1954

Reactions of cobalamins with several protein preparations

Wallace R. Bauriedel
Iowa State College

NOTE TO USERS

REACTIONS OF COBALAMINS WITH SEVERAL PROTEIN PREPARATIONS

by

Wallace R. Bauriedel

A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of
The Requirements for the Degree of
DOCTOR OF PHILOSOPHY

Major Subject: Biochemistry

Approved:

Signature was redacted for privacy.

In Charge of Major Work

Signature was redacted for privacy.

Head of Major Department

Signature was redacted for privacy.

Dean of Graduate College

Iowa State College
1954

TABLE OF CONTENTS

INTRODUCTION

The isolation of vitamin B_{12} in 1948 concluded an intensive twenty-two year search for the antipernicious anemia principal or extrinsic factor of liver, and initiated one of the most intriguing and complex problems of contemporary biochemistry. The concerted efforts of scientists of many disciplines have, in the six intervening years, produced only a partial, and in some areas, actually superficial understanding of the chemistry and physiology of this remarkable vitamin.

Vitamin B_{12} is only one of a family of closely related compounds, the cobalamins, and although the structural relationships between the several cobalamins have been tentatively defined, only about two-thirds of the structure of the parent molecule has been elucidated. As a result the present concepts of the biological reactions of cobalamins are based on the known structural features and may have to be considerably revised when the structure of the remainder of the molecule has been determined.

The vitamin appears to be synthesized exclusively by microorganisms and to have an essential function in many, if not all, forms of life. The vitamin is a dietary essential

for many higher animals including man, and appears to require a substance present in normal gastric juice and gastric mucosa, the intrinsic factor, for transport through the intestinal wall.

The extreme potency of the vitamin indicates that it functions as a prosthetic group in an enzyme system. Although the fundamental reactions controlled by this enzyme system remain obscure, studies of deficient organisms have implicated the vitamin in nucleoprotein synthesis, labile methyl group synthesis and disulfide reduction. Metabolic interrelationships with other vitamins, notably folic acid, have been discovered.

The vitamin appears to be tenaciously retained by higher animals, and a considerable capacity to store the vitamin in the liver and other organs has been demonstrated. The vitamin exists primarily in a bound form in all tissues, the binding substances generally appearing to be protein in nature. Several mechanisms have been proposed for the binding of cobalamins by proteins. Most of the prior evidence concerning the nature of these reactions has been based on the differential response of selected bacteria toward free and bound cobalamins.

The purpose of this study was to examine the reactions of cobalamins with proteins, using a method which accomplished

the separation and determination of free and bound cobalamins under mild conditions and without the use of microbiological systems. Of particular interest were the structural features of the cobalamin molecule which participate in the reactions, and the possible structures of the binding sites on the proteins. Although most of the work involved the use of serum and gastric extract, systems which appear to function primarily in the transport of the vitamin, the information gained may be of value in anticipating the nature of the reactions which are involved in the storage and enzymatic function of the vitamin.

LITERATURE REVIEW

Chemistry of the Cobalamins

The isolation of the long sought antipernicious anemia principle of liver was announced by two groups of workers almost simultaneously in 1948. Rickes and co-workers (1) reported the crystallization of an intensely red compound, tentatively designated vitamin B_{12}, which showed remarkably high hematopoietic activity for Addisonian pernicious anemia. Their isolation procedures were guided by the LLD microbiological assay method of Shorb (2), who had observed that the response of Lactobacillus lactis Dorner to liver extracts was proportional to the potency of these extracts in the treatment of pernicious anemia (3). Independently, E. L. Smith reported the purification (4), and later crystallization (5, 6) of vitamin B_{12} from liver using clinical assays on pernicious anemia patients to guide the fractionation. These two products were later shown to be identical (6,7,8).

The new vitamin was reported to contain cobalt, phosphorous and nitrogen, but no sulfur (6, 8, 9, 10, 11), and no alpha-amino acids were liberated on acid hydrolysis (7). The molecular weight was estimated as about 1350 (6, 7, 12)

and the formula $C_{61-64}H_{86-92}N_{14}O_{13}PCo$ was proposed (6, 7). Several weakly basic groups, titrable in glacial acetic acid, were reported (6, 7). Absorption maxima in aqueous solution were observed at 278, 361 and 550 mμ. (6, 7, 13), and were not affected by changes of pH (7).

After the report that vitamin B_{12} could be isolated from the fermentation broth of Streptomyces griseus (14), the vitamin was found and isolated as a by-product of other antibiotic fermentations, resulting in an ample supply of the vitamin for investigational and therapeutic uses.

Other closely related compounds were soon isolated and designated vitamin B_{12a} (15, 16), vitamin B_{12b} (17, 18, 19) and vitamin B_{12c} (20, 21, 22). It was then demonstrated that vitamin B_{12} contained a cyano- group attached to the cobalt (23), and that this cyano- group was replaced by a hydroxo- group in vitamin B_{12a} (16), and by a nitrito- group in vitamin B_{12c} (20). Vitamins B_{12a} and B_{12b} were later shown to be identical (21, 24, 25, 26). In order to simplify the nomenclature of this fast-growing group of compounds it was proposed (16) that the name cobalamin be used to designate all of the vitamin B_{12} molecule except the cyano- group, so that vitamins B_{12}, B_{12a} (B_{12b}) and B_{12c} would be cyanocobalamin, hydroxo- (or later, aquo-) cobalamin and nitritocobalamin respectively. A few of the older terms

such as B_{12}, B_{12b} and B_{12c} continue to be used, and often, unfortunately, vitamin B_{12} is used in the generic sense or for undefined mixtures of the cobalamins. A description of the many cobalamins that have been studied will be presented after a summary of the present knowledge concerning the parent cobalamin structure.

Acid hydrolysis of vitamin B_{12} yields a mixture of amorphous cobalt-containing fragments of about two-thirds the size of the original molecule, and a variety of smaller fragments, depending upon the experimental conditions. On heating at 100-150° C. in concentrated hydrochloric acid for several hours, ammonia, phosphoric acid, the red fragments, and two bases were obtained (10, 13, 27). One of the bases, originally thought to be 2-amino-1-propanol (27, 28), was identified as Dg-1-amino-2-propanol (29). The other basic compound resulting from this drastic hydrolysis was shown to be 5,6-dimethyl-benzimidazole (30, 31, 32, 33). Milder hydrolysis procedures yielded two N-substituted 5,6-dimethyl-benzimidazoles (33, 34). These compounds were found to be 1-α-D-ribofuranoside-5,6-dimethylbenzimidazole (35), and a phosphate ester of this substituted benzimidazole (36, 37). The phosphoryl group is attached to either the second or third carbon atom of the ribose moiety, the exact position being uncertain because of cyclization during hydrolysis

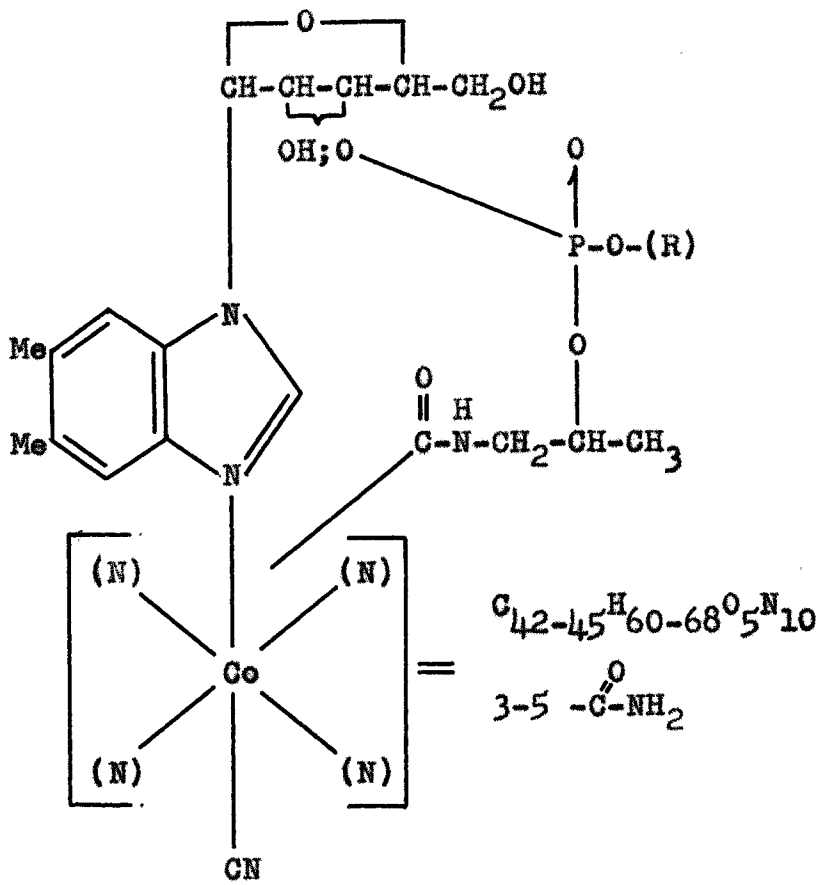
(38). There is one mole of the benzimidazole N-glycoside present per mole of the vitamin (33), and it has been postulated from spectroscopic evidence that in the intact structure a coordinate linkage exists between the nitrogen of the benzimidazole and the cobalt atom (39, 40, 41). There are either one (42, 43) or two (44, 45) aminopropanol groups in the molecule, with the evidence perhaps favoring the latter. It appears probable that one aminopropanol is joined by ester linkage to the phosphoric acid group, and by amide linkage to a carboxyl group of the cobalt-containing part of the molecule (36, 40, 42, 43). The phosphate must be fully esterified, either by a second aminopropanol (36), or by some group in the cobalt-containing fragment (43).

Only a few features of the remaining structure have been elucidated. Magnetic susceptibility measurements of vitamins B_{12} and B_{12b} (46, 47, 48, 49) and polarographic evidence (22, 49, 50, 51) indicate that the cobalt is in the trivalent state and is hexacoordinately bound. Failure of the cobalt of vitamin B_{12} to exchange with ionic radioactive cobalt (6, 52, 53) or to be liberated by drastic acid hydrolysis or treatment with excess cyanide (49) supports this conclusion. The facile removal of the cobalt by relatively mild reducing agents such as ascorbic acid, cysteine, thiamine and hydroquinone (54, 55, 56, 57), providing the stabilizing cyano- group is removed first, is not

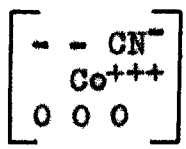
unexpected considering the instability in aqueous solution of hexacovalent cobaltous complexes containing benzimidazole (58).

The rest of the planar structure (12) around the cobalt contains no primary amino groups (59), but does contain either three (43) or five (60) primary amide groups. Prolonged acid hydrolysis resulted in a mixture of fragments containing five, six and seven acidic groups (43) and apparently retaining four nitrogen atoms (45). Alkaline permanganate oxidation of the cobalt-containing fragments (61) permitted the isolation of acetic, oxalic, succinic, methylsuccinic and dimethylmalonic acids, and four unidentified acids. The structure around the cobalt is probably not a porphyrin (43), although some of the reactions of synthetic cobalti-porphyrins are strikingly similar to the reactions of cobalamins (62).

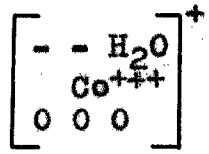
Based on the above findings, a partial structure of vitamin B_{12} can be presented in the following manner:



The many cobalamins that have been prepared can be conveniently classified into three groups, neutral, basic and acidic (63, 64). Using the notation of Buhs (65), examples of these three groups can be formulated as follows (64):



I
cyanocobalamin



II
aquacobalamin

III
dicyanocobalamin

The negative sign denotes a group contributing a net negative charge, and the zero sign denotes a neutral group, such as the substituted benzimidazole.

The neutral cobalamins are relatively stable and can be isolated in crystalline form. Cyanocobalamin (B_{12}) is an exceptionally stable complex, and all of the cobalamins studied so far can be converted to cyanocobalamin by means of either cyanide salts or hydrogen cyanide at a pH range of 5.5 to 9 (16, 20, 23, 25, 64, 66, 67, 68, 69, 70). The cyanide can be removed by catalytic reduction with hydrogen in neutral solution followed by reoxidation of the cobalt by air (15, 19, 49), or by photolysis in acid solution accompanied by aeration to remove the hydrogen cyanide (6, 65, 67, 71, 72). By either method aquocobalamin (B_{12b}) is formed, and can be used to form other neutral cobalamins such as nitritocobalamin (B_{12c}) (20, 21, 22), cyanatocobalamin (69) and thiocyanatocobalamin (65) by reaction of vitamin B_{12b} in aqueous solution with salts of nitrite, cyanate and thiocyanate, respectively. By exchange studies using

radioisotopically labeled anions, Smith (64) demonstrated that the cyanide of cyanocobalamin exchanges rapidly with cyanide ions in alkaline solution but very slowly in acid solution, and that very little replacement of the cyano-group by thiocyanate ion occurs, even in alkaline solutions. On the other hand, there is rapid exchange of the thiocyanato-group of thiocyanatocobalamin with the thiocyanate ion. From this evidence Smith postulates that cyano-cyanide exchange occurs principally through the pH-labile dicyano-cobalamin, implying that the two cyano groups of this compound are equivalent.

The basic cobalamins are characterized as having the cyano- group of cyanocobalamin replaced by a neutral molecule, the water of aquocobalamin (B_{12b}) for example. This compound is a weak base, with a pK_a of about 7 (22, 65) and is considered an equilibrium mixture of aquocobalamin and hydroxocobalamin, the ratio of the two being dependent upon the pH of the solution (65, 68), viz:

$$\left[\begin{matrix} - & - & H_2O \\ & Co^{+++} & \\ 0 & 0 & 0 \end{matrix}\right]^{+} + OH^{-} \rightleftharpoons \left[\begin{matrix} - & - & OH^{-} \\ & Co^{+++} & \\ 0 & 0 & 0 \end{matrix}\right] + H_2O$$

Aquocobalamin reacts with strong acids such as sulfuric, hydrochloric and perchloric to give isolable salts (64, 73, 74), which are named aquocobalamin sulfate, chloride and perchlorate.

Also considered as basic cobalamins were two compounds prepared by Cooley and co-workers (68), ammonia cobalichrome (analogous to the hemochromes) and histidine cobalichrome, in which the water of aquocobalamin was replaced by ammonia or histidine. The authors proposed that cobalamin may be stored in tissues in a cobalichrome type of linkage with protein or peptide.

Cobalamins of the third (acidic) class arise from the addition of two univalent anions or one bivalent anion to the cobalamin residue. These compounds are stable only in the presence of excess anion, and have therefore not been crystallized. Dicyanocobalamin was the first of this group to be prepared (39, 40, 75), and clearly posed a question as to the position of the second cyano- group. Based on absorption spectra and other considerations Cooley and co-workers (68) have suggested that the second cyanide, and therefore any second acidic group of this class, displaces the benzimidazole nitrogen from coordination with the cobalt. Direct evidence concerning this mechanism has not been presented. The addition of the second cyanide is reversible, occurring only in basic solutions (39, 40). A reported addition of two cyanide groups to cyanocobalamin (75) seems to have been discounted in favor of rather strong evidence that only one cyanide can be added to cyanocobalamin (49).

Other cobalamins of the acidic group, both simple (dithiocyanatocobalamin) and mixed (thiocyanatocyanocobalamin), have been studied (68). Sulfitocobalamin, prepared by treating aquocobalamin with sulfite (16) appears to belong to this group (68).

In addition to the above "authentic" cobalamins, there is a growing group of naturally occurring vitamin B_{12} analogs, the pseudovitamins B_{12}. These compounds are structurally similar to the cobalamins, except for the nucleotide moiety, which may be missing entirely (76, 77, 78), or in which adenine (79, 80), 2-methyladenine (78, 80) or 2-methylhypoxanthine (78) are present instead of the 5,6-dimethylbenzimidazole. It appears likely that these factors are not active in the metabolism of higher animals (81).

Reactions of Cobalamins with Natural Materials

Before reviewing the evidence concerning the reactions of cobalamins with natural materials, it may be well to describe briefly the methods which have been used to detect and study such reactions. Essentially, these methods depend upon two techniques for the measurement of cobalamins: the microbiological assay method and the radio assay of isotopically labeled cobalamins.

The microbiological assay is subject to many errors,

only a few of which will be pointed out here. The common assay organisms, Lactobacillis lactis Dorner (very seldom used at present), Lactobacillis leichmannii 4797 and 9830, Euglena gracilis var. bacillaris, and a mutant, Escherichia coli 113-3 are not entirely specific in their requirement of cobalamins. For example, the Lactobacilli respond to desoxyribosides, the E. coli to methionine, and all to pseudovitamins B_{12}. The growth of these organisms may be inhibited or modified by such diverse factors as oxygen tension, salt concentration, light and naturally occurring organic growth inhibitors.

The radio assay using Co^{60}-labeled cobalamins (82) is certainly the preferred method, even though it is not usually as sensitive as the microbiological assay, and the soft (0.308 m.e.v.) beta-emission of Co^{60} presents some difficulties in counting.

The most widely used method for detecting and measuring reactions of cobalamins with natural materials is the bacterial inhibition method, originally proposed by Ternberg and Eakin (83). The method is based on the assumption that the microbiological assay organisms can use only free cobalamins, and not cobalamins which have reacted with, or have been bound by some substance. The test substance is usually added aseptically to sterile assay medium containing a known amount of cobalamin, and the amount of inhibition of

bacterial growth relative to cobalamin alone is taken as an indication of cobalamin binding. Several modifications for titrating the inhibitory substance and for correcting for the presence of irrelevant growth and inhibitory factors have been proposed, but at best, the method can be considered only semi-quantitative.

The above method was actually based on earlier observations that the amount of activity present in many natural materials, as measured microbiologically, could be increased by heating, treating with proteolytic enzymes (84) or treating with cyanide, sulfite or nitrite (85, 86, 87, 88), indicating the presence of bound forms of the vitamin. A general feeling arose that the vitamin was often bound to protein material, and some workers have presented evidence for cobalamin-peptide conjugates (25, 89). There seems therefore, to be an adequate basis for the bacterial inhibition method, but in practice the method leaves much to be desired.

A more direct approach has been to separate the free cobalamins from the bound cobalamins by dialysis, since most of the vitamin binding substances studied have been non-dialysable, whereas cobalamins are dialysable. A quantitative dialysis method was first reported by Bird and Hoevet (90). When the amount of vitamin dialysed or amount retained, or both, are measured microbiologically the method

is subject to the errors mentioned previously. Isotopically labeled cobalamins have been used to some extent. Probably because of mechanical difficulties, such as the large volumes of liquids encountered and length of time required, this method has not been widely used.

A third method, proposed by Burkholder (91, 92) is based on the rapid adsorption or absorption of free cobalamins by several strains of bacteria (93, 94). The general method consists of mixing solutions of the test substance and cobalamins, adding a heavy suspension of suitable bacteria, incubating at 25-37° C. for from one to several hours, then separating the bacteria by centrifugation. The amount of cobalamin in the supernatant or with the bacterial mass is measured, and the amount of cobalamin bound by the test substance is obtained either directly or by difference. For microbiological assay procedures, the cobalamins are liberated from the cells and binding substance by heating. The radio assay technique has been applied quite successfully to this method because of the concentration of the activity to a relatively small volume of cells (95, 96, 97).

A fourth method, suggested by Latner (98, 99) makes use of the paper electrophoresis technique for separating components of a system by differences in their electrophoretic mobilities. This has been used principally as a qualitative means of locating the components responsible for

binding cobalamins and for isolating the desired components by elution from the paper.

A fifth method, spectrophotometric differentiation of free and bound cobalamins has been used by Wijmenga and co-workers (25, 100, 101). Usually the amounts of cobalamins reacting are too low to be detected spectrophotometrically. As substances which react with cobalamins are obtained in greater purity this will certainly become a valuable method.

In interpreting the evidence using these five general methods one is forced to conclude that usually the cobalamin involved was not defined, even though the term vitamin B_{12} was used. There has been an apparent disregard of the well known conversion of cyanocobalamin to aquocobalamin by light, and of the reports of a conversion of cyanocobalamin to an unidentified cobalamin by heat-sterilization in the microbiological assay medium (102, 103). Therefore, unless suitable precautions were reported, such as performing the experiments in the dark and in the case of the bacterial inhibition method, aseptic addition of cyanocobalamin, the results will be reported as having involved cobalamins, not specifically vitamin B_{12}.

Considerable evidence has been presented for the reaction or binding of cobalamins with gastric juice and gastrointestinal tract mucosal extracts, serum, whey from sow's milk, human saliva, lysozyme, bacterial cells, egg yolk,

heparin and other substances. The impetus for much of this work was derived from an intensive search for the identity and mode of action of the intrinsic factor. This factor is present in normal human gastric juice and in extracts of hog gastric and duodenal mucosa, and is involved in some way with the transfer of the extrinsic factor (cobalamins) through the intestinal wall. The importance of cobalamin binding to intrinsic factor activity is at present uncertain.

Ternberg and Eakin (83) were the first to report that unheated gastric juice inhibited the growth of L. lactis Dorner, L. leichmannii and E. coli, apparently by a stoichiometric binding of cobalamins, and that the complex formed was non-dialysable. Binding did not occur if the gastric juice was heated first, and the complex was dissociated by heating at 120° C. for 15 minutes, making the cobalamins available to the assay organisms. Aqueous extracts of hog gastric mucosa exhibited a similar binding activity, whereas little activity was found in commercial pepsin preparations. The activity from hog gastric mucosa was precipitated by alcohol, acetone and ammonium sulfate at 80 per cent saturation.

The findings of Ternberg and Eakin have been confirmed and elaborated upon by several groups. Prusoff and co-workers (104) prepared several fractions of a hog gastric mucosal extract by salting-in and salting-out procedures.

The amount of cobalamin bound per gram of protein by these fractions was determined by the bacterial inhibition method and a dialysis method using L. leichmannii as the assay organism. The dialysis method gave values roughly ten times higher than the inhibition method, although both methods indicated a progressive increase in binding activity of fractions obtained at 35, 55 and 100 per cent saturation with ammonium sulfate. Subfractionation by extended dialysis indicated that the binding substance was present in the water soluble fractions. Clinical tests revealed no correlation between binding capacity and intrinsic factor activity.

