyano-group.

That the cyano-group of vit. B_{12} is not displaced by the binding reaction is indicated by data presented in Table V. The cyanide method of Boxer and Rickards(11) was used. Gastric mucosal extract was first freed of cyanide by nitrogen aeration at pH 5. Labeled vit. B_{12} was then added and cyanide again determined, first after aeration in the dark, then while being illuminated. The amount of vit. B_{12} which had actually been bound by gastric extract was subsequently determined by partial dialysis. It appears that the first cyano-group is not displaced by the binding reaction, but remains in a photolabile position, presumably still with the cobalamin structure.

The effect of several treatments on vit. B_{12}-gastric mucosal extract reaction, as studied by partial dialysis, is shown in Table VI. The reaction was not affected by type or concentration of buffer. The effect of heat was similar to that reported by others using bacterial assay methods(2,12,13) and indicates that the complex is as heat-labile as the unreacted binding substance at pH 6.6. The report that cobalt-benzimidazole mixtures inhibit the reaction(2) could not be confirmed by this method. The suggestion that protein sulfhydryl groups may be involved in binding was shown to be unlikely, for the reaction was unaffected by excess para-chloromercuribenzoate. A cobalt porphyrin, reported to promote growth of vit. B_{12}-requiring microorganisms(14) was found to have no marked effect on the reaction.

Discussion. The marked reactivity of vit. B_{12b} suggests that suitable precautions should be taken against photoconversion when attempting to study vit. B_{12}-protein reactions. For example, heparin and nucleic acids were found to bind vit. B_{12b}, and not vit. B_{12} as had been reported(15). The binding or adsorption of vit. B_{12b}, by substances such as cellophane and filter paper point up difficulties involved in ascribing a physiological function to substances showing vit. B_{12b}-binding activity. Data presented in Tables I and

TABLE VI. Effect of Various Treatments on Binding of Vit. B_{12} by the Gastric Extract.

Treatment		Bound vit. B_{12}, mμg/mg N
Effect of buffer at pH 6.6		
No buffer		350
.1 M phosphate		320
.2 "		330
.4 "		330
.2 M acetate		340
Heating the gastric extract		
60°C, 1 hr		330
100°C, "		180
Heating the B_{12}-gastric complex		
60°C, 1 hr		330
100°C, "		180
Reagents	Molar excess*	
$CoCl_2$	2×10^6	320
Benzimidazole	3×10^5	330
" , $CoCl_2$	" , 2×10^6	320
" , " , heat†	" "	270
p-Chloromercuribenzoate	1×10^4	340
Cobalt-hematoporphyrin	1×10^5	310

* Relative to original B_{12} concentration, 7.6 mμg per ml or 5.6×10^{-9} M.

† Heated at 100°C for 10 min. prior to addition of B_{12}. Gastric extract alone given same heat treatment bound 290 mμg B_{12}/mg N.

II suggest that binding of vit. B_{12b} by proteins, possibly via a cobalichrome linkage with histidine residues, is a rather general reaction involving little structural specificity of the proteins involved, and may therefore be of little physiological significance.

The limited study of the cobalamin-gastric mucosal extract reaction reported here is in general agreement with the more extensive study of Gregory and Holdsworth(4) who used ultrafiltration to study binding of vit. B_{12} by an isolated milk protein. However, there appears to be some discrepancy concerning the effect of excess cyanide on the reaction or reaction product. Gregory and Holdsworth observed no changes in absorption spectrum of cyanocobalamin-milk protein complex in presence of excess cyanide at pH values as high as pH 11. The data presented here indicate that dicyanocobalamin, in presence of excess cyanide, does not react completely with gastric extract, that excess cyanide dissociates pre-formed cyanocobalamin-gastric extract complex, and that these reactions are dependent upon cyanide concentration and pH. These results may represent a difference in nature of the binding reaction by the 2 preparations, or a difference in molar excess of cyanide used in the two situations. Although there have been reports (16,17) that cobalamin-peptide complexes are split by cyanide in neutral solution, the possibility exists that these are cobalichrome type structures and thus more susceptible to cyanolysis.

Summary. 1. A short-term dialysis procedure has been developed for studying cobalamin-protein reactions. 2. Aquocobalamin (vit. B_{12b}) was much more reactive than cyanocobalamin, being bound in rather large amounts by several protein preparations of diverse origin. In several instances binding was increased by denaturation and decreased in acid media. It is suggested that this type of binding may occur via cobalichrome formation. 3. A gastric mucosal extract bound large and equal amounts of aquocobalamin and cyanocobalamin, apparently at the same binding site. Excess cyanide has no effect on the cyanocobalamin-gastric extract reaction in neutral media, but exhibits an inhibiting effect at high pH levels. In this reaction the cyano-group of cyanocobalamin is not released, but remains in a photolabile position. It is suggested that the cobalt atom participates in the reaction and that it is the position occupied by the substituted benzimidazole moiety that is involved.

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