Bird and Hoevet (90) attempted to devise a quantitative method for measuring the binding capacity of a hog mucosal extract. A comparison of three methods: (1) bacterial inhibition with constant cobalamins and increasing inhibitor; (2) bacterial inhibition with constant inhibitor and increasing cobalamins; and (3) dialysis with constant inhibitor and increasing cobalamins, gave values of 3.5, 9.0 and 83 µg. of cobalamins bound per gram of protein respectively. They interpreted these results as indicating that the assay organism, L. leichmannii, was capable of utilizing all but a small part of the bound vitamin. By the dialysis procedure the cobalamin binding appeared to be stoichiometric.

Recognizing the shortcomings of the bacterial inhibition method, Burkholder (92) and Hoff-Jørgensen (105) showed that a fairly quantitative measure of cobalamin binding could be obtained with the bacterial inhibition method, using E. coli to adsorb the free cobalamins and either E. coli or L. leichmannii as assay organism. The data of Burkholder indicated a stoichiometric binding of cobalamins by a hog gastric mucosal extract. In a similar study by Chow (95) using the radio assay method of determining the free cobalamins adsorbed by bacteria, a non-stoichiometric relationship appeared to exist between gastric juice and cobalamins.

There have been several studies of the effect of heat on the binding capacity of gastric juice and gastric mucosal extracts, and of the effect of heat on the stability of the cobalamin complex with these substances. Ternberg and Eakin claimed that heated gastric juice showed no binding activity, and that the complex with cobalamin was dissociated by heating at 120° C. for 15 minutes (83). Spray (106) confirmed these results using 100° C. for 5 minutes. However, when the gastric juice was neutralized, heating at 127° C. for 30 minutes was required to destroy the binding effect. The cobalamin-gastric juice complex was also much more stable at a neutral pH. A similar heat-stability of hog mucosal extracts has been reported by Bethell and co-workers

(107) and by Shaw (108).

Van der Zant and Underkofler (109, 110) have reported the apparent stability of the binding substance present in hog gastric mucosal extracts to heating at 100° C. for 10 minutes or 121° C. for 5 minutes over a pH range of 2.5 to 7.0. A similar heating period at pH 11 or in the presence of the microbiological assay medium destroyed the binding capacity. Histidine and Fe(II) were implicated in the destruction of binding power by heating with the basal medium, and it was found that heating with histidine and Co(II), or benzimidazole and Fe(II) or Co(II) also destroyed the binding capacity. It was suggested that these combinations react with and block the reactive sites of the binding substance, preventing the binding of cobalamins, and that the bonds involved in cobalamin binding are with the benzimidazole moiety and the cobalt atom. The evidence cited above concerning the effect of heat was obtained using bacterial inhibition methods.

Evidence concerning the effect of proteolytic enzymes on the binding capacity of mucosal extracts is rather inconclusive, but indicates that a considerable part of the activity survives enzymatic digestion with trypsin, pepsin and papain (92, 110), and that the cobalamin-mucosal extract complex is not dissociated by tryptic digestion (107).

Latner and co-workers have done a considerable amount of work trying to isolate the component of gastric juice responsible for intrinsic factor activity. Using a preparative paper electrophoresis technique (98) they observed that all of the components of gastric juice showed binding power as measured by the bacterial inhibition method, whereas only two components showed intrinsic factor activity (99). Recent reports described the isolation of a mucoprotein of high intrinsic factor activity, but the binding capacity of this preparation has not been reported (111, 112, 113). In a similar study, Shilling and Driess (114) observed that one component of gastric juice appears to contain most of the cobalamin binding activity. They separated the components of a gastric juice-radiotracer labeled cobalamin mixture by paper electrophoresis and determined the position and amount of cobalamins by radio assay. They also observed that boiling the gastric juice-cobalamin mixture before electrophoresis considerably diminished the localization of activity.

Roland and co-workers (115) have used a 10-cell isoelectric electrophoresis apparatus to distribute the components of a hog mucosal extract and have measured the binding activity of the contents of each compartment by a dialysis method using L. leichmannii as the assay organism. The fractions which were isoelectric at pH 3.5 to 5.0 contained most

of the cobalamin binding activity. This work was performed in the dark, so it is probable that cyanocobalamin binding activity was actually being measured.

Chow and Yamamoto (116) have attempted to isolate a gastric juice-cobalamin complex. They used radioisotopically labeled cobalamins and followed the separation by radio assay. They reported the isolation of a "substance X" which was isoelectric at pH 3.5-4.0, had an approximate molecular weight of 5×10^5, and was precipitated by 60 per cent ethanol, but not by 40 per cent ethanol. The cobalamin content of this substance has not been reported.

Perhaps the most convincing evidence that cobalamins react with some specific component of gastric juice or mucosa has been presented by Wijmenga and co-workers (100, 101). They isolated several pink "cobalamin proteins" from a cyanocobalamin-hog mucosal extract mixture that contained up to 18.5 µg. of cobalamin per milligram of protein, as determined spectrophotometrically. These preparations appeared to be mucoproteins of 70,000-100,000 molecular weight. The observed absorption maxima at 363 and 550 mµ. with inflections at 410 and 520 mµ. indicated that the cyano- group was still attached to the cobalamin. Only preliminary reports of this work have been published.

Cobalamins present in serum appear to be primarily in a bound form. Ross (117, 118) reported that heating serum at

100° C. for 30 to 60 minutes was required for maximum release of cobalamins as measured with E. gracilis. These observations have been confirmed by several groups (119, 120, 121). Rosenthal and Sarett (121) further demonstrated that the cobalamins present in serum were not dialysable, and that additional amounts of cobalamins could be bound by serum. Independently, Wolff and co-workers (122) reported that serum bound limited amounts of cobalamins, as determined by the bacterial inhibition method. This finding was not unexpected, since Bird and Hoevet (90) had previously reported the apparent binding of cobalamins by serum globulin fractions. Reports that cobalamins added to serum were freely dialysable were apparently the result of using levels of cobalamins greatly exceeding the binding capacity of the serum (123, 124).

Roland and co-workers (115) using a dialysis method obtained evidence that the binding of added amounts of cobalamins by serum was not stoichiometric, suggesting that the reaction may be reversible to a considerable extent.

Shilling reported that added Co^{60}-labeled cobalamins could be detected only in the albumin fraction of serum subjected to paper electrophoresis (114). On the contrary, Pitney and co-workers (125, 126) have presented evidence that the cobalamin activity of serum was present principally in the alpha globulin fraction, and that added cobalamins

were bound by the alpha and beta globulin fractions. These workers used paper electrophoresis to separate the serum proteins, and the E. gracilis microbiological assay, differentiating free and bound activity by the method of Ross (117). Their microbiological assay values appeared to be too erratic to support more than the qualitative conclusions given above.

Davis and Chow (127) have added further testimony that serum is capable of binding additional amounts of cobalamins, and have described a quantitative procedure using the bacterial adsorption method and radioisotopically labeled cobalamins.

Unglaub and co-workers have reported an unexpected increase in the cobalamin binding capacity of serum when small amounts of normal gastric juice were added to the serum (128, 129). The experimental details of this work have not been published.

A cobalamin binding substance present in sow's milk, but not in cow's milk has been reported by Gregory and co-workers (130). A dialysis method was employed in this investigation. The substance was reported to be heat stable at 100° C., labile to tryptic digestion, non-dialysable and precipitated by 35 to 55 per cent saturated ammonium sulfate. Further studies by this group (131, 132) indicated that hog gastric mucosal extracts and sow's milk preparations bound

three pseudovitamins B_{12} to approximately the same degree as cobalamins. A bacterial inhibition method using the E. coli mutant was used in the latter experiments.

It has been reported that human saliva contains a cobalamin binding substance (133) as detected by the bacterial inhibition method. This substance was found to be more heat labile than gastric preparations, showing no binding activity after heating at 70° C. for one minute (134).

Meyer and co-workers (135) reported that lysozyme appeared to bind cobalamins, as measured by the bacterial inhibition method. Bird and Hoevet (90) in a more detailed study observed that the inhibitory action of lysozyme was not reversed by relatively large amounts of cobalamins or by thymidine, a cobalamin replacing factor for their assay organism, L. leichmannii. Furthermore, lysozyme did not prevent the dialysis of cobalamins or of thymidine. Thus, it appears that lysozyme does not bind cobalamins and that in this case the bacterial inhibition method gave completely erroneous results. This was substantiated by Hoff-Jørgensen (105) with the demonstration that lysozyme did not inhibit the adsorption of cobalamins by E. coli cells. Chow has also reported that by bacterial uptake and dialysis methods using Co^{60}-cobalamins, lysozyme apparently does not possess binding activity (95).

It was stated earlier that several strains of bacteria rapidly remove free cobalamins from solution. This behavior has been variously referred to as absorption and adsorption. Possibly a loose differentiation can be made depending upon the conditions of incubation, the adsorption being rapid in comparison to absorption. The process does not appear to be related to the need for an exogenous source of vitamin B_{12}, and the binding capacity appears to be a strain characteristic (136). Several, but not all strains of E. coli, including the mutant strain which requires cobalamins, have been shown to possess this property (93, 94, 105, 137). Cobalamin uptake was also exhibited by L. leichmannii and L. lactis Dorner, which require cobalamins, and by S. lactis R, B. subtilis, P. mirabilis, B. mycoides, E. freundii and S. dobson, none of which require cobalamins. Uptake was not exhibited by L. arabinosus which does not require cobalamins (94, 136).

It appeared that if the incubation time was short (30 to 60 minutes) the cobalamins could be released by heating (138) or by exchange with additional amounts of cobalamins in the solution (137). If prolonged periods of incubation were used disintegration of the cells was necessary to release the cobalamins (105, 136).

Oginsky and Smith (139) reported that 4,5-dimethyl-benzimidazole inhibited the uptake of cobalamins by E. coli,

while benzimidazole and 2,5-dimethylbenzimidazole were without effect. This suggested that the uptake is of a specific nature. Davis and Chow (138) reported that the uptake of cobalamins was maximal in the pH range from 3 to 9, but was reduced by higher pH levels and by sodium chloride concentrations above 1.7 per cent. Although Davis and Chow have often referred to this uptake as occurring with "resting" cells, no evidence has been presented on the possible uptake by killed but intact cells, or by disintegrated cells.

Several other substances have been reported to bind cobalamins. In a recent report Couch and co-workers (140) claimed that egg yolk contained a substance which inhibited L. leichmannii, formed a non-dialysable complex with cobalamins, and was stable to prolonged autoclaving.

Chow and Davis (95) have studied some non-protein systems, namely heparin, ribonucleic acid and chondroitin sulfuric acid, and reported that all had binding activity as evidenced by bacterial uptake and dialysis methods using Co^{60}-labeled cobalamins. However, a recent report by the same group (141) indicated that L. leichmannii, the organism used in their earlier study, and two strains of E. coli differ qualitatively and quantitatively when used to measure cobalamin binding by these and similar substances by the bacterial uptake method. For example, heparin did not

affect the uptake of cobalamins by the E. coli strains. Earlier Latner had reported, without detail, that heparin inhibited the growth of E. coli (99). What possible role these substances may have in the physiological reactions of cobalamins is uncertain.

EXPERIMENTAL METHODS AND RESULTS

Materials

Chemicals

Cobalamins. The United States Pharmacopeia Cyanocobalamin Reference Standard, obtained from the Board of Trustees of the United States Pharmacopoeial Convention, Incorporated, was used as the standard for the microbiological assay of vitamin B_{12}. This material was in powdered form and was labeled as containing 530 mμg. cyanocobalamin per mg.

Bevidox, prepared by Abbott Laboratories and specified as containing 1 mg. of vitamin B_{12} per ml., was obtained from a local drug store, and was used for spectrophotometric, dialysis and cyanide studies.

Isotopically labeled cobalamins. Vitamin B_{12} labeled with Co^{60} prepared by the fermentation method of Chaiet (82) was obtained from Merck and Company, Incorporated, by permission of the United States Atomic Energy Commission, through the Institute for Atomic Research, Iowa State College. The material was received in vials labeled as containing 40 μg. of vitamin B_{12} in 5 ml. of 10 per cent aqueous methanol. The specific activity of the material was

225 μc. per mg. of vitamin B_{12}.

Stock solutions containing approximately 200 mμg. of cobalamins per ml. were prepared by dilution of this material with water. These stock solutions were either converted entirely to vitamin B_{12} by treatment with excess sodium cyanide followed by nitrogen aeration at pH 6 in the dark, or completely to vitamin B_{12b} by nitrogen aeration at pH 5 under strong tungsten illumination.

Special chemicals. Lysozyme and crystalline preparations of trypsin and pepsin were obtained from Nutritional Biochemicals Corporation. Ventriculin, a desiccated stomach preparation manufactured by Parke, Davis and Company, was obtained from a local drug store. Heparin was obtained from Connaught Medical Research Laboratories. Para-chloromercuribenzoic acid was obtained from the Sigma Chemical Company. Imidazole and 3-methyl-1-phenyl-5-pyrazolone were obtained from Distillation Products Industries. Bis-3-methyl-1-phenyl-5-pyrazolone was prepared from the preceding ketone by the method used by Epstein (166). A bovine albumin preparation was obtained from the Armour Laboratories. A high molecular weight dextran preparation was obtained from the Northern Utilization Research Branch of the Agricultural Research Service, United States Department of Agriculture.

General chemicals. The biological chemicals used in this investigation were obtained from Nutritional Biochemicals Corporation. Other chemicals were obtained from the Iowa State College Chemistry Stores or from commercial chemical firms.

Microorganism

Lactobacillus leichmannii 7830 was obtained in lyophilized form from the American Type Culture Collection.

Instruments

Radioisotope instruments. Liquid samples were counted using a Nuclear model D-52 G-M tube used with an Instruments Development Laboratories model 161 scaler and a Production Instrument Company model EC-84 register.

The counting tube was held vertically in a glass tube of such a size that 20 ml. of solution exactly filled the annular space. The outside tube was open at the top for filling, and had a stopcock at the bottom for emptying. This counting tube assembly was housed in a light-tight box. The background count for this tube was about 42 c.p.m., and remained constant to within one or two c.p.m. throughout the experimental period.

Solid samples were counted using a Nuclear model D-34 end-window G-M tube in a Technical Associates model L-56

lead shield used with a Nuclear model 165 scaler. Samples were applied to 1 inch copper planchets and were placed as close to the counting tube as possible. The input voltages of the scaling units were held constant with a Sorensen model 1750 voltage regulator.

Electrophoresis apparatus. Moving boundary electrophoresis determinations were performed on an Aminco Portable Electrophoresis unit using a 6 ml. analytical cell.

Paper electrophoresis apparatus was constructed in this laboratory for this investigation.

Dialysis cells. The original dialysis cell was constructed in this laboratory. Three identical cells were later constructed by the College Instrument Shop. The rotating assembly was constructed in this laboratory.

Spectrophotometers. A Beckman model DU spectrophotometer was used in the work involving high levels of cobalamins. A Coleman model 6A spectrophotometer was used in the colorimetric protein and sulfhydryl determinations.

Reactions of Cobalamins with Amino Acids and Other Small Molecules

Introduction

Aquocobalamin (vitamin B_{12b}) has been shown to react

with two amino acids, histidine and cysteine. Cooley and co-workers (68) prepared histidine cobalichrome by mixing 1 ml. of DL-histidine and 1 mg. of B_{12b} in 0.25 ml. water, separating the cobalichrome from the excess amino acid by paper chromatography, and eluting the cobalichrome with water. The absorption spectrum of histidine cobalichrome showed maxima at 275, 357, 410 and 535 mμ. at pH 2 and showed a slight bathochromic shift toward longer wavelengths as the pH of the solution was raised. Chromatographic evidence indicated that the treatment of the compound with cyanide removed the histidine. It was not demonstrated that complete conversion of B_{12b} to the cobalichrome had been effected, and no mention was made of the results of similar experiments using cyanocobalamin (vitamin B_{12}) and histidine or with these cobalamins and other amino acids.

The above rather limited evidence has been widely cited as suggesting cobalichrome formation as a mode of linkage between proteins and cobalamins. No further information has appeared in the literature concerning this or similar reactions.

The reaction of vitamin B_{12b} with cysteine was first reported by Lang and Chow (54), and appears to be similar to the reaction of B_{12b} with other reducing agents such as thiamine, ascorbic acid, hydroquinone, hydrogen peroxide, sodium thioglycolate, thiomalic acid and thiosorbitol (55,

56, 57, 142, 143). Heating B_{12b} with any one of the above compounds in solution destroyed the microbiological activity of the cobalamin. Vitamin B_{12} is unaffected by similar treatment. The reaction of B_{12b} with ascorbic acid results in complete loss of color and liberation of the cobalt atom from the B_{12b} molecule (55). Presumably, the other reducing agents react in the same manner. The reaction is not reversible, but can be prevented by the presence of cyanide, sulfite, nitrite (all of which replace the aquo- group of aquocobalamin) and ferric iron (55).

It was the lack of information concerning the reactions of cobalamins with amino acids that prompted the limited studies reported below, for if histidine is the only amino acid which will combine with cobalamins, and if histidine will not react with cyanocobalamin, some further mechanism must be sought to explain the apparent reaction of cyanocobalamin with protein.

General method

A shift of the absorption spectrum of the cobalamin in the presence of the amino acid was taken as evidence for a reaction. Obviously, a reaction which did not affect the chromophoric part of the molecule would not have been observed by this method. Most of the reactions were carried out in 0.1 M primary sodium phosphate (pH 4.5) using approximately

50 µg. cobalamin and 6 mg. of the test substance per milliliter. After allowing the solution to stand at room temperature for at least one hour, the positions of the three principal maxima of the absorption spectrum were determined. Some reactions required several hours to reach equilibrium.

Two cobalamins were used in this study. Vitamin B_{12} as obtained in Bevidox (Abbott) was found to be essentially pure, spectrophotometrically. Vitamin B_{12b} was prepared from vitamin B_{12} by aeration with nitrogen at pH 4 while being illuminated with a 500 watt light bulb. The reaction was considered complete when the ratio of absorbancies at 351 and 361 mµ. had risen from 0.62 to a maximum of approximately 1.5 (68).

Results

The vitamin B_{12b} solution showed absorption spectrum maxima at 274, 351 and 524 mµ. at pH 4.5. In the presence of a large molar excess of histidine, histidyl histidine, pyridine, histamine, carnosine (β-alanyl histidine) or imidazole the maxima were shifted to the longer wavelengths, indicating a reaction of the compound with B_{12b}. The absorption spectra maxima of these solutions are given in Table 1. Vitamin B_{12b} did not appear to react with any of

Table 1. Principal visible and ultraviolet absorption spectra maxima of derivatives of vitamin B_{12b}

Mixture	Maxima, mμ.		
Vitamin B_{12b}	274	351	524
Vitamin B_{12b} plus:			
Histidine	277	358	538
Histidyl-histidine	277	358	538
Histamine		358	538
Carnosine	276	358	536
Imidazole	278	358	542
Pyridine		359	533

the following amino acids: glycine, alanine, arginine, aspartic acid, cystine, glutamic acid, hydroxyproline, lysine, methionine, isoleucine, leucine, norleucine, proline, serine, threonine, asparagine, valine and glutamine. Further, B_{12b} apparently did not react with any of the following compounds: glycyl-glycyl-glycine, benzimidazole, nicotinic acid, pyridoxine, uracil, adenine, creatinine, nicotinamide, kynurenic acid and nicotine. The visible and near ultraviolet absorption maxima of B_{12b} were destroyed when allowed to react under the same conditions with cysteine, ascorbic acid, thiamine or glutathione. The absorption spectra of B_{12} remained unchanged in the presence of any of the above compounds.

The observation that benzimidazole did not react with B_{12b} was unexpected, for benzimidazole is not much weaker in basicity, with a pK_a of 5.5 (144), than the imidizole of histidine, with a pK_a of 6.0. Even at pH 7 there was no evidence of a reaction between benzimidazole and B_{12b}. Compounds which would permit a differentiation of basicity and steric effects have been prepared (144) but were not available for this investigation.

It is apparent from the compounds which react with B_{12b} that the ring nitrogen atom of histidine is the one involved in the formation of histidine cobalichrome. This would substantiate the proposed nature of this reaction by Cooley and co-workers (68). However, if this reaction involves the formation of a coordinate bond between the ring nitrogen and the cobalt atom, as proposed, one would expect hydrogen ions to compete for the basic nitrogen, i.e., the reaction should be reversible by lowering the pH. Qualitatively, this was found to be the case, and at about pH 3 even a 10^4 molar excess of histidine was insufficient to effect a conversion to the cobalichrome.

It was decided to determine the magnitude of the equilibrium constant so that a better estimate of the effect of pH on the extent of the reaction could be made. Varying dilutions of histidine (free base) were added to a given amount of B_{12b} in 0.2 M phosphate buffer at pH 7 and 25° C.

The absorbancies were determined at 370 mμ., a wavelength which gave a satisfactory absorbancy differential between the two compounds. These data are presented in Table 2. Using these absorbancy values the amount of histidine cobalichrome formed was calculated. The equilibrium constant for the reaction:

$$\text{Histidine} + B_{12b} \rightleftharpoons \text{Histidine cobalichrome} + H_2O$$

was then calculated by means of the following equation:

$$K = \frac{[HC]\left(1 + \frac{[H^+]}{10^{-6}}\right)}{([Hist] - [HC])\,([B_{12b}] - [HC])}$$

where $[HC]$ is the concentration of histidine cobalichrome at equilibrium, $[Hist]$ is the original histidine concentration and $[B_{12b}]$ is the original B_{12b} concentration. The term involving the hydrogen ion concentration is in consequence of the second ionization reaction of histidine ($pK_a = 6.0$). The above equation was derived on the assumption that only the concentrations of the monocationic and isoionic forms of histidine were present, and that the isoionic form was the reactive species. The equilibrium constants for the values between one-tenth and nine-tenths conversion are given in Table 2. It can be seen that the reaction requires a considerable excess of histidine even at pH 7 to accomplish a considerable conversion. At pH 4 it would require an approximately 0.1 M histidine to effect the same conversion that

Table 2. Estimation of the equilibrium constant for the vitamin B_{12b}-histidine reaction

No.	Initial histidine conc. M x 10^4	Absorbancy 370 mμ.	Calculated cobalichrome conc.[a] M x 10^5	Equilibrium constant[b] K x 10^{-3}
A	750.	0.477		
B	500.	0.469		
C	50.	0.459	3.00	3.5
D	10.	0.428	2.42	3.5
E	7.5	0.419	2.28	3.8
F	5.0	0.401	1.99	3.8
G	3.0	0.378	1.61	3.9
H	2.0	0.358	1.30	4.0
I	1.0	0.330	0.85	4.3
J	0.50	0.308	0.50	4.2
K	0.00	0.277		

[a] Initial vitamin B_{12b} concentration; 3.20 x 10^{-5} M.

[b] $K = \frac{[\text{Histidine cobalichrome}]}{[\text{Histidine}]\ [B_{12b}]}$

0.001 M histidine does at pH 7.

These results appear to open to question the reported determination of the absorption spectrum maxima of histidine cobalichrome at pH 2 (68). However, it was found that once the cobalichrome was formed, the rate of removal of the histidine group by acid was slow enough to permit spectrophotometric examination even at pH 2. This situation is somewhat analogous to the acid stability of B_{12}, although the equilibrium constant for the reaction of B_{12b} with cyanide is much larger than for the above reaction (65).

Summary

Vitamin B_{12b} has been found to react with histidylhistidine, carnosine, histamine, imidazole and pyridine to form cobalichromes analogous to histidine cobalichrome, as evidenced by characteristic shifts in absorption spectra maxima. The characteristic absorption spectrum of B_{12b} was destroyed by several reducing agents, including cysteine and glutathione. Vitamin B_{12} did not appear to react with any of the compounds tested.

The equilibrium constant for the reaction between B_{12b} and histidine was estimated to be 4×10^3 l. per mole at 25° C. The reaction was found to be markedly pH dependent.

Development and Examination of a Dialysis Method for Determining Bound Cobalamins

Introduction

A careful consideration of the published methods for measuring the extent of cobalamin-protein or similar reactions led to the conclusion that separation of the free cobalamin from the bound cobalamin by dialysis followed by radio-assay of each, offered the greatest promise of being an uncomplicated, straightforward approach. The one reported attempt (95) to devise such a procedure resulted in a cumbersome, theoretically unsound method. This method consisted of dialysing 11 ml. of a labeled cobalamin-test substance mixture against 10 ml. of water for 24 hours, removing 5 ml. of the dialysate and replacing it with 5 ml. of water, dialysing 24 hours, removing 5 ml. of the second dialysate and again replacing it with 5 ml. of water, dialysing for a third 24 hour period and again removing 5 ml. of the dialysate. The 5 ml. dialysate aliquots were added successively to a planchet, dried and counted after each addition. It was stated (95) that "the addition of the third portion of the dialysate did not materially increase the activity". Theoretically this cannot be so. Assuming an equilibrium had been reached at the end of each

24 hour period, the third addition should have increased the activity by 32.9 per cent, and even a fourth dialysis should have increased the activity by 9.5 per cent. One explanation might be offered: That the increase of self-absorption of the weak β-radiation of Co^{60}, by an accumulation of salts for example, roughly compensated for the activity added to the planchet.

All of the other reported dialysis methods involved exhaustive dialysis and measurement of activity by microbiological assay. Exhaustive dialysis was considered undesirable because of the time involved and the extent of dilution of the dialysate, necessitating either a concentration step, or the use of high activities. A review of dialysis procedures used for other purposes did not reveal any which appeared to be particularly suited to this problem.

Development of the dialysis method

It seemed desirable to obtain a mathematical expression of the dialysis process for use as a basis for selecting the most desirable conditions. Since dialysis is essentially a diffusion process, the rate of dialysis is dependent on the area involved and the concentration gradient. Assuming that the only significant concentration gradient is across the membrane, that the temperature is constant, and that the membrane is of constant thickness, the rate of dialysis of a

substance can be expressed (145) as:

$$\frac{dg}{dt} = KA\ (C_o - C_i)$$

where g is the amount of solute dialysed, K is the permeability constant for the membrane, A is the area of the membrane, and C_o and C_i are concentrations of the solute outside and inside the membrane respectively. Expressing concentrations as amount of solute per volume and integrating this expression from zero time to time t the following formula was obtained:

$$t = \frac{v_o v_i}{KAV} \log \frac{Gv_o}{Vg - v_i G}$$

where v_o and v_i are the volumes outside and inside respectively, V is the total volume $(v_o + v_i)$, G is the amount of solute inside originally, g is the amount of solute inside at time t, and K is a lumped constant term. By substituting arbitrary values in this equation it was found that for a constant membrane area, the time required to reach a given concentration ratio (i.e., the rate at which equilibrium is approached) increases if the volume of either side is increased. This time is of course, decreased by increasing membrane area, and is unaffected by the original amount of solute. It was thus concluded that if a certain minimum volume was required of each side for samples, the ideal apparatus would consist of equal volumes of liquids on

either side of a membrane of as large an area as possible. For a system of equal volumes the second equation reduces to:

$$\log \frac{G}{2g-G} = \frac{K'At}{v}$$

The minimum volume of solution required for radioisotope counting was the 20 ml. necessary to fill the liquid counter. Previous experiences with Co^{60} indicated that counting dried samples on planchets was very unsatisfactory if there were any dissolved salts or other non-volatile solutes present. Liquid counting, even though inefficient, was found to be very reproducible, unaffected by moderate amounts of other solutes, and certainly less time-consuming than plating out cobalt (146) or attempting to correct for self-absorption of solid samples.

The original plan to meet these requirements consisted of using dialysis tubing of small diameter, stirring both solutions, dialysing against as small a volume of outside liquid as possible, and allowing the dialysis to go to within one per cent of equilibrium. Using 16/32 inch diameter Visking Dialysis Tubing, 25 ml. inside and outside volumes, and vertical motion of the bag for stirring, dialysis was 99 per cent complete in 12 hours. These runs were performed at room temperature. Difficulties with the agitation apparatus and the probable necessity of running the dialysis at lower temperature to prevent microbial

growth suggested that this method was not very practical.

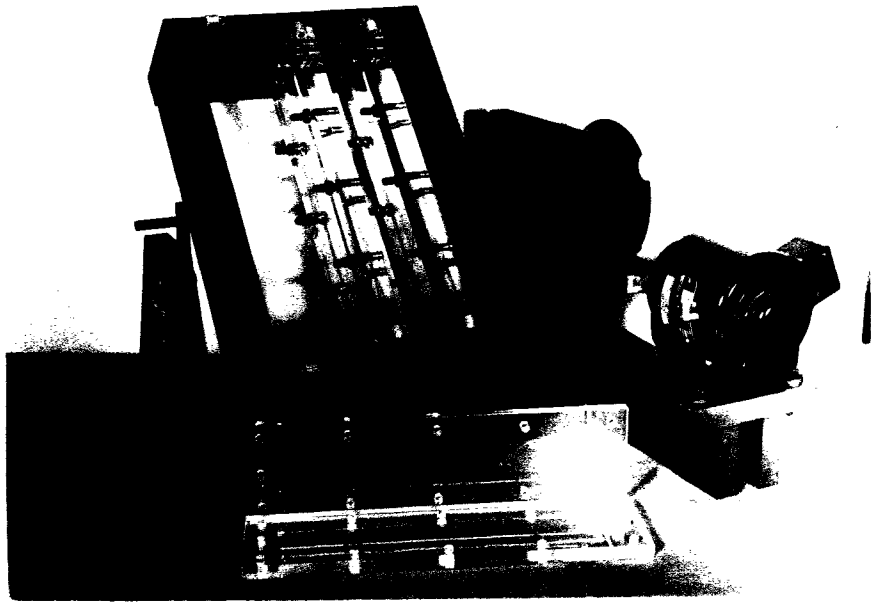
It was reasoned that if the area of the membrane could be kept constant from one run to another, dialysis for a given period of time should lead to a constant molar ratio on each side, and that extended dialysis to a point close to equilibrium would be unnecessary. Taking into account the desirability of a large membrane area to volume ratio and the necessity of uniform and reproducible agitation, an apparatus consisting of two channels, separated by a sheet of cellophane, was constructed of Plexiglas. This cell was rotated at the rate of approximately three revolutions per minute by an electric motor-reduction gear assembly. This apparatus was found to give surprisingly reproducible results, and preliminary studies with several protein preparations indicated that a fairly good estimation of the extent of reactions making cobalamins non-dialysable could be made.

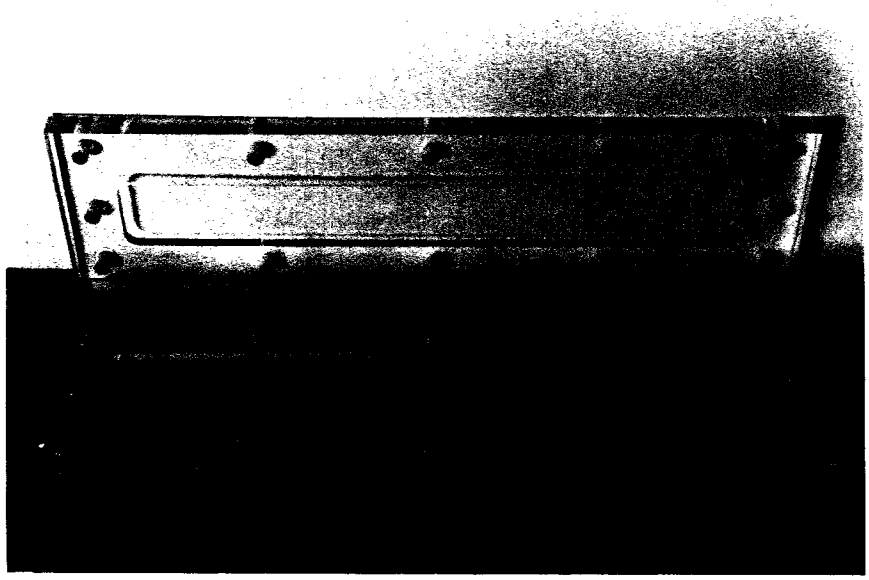
Based on these promising results, and with some design modifications, three identical dialysis cells were constructed and the rotating mechanism modified to accept the three cells. The apparatus is shown in Figure 1. Each cell half was 3 1/4 by 12 by 3/8 inches in size and contained a milled out channel of 1 1/4 by 10 by 1/8 inches. A hole for filling and emptying each channel was drilled from the outside edge

to the channel, and the outside third of this hole was tapped to accept a small bolt. Appropriate holes were drilled to accept the 1/4 inch bolts holding the two cell halves together. The membrane was obtained by lengthwise splitting of 36/32 inch diameter Visking Dialysis Tubing, and was stretched taut while being clamped between the two sections. A thin layer of grease applied around the edge of the inside face of both cell sections prevented any leakage.

The two channels of each unit were designated as side A and side B, and were filled simultaneously using two 25 ml. transfer pipets connected to 3 inch, 17 gauge needles with short pieces of rubber tubing. The solutions used for both sides were identical, except for the cobalamins and non-dialysable test substances, which were present in the solution used for side A only. The 25 ml. left an air space of about 5 ml. on each side. When filled, the small bolts were inserted and the cell was rotated for four hours. The contents of each side were then removed with a syringe and needle, and 20 ml. of each side was counted to a total of 10^4 counts, using the G-M dip tube. The same membrane was used for several runs, and the unit was flushed out eight or ten times with distilled water and kept filled between runs. The results were most easily tabulated as the per cent of the total activity which was present in side B at the end of four hours, designated as %D. This value

Figure 1. The partial dialysis apparatus used in this investigation.





approaches 50 per cent as the maximum. From this value actual concentrations can be calculated if the count per minute per amount of cobalamin has been determined.

Results of studies to determine the validity of the dialysis method

The requirements placed on this method were initially rather simple, as preliminary work with it was primarily qualitative in nature--that is, to determine whether or not a protein preparation binds a cobalamin. As the work progressed a more quantitative answer was desired, leading to further testing of the method, so that the data reported in this section represent only a partial and somewhat disconnected survey of the factors which affect the method.

Effect of cobalamin concentration. Clearly, the %D as defined above should be independent of original cobalamin concentration for a system containing no bound (non-dialysable) cobalamin. This was found to be generally true with one reservation: The dialysis rate of vitamin B_{12b} was lower than that of vitamin B_{12} when the dialysis was performed in distilled water. Using buffered solutions, B_{12} and B_{12b} gave approximately the same %D values, and their concentrations had no apparent effect on the dialysis rate, as shown by the results listed in Table 3. An asterisk (*) has been used in the tables to indicate Co^{60} labeled cobalamins.

Table 3. Effect of cobalamin concentration on the apparent rate of dialysis

Initial contents of Side A[a]	%D[b]
Original cell	
1 ml. B_{12*}-B_{12b*} mixture[c]	44.3
2 ml. B_{12*}-B_{12b*} mixture	44.5
4 ml. B_{12*}-B_{12b*} mixture	44.2
Identical cells	
1 ml. B_{12*}, no buffer	41.4
1 ml. B_{12b*}, no buffer	36.5
1 ml. B_{12b*}, 0.2 M buffer	40.6
1 ml. B_{12*}	40.2
1 ml. B_{12b*}	40.0
1 ml. B_{12b*}, 50 μg. B_{12b}	39.8
1 ml. B_{12*}	41.5
2 ml. B_{12*}	42.1
2 ml. B_{12*}, 50 μg. B_{12}	41.5
1 ml. B_{12b*}	40.6
2 ml. B_{12b*}	40.6
2 ml. B_{12b*}, 50 μg. B_{12b}	40.3

[a]Dialysis was carried out in 0.2 M phosphate buffer, pH 6.6, except where noted otherwise.

[b]The per cent dialysed, %D, is the per cent of the total activity found in Side B at the end of the four hour period of dialysis.

[c]One ml. of the Co^{60}-labeled cobalamin solutions contained approximately 200 mμg. The labeled cobalamin solutions are denoted with an asterisk (*).

The slower dialysis rate of vitamin B_{12b} in water than in buffer solution may be partially explained by the observation that cellophane binds B_{12b} to a considerable extent, but less B_{12b} is bound in 0.2 M phosphate buffer than in water. This was demonstrated by mixing cobalamins and cellophane in the amounts present in the dialysis cell, and, after allowing 30 minutes for the binding to go to completion, measuring the activity of the supernatant solution. The results are given in Table 4. The bound vitamin B_{12b}

Table 4. Binding of vitamin B_{12b} by cellophane

Mixture	Activity of solution, cpm.
1 ml. B_{12*} in 25 ml. H_2O	347
1 ml. B_{12*}, 0.28 g. cellophane in H_2O	348
1 ml. B_{12b*} in 25 ml. H_2O	364
1 ml. B_{12b*}, 0.28 g. cellophane in 25 ml. H_2O	335
1 ml. B_{12b*} in 0.2 M buffer	363
1 ml. B_{12b*}, 0.28 g. cellophane in 25 ml. 0.2 M buffer	345

could be liberated by the addition of a small amount of sodium cyanide. Apparently this binding does not interfere with the dialysis of B_{12b} in buffered solution, for the addition of large amounts of non-labeled vitamin B_{12b} in the dialysis run resulted in complete recovery of activity, but no change in dialysis rate as shown in Table 3.

Although the binding of B_{12b} by cellophane has not been mentioned in the literature, it was reported (147) that cobalamins present in sea water appeared to be adsorbed by a cellulose acetate membrane after prolonged dialysis. It appears probable that B_{12b}, not B_{12} was the form adsorbed.

Effect of temperature. A constant temperature room was not available for these studies, necessitating the use of one cell as a control run where quantitative results were desired. It was of interest however, to determine the effect of temperature on dialysis rate as a basis for possible construction of a large thermostat. Triplicate determinations at five temperatures were performed, using the same cobalamin and buffer solution and the same three membranes. Temperature variations during those runs did not exceed $\pm 1^{\circ}$ C. These data, given in Table 5, also illustrate the reproducibility attained in the three identical cells. It was interesting to note whether the effect of temperature on the dialysis constant followed the Arrhenius equation:

Table 5. Effect of temperature on the dialysis rate of vitamin B_{12}

Temperature °C.	Cell no.	Activity after 4 hours[a] cpm. A	B	Total	%D
23	1	240	170	410	41.5
	2	245	168	413	40.7
	3	248	171	419	40.8
24	1	245	169	414	40.8
	2	243	171	414	41.3
	3	244	174	418	41.6
28	1	237	177	414	42.8
	2	235	178	413	43.1
	3	237	177	414	42.8
33	1	230	184	414	44.4
	2	230	186	416	44.7
	3	228	184	412	44.7
38	1	221	190	411	46.2
	2	221	192	413	46.5
	3	219	192	411	46.7

[a]Initially each cell contained:
Side A: 1.25 ml. B_{12}* in 25 ml. of 0.2 M phosphate buffer, pH 6.6
Side B: 25 ml. of 0.2 M phosphate buffer, pH 6.6.

$$\ln K = - \frac{E}{RT} + C.$$

Since the area and time were contant for all runs, it was sufficient to plot

$$\log \log \frac{G}{2g-G}$$

against reciprocal absolute temperature to test for the applicability of the Arrhenius equation to this situation. Such a plot, given in Figure 2, revealed that the data were rather insufficient to decide this point.

Correspondence of the dialysis method with the dialysis rate equation. Although it was not necessary for the dialysis method to follow the rate formulation to serve the purposes of this investigation, the fact that it does appear to follow the equation lends some respectability to the method, and allows one to calculate with some certainty the time required to reach a given concentration ratio. Two series of cells containing identical solutions were used for this study. One cell of each series was taken off at the end of two hours, one at three hours, and the third at four hours. Calculation of $\frac{K'A}{V}$ for each of the cells gave a relatively constant value, as shown in Table 6. From this value of $\frac{K'A}{V}$ the time required to reach to within one per cent of equilibrium was calculated and found to be 8.3 hours. This is not an inordinate length of time, and makes feasible

Figure 2. The effect of temperature on the dialysis rate constant. Individual determinations were plotted from the data given in Table 5.

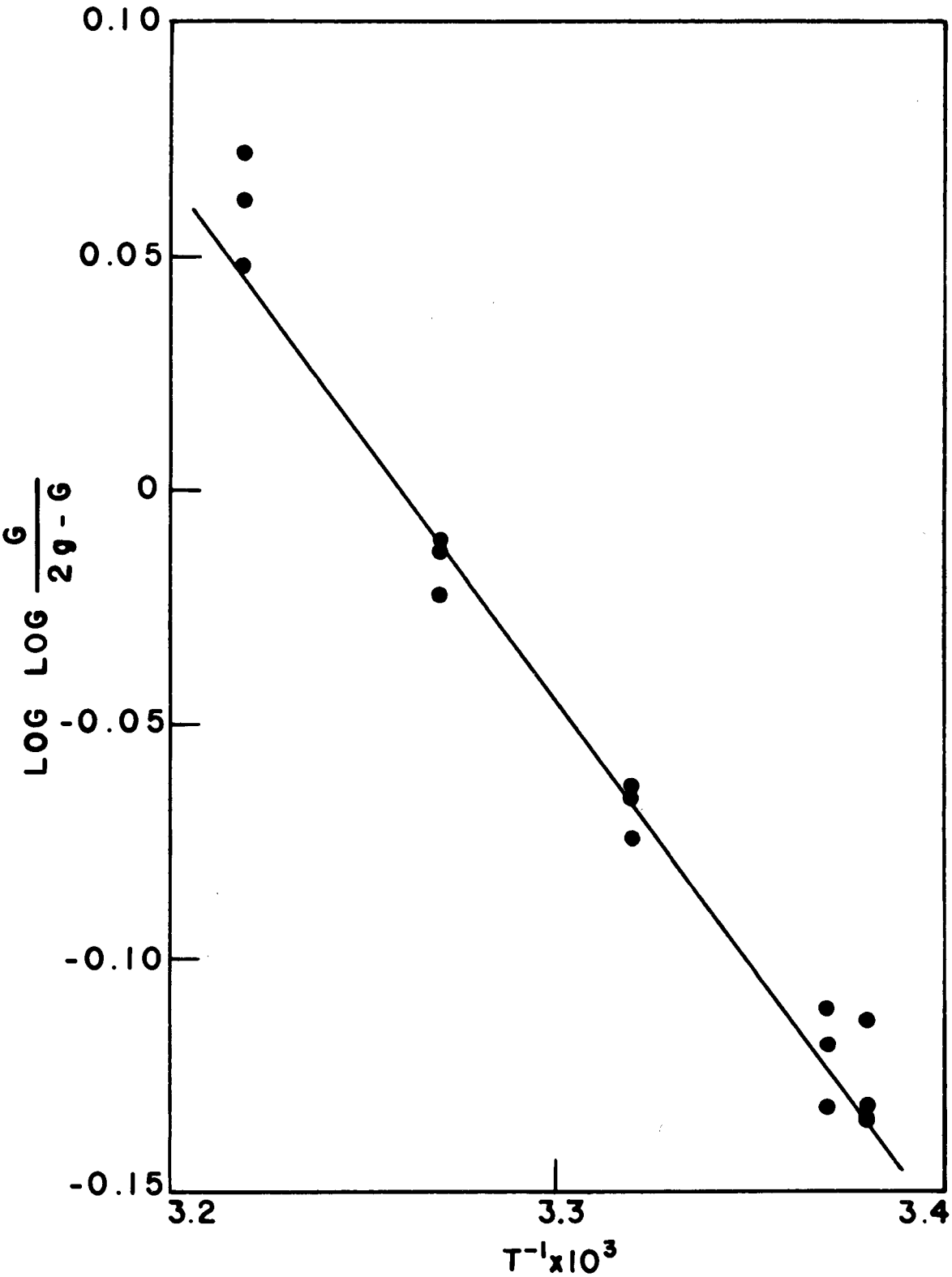

0.10
0.05
0
-0.05
-0.10
-0.15
LOG LOG G/(2g-G)
3.2
3.3
3.4
T⁻¹x10³

Table 6. Correspondence of the partial dialysis method with the dialysis rate equation

Time hr.	Cell no.	Activity after given time[a] cpm. A	B	Total	%D	$\frac{K'A}{v}$[b]
2	1	338	172	510	33.7	0.243
3	2	307	207	514	40.3	0.237
4	3	286	231	517	44.7	0.244
3	1	306	210	516	40.7	0.242
4	2	289	229	518	44.2	0.234
2	3	337	171	508	33.7	0.243

[a]Initially each cell contained:
Side A: 1.5 ml. B_{12*} in 25 ml. 0.2 M buffer.
Side B: 25 ml. 0.2 M buffer.

[b]$\frac{K'A}{v} = \frac{1}{t} \log \frac{G}{2g-G} = \frac{1}{t} \log \frac{A + B}{A - B}$.

extending the dialysis period whenever the precision at four hours is considered insufficient.

Other factors which may affect the rate of dialysis. Limited data will be presented later which indicate that the rate of dialysis of cyanocobalamin is independent of pH between pH 2 and 10, but that at pH 11 to 12 the dialysis rate apparently decreases. Several other factors, such as the variability of pore size of the membrane and the viscosity of the test solutions, were not investigated.

Summary

A workable and reproducible partial dialysis method was developed for the purpose of measuring the amount of free (dialysable) and thus the amount of bound (non-dialysable) cobalamin. A dialysis cell possessing a rather large membrane area to volume ratio was constructed. The use of three identical cells permitted simultaneous determinations to be made. Several factors which affect the rate of the dialysis were studied. It was concluded that the rate of dialysis is independent of the cobalamin concentration, that the rate is directly proportional to the time of dialysis, and that the rate is markedly temperature dependent. The rate of dialysis of vitamins B_{12} and B_{12b} were found to be essentially the same in buffered solutions. The cellophane dialysis tubing used as the membrane was found to bind vitamin B_{12b}, but to a lesser extent in buffer solution than in water.

Reactions of Cobalamins with Blood Proteins

Introduction

Most of the reported values for the amount of cobalamin present in mammalian serums range from 0.1 to 0.6 mμg. per ml. (117, 118, 120, 125), while values for whole blood are

reported to range from 0.3 to 1.5 mμg. per ml. (148, 149, 150), as measured by microbiological assay procedures. It has been claimed that additional amounts of cobalamins, in the order of 0.5 mμg. per ml. can be bound by normal human serum, and that alpha-globulin proteins are primarily involved in the binding of cobalamins (122, 125, 126). It has also been reported (151) that cobalamins incubated with whole blood appear not to enter or be bound by the red cells. It was quite unexpected therefore, when results indicating a binding in the order of 150 mμg. of cobalamin per ml. of whole hemolysed blood were obtained during some early trials with dialysis bags. This was later confirmed using the dialysis cells, and further studies were carried out in order to find the cause of this large discrepancy between reported results and results obtained by the dialysis method. Bovine blood preparations were used in this investigation.

Studies using the dialysis method

The first studies of the effect of whole blood on the dialysis systems, using the original dialysis cell, indicated that over half of the cobalamins were rendered non-dialysable, and that this effect could be prevented by an excess of cyanide. These results are given in Table 7. Several subsequent attempts to repeat these observations, using a

Table 7. Effect of several blood preparations on the dialysis of cobalamins

Initial contents of Side A	%D
Old Co^{60}-cobalamin stock solution	
2 ml. stock soln.	44.3
2 ml. stock soln., 1 ml. blood	20.7
2 ml. stock soln., 1 ml. blood, 5 mg. NaCN	44.9
2 ml. stock soln., 1 ml. blood	17.9
Fresh Co^{60}-cobalamin stock solution	
1.5 ml. stock soln.	44.4
1.5 ml. stock soln., 1 ml. blood	43.0
1.5 ml. stock soln., 1 ml. hemolysed red cells	44.1
1.5 ml. stock soln., 1 ml. plasma	44.3

newly prepared dilution of Co^{60} cobalamin, failed to show any evidence of cobalamin binding. These results are also shown in Table 7.

A possible explanation for this discrepancy was that the old solution of cobalamins, which had been kept in the refrigerator except for removing portions for experiments, had been exposed to enough light to effect a considerable conversion from the original form, B_{12} to B_{12b}. This was easily shown to be the case by converting a portion of the newer dilution to B_{12b} by illumination and nitrogen aeration at pH for 8 hours, and then repeating the previous experiments with this preparation. The results are given in Table 8. This great difference in the reactivity of B_{12} and B_{12b},

Table 8. Effect of several blood preparations on the dialysis of vitamin B_{12b}

Initial contents of Side A	%D
1.5 ml. B_{12b}*	44.2
1.5 ml. B_{12b}*, 1 ml. blood	21.3
1.5 ml. B_{12b}*, 1 ml. hemolysed red cells	30.9
1.5 ml. B_{12b}*, 1 ml. plasma	42.4

although perhaps not unexpected, has not been reported in the literature.

Since such a difference does exist, considerable modification of the experimental procedure was indicated. Thereafter, solutions of Co^{60} cobalamins were converted either to B_{12b} by the above method, or completely to B_{12} by adding an excess of sodium cyanide and removing the excess cyanide by nitrogen aeration. The B_{12} solution was kept in a perfectly light-tight flask in a refrigerator. All operations involving the use of B_{12}, including the actual dialysis runs, were carried out in a photographic darkroom under minimum light conditions.

The results of further studies of the effect of blood preparations on the dialysis of vitamins B_{12} and B_{12b} are summarized in Table 9. It is clear that a considerable amount of B_{12b} is rendered non-dialysable by whole blood,

Table 9. Effect of several blood preparations on the dialysis of vitamins B_{12} and B_{12b}

Initial contents of Side A	%D
Original cell	
1.5 ml. B_{12b*}	44.8
1.5 ml. B_{12b*}, 1 ml. blood (170 mg. protein)	17.4
1.5 ml. B_{12b*}, 0.5 ml. red cells (170 mg. protein)	32.4
1.5 ml. B_{12b*}, 2.7 ml. plasma (170 mg. protein)	24.4
1 ml. B_{12b*}	43.4
1 ml. B_{12b*}, 2.5 ml. plasma	22.5
1 ml. B_{12b*}, 2.5 ml. plasma, heat-denatured (100° C.)	18.8
1 ml. B_{12*}, 2.5 ml. plasma	43.5
1 ml. B_{12b*}	44.7
1 ml. B_{12b*}, 150 mg. bovine albumin (Armour)	23.7
1 ml. B_{12b*}, 16 mg. purified globin	24.8
1 ml. B_{12*}, 16 mg. purified globin	44.6
Identical cells	
1 ml. B_{12*}	44.2
1 ml. B_{12*}, 2.5 ml. serum	42.0
1 ml. B_{12b*}, 2.5 ml. serum	17.9
1 ml. B_{12*}	43.1
1 ml. B_{12*}, 2.5 ml. serum, heat-denatured (100° C.)	41.8
1 ml. B_{12b*}, 2.5 ml. serum, heat-denatured (100° C.)	12.7

hemolysed red cells, serum (and plasma), globin, and purified albumin, whereas, under the same conditions the dialysis of B_{12} is essentially unaffected. It is interesting to note that heat-denatured serum appears to bind increased amounts of B_{12b}, for heating serum to release bound cobalamins for microbiological assay has become a widely used procedure.

This method of partial dialysis provides little information concerning the firmness with which the cobalamins are bound by the protein. To estimate this, exhaustive dialysis of serum-cobalamin mixtures against relatively large volumes of saline were performed, and the progress followed by radio assay of the protein solution. It was found that a 1:10 dilution of serum containing an excess of Co^{60}-B_{12b} retained 110 c.p.m., or approximately 25 mμg. of B_{12b} per ml. of serum, while a 4:5 dilution of serum containing an excess of Co^{60}-B_{12} retained only 30 c.p.m., or approximately 0.9 mμg. of B_{12} per ml. of serum. From the partial dialysis results given in Table 9, roughly half of the 200 mμg. of B_{12b} was bound by 2.5 ml. of serum, or approximately 40 mμg. B_{12b} per ml., indicating fair agreement between the two methods. The values obtained by exhaustive dialysis for serum-B_{12} mixtures indicate that only about one per cent of the amount of B_{12} used in the partial dialysis experiments would be bound by the amount of serum used. This is definitely below the sensitivity of the dialysis method.

Table 10. Effect of pH on the binding of vitamin B_{12b} by serum and serum albumin

Initial contents of Side A			pH	%D
B_{12b}*, ml.	Serum, mg.	Albumin, mg.		
1			4	44.7
1	2.5		4	36.8
1	2.5		6.6	17.7
1			10	43.5
1	2.5		10	27.9
1	2.5		6.6	23.6
1			4	44.7
1		150	4	41.6
1		150	6.6	22.8

If the binding of B_{12b} by serum and other preparations is the result of cobalichrome formation with the histidine side chains of the protein, one could expect the reaction to show a pH dependency similar to that of the reaction between B_{12b} and histidine. This point was examined by conducting the dialysis experiments at different pH levels using 0.2 M phosphate buffer solutions. The results of these experiments, given in Table 10, indicated that the binding of B_{12b} by serum and serum albumin was definitely reduced at pH 4, but was not greatly affected by raising the pH to 10. These results only serve to indicate that histidine side chains may be involved and, of course, do not eliminate other possibilities.

Position of vitamin B_{12b} bound by serum

Pitney and co-workers (125, 126) have presented evidence that the α - and β - globulin fractions of human serum are primarily involved in the binding of cobalamins. Although the cobalamin involved was referred to as vitamin B_{12}, no precautions to prevent conversion to B_{12b} were mentioned in the report. In fact, the work is in disagreement with the less ambiguous work of Shilling (114), who reported that added Co^{60}-cobalamins are bound primarily by the albumin component of serum. With the demonstration, described in the preceding section, that relatively large amounts of B_{12b} are bound to serum as compared with the amount of additional B_{12} which can be bound, it became important to determine whether a single component or all components of serum were involved in the binding of B_{12b}. Four pieces of evidence concerning this point were obtained.

Salt fractionation of serum labeled with Co^{60}-B_{12b}. The general purpose of this experiment was to determine if an arbitrary fractionation of a labeled serum resulted in any concentration of activity. Twenty ml. of serum were mixed with 100 ml. (2000 mμg.) of Co^{60}-B_{12b} and dialysed exhaustively against distilled water at 0° C. A precipitate (euglobins) was removed by centrifugation, re-dissolved in saline and designated Fraction A. The supernatant was

slowly made 2 M in ammonium sulfate at room temperature and the precipitate (Fraction B) was removed by centrifugation, re-dissolved in water, and dialysed against saline overnight. The supernatant (Fraction C) was dialysed against saline, and concentrated by pervaporation. The final volumes of the three fractions were 25, 50 and 100 ml. respectively. Twenty ml. of each fraction were counted in the GM-dip tube assembly.

Protein nitrogen of the original serum was determined by micro-Kjeldahl using a copper selenite catalyst for the digestion step, and distillation into 2 per cent boric acid, followed by titration with 0.05 N hydrochloric acid using a mixed indicator. The protein contents of the fractions were determined by a colorimetric biuret procedure (152) using the original serum as a standard. The standard curve obtained for this determination is given in Figure 3. Portions of each fraction and of the original serum were subjected to moving boundary electrophoresis and the relative amounts of albumin and the globulins were determined. The collected results of this experiment, presented in Table 11, indicate that no concentration of activity was effected by this separation procedure. Since Fraction A contained very little albumin, and Fraction C contained no measurable γ-globulins, these components can be ruled out as possessing exclusive binding power. Conceivably, the cobalamin binding

Figure 3. Standard curve for the biuret protein determination.

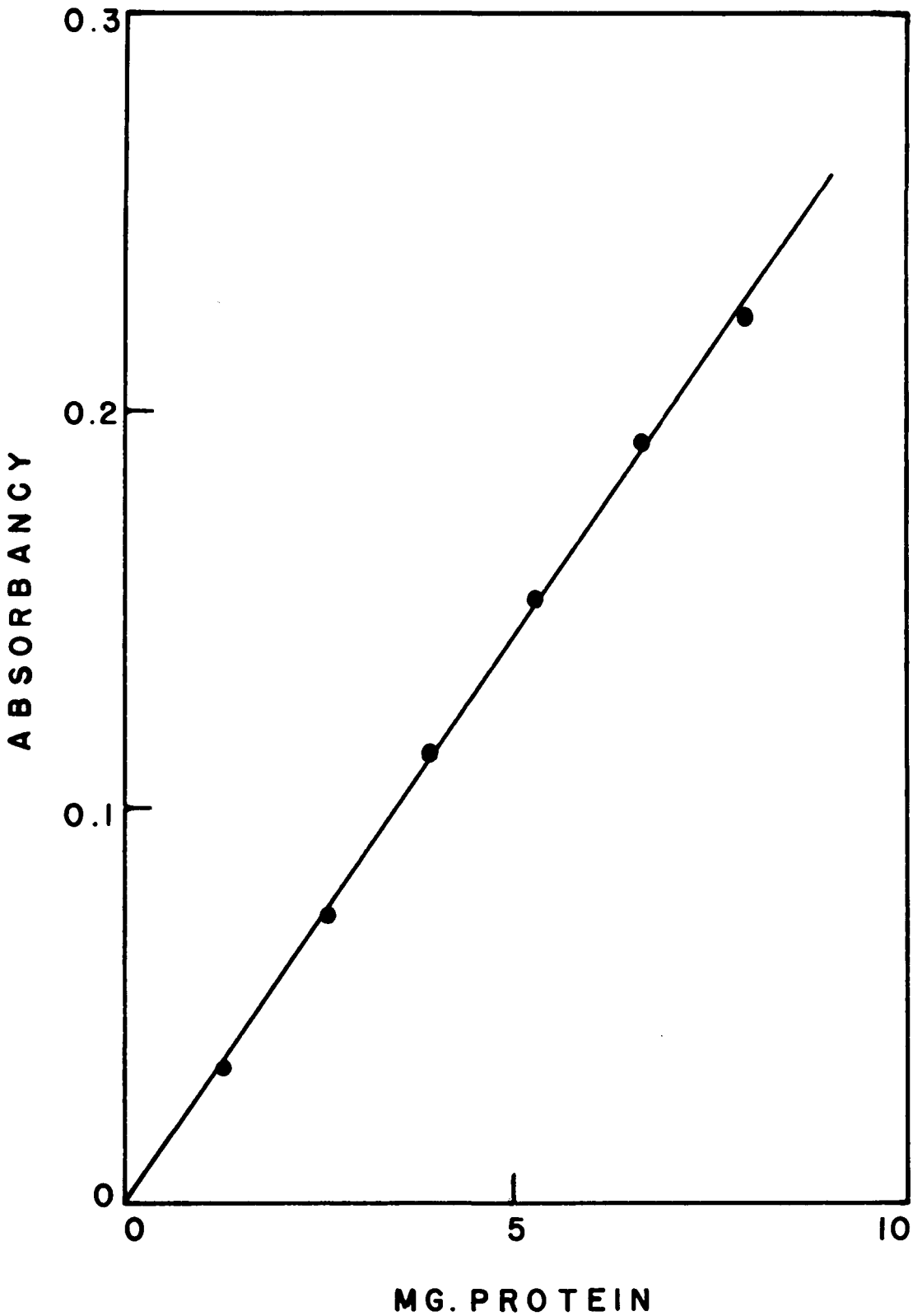

0.3
0.2
0.1
0
ABSORBANCY
0
5
10
MG. PROTEIN

Table 11. Position of labeled vitamin B_{12b} in serum fractions obtained by salt fractionation

Quantities determined	Original serum	Fractions A	Fractions B	Fractions C
Total volume, ml.	20	25	50	100
Total counts per 20 ml.	-	135	200	188
Protein				
mg. per ml.	66.5	6.0	8.0	7.8
total, g.	1.33	0.15	0.40	0.78
Counts per min. per mg. protein per ml.	-	23	25	24
Electrophoretic analyses				
Albumin, per cent	51.6	6.6	7.2	75.0
total g.	0.69	0.01	0.03	0.59
α-Globulin, per cent	19.3	23.5	26.7	16.3
total, g.	0.26	0.03	0.11	0.13
β-Globulin, per cent	9.5	45.1	11.0	8.7
total, g.	0.13	0.07	0.04	0.07
γ-Globulin, per cent	19.6	24.8	55.1	0.0
total, g.	0.26	0.04	0.22	0.0

activity could be present solely in the α-globulin or β-globulin fractions and still be more or less equally distributed by this procedure. Therefore, these results are only suggestive of a generalized reaction of B_{12b} with serum proteins.

Paper electrophoresis of serum labeled with Co^{60}-B_{12b}.

Paper electrophoresis appeared to offer a method simpler than fractional precipitation for effecting a complete separation of serum proteins. An apparatus was constructed which provided free suspension over a span of 12 inches for either five 1 inch strips or one 6 inch sheet of filter paper. The ends of the paper were dipped directly into the electrode chambers at each end. Distillation from the paper was prevented by using buffer solutions containing 15 to 20 per cent glycerol (153), which permitted the process to be carried on at room temperature.

A labeled serum (the same serum used in the previous experiment) was prepared by dialysis against saline, and was concentrated to one-half of the original volume by pervaporation. Three essentially identical determinations were performed on this preparation.

A 6 inch sheet of Whatman #1 filter paper was equilibrated with the buffer, which was composed of 15 parts of glycerol and 85 parts of veronal buffer of pH 8.6 and 0.1 ionic strength. Then a 0.2 ml. sample (0.1 ml. for the third determination) of the labeled serum was applied and the sheet subjected to about 3 ma. (250 v.) D. C. current for 24 hours. The sheet was then air-dried, and submerged for a few minutes in 0.2 per cent ninhydrin solution in

acetone containing 1 per cent glacial acetic acid. Heating at 100° C. for 10 minutes was sufficient to develop the colored protein zones. Figure 4 is a photograph of one of these sheets.

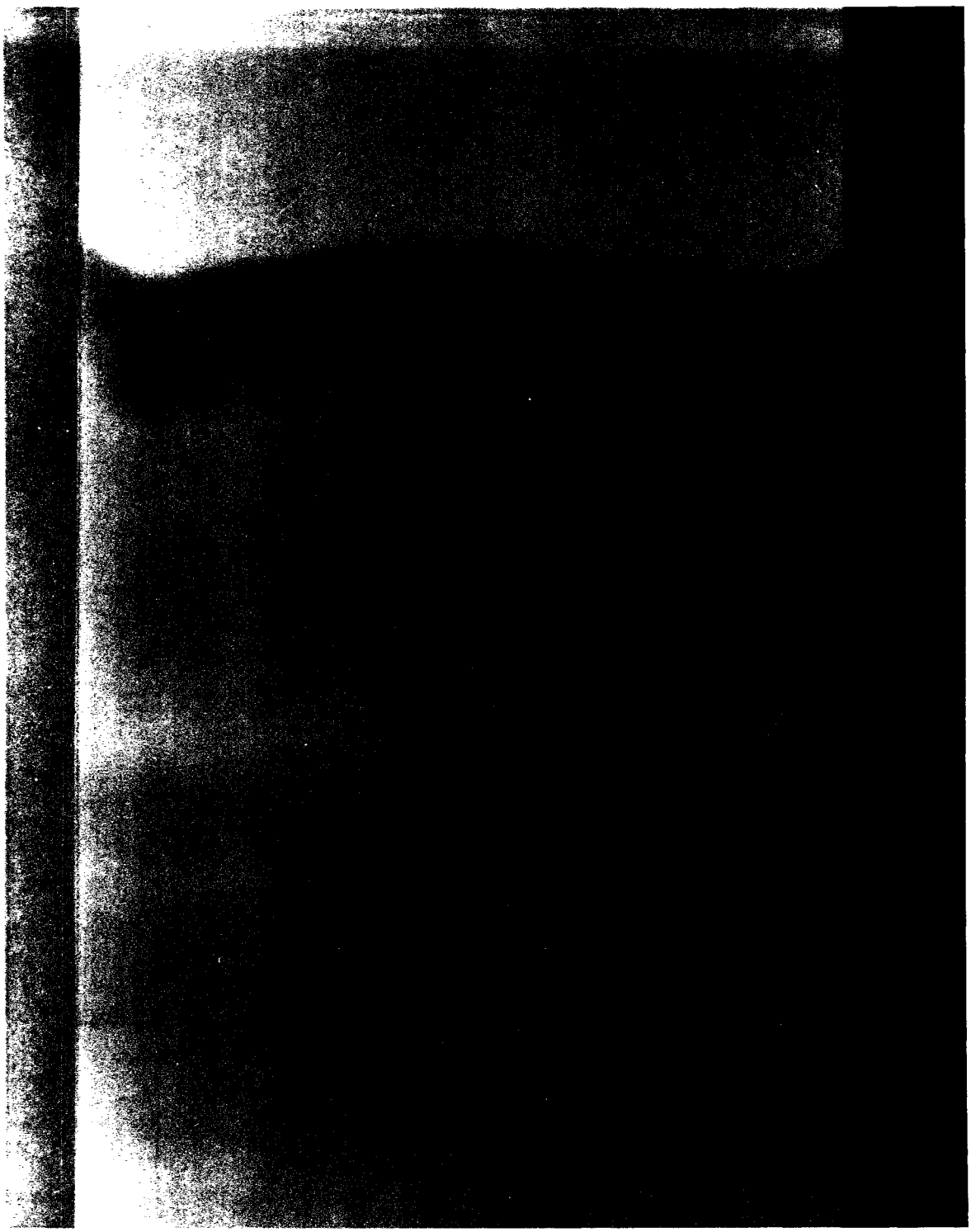
The paper was cut into four sections containing the principal components, as indicated in Figure 4, and ashed at 600° C. in separate crucibles. The resulting ash was transferred to planchets with 0.7 ml. of 0.1 N hydrochloric acid and dried. The planchets were counted with the end-window GM tube in the lead shield. The results of these determinations, given in Table 12, were calculated to per cent of the total counts (494, 545 and 207 counts per minute for the

Table 12. Position of labeled vitamin B_{12b} in serum fractions obtained by paper electrophoresis

Determination no.	Total cpm.	Per cent of total counts per minute[a]			
		Albumin	Globulins		
			α	β	γ
1	494	8	34	21	38
2	545	11	32	22	37
3	207	11	28	18	43
Average		10	31	20	39

[a]The original serum contained 51.6 per cent albumin, 19.3 per cent α-globulins, 9.5 per cent β-globulins, and 19.6 per cent γ-globulins.

Figure 4. Paper electrophotogram of serum labeled with Co^{60}-vitamin B_{12b}. The zones were developed with ninhydrin.



three runs respectively) to allow direct comparison. They actually have little quantitative significance due to the great amount of self-absorption of the salt-encrusted samples. They do indicate, however, that activity is present in all four fractions, with considerable preference toward the globulins.

It will be recalled that by the dialysis method, a purified albumin preparation exhibited considerable binding activity. This albumin preparation was found to be at least 97 per cent albumin by electrophoretic analysis. It will also be recalled that heat-denatured serum appeared to bind more Co^{60}-B_{12b} than native serum, a situation which can be interpreted as an argument against a highly specific structural requirement for B_{12b} binding. These four pieces of evidence thus suggest that a specific protein or type of protein is not responsible for the binding of B_{12b} by serum.

Position of cyanocobalamin bound by serum

An attempt was made to determine the position of the small amount of Co^{60}-B_{12} bound by serum. A mixture of serum and Co^{60}-B_{12} was dialysed exhaustively in the dark, first against saline containing 0.5 mg. of sodium cyanide per liter, then against saline. By radio assay the serum bound 1.4 mμg. B_{12} per milliliter. The labeled serum was concentrated to one-fifth volume by lyophilization, and the

Table 13. Position of labeled vitamin B_{12} in serum fractions obtained by paper electrophoresis

	Fractions			
	Albumin	Globulins		
		α	β	γ
Counts per minute	2.6	2.0	5.8	8.8
Per cent of total counts per minute	13.6	10.4	30.2	45.8
Protein, per cent (moving boundary electrophoresis)	53.8	18.3	8.0	19.9

components were separated by two paper electrophoresis runs in complete darkness, using 0.10 and 0.16 ml. per 6 inch sheet of Whatman #3M paper. The zones were developed by the ninhydrin procedure described previously, cut apart, and similar sections of the two runs placed in a single crucible for ashing. The samples were ashed, transferred to planchets and counted as before. The results are given in Table 13. No conclusion can be drawn from this experiment because the small amount of activity and the large amount of ash present on the planchets preclude any good estimation of activity. With considerable refinement of technique it is believed that a more valid determination of the position of bound B_{12} can be made in this way, than by the bacterial assay method.

Summary

It was found by the partial dialysis method that unexpectedly large amounts of vitamin B_{12b} were bound by whole blood, serum (and plasma), hemolysed red cells, purified globin, purified albumin and heat-denatured serum. For serum this binding was in the order of 30 mμg. of B_{12b} per ml., and was stable to prolonged dialysis. Serum and purified albumin were found to bind considerably less vitamin B_{12b} at pH 4 than at pH 6.6. Salt fractionation of serum into three crude fractions did not effect any concentration of bound B_{12b}. Fractionation of serum by paper electrophoresis indicated that B_{12b} was bound by all fractions, with a possible predominance in the globulins. It was concluded that there is little protein specificity involved in the binding of B_{12b} by serum, perhaps indicating that a cobalamin type of linkage with histidyl side chains is involved.

In contrast, vitamin B_{12} was not bound to a measurable extent by the above preparations, as determined by the partial dialysis method. By exhaustive dialysis it appeared that about 1 mμg. of B_{12} could be bound per ml. of serum. The position of B_{12} bound by serum could not be determined using the present methods.

Reactions of Cobalamins with Lysozyme

Introduction

Early work using bacterial inhibition methods indicated that cobalamins may be bound by lysozyme (135). This has been largely refuted by further examination of this system using bacterial uptake and dialysis methods (90, 95, 105). However, it has been pointed out (115) that lysozyme is not completely retained by cellophane membranes (154), so that there still exists some possibility of a lysozyme-cobalamin interaction. Furthermore, in the light of the work with blood proteins described in the previous section, a reaction of lysozyme with B_{12b} appeared to be quite likely, for lysozyme contains one per cent histidine (155), and the imidazole side chain may be accessible for it appears to be involved in the lytic activity of lysozyme (154). It was therefore decided to study this system using the methods described in the preceding section.

Effect of lysozyme on the dialysis of cobalamins

The lysozyme used in this work showed only a single peak by moving boundary electrophoresis at pH 8.6 (veronal buffer) and 7.8 (phosphate buffer). As shown in Table 14, relatively large amounts of lysozyme failed to bind either

Table 14. Effect of lysozyme on the dialysis of vitamins B_{12} and B_{12b}

Initial contents of Side A	%D	
	Cobalamins	Lysozyme
Original cell		
1 ml. B_{12b*}	44.7	
1 ml. B_{12b*}, 100 mg. lysozyme	42.1	
1 ml. B_{12b*}, 50 mg. lysozyme in saline	43.9	16.0
1 ml. B_{12}, 50 mg. lysozyme	45.2	6.0
Identical cells		
1 ml. B_{12*}	45.2	
1 ml. B_{12b*}, 250 mg. lysozyme	42.2	
1 ml. B_{12*}, 250 mg. lysozyme	44.3	

vitamin B_{12} or B_{12b} to any appreciable extent. The amount of lysozyme which dialysed through the membrane during the 4 hour period was estimated for two runs by determining the final protein content of both sides by the biuret method, using the original lysozyme solution as a standard. Side B contained in one instance 16 per cent, and in the other, 6 per cent of the original protein. Thus the dialysability of lysozyme is at least lower than cobalamins, and any appreciable binding of cobalamins by lysozyme would certainly be detectable.

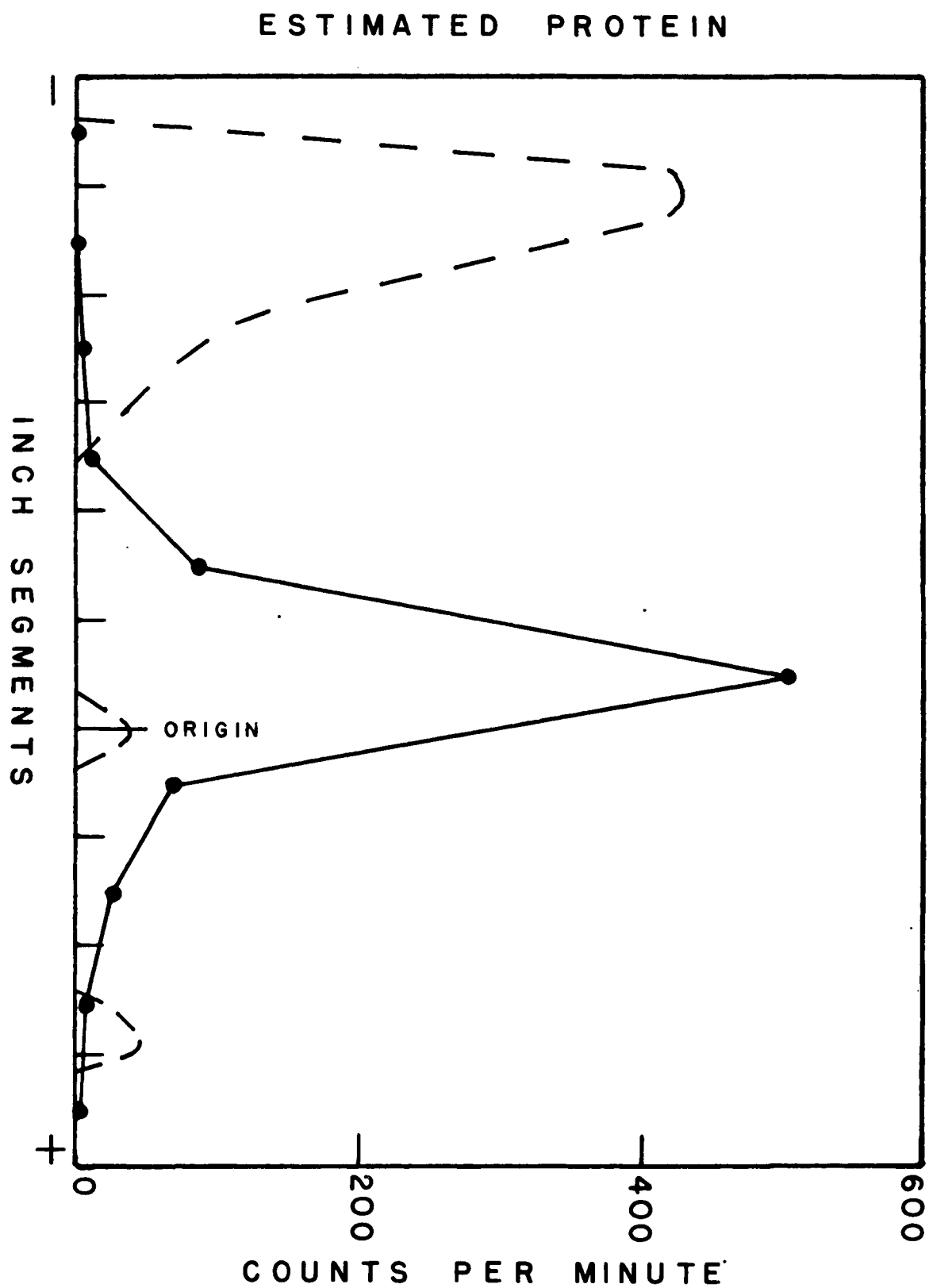
Paper electrophoresis of cobalamin-lysozyme mixtures

Preliminary paper electrophoresis of combinations of lysozyme with excess Co^{60}-B_{12} or Co^{60}-B_{12b} indicated that both cobalamins moved slightly toward the cathode and showed considerable spreading, and lysozyme moved a considerable distance toward the cathode. By using a 6 inch wide sheet of filter paper (Whatman #1) 15 mg. of lysozyme dissolved in 0.15 ml. of a 1:1 mixture of stock Co^{60}-B_{12} and Co^{60}-B_{12b} (ca. 200 mμg. per ml.) could be applied. Electrophoresis was carried on in the dark for 14 hours at 4 m.a. (320 v.), using pH 6.8 veronal buffer containing 20 per cent glycerol. The sheet was developed with ninhydrin and cut crosswise into one inch strips starting from the line of application. Each strip was ashed, transferred to planchets and counted. The resulting counts and a visual estimate of the protein distribution are plotted against distance from the point of application in Figure 5. It is apparent that no measurable activity migrated with the principal protein component. The identity of the two minor protein components is unknown.

Summary

Lysozyme does not appear to react with either vitamin B_{12} or B_{12b}, at least in amounts measurable by partial dialysis and paper electrophoresis techniques. Thus, either

Figure 5. Distribution of radioactivity and protein resulting from paper electrophoresis of a mixture of lysozyme and Co^{60}-vitamins B_{12} and B_{12b}. The solid line connects radioactivity values for each 1 inch segment. The dashed line represents a visual estimate of the distribution of protein as detected with ninhydrin.


ESTIMATED PROTEIN
INCH SEGMENTS
ORIGIN
0
200
400
600
COUNTS PER MINUTE

the imidazole side chain of histidine is covered up or is sterically unapproachable by B_{12b}, or there are further structural requirements for protein-B_{12b} reactions.

Reactions of Cobalamins with a Gastric Mucosal Extract Preparation

Introduction

There is ample evidence in the literature that gastric juice and gastric and duodenal mucosa contain a substance or substances which bind large amounts of cobalamins, rendering the cobalamins non-dialysable (83, 90, 104) and unavailable for growth of cobalamin-requiring microorganisms (83, 90, 104). Early speculations that the intrinsic factor activity of these substances may be indicated by their cobalamin-binding activity (83, 92, 105) were not borne out by experimental evidence (95, 99, 104). At the present time the exact relationship between these two biological activities of these substances is unknown. As a result, newer methods of measuring intrinsic factor activity based on intestinal absorption have been developed for use in the search for the identity of the intrinsic factor (156, 157, 158, 159, 160), and the reactions of these substances with cobalamins have been placed in a position of secondary importance.

Evidence concerning the nature of the reactions between these substances and cobalamins is limited. There is some doubt that the reaction is stoichiometric, and conflicting evidence regarding the heat-lability of the substances has been presented. Heating the substances in the presence of cobaltous chloride and benzimidazole has been reported to destroy the binding power of the substances (109, 110). The pH dependency of the reaction has not been studied, but it has been suggested that acid groups may be involved in the reaction (95, 116). The sulfhydryl group has been suggested as a possible reactive site (161). It has also been suggested that a peptide bond may be formed through one of the primary amides of the cobalamin structure (161). Actually, the question of which cobalamins are able to participate in these reactions has not been studied. It was therefore of interest to apply the relatively uncomplicated partial dialysis method to the study of these reactions.

Preparation of a gastric extract

A preliminary extraction of Ventriculin with 1.0 per cent sodium chloride, precipitation of protein by slow saturation with ammonium sulfate, and re-solution of the precipitate in distilled water gave a solution of very high Co^{60}-B_{12} binding activity. The activity was not diminished by exhaustive dialysis or by clarification by centrifugation

at 30,000 r.p.m. in a Spinco preparative centrifuge. This preliminary trial being successful, an extraction designed to collect all of the extractable protein while attempting to eliminate as much non-protein material as possible was undertaken.

One hundred grams of Ventriculin were ground in a mortar and extracted at pH 7.5 with 400 ml. of 0.2 per cent sodium chloride, then extracted again with two 200 ml. portions of water, separating the insoluble matter by centrifugation. The combined supernatant was slowly made 2 M in sulfate and 2 M in phosphate by adding the sodium salts (final pH 5.9) at room temperature. The resulting precipitate was then harvested by centrifugation, washed with a 4 M sodium phosphate solution (pH 6), and re-dissolved in 100 ml. of water. This solution was extensively dialysed at 0° C. against one per cent sodium chloride, brought to pH 9.5 for an hour to destroy any pepsin, returned to pH 7.4 and clarified somewhat by centrifugation at 11,000 r.p.m. in a Sorval centrifuge. The supernatant was brought to 0° C. and the protein was precipitated by the slow addition of cold ethanol to reach an 80 per cent ethanolic solution. The precipitate was harvested by centrifugation at 0° C., re-dissolved in cold water, and dialysed against three changes of water at 0° C. The solution was then pulled through a Selas No. 02 bacterial filter, to give a clear, light brown

solution. Five ml. aliquots of this solution were frozen in test tubes and served as samples for all of the work reported in the following sections.

This preparation was found to contain 2.16 mg. of nitrogen per ml. by the micro-Kjeldahl method, or 13.5 mg. protein per ml. using the factor 6.25. Using only one 5 ml. sample, the solution contained 18 per cent solids when evaporated and dried at 110° C., and 0.9 per cent ash. As determined by the biuret method using a serum as the standard, this solution contained 10.1 mg. of protein per ml. By moving boundary electrophoresis in phosphate buffer at pH 7.8 at least six components were observed, with the bulk of the material exhibiting low anodic mobility.

Dialysis experiments with the gastric extract

This gastric extract (GE) was found by the dialysis method to bind rather large amounts of Co^{60}-B_{12} in an apparently stoichiometric manner. Unfortunately, the magnitude of the temperature effect on the dialysis rate was not realized at the time many of these runs were made, and a control determination using only the cobalamin was not included in each set. In calculating the amount of B_{12} or B_{12b} bound, an assumed value, based on the results of the most recent control runs, was used. In all of this work the newer cells were used, and the data are presented in groups

of two or three, indicating simultaneous runs.

Effect of the gastric extract on the dialysis of cobalamins. It was found that 0.15 ml. of the GE bound approximately half of the activity present in 1 ml. of Co^{60}-B_{12} or Co^{60}-B_{12b}, so this amount of GE was used most often. By microbiological assay using L. leichmannii (162) the radio-cobalamin solutions were found to contain 190 mμg. as B_{12} per ml. Using two levels of GE it was found that about 730 mμg. of B_{12} and 780 mμg. of B_{12b} were bound by 1 ml. of GE. These data are presented in Table 15.

As a check on the possibility that the gastric extract was retarding the dialysis by some means other than by binding cobalamins, one run was continued for eight hours. Using the dialysis equation (page 45), 49.4 per cent of the free B_{12} should be found in side B at the end of eight hours. The calculated amount of B_{12} bound by 1 ml. of GE using this value for the 8 hour run agreed well with the 4 hour values. To further establish this point, a cell containing an excess of the gastric extract on one side of the cell and the Co^{60}-B_{12} on the other side, was dialysed for four hours, giving a %D of 64.9 per cent. An expected value of 65.1 per cent was calculated from the general dialysis equation (page 44) by assuming a very large (1,000 times) side B volume. Thus, it can be concluded that the system is actually measuring free cobalamins.

Table 15. Effect of gastric extract (GE) on the dialysis of vitamins B_{12} and B_{12b}

Initial contents of Side A			%D	Bound cobalamin mμg. per ml. GE
B_{12}* ml.	B_{12b}* ml.	GE ml.		
1			44.1	
1			44.5	
1		0.1	27.3	730
	1	0.1	25.0	829
1		0.1	27.5	722
	1	0.15	17.1	722
1		0.15	18.0	751
	1	0.15	17.1	772
1		0.15	18.5	733
1		0.15	21.5[a]	726
1			64.9[b]	

[a]This run was dialysed 8 hours.

[b]The side B of this run contained 0.5 ml. GE.

The slightly higher values for bound B_{12b} as compared to B_{12}, cited above, were investigated further. If these two cobalamins are bound at the same sites by the binding substance, they should show mutual blocking of these sites. By adding excess non-labeled B_{12} or B_{12b} to the gastric extract before adding the labeled cobalamins, the sites of combination, if identical, should be filled and a normal dialysis value should be obtained. As shown in Table 16, excess B_{12b} adequately blocked the B_{12} sites, but perhaps

Table 16. Effect of excess non-labeled vitamins B_{12} and B_{12b} on the binding of labeled vitamins B_{12} and B_{12b} by gastric extract

Initial contents of Side A[a]	%D
0.15 ml. GE, 1 ml. B_{12*}	17.7
0.15 ml. GE, 50 µg. B_{12}, 1 ml. B_{12*}	44.0
0.15 ml. GE, 50 µg. B_{12b}, 1 ml. B_{12*}	43.5
0.15 ml. GE, 1 ml. B_{12b*}	17.0
0.15 ml. GE, 50 µg. B_{12b}, 1 ml. B_{12b*}	44.0
0.15 ml. GE, 50 µg. B_{12}, 1 ml. B_{12b*}	41.4
0.15 ml. GE, 1 ml. B_{12b*}	18.2
0.15 ml. GE, 50 µg. B_{12b}, 1 ml. B_{12b*}	44.0
0.15 ml. GE, 50 µg. B_{12}, 1 ml. B_{12b*}	42.7

[a]The components are listed in order of addition to the buffered solution. Solutions containing non-labeled cobalamins were incubated 1 hour at room temperature before adding the labeled cobalamins.

not all of the B_{12b} sites were blocked by excess B_{12}.

These differences in the amount of B_{12} and B_{12b} bound are probably only a little larger than the sensitivity of the method, so that further refinements in the method will have to be made before effects of this order can be adequately examined. It can be concluded from these data, however, that it is quite unlikely that B_{12} and B_{12b} react at different sites entirely, and therefore their structures probably have a common reactive group or groups.

Effect of pH and excess cyanide on cobalamin binding by the gastric extract. If acid groups in the protein molecule are primarily responsible for cobalamin binding, as suggested by Chow (95, 116), one would expect the reaction to be pH dependent. This has not been studied previously because the pH must be kept relatively constant for the bacterial inhibition or bacterial adsorption method. The dialysis method is not limited by such restrictions.

Using phosphoric acid and mono-, di-, and tri-sodium phosphates, 0.2 M buffer solutions varying from pH 2 to 12 were prepared, and the effect of increasing or decreasing the pH relative to the routine pH of 6.6 was studied. It was found that the amount of either B_{12} or B_{12b} bound by the gastric extract was essentially unaffected by pH changes in the range of pH 2 to 10, with the possible exception that slightly less binding of B_{12b} may have occurred at pH 2. These results are recorded in Table 17.

By adding an excess of cyanide to a B_{12} solution at above pH 7 the dicyanocobalamin form predominates (39, 40, 75). If the proposed structure of this cobalamin is correct (68), and if the reaction between the binding substance and B_{12} involves coordination with the cobalt atom, one might expect cyanide to compete with the binding substance for one or both of these coordination sites. Dialysis experiments examining this possibility are presented in Table 18. It

Table 17. Effect of pH on the binding of vitamins B_{12} and B_{12b} by gastric extract

Initial contents of Side A			pH	%D
B_{12*}, ml.	B_{12b*}, ml.	GE, ml.		
1		0.15	2	21.7
1		0.15	4	20.2
1		0.15	10	20.3
1		0.15	6	19.0
1		0.15	7	19.5
1		0.15	8	20.2
1			2	44.2
1		0.15	2	19.7
1		0.15	6.6	19.8
	1	0.15	2	23.5
	1	0.15	6	16.2
	1	0.15	10	17.4

appears that at high pH values there is partial interference with the binding by cyanide ions. At pH 6.6 there is no apparent interference by cyanide, or rather, hydrocyanic acid (pK_a = 10), even though complete conversion of B_{12b} to B_{12} can be effected at this pH. It was found that, at least at pH 10, the same degree of interference was obtained whether the cyanide was added to the B_{12} solution before or after addition of the gastric extract. Thus, dicyanocobalamin also reacts with the gastric extract, to an extent probably determined by the cyanide ion concentration, and bound

Table 18. Effect of excess sodium cyanide on the binding of vitamin B_{12} by gastric extract

Initial contents of Side A			pH	%D
B_{12*}, ml.	NaCN, mg.	GE, ml.		
1			6.6	44.1
1		0.15	6.6	18.7
1	2.5	0.15	6.6	18.5
1		0.15	10	19.5
1	2.5	0.15	10	25.5
1	2.5[a]	0.15	10	25.0
1			10	45.0
1		0.15	10	20.6
1	2.5[a]	0.15	10	25.7
1		0.15	10	20.0
1	2.5	0.15	10	25.8
1	10.0	0.15	10	29.9
1			11	42.9
1		0.15	11	27.0
1	2.5	0.15	11	37.2
1			12	40.3
1		0.15	12	33.6
1	2.5	0.15	12	41.7

[a]In these two samples the GE and B_{12*} were mixed and allowed to stand 1 hour before the NaCN was added. In all others, the B_{12*} and NaCN were mixed before the addition of the GE.

cyanocobalamin can apparently be liberated by cyanide ions. Qualitatively then, it appears that there is competition between the gastric extract and cyanide ions for coordination with the cobalt. These results suggest that coordination with the cobalt is an essential feature of the cobalamin-gastric extract reaction, and that this coordination may occur only at the position taken by the second cyano- group of dicyanocobalamin.

It will also be noted in Table 18 that the dialysis rate of B_{12} decreases above pH 10, and that the binding of B_{12} by the gastric extract is quite markedly reduced above pH 10. The structural significance of these data is not apparent.

Effect of buffer type and concentration on cobalamin binding by gastric extract. As a check against the possible involvement of the phosphate ion in the gastric extract-cobalamin reaction, the concentration of phosphate was varied, and an acetate buffer was used for one run. The results, listed in Table 19, indicate that the binding is unaffected by these changes in buffer concentration.

Effects of heating and of cobalt and benzimidazole on the binding of cobalamins by gastric extract. Van der Zant and Underkofler, using the bacterial inhibition method, have found that heating a similar gastric extract with Co (II) or Fe (II) and benzimidazole or histidine greatly reduces the

Table 19. Effect of buffer type and concentration on the binding of vitamin B_{12} by gastric extract

Initial contents of Side A		Buffer	pH	%D
B_{12}*, ml.	GE, ml.			
1	0.15	0.2 M Phosphate	6.6	19.1
1	0.15	0.2 M Acetate	6	18.7
1	0.15	none	6.4	17.5
1	0.15	0.1 M Phosphate	6.6	19.6
1	0.15	0.4 M Phosphate	6.6	19.1

capacity of the material to bind cobalamins (109, 110). It was proposed that the cobalt and benzimidazole blocked the cobalamin-reactive sites of the binding material, and therefore the cobalamins may be bound by both a linkage to the cobalt, and to the benzimidazole moiety. This proposal is very attractive in the light of the work described in the preceding section, and was therefore investigated with the partial dialysis method.

It was found that heating the gastric extract at 100° C. in the phosphate buffer solution (pH 6.6) either before or after addition of Co^{60}-B_{12} decreased the observed binding. The presence of Co (II) or benzimidazole or both had no effect without heating, and the effect with heating was no more than obtainable by heating alone. These data are given in Table 20.

Table 20. Effect of heat, cobalt and Benzimidazole (Bz.) on the binding of vitamin B_{12} by gastric extract

Initial contents of Side A[a]	%D
0.15 ml. GE, 1 ml. B_{12*}	19.2
0.15 ml. GE, 60° C. 1 hour, 1 ml. B_{12*}	19.5
0.15 ml. GE, 100° C. 1 hour, 1 ml. B_{12*}	30.9
0.15 ml. GE, 1 ml. B_{12*}	19.5
0.15 ml. GE, 1 ml. B_{12*}, 60° C. 1 hour	19.4
0.15 ml. GE, 1 ml. B_{12*}, 100° C. 1 hour	31.4
1 ml. B_{12*}	45.5
0.15 ml. GE, 50 mg. Bz., 1 ml. B_{12*}	19.6
0.15 ml. GE, 50 mg. $CoCl_2$, 1 ml. B_{12*}	20.7
0.15 ml. GE, 100 mg. Bz., 100 mg. $CoCl_2$, 1 ml. B_{12*}	20.2
0.15 ml. GE, 100 mg. $CoCl_2$, 100 mg. Bz., 1 ml. B_{12*}	20.3
0.15 ml. GE, 100 mg. Bz., 100° C. 5 min., 100 mg. $CoCl_2$, 100° C. 5 min., 1 ml. B_{12*}	23.6
0.15 ml. GE, 100° C. 10 min., 1 ml. B_{12*}	22.5
0.15 ml. GE, 60 mg. $CoCl_2$, 30 mg. Bz., 100° C. 10 min., 1 ml. B_{12*}	24.2
0.15 ml. GE, 60 mg. $CoCl_2$, 17 mg. Imidazole, 100° C. 10 min., 1 ml. B_{12*}	23.6

[a]Components are listed in order of addition and treatment.

This system proved to be unworkable because of a pink precipitate which formed when the cobalt and benzimidazole were added to the gastric extract. When the mixture was heated, the precipitate turned blue and the amount of precipitate appeared to increase. Thus the amount and nature of the cobalt-benzimidazole complexes in solution were not defined. The precipitate interfered with the transfer of samples into and out of the dialysis cells, and rapidly settled to the bottom of the counting chamber.

Effect of p-chloromecuribenzoic acid on the binding of cobalamins by gastric extract. It has been suggested by Smith that sulfhydryl groups of natural materials which bind cobalamins may be involved in the coordination with the cobalt atom of cobalamins, possibly by displacing the benzimidazole moiety (161). Although this proposal appears unlikely in several respects, it is one which can be readily examined by the use of p-chloromercuribenzoic acid (PCMB). This reagent is highly selective for sulfhydryl groups and is able to react with sulhydryl groups of proteins which are not accessible to other sulfhydryl reagents such as oxidizing agents (163).

The colorimetric method of Anson (164) for determining the sulfhydryl content of proteins was used in a preliminary effort to estimate the amount of PCMB needed to react with the gastric extract. Glutathione (GSH) was used as the

sulfhydryl standard for this determination. The standard curve was obtained by using from 0 to 1 ml. of 0.001 M PCMB made up to 7.5 ml. with water, adding 1.0 ml. of 0.001 M GSH, 0.2 ml. of 0.1 M phosphate buffer (pH 7) and 0.5 ml. of 0.2 M potassium ferricyanide. The mixture was allowed to react at 37° C. for five minutes, and then 0.5 ml. of 2 N sulfuric acid and 0.5 ml. of 0.5 M ferric chloride were added. The resulting Prussian blue color was read at 650 mμ. after 20 minutes. Determination of the sulfhydryl content of a protein sample consisted of adding an amount of protein denatured with detergent to 1 ml. of the PCMB solution, then adding 1 ml. of the GSH solution, making the volume up to 8.5 ml. with water and carrying out the above procedure to determine the amount of unreacted GSH. However, when this was tried with 1 ml. of the gastric extract, a precipitate was obtained at the acidification step. Using as little as 0.2 ml. of the gastric extract produced considerable turbidity. Therefore, a visual estimation of the color produced was resorted to, and it was estimated that 0.2 ml. of gastric extract did not react with more than 0.1 ml. of 0.001 M PCMB. It was therefore planned to start with amounts of PCMB greatly in excess of this and if binding was impaired, to work downward toward this value. A spectrophotometric sulfhydryl method which can be carried out at a neutral pH has been recently reported (165).

Table 21. Effect of para-chloromercuribenzoic acid (PCMB) on the binding of vitamin B_{12} by gastric extract

Initial contents of Side A[a]			%D
B_{12*}, ml.	GE, ml.	10^{-3} M PCMB, ml.	
1			43.8
1	0.15		18.6
1	0.15	1.5	18.8
1			43.4
1	0.10		26.1
1	0.10	1.5	26.0

[a]When PCMB was used it was added to the gastric extract and incubated for 2 hours at 30° C. before the labeled B_{12} was added.

The two experiments carried out on this point, summarized in Table 21, indicated that PCMB in great excess had no effect on the binding of B_{12} by the gastric extract. In these experiments the gastric extract was allowed to incubate with the PCMB for 2 hours at 30° C. before the Co^{60}-B_{12} was added. It thus appears highly unlikely that sulfhydryl groups participate in cobalamin binding in this system.

Fate of the cyano- group during the reaction of vitamin B_{12} with gastric extract

If coordination with the cobalt of cobalamin constitutes part of the reaction of gastric extract with vitamin B_{12}, either the cyano- group or the benzimidazole moiety must be

displaced from the cobalt coordination system. If it is the cyano- group that is displaced, performing the reaction in acid media should result in evolution of hydrocyanic acid. If the cyano- group is not displaced during the reaction, it appeared possible that it might still be photo-labile, as it is in free vitamin B_{12}.

To examine these possibilities use was made of the elegant and sensitive cyanide determination of Epstein (166) as adapted to the determination of vitamin B_{12} by Boxer and co-workers (71, 167, 168, 169). Essentially this method consists of isolating the cyanide by nitrogen aeration into a small volume of dilute sodium hydroxide, and converting the cyanide to cyanogen chloride with chloramine-T. Cyanogen chloride is then reacted with aqueous pyridine to form glutaconic aldehyde, which will then react with 3-methyl-1-phenyl-5-pyrazolone (MPP) to form a blue dye. This dye was found to be stabilized by the presence of bis-3-methyl-1-phenyl-5-pyrazolone (bisMPP). Boxer and Rickards have claimed that this method is capable of determining one part of cyanide in 10^{11} parts of solution with a precision of ± 1.5 per cent (167).

The procedure of Boxer and Rickards for aeration of samples and for color development was followed as closely as possible. A 0.1 N sodium cyanide was prepared, standardized against silver nitrate (170), and diluted in 0.1 N sodium

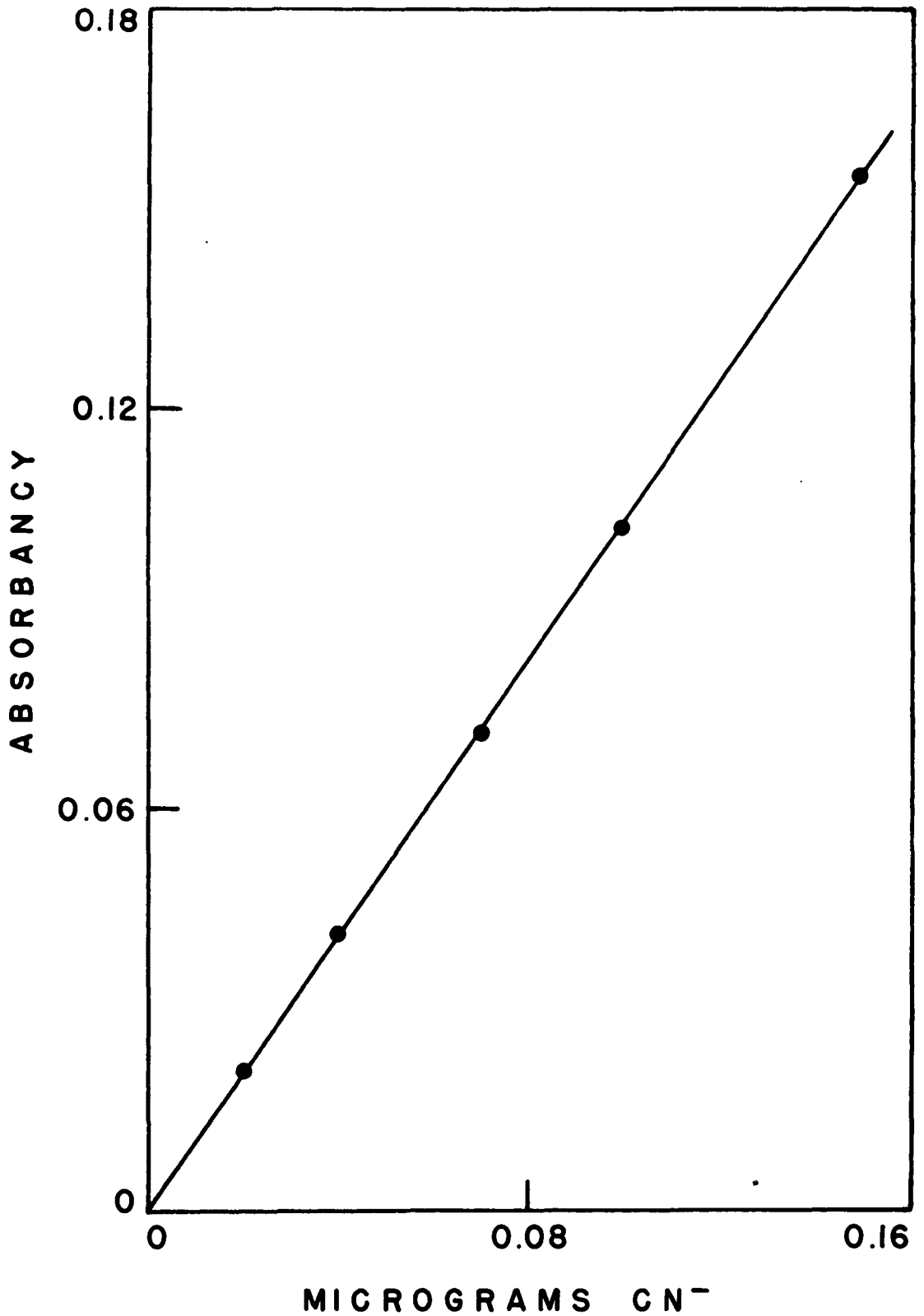
hydroxide to six concentrations (0.02 to 0.20 μg CN^- per ml.) to serve as standards. An aeration train capable of handling six samples was constructed. This consisted of a nitrogen tank, distilled water trap, a trap containing 0.05 N ceric sulfate in 1 N sulfuric acid, a trap containing saturated silver sulfate in 20 per cent sulfuric acid, a trap containing 0.1 N sodium hydroxide, and an empty trap followed by the sample assemblies in series. Each sample assembly consisted of the sample tube (38 by 200 mm.) containing 20-25 ml. of solution at pH 5 or less, followed by two successive collecting tubes (18 by 150 mm.), each containing 1 ml. of 0.1 N sodium hydroxide. At the end of the train a gas flow meter was installed. The sample tubes and collection traps were treated with Dri-Film No. SC-87 to avoid creeping of the solution. The system was easily tested for leaks by putting the chain under positive gas pressure and noting any continued bubbling. Samples to be run in the dark were encased in tubes of black construction paper, and samples to be illuminated were placed in the light beam of a Bell and Howell slide projector. Aeration was carried on at the rate of 400 ml. per minute for 3 hours, this being about twice the minimum suggested by Boxer and Rickards.

Color development consisted of chilling the collection and standard tubes to 0° C., adding 0.2 ml. of the fresh,

chilled chloramine-T reagent (prepared by adding 1 part of 0.25 per cent chloramine-T in water to 3 parts of 1 M primary sodium phosphate), mixing, and allowing to stand for from 2 to 10 minutes at 0° C. To this solution were added 3 ml. of fresh pyrazolone reagent (prepared by mixing 5 parts of filtered, saturated MPP in water with 1 part of 0.1 per cent bisMPP in pyridine), followed by incubation in a 50° C. water bath for 30 minutes. The absorbancies of the solutions were read within an hour at 620 mμ using the Beckman spectrophotometer. A standard curve for this method is presented in Figure 6. Good agreement was obtained between duplicate tubes of the standard series.

The method was tested by aeration of the standard cyanide solutions and by illumination-aeration of a vitamin B_{12} solution standardized spectrophotometrically. The results were somewhat disappointing, ranging from 96 to 112 per cent recovery. The principal difficulty was finally traced to the sample collection tubes. There appeared to be a considerable carry-over of hydrocyanic acid from the first tube to the second, amounting in some samples to 20 per cent, and quite variable. This apparently was the result of bumping of a large part of the 0.1 N sodium hydroxide onto the sides of the tube, leaving less solution for the gas to bubble through. In addition, there was a small, but also variable amount of blank color, possibly due to something in

Figure 6. Standard curve for the cyanide determination.


0.18
0.12
0.06
0
ABSORBANCY
0
0.08
0.16
MICROGRAMS CN⁻

the silicone coating. Other coatings such as Desicote and paraffin, and different methods of washing the treated tubes were tried without success. It was finally decided to proceed, recognizing that the results would be good to only about ±10 per cent. This proved to be adequate for deciding the original question.

The plan of the experiment was to aerate 5 ml. of the gastric extract both in the dark and in the light to remove all free and cobalamin-bound cyanide, then to add a known amount of vitamin B_{12}, slightly in excess of the amount expected to react, and to aerate again, first in the dark (to obtain any cyanide released by the reaction) and then during illumination. As a control, the same amount of B_{12} that was used for the sample was aerated, first in the dark and then while being illuminated.

To determine the extent of the B_{12} actually bound, in the event that the nitrogen aeration destroyed the binding activity, small amounts of Co^{60}-B_{12} were added to the non-labeled B_{12}, and this mixture used in three of the determinations. After the last aeration, 25 ml. of the sample were transferred to a dialysis cell and the amount of free B_{12} was determined by the partial dialysis method.

It was found that an average of 700 mμg. of B_{12} remained bound by 1 ml. of the gastric extract after the aeration process as compared to an average of 730 mμg. of

B_{12} bound by 1 ml. of gastric extract which had not been subjected to the aeration treatment.

The results of five such experiments are given in Table 22, and illustrate the considerable variation obtained with this method. The cyanide values were obtained by adding the cyanide recovered in both collection tubes, but no attempt was made to correct for blank color, which varied from none to 0.01 µg. as cyanide.

It is clear, however, that the results indicate that cyanide was not released by the reaction between B_{12} and the gastric extract. The amount of cyanide recovered from the B_{12}-gastric extract mixture on illumination was essentially the same as recovered from the same amount of B_{12} alone. As determined by the partial dialysis method, the amount of B_{12} which reacted amounted to about three-fourths of the total B_{12} used, so certainly, the equivalent amount of cyanide, had it been released, would have been detected.

There exists the possibility that the cyanide was released by the reaction and was bound elsewhere, to be subsequently released by light. This is unlikely for two reasons. At the pH used (pH 5), the cyano- group would be immediately converted to hydrocyanic acid, which is relatively unreactive. Even vitamin B_{12b} will not react with hydrocyanic acid at pH 5 (25, 68, 71). Secondly, examination of many different biological materials have indicated that

Table 22. Determination of the cyanide of vitamin B_{12} and mixtures of vitamin B_{12} and gastric extract

Exp. no.	GE ml.	Initial cyanide removal by aeration			Added B_{12} calc. as:		Bound B_{12}[a] calc. as:		Cyanide recovered by aeration	
		dark	light	light	B_{12}	CN^-	B_{12}	CN^-	dark	light
	ml.	mμg.	mμg.	mμg.	μg.	mμg.	μg.	mμg.	mμg.	mμg.
I	5.0	62	22	10	4.3	82			13	75
	0.0				4.3	82			-	76
II	5.0	-	28	29	4.5	86	3.6	68	10	73
	0.0				4.5	86			1	129
III	5.0	25	19	10	4.5	86	3.5	67	10	78
	0.0				4.5	86			2	91
IV	5.0	86	15	8	4.5	86	3.4	64	14	79
	0.0				4.5	86			12	83
V	5.0	40	24	6	4.2	80			8	85
	0.0				4.2	80			10	86

[a]The amount of B_{12} bound was determined by partial dialysis.

release of cyanide by illumination appears to be a specific reaction of vitamin B_{12} (71, 168, 169).

With the above possibility in mind, it appears most likely that the binding of B_{12} by gastric extract does not rupture the cyano-cobalt linkage.

Summary

An extract of Ventriculin was prepared which exhibited a relatively large cobalamin binding capacity. It was found that both B_{12} and B_{12b} were bound by the gastric extract by approximately the same amount, and that an excess of either cobalamin provided an essentially complete block for the other. It was concluded that B_{12} and B_{12b} react at the same sites on the binding substances present in the gastric extract.

The binding of B_{12} was essentially the same over the range of pH 2 to 10, but decreased above pH 10. Excess cyanide inhibited the reaction at pH 10 to 12, but was without effect at pH 6.5, providing some evidence that coordination with the cobalamin cobalt is involved in the reaction. Buffer concentration had no effect on the reaction, but heating at 100° C. definitely decreased the extent of the reaction. The presence of cobalt chloride and benzimidazole appeared to have no effect on the reaction. Excess p-chloromercuribenzoic acid did not inhibit the reaction,

indicating that sulfhydryl groups are probably not involved in the binding reaction.

Using a cyanide method it was found that apparently no cyanide was released by the reaction of B_{12} with gastric extract, but that an amount of cyanide roughly equivalent to the amount of B_{12} added could be recovered by illumination.

Reactions of Cobalamins with Other Preparations

Introduction

Chow has presented evidence that cobalamins are bound by heparin, yeast nucleic acid and chondroitin sulfuric acid (95, 141), based on dialysis and bacterial uptake methods. These substances are of considerable interest, since they are non-protein in nature, whereas most of the speculations concerning cobalamin binding reactions have assumed that protein is involved in some way. It was of interest, therefore, to examine some of these compounds using the partial dialysis method.

The work with blood preparations reported earlier, indicated that B_{12b} is bound quite unspecifically by all of the preparations, suggesting that perhaps almost any protein will show some binding of B_{12b}. As a limited test of this possibility, three other protein preparations were tested for cobalamin binding activity by the partial dialysis method.

Effect of several protein and non-protein preparations on the dialysis rate of cobalamins

It has been mentioned that cellophane binds B_{12b}, but not B_{12}, as one example of non-protein binding. Three other examples of binding by non-protein substances are given in Table 23. Heparin and two nucleic acid preparations appeared to bind limited amounts of B_{12b} but not B_{12}. A dextran preparation showed no evidence of binding either form of the vitamin. The possibility that these substances may exhibit some ability to pass through the membrane was not investigated because only a qualitative answer was desired.

Also given in Table 23 are the results of a comparison of the binding activity of "resting" bacterial cells for B_{12} and B_{12b}. Apparently more B_{12} is bound than B_{12b}, even though a considerable amount of B_{12b} is bound. The same qualitative results were obtained by direct measurement of the "free" cobalamins after removal of the bacterial cells by centrifugation.

Three protein preparations of at least some degree of purity, crystalline trypsin and pepsin, and lactalbumin, were found to bind considerable amounts of B_{12b}, but not B_{12}. These data are shown in Table 24. It will be noted that trypsin, like serum and serum albumin, showed decreased

Table 23. Effect of non-protein substances and bacterial cells on the dialysis of vitamins B_{12} and B_{12b}

Initial contents of Side A	%D
1 ml. B_{12b}*	44.1
1 ml. B_{12b}*, 100 mg. heparin	38.3
1 ml. B_{12}*, 100 mg. heparin	44.2
1 ml. B_{12b}*, no buffer	34.8
1 ml. B_{12b}*, 100 mg. heparin, no buffer	17.7
1 ml. B_{12}*, 100 mg. heparin, no buffer	44.6
1 ml. B_{12b}*	43.6
1 ml. B_{12b}*, 200 mg. ribonucleic acid	41.8
1 ml. B_{12}*, 200 mg. ribonucleic acid	44.6
1 ml. B_{12b}*	42.9
1 ml. B_{12b}*, 200 mg. desoxyribonucleic acid	40.4
1 ml. B_{12}*, 200 mg. desoxyribonucleic acid	42.8
1 ml. B_{12}*	41.8
1 ml. B_{12}*, 50 mg. dextran	42.3
1 ml. B_{12b}*, 50 mg. dextran	42.1
1 ml. B_{12b}*	44.7
1 ml. B_{12b}*, resting L. leichmannii cells	16.6
1 ml. B_{12}*, resting L. leichmannii cells	3.6

Table 24. Effect of protein preparations on the dialysis of vitamins B_{12} and B_{12b}

Initial contents of Side A	pH	%D
1 ml. B_{12*}	4.0	44.5
1 ml. B_{12*}, 150 mg. crystalline trypsin	4.0	43.8
1 ml. B_{12b*}, 150 mg. crystalline trypsin	4.0	43.5
1 ml. B_{12b*}	6.6	43.5
1 ml. B_{12b*}, 150 mg. crystalline trypsin	6.6	39.2
1 ml. B_{12*}, 150 mg. crystalline trypsin	6.6	43.5
1 ml. B_{12b*}	6.6	44.2
1 ml. B_{12b*}, 150 mg. crystalline pepsin	6.6	21.9
1 ml. B_{12*}, 150 mg. crystalline pepsin	6.6	42.0
1 ml. B_{12b*}	6.6	43.9
1 ml. B_{12b*}, 150 mg. lactalbumin	6.6	29.7
1 ml. B_{12*}, 150 mg. lactalbumin	6.6	44.1

binding activity at lower pH levels.

Summary

Heparin, desoxyribonucleic acid and ribonucleic acid preparations were found to bind limited amounts of vitamin B_{12b} but not B_{12}. Pepsin, trypsin and lactalbumin bound considerable amounts of B_{12b} but not B_{12}. Both vitamins were bound by "resting" bacterial cells, while a dextran preparation showed no evidence for binding either form of the vitamin.

DISCUSSION

This investigation has confirmed and elaborated upon the reported reaction of vitamin B_{12b} with histidine and the binding of vitamin B_{12} by blood plasma and gastric extracts. In addition, the binding of B_{12b} by blood proteins, gastric extract and other protein and non-protein substances has been observed, and some features of the B_{12}-gastric extract reaction have been studied. As a result some tentative conclusions can be made concerning the specific structures involved in these reactions, and suggestions for future investigations can be proposed.

It was found that several nitrogenous compounds and protein preparations reacted with vitamin B_{12b} but not with vitamin B_{12}. Since the proposed structures of these two forms of the vitamin differ only in the nature of the small group coordinated with the cobalt atom, it may be concluded that the reactions entered into by B_{12b} but not B_{12} involve this position primarily. Specifically, it appears that the reaction of B_{12b} with histidine and other compounds containing a cyclic tertiary nitrogen and with the several different protein preparations studied may involve a replacement of the aquo- group by a suitable basic ligand, resulting in

what has been termed a cobalichrome type of structure.

There is no direct evidence to indicate which group or groups present in protein may be responsible for this binding of vitamin B_{12b}. The observations that all fractions of serum, that denatured serum, and that such diverse proteins as globin, trypsin, pepsin and lactalbumin exhibit B_{12b}-binding indicate that the groups capable of replacing the aquo- group of vitamin B_{12b} are certainly not uncommon. Since histidine was the only amino acid which appeared to enter into cobalichrome formation with B_{12b}, it is tempting to consider the binding of B_{12b} by these proteins as involving cobalichrome formation with histidine side chains. However, it was found that lysozyme did not bind B_{12b} even though this protein contains about 1 per cent histidine. Further, the amount of B_{12b} bound by serum proteins, although in great excess of the physiological level, was certainly below the amount expected to be bound by the approximately 3 per cent histidine present in these proteins. This situation was also encountered in the other proteins examined, and suggests that only part of the histidine side chains may be available for cobalichrome formation. In addition, the possible participation by other groups cannot be denied on the basis of present evidence. The involvement of histidine in the binding of B_{12b} by these proteins can therefore be considered only a tentative proposal at the

present time.

An entirely different picture was presented by the reactions of cobalamins with a gastric extract preparation. Both forms of the vitamin (B_{12} and B_{12b}) were found to react to approximately the same extent, and probably at the same site on the binding substance. The observed inhibition by cyanide at high pH levels indicated that coordination with the cobalt atom may be a necessary feature of this reaction. However, the cyano- group of vitamin B_{12} did not appear to be displaced by the reaction, but remained in a photo-labile position, and the reaction was not affected by a wide range of pH levels or buffer concentrations. Although these results effectively eliminate cobalichrome formation as a possible mechanism for this reaction, they are insufficient to permit a decision to be made between several other possible mechanisms. The proposal that the reaction involves a displacement of the benzimidazole group and bond formation between the binding substance and both the benzimidazole group and the cobalt atom appears to be supported, but certainly not proved by the results of this investigation. Conceivably, displacement of the benzimidazole group and only coordination with the cobalt by a suitable group could occur. Other proposals, such as the formation of a peptide linkage through the primary amide groups of the cobalamins, and indeed, reactions involving any other structural feature of

the cobalamin molecule except the cobalt atom must still be considered possible, even though unlikely as judged by the present evidence.

The protein groups of the gastric extract responsible for the binding of both B_{12} and B_{12b} remain undetermined. Participation by sulfhydryl groups was shown to be very unlikely, and the involvement of the side chains of other common amino acids would appear to be improbable, since vitamin B_{12} did not appear to react with any of the free amino acids. Actually, there is no need to depend upon the reactivity of the amino acid side chains, for all of the gastric juice and mucosal extract preparations which have been reported to possess cobalamin binding activity have contained considerable amounts of glycoproteins, offering rather unlimited possibilities for reactive groups. Whatever the responsible groups may be, it appears probable that the binding of cobalamins by gastric extracts involves a more specific or specialized structure than the vitamin B_{12b}-binding structure present in serum and other proteins.

In proposing further work on the reactions of cobalamins with natural materials the question of biological significance should be considered. Although vitamin B_{12b} was found to be more reactive than vitamin B_{12} in several <u>in vitro</u> systems, the two forms of the vitamin have repeatedly been shown to be physiologically equivalent, regardless of

the route of administration. Thus, either the cyano- group is not essential for biological activity and must be removed from the vitamin B_{12} molecule, or the cyano- group is compatible with or participates in the biological activity of the vitamin. The available evidence appears to favor the second alternative. Assuming this to be the case, further studies using systems which react only with vitamin B_{12b} would appear to be of less value than studies with systems which react with both forms of the vitamin. It is of obvious importance in future studies to define as nearly as possible the form of the vitamin being used.

The gastric extract system presents further opportunities for obtaining information concerning the nature of the binding reaction. Of particular interest would be a study of the possible binding by gastric extracts of degradation products of vitamin B_{12} and of substances such as cobaltiporphyrins, for it may be possible by such studies to demonstrate the participation of the cobalt atom in the binding reaction.

It would be desirable to isolate the components of gastric extracts responsible for the binding of cobalamins, and several groups of workers are apparently engaged in this problem. It may be suggested that if the separation is followed by radioisotope assay of bound (labeled) cobalamins, it may be possible to remove the cobalamins and recover the

active binding substance by dialysis at high cyanide concentrations and high pH levels.

The isolation and study of the vitamin B_{12}-binding substances in serum presents a formidable problem of concentration, for if these substances are capable of binding only about 1.5 mμg. of B_{12} per ml. of serum, the concentration of the binding substances in serum is probably not more than 1 μg. per ml.

Perhaps the greatest opportunity for future work lies in the isolation of the cobalamin-protein complexes present in liver tissue, for it is here that the vitamin exists in storage and functional states. There have been no reported attempts to isolate these complexes even though the problem would appear to be no more difficult than the isolation of the intrinsic factor or the gastric extract binding substances.

SUMMARY AND CONCLUSIONS

1. The effect of amino acids and other nitrogenous compounds on the visible and ultraviolet absorption spectra maxima of vitamins B_{12} and B_{12b} in aqueous solution was studied. It was found that vitamin B_{12b} entered into cobalichrome formation with histidine, histidyl-histidine, carnosine, histamine, imidazole, and pyridine. The absorption spectrum of vitamin B_{12b} was destroyed in the presence of several reducing agents including cysteine and glutathione. Vitamin B_{12b} did not appear to react with any other of the eighteen amino acids tested or with benzimidazole, glycyl-glycyl-glycine, or several other compounds. The absorption spectrum of vitamin B_{12} was found to be unchanged in the presence of any of the above compounds.

2. The reaction of vitamin B_{12b} with histidine was found to be pH dependent, and the equilibrium constant for the reaction at 25° C. and at pH 7 was estimated to be 4×10^3 l. per mole.

3. A partial dialysis method was devised for the purpose of determining the extent of the reactions between cobalamins and non-dialysable substances such as proteins. By constructing an apparatus in which the volume, membrane

area and agitation were held constant, reproducible short-term dialysis of cobalamins was obtained. Cobalamins labeled with Co^{60} were used for this work, and a dependable method of radio-assay of liquid samples was devised. It was demonstrated by using this method that the rates of dialysis of vitamin B_{12} and vitamin B_{12b} in buffered solutions were identical, and could be adequately expressed as functions of time, concentration and temperature.

4. Using the partial dialysis method it was found that unexpectedly large amounts of vitamin B_{12b} were bound by whole hemolysed blood, plasma and serum, hemolysed red cells, purified globin, purified serum albumin and heat denatured serum. No binding of vitamin B_{12} by these preparations could be detected by this method. By exhaustive dialysis serum was found to bind approximately 30 mμg. of vitamin B_{12b} and 1 mμg. of vitamin B_{12} per milliliter. Considerably less vitamin B_{12b} was bound by serum at pH 4 than at pH 6.6.

5. Serum containing bound labeled vitamin B_{12b} was fractionated by salt precipitation and by paper electrophoresis. All fractions retained considerable radioactivity, indicating that no one serum protein component was responsible for the binding of vitamin B_{12b}. A similar attempt to determine the position of bound vitamin B_{12} was inconclusive.

6. It was concluded that the reaction of vitamin B_{12b} with the several blood protein preparations may be the

result of cobalichrome formation, possibly with exposed or available histidine side chains.

7. Lysozyme was shown by the partial dialysis method and by paper electrophoresis to have no binding activity for either vitamin B_{12} or vitamin B_{12b}. It was suggested that the histidine residue of lysozyme may not be accessible for cobalichrome formation by vitamin B_{12b}.

8. A gastric mucosal extract exhibiting high cobalamin binding activity was prepared from a commercial dried stomach preparation by aqueous extraction and successive salt and alcohol precipitation of the protein constituents.

9. The gastric extract was found by the partial dialysis method to bind approximately equal amounts of vitamins B_{12} and B_{12b}. Addition to the gastric extract of an excess of either form of the vitamin provided an essentially complete block for the binding of the other form. It was concluded that apparently both forms of the vitamin react at the same site on the binding substance present in the gastric extract.

10. The binding of vitamins B_{12} and B_{12b} by the gastric extract was found to be essentially unaffected by changes of hydrogen ion concentration from pH 2 to pH 10. The binding of vitamin B_{12} by the gastric extract appeared to be unaffected by changes in buffer anion and concentration.

11. The binding of vitamin B_{12} by the gastric extract

was found to be inhibited by the presence of cyanide at pH 10 or above, but not at pH 6.6. Evidence was presented which indicated that the cyano- group of vitamin B_{12} was not displaced by the reaction of vitamin B_{12} with the gastric extract, but that this cyano- group remained in a photo-labile position. It was concluded that these observations are consistent with the proposal that the gastric extract-vitamin B_{12} reaction involves displacement of the benzimidazole group by a group which enters into coordination with the cobalt atom of vitamin B_{12}.

12. The binding of vitamin B_{12} by the gastric extract did not appear to be inhibited by the presence of cobaltous chloride and benzimidazole.

13. The binding of vitamin B_{12} by the gastric extract was unimpaired by the presence of an excess of p-chloromercuribenzoic acid, indicating that sulfhydryl groups do not participate in the reaction.

14. Several other substances were noted to bind vitamin B_{12b} but not vitamin B_{12}. Included among these were cellophane, heparin, nucleic acids, trypsin, pepsin and lactalbumin.

LITERATURE CITED

1. Rickes, E. L., Brink, N. G., Koniuszy, F. R., Wood, T. R., and Folkers, K. Crystalline Vitamin B_{12}. Science 107: 396 (1948).

2. Shorb, M. S. Unidentified essential growth factors for Lactobacillus lactis found in refined liver extracts and in certain natural materials. J. Bact. 53: 669 (1947).

3. Shorb, M. S. Unidentified growth factors for Lactobacillus lactis in refined liver extracts. J. Biol. Chem. 169: 455 (1947).

4. Smith, E. L. Purification of antipernicious anemia factors from liver. Nature 161: 638 (1948).

5. Smith, E. L., and Parker, L. F. J. Purification of antipernicious anemia factor. Biochem. J. 43: viii (1948).

6. Fantes, K. H., Page, J. E., Parker, L. J. F., and Smith, E. L. Crystalline antipernicious anemia factor from liver. Proc. Roy. Soc. (London) B136: 592 (1950).

7. Brink, N. G., Wolf, D. E., Kaczka, E., Rickes, E. L., Koniuszy, F. R., Wood, T. R., and Folkers, K. Vitamin B_{12}. IV Further characterization of vitamin B_{12}. J. Am. Chem. Soc. 71: 1854 (1949).

8. Smith, E. L. Vitamin B_{12}. J. Pharm. Pharmacol. 1: 500 (1949).

9. Smith, E. L. Presence of cobalt in the antipernicious anemia factor. Nature 162: 144 (1948).

10. Ellis, B., Petrow, V., and Snook, G. F. Chemistry of antipernicious anemia factor. I Liberation of phosphorous as phosphate from B_{12} by acid hydrolysis. J. Pharm. Pharmacol. 1: 287 (1949).

11. Rickes, E. L., Brink, N. G., Koniuszy, F. R., Wood, T. R., and Folkers, K. Vitamin B_{12}, a cobalt complex. Science 108: 134 (1948).

12. Hodgkin, D. C., Porter, M. W., and Spiller, R. C. Crystallographic measurements of the antipernicious anemia factor. Proc. Roy. Soc. (London) B136: 609 (1949).

13. Ellis, B., Petrow, V., and Snook, G. F. The isolation of the crystalline antipernicious anemia factor from liver. J. Pharm. and Pharmacol. 1: 60 (1949).

14. Rickes, E. L., Brink, N. G., Koniuszy, F. R., Wood, T. R., and Folkers, K. Comparative data on vitamin B_{12} from liver and from a new source, Streptomyces griseus. Science 108: 634 (1948).

15. Kaczka, E., Wolf, D. E., and Folkers, K. Vitamin B_{12}. V Identification of crystalline vitamin B_{12a}. J. Am. Chem. Soc. 71: 1514 (1949).

16. Kaczka, E. A., Wolf, D. E., Kuehl, F. A. Jr., and Folkers, K. Vitamin B_{12}. Reactions of cyanocobalamin and related compounds. Science 112: 354 (1950).

17. Pierce, J. V., Page, A. C. Jr., Stokstad, E. L. R., and Jukes, T. H. Crystallization of vitamin B_{12b}. J. Am. Chem. Soc. 71: 2952 (1949).

18. Pierce, J. V., Page, A. C. Jr., Stokstad, E. L. R., and Jukes, T. H. Studies of some characteristics of vitamin B_{12b}. J. Am. Chem. Soc. 72: 2615 (1950).

19. Brockman, J. A. Jr., Pierce, J. V., Stokstad, E. L. R., Broquist, H. P., and Jukes, T. H. Some characteristics of a crystalline compound derived from vitamin B_{12}. J. Am. Chem. Soc. 72: 1042 (1950).

20. Smith, E. L., Fantes, K. H., Ball, S., Ireland, D. M., Waller, J. G., Emery, W. B., Anslow, W. K., and Walker, A. D. Vitamin B_{12}. Additional factors. Biochem. J. 48: 1 (1951).

21. Anslow, W. K., Ball, S., Emery, W. B., Fantes, K. H., Smith, E. L., and Walker, A. D. The nomenclature of the vitamins B_{12}. Chemistry and Industry 574 (1950).

22. Smith, E. L., Fantes, K. H., Ball, S., Waller, J. G., Emery, W. B., Anslow, W. K., and Walker, A. D. B_{12} vitamins (cobalamins). I Vitamin B_{12c} and B_{12d}. Biochem. J. 52: 389 (1952).

23. Brink, N. G., Kuehl, F. A. Jr., and Folkers, K. Vitamin B_{12}. The identification of vitamin B_{12} as a cyano-cobalt complex. Science 112: 354 (1950).

24. Kaczka, E. A., Denkewalter, R. G., Holland, A., and Folkers, K. Vitamin B_{12}. XIII Additional data on vitamin B_{12a}. J. Am. Chem. Soc. 73: 335 (1951).

25. Wijmenga, H. G., Veer, W. L. C., and Lens, J. Vitamin B_{12}. II The influence of HCN on some factors of the vitamin B_{12} group. Biochim. et Biophys. Acta 6: 229 (1950).

26. Cooley, G., Ellis, B., Petrow, V., Beaven, G. H., Holiday, E. R., and Johnson, E. A. Some observations on the relationship between vitamins B_{12a} and B_{12b}. J. Pharm. Pharmacol. 3: 607 (1951).

27. Ellis, B., Petrow, V., and Snook, G. F. The chemistry of antipernicious anemia factor. II The "ninhydrin-reacting" hydrolytic fragment of vitamin B_{12}. J. Pharm. Pharmacol. 1: 950 (1949).

28. Ellis, B., Petrow, V., and Snook, G. F. The "ninhydrin-reacting" hydrolytic fragment of vitamin B_{12}. J. Pharm. Pharmacol. 1: 735 (1949).

29. Wolf, D. E., Jones, W. H., Valiant, J., and Folkers, K. Vitamin B_{12}. XI Degradation of vitamin B_{12} to Dg-1-amino-2-propanol. J. Am. Chem. Soc. 72: 2820 (1950).

30. Brink, N. G. and Folkers, K. Vitamin B_{12}. VI 5,6-dimethylbenzimidazole, a degradation product of vitamin B_{12}. J. Am. Chem. Soc. 71: 2951 (1949).

31. Brink, N. G. and Folkers, K. Vitamin B_{12}. X 5,6-dimethylbenzimidazole, a degradation product of vitamin B_{12}. J. Am. Chem. Soc. 72: 4442 (1950).

32. Holiday, E. R. and Petrow, V. Vitamin B_{12} as a 5,6-dimethylbenzimidazole derivative. J. Pharm. Pharmacol. 1: 734 (1949).

33. Beaven, G. R., Holiday, E. R., Johnson, E. A., Ellis, B., Mamalis, P., Petrow, V., and Sturgeon, B. The chemistry of antipernicious anemia factors. III 5,6-disubstituted benzimidazoles as products of acid hydrolysis of vitamin B_{12}. J. Pharm. Pharmacol. 1: 957 (1949).

34. Cooley, G., Ellis, B., Mamalis, P., Petrow, V., and Sturgeon, B. The chemistry of antipernicious anemia factor. V Interrelationship and structure of the α-, β- and γ-components. J. Pharm. Pharmacol. 2: 579 (1950).

35. Brink, N. G., Holly, F. W., Shunk, C. H., Peel, E. W., Cahill, J. J., and Folkers, K. Vitamin B_{12}. IX 1-α-D-ribofuranosido-5,6-dimethylbenzimidazole, a degradation product of vitamin B_{12}. J. Am. Chem. Soc. 72: 1866 (1950).

36. Buchanan, J. G., Johnson, A. W., Mills, J. A., and Todd, A. R. Isolation of a phosphorous-containing degradation product from vitamin B_{12} and B_{12a}. Chemistry and Industry 426 (1950).

37. Buchanan, J. G., Johnson, A. W., Mills, J. A., and Todd, A. R. Chemistry of the vitamin B_{12} group. I Acid hydrolysis studies. Isolation of a phosphorous-containing degradation product. J. Chem. Soc. 2845 (1950).

38. Todd, A. R. and Johnson, A. W. Vitamin B_{12}. Revs. Pure and Applied Chem. (Australia) 2: 23 (1952).

39. Beaven, G. H., Holiday, E. R., Johnson, E. A., Ellis, B., and Petrow, V. The mode of linkage of component α in vitamin B_{12}. J. Pharm. Pharmacol. 2: 733 (1950).

40. Beaven, G. H., Holiday, E. R., Johnson, E. A., Ellis, B., and Petrow, V. The chemistry of antipernicious anemia factor. VI The mode of combination of component α in vitamin B_{12}. J. Pharm. Pharmacol. 2: 944 (1950).

41. Beaven, G. H. and Holiday, E. R. Spectroscopic evidence for the presence of the benzimidazole chromophore in intact vitamin B_{12}. J. Pharm. Pharmacol. 4: 342 (1952).

42. Cooley, G., Davies, M. T., Ellis, B., Petrow, V., and Sturgeon, B. The chemistry of antipernicious anemia factors. XII The 1-amino-2-propanol component of cyanocobalamin. J. Pharm. Pharmacol. 5: 257 (1953).

43. Armitage, J. B., Cannon, J. R., Johnson, A. W., Parker, L. F. J., Smith, E. L., Stafford, W. H., and Todd, A. R. Chemistry of the vitamin B_{12} group. III The course of hydrolytic degradations. J. Chem. Soc. 3849 (1953).

44. Chargaff, E., Levine, C., Green, C., and Kream, J. Study of some constituents of vitamin B_{12}. Experientia 6: 229 (1950).

45. Brierly, J. M. Chemical studies on vitamin B_{12}. Unpublished Ph. D. Thesis. Ames, Iowa, Iowa State College Library (1953).

46. Diehl, H., Vander Haar, R. W., and Sealock, R. R. The magnetic susceptibility of vitamin B_{12}. J. Am. Chem. Soc. 72: 5312 (1950).

47. Grün, E. and Menasse, R. The determination of the magnetic susceptibility of vitamin B_{12}. Experientia 6: 263 (1950).

48. Wallman, J. C., Cunningham, B. B., and Calvin, M. The magnetic susceptibility of vitamin B_{12} and B_{12b}. Science 113: 55 (1951).

49. Diehl, H. Physiochemical studies on vitamin B_{12}. Record Chem. Prog. (Kresge-Hocker Sci. Lib.) 13: 9 (1952).

50. Diehl, H., Morrison, J. I., and Sealock, R. R. Polarographic behavior of vitamin B_{12} in potassium cyanide supporting electrolyte. Experienta 7: 60 (1952).

51. Boos, R. N., Carr, J. E., and Conn, J. B. The reduction of vitamin B_{12}. Science 117: 603 (1953).

52. Baldwin, R. R., Lowry, J. R., and Harrington, R. V. The failure of crystalline vitamin B_{12} to exchange with Co^{60} in acidic and neutral aqueous solutions. J. Am. Chem. Soc. 73: 4968 (1951).

53. Boos, R. N., Rosenbloom, C., and Woodbury, D. T. The exchange stability of cobalt in vitamin B_{12}. J. Am. Chem. Soc. 73: 5446 (1951).

54. Lang, C. A. and Chow, B. F. Inactivation of microbiological activity of crystalline vitamin B_{12} by reducing agents. Proc. Soc. Exp. Biol. Med. 75: 39 (1950).

55. Frost, D. V., Lapidus, M., Armstrong, K., Plaut, K. A., Scherfling, E., and Fricke, H. H. Differential stability of various analogs of cobalamin to vitamin C. Science 116: 119 (1952).

56. Campbell, J. A., McLaughlan, J. M., and Chapman, D. G. A method for the differentiation of hydroxocobalamin from cyanocobalamin employing the ascorbic acid reaction. J. Am. Pharm. Assoc. 41: 479 (1952).

57. Trenner, N. R., Buhs, R. P., Bacher, F. A., and Gakenheimer, W. C. A note concerning the incompatibility of vitamin B_{12} and ascorbic acid. J. Am. Pharm. Assoc. 39: 361 (1950).

58. Davies, M. T., Mamalis, P., Petrow, V., Sturgeon, B., Beaven, G. H., Holiday, E. R., and Johnson, E. A. The chemistry of antipernicious anemia factors. IX The preparation and spectrographic properties of some benzimidazole-cobalt coordinated compounds. J. Pharm. Pharmacol. 4: 448 (1952).

59. Diehl, H. and Morrison, J. I. Determination of amino nitrogen in vitamins B_{12} and B_{12a}. Iowa State Coll. J. Sci. 26: 589 (1952).

60. Diehl, H. and Ellingboe, J. L. Determination of amide nitrogen in vitamins B_{12} and B_{12a}. Iowa State Coll. J. Sci. 27: 421 (1953).

61. Schmid, H., Ebnother, A., and Karrer, P. A contribution to the knowledge of vitamin B_{12}. Helv. Chim. Acta 36: 65 (1953).

62. McConnel, R. J., Overell, B. G., and Petrow, V. The chemistry of antipernicious anemia factors. XI The preparation and properties of some cobalt porphyrins. J. Pharm. Pharmacol. 5: 179 (1953).

63. Smith, E. L. Structure and stability of some cobalamins. Biochem. J. 50: xxxvi (1952).

64. Smith, E. L., Ball, S., and Ireland, D. M. B_{12} vitamins (cobalamins). II Neutral, basic and acidic cobalamins. Biochem. J. 52: 395 (1952).

65. Buhs, R. P., Newstead, E. G., and Trenner, N. R. Analog of vitamin B_{12}. Science 113: 625 (1951).

66. Ellis, B., Petrow, V., Beaven, G. H., Holiday, E. R., and Johnson, E. A. Conversion of vitamin B_{12b} into vitamin B_{12}. J. Pharm. Pharmacol. 2: 735 (1950).

67. Veer, W. L. C., Edelhausen, J. H., Wijmenga, H. G., and Lens, J. Vitamin B_{12}. I The relation between vitamin B_{12} and B_{12b}. Biochim. et Biophys. Acta 6: 225 (1950).

68. Cooley, G., Ellis, B., Petrow, V., Beaven, G. H., Holiday, E. R., and Johnson, E. A. The chemistry of antipernicious anemia factors. VII Some transformations of vitamin B_{12}. J. Pharm. Pharmacol. 3: 271 (1951).

69. Kaczka, E. A., Wolf, D. E., Kuehl, F. A. Jr., and Folkers, K. Vitamin B_{12}. XVI Modifications of cyanocobalamin. J. Am. Chem. Soc. 73: 3569 (1951).

70. Conn, J. P. and Wartman, T. G. Kinetics of reaction of certain vitamin B_{12} analogs with cyanide ion. Science 115: 72 (1952).

71. Boxer, G. E. and Rickards, J. C. Chemical determinations of vitamin B_{12}. III Methods for the quantitative and specific release of the cyano- group of vitamin B_{12}. Arch. Biochem. 30: 382 (1951).

72. Wokes, F., Baxter, N., Horsford, J., and Preston, B. Effect of light on vitamin B_{12}. Biochem. J. 53: xix (1953).

73. Alicinco, J. F. Perchloric acid salt of vitamin B_{12}. J. Am. Chem. Soc. 73: 4051 (1951).

74. Ellis, B. and Petrow, V. Some observations on vitamin B_{12}, B_{12b} and chlorocobalamin. J. Pharm. Pharmacol. 4: 152 (1952).

75. Conn, J. B., Norman, S. L., and Wartman, T. G. Equilibrium between vitamin B_{12} (cyanocobalamin) and cyanide ion. Science 113: 658 (1951).

76. Ford, J. E. and Porter, J. W. G. Some properties of vitamin B_{12}-like factors from calf faeces. I Characteristics of different fractions. Biochem. J. 51: v (1952).

77. Ford, J. E. and Holdsworth, E. S. Biosynthetic relationships between vitamin B_{12}-like factors. Biochem. J. 56: xxxv (1954).

78. Brown, F. B. and Smith, E. L. New purines in B_{12} vitamins. Biochem. J. 56: xxxiv (1954).

79. Dion, H. W., Calkins, D. G., and Pfiffner, J. J. Hydrolysis products of pseudovitamin B_{12}. J. Am. Chem. Soc. 74: 1108 (1952).

80. Pfiffner, J. J., Calkins, D. G., and Dion, H. W. Characterization of pseudovitamin B_{12} pigments. Fed. Proc. 13: 274 (1954).

81. Ford, J. E., Holdsworth, E. S., and Porter, J. W. G. The occurrence of cyanocobalamin and related compounds in natural materials. Proc. Nutrition Soc. (Eng. and Scot.) 12: xi (1953).

82. Chaiet, L., Rosenblum, C., and Woodbury, D. T. Biosynthesis of radioactive vitamin B_{12} containing cobalt^{60}. Science 111: 601 (1950).

83. Ternberg, J. L. and Eakin, R. E. Erythein and apoerythein and their relation to the antipernicious anemia principle. J. Am. Chem. Soc. 71: 3858 (1949).

84. Scheid, H. E. and Schweigert, B. S. Liberation and microbiological assay of vitamin B_{12} in animal tissues. J. Biol. Chem. 193: 299 (1951).

85. Cooperman, J. M., Drucker, R., and Tabenkin, B. Microbiological assays for vitamin B_{12}: A cyanide enhancement effect. J. Biol. Chem. 191: 135 (1951).

86. Soars, M. H. and Hendlin, D. The use of potassium cyanide in the Lactobacillus leichmannii assay for vitamin B_{12}. J. Bact. 62: 15 (1951).

87. Prier, R. F., Derse, P. H., and Krieger, G. H. Effect of bisulfite and nitrite on vitamin B_{12} microbiological assays. Arch. Biochem. Biophys. 40: 474 (1952).

88. Denton, C. A. and Kellogg, W. L. The vitamin B_{12} activity of eggs and some materials as affected by extraction in the presence of sodium cyanide or sodium bisulfite. Arch. Biochem. Biophys. 46: 105 (1953).

89. Hausmann, K. and Mulli, K. The hemopoietic effect of vitamin B_{12} prepared from fish. Lancet 262: 185 (1952).

90. Bird, O. D. and Hoevet, B. The vitamin B_{12}-binding power of protein. J. Biol. Chem. 190: 181 (1951).

91. Burkholder, P. R. Microbiological studies on the intrinsic factor of Castle. Science 114: 478 (1951).

92. Burkholder, P. R. Microbiological studies on materials which potentiate oral vitamin B_{12} therapy in Addisonian anemia. Arch. Biochem. Biophys. 39: 322 (1952).

93. Davis, B. D. and Mingioli, E. S. Mutants of Escherichia coli requiring methionine or vitamin B_{12}. J. Bact. 60: 17 (1950).

94. Davis, R. L., Layton, L. L., and Chow, B. F. Uptake of radioactive vitamin B_{12} by bacteria in single and mixed cultures. Fed. Proc. 10: 380 (1951).

95. Chow, B. F. and Davis, R. L. Some observations on substances combining with vitamin B_{12}. Recent Advances in Nutritional Research 5: 17 (1952).

96. Davis, R. L. and Chow, B. F. Determination of vitamin B_{12} by means of its combination with L. leichmannii resting cells. Fed. Proc. 11: 466 (1952).

97. Yamamoto, R. S. and Chow, B. F. A rapid method for the determination of B_{12} binding power in the gastric juice. J. Lab. Clin. Med. 43: 316 (1954).

98. Latner, A. L., Raine, L., Ross, G. I. M., and Ungley, C. C. A preparative paper-electrophoresis technique and its application to vitamin$_{12}$-binding in serum. Biochem. J. 52: xxxiii (1952).

99. Latner, A. L., Ungley, C. C., Cox, E. V., McEvoy-Bowe, E., and Raine, L. Electrophoresis of human gastric juice in relation to Castle's intrinsic factor. Brit. Med. J. 1: 467 (1953).

100. Wijmenga, H. G., Thompson, K. W., Stern, K. G., and O'Connell, D. J. Preparation and properties of a cobalamin protein. Biochim. et Biophys. Acta 13: 144 (1954).

101. Wijmenga, H. G., Stern, K. G., O'Connell, D. J., and Thompson, K. W. Components of gastric mucosa extracts. Fed. Proc. 13: 320 (1954).

102. Broquist, H. P., Stokstad, E. L. R., and Jukes, T. H. Further observations on the microbiological assay for vitamin B_{12} using Lactobacillus leichmannii. Proc. Soc. Exp. Biol. Med. 76: 806 (1951).

103. Loy, H. W. Jr. and Kline, O. L. Microbiological assay for vitamin B_{12}. J. Assoc. Offic. Agr. Chemists 34: 225 (1951).

104. Prusoff, W. H., Welch, A. D., Heinle, R. W., and Meacham, G. C. Concentration of intrinsic factor and vitamin B_{12}-binding activities of fractions of dessicated hog stomach. Blood 8: 491 (1953).

105. Hoff-Jørgensen, E. The effect of "intrinsic factor" on the absorption of vitamin B_{12} by wild type Escherichia coli. Arch. Biochem. Biophys. 36: 235 (1952).

106. Spray, G. H. The effect of heat on the microbiological and anti-anemic properties of human gastric juice mixed with vitamin B_{12}. Biochem. J. 50: 587 (1952).

107. Bethell, F. H., Swendseid, M. E., Miller, S., and Cintron-Rivera, A. A. Cobalamin (vitamin B_{12}) and the intrinsic factor of Castle. Ann. Internal Med. 35: 518 (1951).

108. Shaw, G. E. An intestinal inhibitor of vitamin B_{12}. Biochem. J. 47: xxxv (1950).

109. van der Zant, E. S. and Underkofler, L. A. Some factors affecting the vitamin B_{12}-binding properties of hog mucosal extracts. Proc. Iowa Acad. Sci. 60: 325 (1953).

110. van der Zant, E. S. Effect of protein materials upon the growth promoting activity of vitamin B_{12} for Lactobacillus leichmannii. Unpublished Ph. D. Thesis. Ames, Iowa, Iowa State College Library, (1953).

111. Latner, A. L. and McEvoy-Bowe, E. Observations on the chemical nature of Castle's intrinsic factor. Biochem. J. 55: xxiii (1953).

112. Latner, A. L. and Ungley, C. C. Intrinsic factor activity in solid material. Brit. Med. J. 1: 1448 (1953).

113. Latner, A. L., Merrills, R. J., and Raine, L. C. D. P. Further observations on the isolation and chemical composition of Castle's intrinsic factor. Biochem. J. 57: xix (1954).

114. Schilling, R. F. and Deiss, W. P. Intrinsic factor studies. I Paper electrophoresis of mixture of gastric juice and vitamin B_{12}. Proc. Soc. Exp. Biol. Med. 83: 506 (1953).

115. Roland, J. F. Jr., Millman, I., and Giffer, J. W. Jr. Fractional electrical transport studies of vitamin B_{12} binding by proteins. J. Biol. Chem. 202: 857 (1953).

116. Chow, B. F. and Yamamoto, R. Properties of B_{12} binding substance in gastric juice. Fed. Proc. 12: 189 (1953).

117. Ross, G. I. M. Vitamin B_{12} assay in body fluids. Nature 166: 270 (1950).

118. Ross, G. I. M. Vitamin B_{12} assay in body fluids using Euglena gracilis. J. Clin. Path. 5: 250 (1952). (Original not available for examination: abstracted in Biol. Abstr. 27: 507 (1953)).

119. Callender, S. T. and Lajtha, L. G. On the nature of Castle's hemopoietic factor. Blood 6: 1234 (1951).

120. Girdwood, R. H. Some aspects of the metabolism of antimegaloblastic substances in man. Blood 8: 469 (1953).

121. Rosenthal, H. L. and Sarett, H. P. The determination of vitamin B_{12} activity in human serum. J. Biol. Chem. 199: 433 (1952).

122. Wolff, R., Karlin, R., and Royer, P. Existence of a combining power of blood for vitamin B_{12}. Compt. Rend. Soc. Biol. 146: 1008 (1952).

123. Yamamoto, R., Barrows, C. Jr., Lang, C., and Chow, B. F. Further studies on the absorption of vitamin B_{12} following oral and parenteral administration. J. Nutr. 45: 507 (1951).

124. Conley, C. L., Krevans, J. R., Chow, B. F., Barrows, C. Jr., and Lang, C. A. Observations on the absorption, utilization and excretion of vitamin B_{12}. J. Lab. Clin. Med. 38: 84 (1951).

125. Pitney, W. R., Beard, M. F., and Van Loon, E. J. Observations on the bound form of vitamin B_{12} in human serum. J. Biol. Chem. 207: 143 (1954).

126. Beard, M. F., Pitney, W. R., and Sanneman, E. H. Serum concentrations of vitamin B_{12} in patients suffering from leukemia. Blood 9: 789 (1954).

127. Davis, R. L. and Chow, B. F. Vitamin B_{12} binding substances in human serum. Fed. Proc. 13: 33 (1954).

128. Unglaub, W. G. and Miller, O. N. Studies of serum and gastric factors in the etiology of pernicious anemia: A proposed in vitro test for intrinsic factor activity. J. Clin. Invest. 33: 969 (1954).

129. Miller, O. N., Unglaub, W. G., and Goldsmith, G. A. Interaction among vitamin B_{12} materials with intrinsic factor activity and serum proteins. Fed. Proc. 13: 265 (1954).

130. Gregory, M. E., Ford, J. E., and Kon, S. K. A vitamin B_{12}-binding factor in sow's milk. Biochem. J. 51: xxix (1952).

131. Gregory, M. E. and Holdsworth, E. S. The combination of some vitamin B_{12}-like compounds with sow's whey and "intrinsic factor" concentrates. Biochem. J. 55: ix (1953).

132. Gregory, M. E. and Holdsworth, E. S. The combination of some vitamin B_{12}-like compounds with sow's milk whey and "intrinsic factor" concentrates. Biochem. J. 55: 830 (1953).

133. Beerstecher, E. Jr. and Aitgelt, S. Apoerythein in saliva. J. Biol. Chem. 189: 31 (1951).

134. Beerstecher, E. Jr. and Edmonds, E. J. Anomalous thermal behavior of salivary apoerythein activity. Science 114: 412 (1951).

135. Meyer, C. E., Eppstein, S. H., Bethell, F. H., and Hall, B. E. Nature of the intrinsic factor. Fed. Proc. 9: 205 (1950).

136. Davis, R. L., Layton, L. L., and Chow, B. F. Uptake of radioactive vitamin B_{12} by various microorganisms. Proc. Soc. Exp. Biol. Med. 79: 273 (1952).

137. Oginsky, E. L. Uptake of vitamin B_{12} by Escherichia coli. Arch. Biochem. Biophys. 36: 71 (1952).

138. Davis, R. L. and Chow, B. F. Some applications of the rapid uptake of vitamin B_{12} by resting Lactobacillus leichmannii organisms. Science 115: 351 (1952).

139. Oginsky, E. L. and Smith, P. H. Effect of vitamin B_{12} derivatives on oxidation and vitamin uptake by Escherichia coli. J. Bact. 65: 183 (1953).

140. Couch, J. R., Ener, H. S., and Olcese, O. Observations on the vitamin B_{12} binding substance of egg yolk. Fed. Proc. 13: 194 (1954).

141. Davis, R. L. and Chow, B. F. Compounds inhibiting uptake of vitamin B_{12} by "resting" bacterial cells. Fed. Proc. 12: 440 (1953).

142. Hartley, F., Stross, P., and Stuckey, R. E. Some pharmaceutical aspects of vitamin B_{12}. J. Pharm. Pharmacol. 2: 648 (1950).

143. Loy, H. W., Haggerty, J. F., and Kline, O. L. Stability of vitamins B_{12} and B_{12b}. J. Assoc. Offic. Agr. Chemists 35: 169 (1952).

144. Davies, M. T., Mamlis, P., Petrow, V., and Sturgeon, B. The chemistry of antipernicious anemia factor. VIII The basicity of some benzimidazoles and benzimidazole glycosides. J. Pharm. Pharmacol. 3: 420 (1951).

145. Stauffer, R. E. Dialysis and electrodialysis. In Weissberger, A., ed. Technique of Organic Chemistry, Volume III. p. 313. N. Y., Interscience Publishers, Inc. (1950).

146. Ballentine, R. and Burford, D. D. Radiochemical assay of cobalt-^{60}. Anal. Chem. 26: 1031 (1954).

147. Provasoli, L. and Pitner, I. J. Assay of vitamin B_{12} in sea water. Proc. Soc. Protozoologists 4: 10 (1953).

148. Rosenthal, H. L. and Brown, C. L. Jr. Vitamin B_{12} activity of plasma and whole blood from various animals. Proc. Soc. Exp. Biol. Med. 86: 117 (1954).

149. Couch, J. R., Olcese, O., Witten, P. W., and Colby, R. W. Vitamin B_{12} content of blood from various species. Am. J. Physiol. 163: 77 (1950).

150. Anthony, W. B., Counch, J. R., Rupel, I. W., Henderson, M. B., and Brown, C. L. Jr. Vitamin B_{12} in blood of newborn and colostrum-fed calves and in colostrum and normal milk of Holstein and Jersey cows. J. Dairy Sci. 34: 749 (1951).

151. Chow, B. F. Sequelae to the administration of vitamin B_{12} to humans. J. Nutr. 43: 323 (1951).

152. Weichselbaume, T. E. An accurate and rapid method for the determination of proteins in small amounts of blood serum and plasma. Am. J. Clin. Path. (Tech. Sec.) 16: 40 (1946).

153. Valmet, E. and Svensson, H. Some problems inherent in paper electrophoresis. Science Tools 1: 3 (1954).

154. Fraenkel-Conrat, H. The essential groups of lysozyme, with particular reference to its reaction with iodine. Arch. Biochem. 27: 109 (1950).

155. Lewis, J. C., Snell, N. S., Hirschmann, D. J., and Fraenkel-Conrat, H. Amino acid composition of egg proteins. J. Biol. Chem. 186: 23 (1950).

156. Welch, A. D., Scharf, V., Heinle, R. W., and Meacham, G. C. Assay for intrinsic factor in patients with pernicious anemia in remission given radioactive vitamin B_{12}. Fed. Proc. 11: 308 (1952).

157. Heinle, R. W., Welch, A. D., Scharf, V., Meacham, G. C., and Prusoff, W. H. Excretion and absorption of cobalt^{60} labeled vitamin B_{12} in pernicious anemia. Trans. Assoc. Am. Physicians 65: 214 (1952). (Original not available for examination; abstracted in Chem. Abstr. 47: 10717g 1953.)

158. Schilling, R, F. Intrinsic factor studies. II The effect of gastric juice on the urinary excretion of radioactivity after the oral administration of radioactive vitamin B_{12}. J. Lab. Clin. Med. 42: 860 (1953).

159. Roseblum, C., Woodbury, D. T., Gilfillan, E. W., and Emerson, G. A. Influence of intrinsic factor upon utilization of orally administered vitamin B_{12}. Fed. Proc. 13: 475 (1954).

160. Glass, G. B. J., Boyd, L. J., Gellin, G. A., and Stephanson, L. Test for intrinsic factor activity: Measurement of hepatic uptake of ingested radioactive vitamin B_{12}. Fed. Proc. 13: 54 (1954).

161. Smith, E. L. Water-soluble vitamins. I Ann. Rev. Biochem. 23: 245 (1954).

162. Bauriedel, W. R. Microbiological assay of vitamin B_{12} in blood. Unpublished M. S. Thesis, Ames, Iowa, Iowa State College Library (1952).

163. Hellerman, L., Chinard, F. P., and Deitz, V. R. Protein sulfhydryl groups and the reversible inactivation of the enzyme urease. The reducing groups of egg albumin and of urease. J. Biol. Chem. 147: 443 (1943).

164. Anson, M. L. The sulfhydryl groups of egg albumin. J. Gen. Physiol. 24: 399 (1941).

165. Boyer, P. D. Spectrophotometric study of the reaction of protein sulfhydryl groups with organic mercurials. J. Am. Chem. Soc. 76: 4331 (1954).

166. Epstein, J. Estimation of microquantities of cyanide. Anal. Chem. 19: 272 (1947).

167. Boxer, G. E. and Rickards, J. C. Chemical determination of vitamin B_{12}. II The quantitative isolation and colorimetric determination of millimicrogram quantities of cyanide. Arch. Biochem. 30: 372 (1951).

168. Boxer, G. E. and Rickards, J. C. Chemical determination of vitamin B_{12}. IV Assay of vitamin B_{12} in multivitamin preparations and biological materials. Arch. Biochem. 30: 392 (1951).

169. Boxer, G. E. and Rickards, J. C. Chemical determination of vitamin B_{12}. V A modified procedure for the determination of cobalamins in liver concentrates. Arch. Biochem. Biophys. 39: 281 (1952).

170. Kolthoff, I. M. and Sandell, E. B. Textbook of quantitative inorganic analysis. Rev. ed. p. 478. N. Y., The Macmillin Company (1943).

ACKNOWEDGEMENTS

The author wishes to express his sincere appreciation to Dr. Leland A. Underkofler for his guidance, encouragement and suggestions throughout the course of this investigation; and to Dr. Joseph C. Picken, Jr. for his aid and advice during many phases of the work and in the preparation of the manuscript.